EXPERIMENTAL AND CYTOLOGICAL STUDIES OF MAMMALIAN FERTILITY
AND INFERTILITY WITH PARTICULAR REFERENCE TO THE AGEING OVARY

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A Thesis presented for the Degree of Doctor of Science

University of Edinburgh, 1988
"If politics is the art of the possible, research is surely
the art of the soluble. Both are immensely practical-minded affairs."

Arthur Koestler

(New Statesman 19 June 1964)
DECLARATION

I hereby declare that this Thesis is my own work and that, with the undernoted exceptions, the publications contained herein were either conducted unaided or, in the case of works with other authors, as a leading member of a research team. My contribution to Reference 11 (see Reference list) extended from pp. 251-253 and 258-260 and I was responsible only for the autoradiography and morphology for Reference 24. None of the enclosed publications have been submitted elsewhere for a degree or professional qualification.

20 June 1988

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Precious little serious research can be undertaken nowadays without a
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Scottish Trust, Carnegie Trust for the Universities of Scotland, Eugenics
Modern science is a collective activity based upon the pooling of skills and knowledge rather than being an individual effort, as is still the case for artists, writers and composers. It is an expensive activity requiring specialised tools and facilities and therefore dependent on grants from governments, private agencies and charitable bodies. It is also, pre-eminently, an international enterprise. I acknowledge all these factors in the development of the research programmes represented in this thesis.

I acknowledge with deep gratitude those who have provided opportunities and encouragement for my research work and professional career. Professor Bob Edwards (Cambridge) launched me into research and had a stimulating influence on my thinking long after formal postgraduate training was completed. I wish to give special thanks also to Professors Jack Everett (Duke University) and "Tuck" Finch (University of Southern California) for introducing me to American science and academic life; experience of working with them has been formative for my scientific attitudes as well as being a pleasure. To the Heads of Departments where I have worked in the UK I also owe a debt of gratitude for providing space and facilities for research: Professors Sir Bryan Matthews, FRS, "Bunny" Austin (Cambridge) and Bill Watson (Edinburgh).

Precious little serious research can be undertaken nowadays without a grant and I am grateful for the financial support I have received over the years. In particular I wish to acknowledge scholarships and fellowships from the Medical Research Council and the Population Council (New York) and research and travel grants from the following: Medical Research Council, Wellcome Trust, Carnegie Trust for the Universities of Scotland, Eugenics Society, International Planned Parenthood Federation and the Moray Fund
(University of Edinburgh). My work in the USA was supported by the National Institutes of Health and the National Science Foundation. I also wish to acknowledge with thanks those who have supported my applications, whether successful or otherwise, and editors and anonymous referees of my papers whose efforts have improved the presentation of my publications.

This section would be incomplete without thanks for support and encouragement from my wife, Carole, and sons, Matthew and Thomas, who have patiently endured unsociable working hours and shared disappointments when experiments failed. To them, and to all technicians and research students who have shared my work, I give heartfelt thanks.

Finally, I am grateful to Academic Press (London) for permission to photocopy a monograph for inclusion within this volume.
Reproduction and ageing are processes in which biological order and organization vary over time. Arguably, neither is a biological discipline in its own right but both involve many branches of science: from molecular aspects, through studies of the whole organism to an appreciation of the behaviour of communities. In both fields, therefore, an interdisciplinary attitude is advantageous and has influenced the course of my research programme. Investigations of the ageing reproductive system and the developmental biology of ovarian follicles have involved cytology and cytogenetics, endocrinology, experimental surgery and mathematical modelling.

Maternal age is a major factor affecting the prospect and outcome of pregnancy in animals and man. Inbred mice have been used as subjects of most studies for practical reasons and because they are good models for mammalian development and ageing. Pregnancy losses in mid-life are attributable to increased numbers of cytogenetically abnormal embryos and a decreased uterine capacity for maintaining conceptuses to full term. The cause(s) of aneuploidy in older mothers remains unknown although the integrity of the meiotic spindle is in question since it disintegrates during "overripening" of oocytes in the fallopian tube and is altered in preovulatory oocytes of older mothers. Experimental delay of ovulation does not affect the incidence of anomalies but unilateral ovariectomy causes the peak incidence of anomalies and subsequent sterility to occur prematurely, indicating that biological and chronological age can be uncoupled. However, many normal conceptuses fail to thrive in older mothers. The uterus undergoes structural changes as a result of ageing and parity, but the functional significance of these, if any, is not known. The endocrinology of pregnancy requires further study for, while luteal function may be unimpaired, there is mounting evidence of age changes
in tissue responsiveness and of detrimental effects of long-term exposure to cycles of ovarian hormone stimulation.

Ovarian oocytes disappear continuously throughout life because of recruitment for growth and ovulation, and cell death. Whereas sterility in some rodents is due to inability to produce an ovulatory surge of gonadotrophic hormones, in others it is depletion of oocytes, as in humans, that is primary. The rate of oocyte depletion and the size of the initial store define the ultimate limit for fecundity. Exponential functions which define the rates of follicle attrition change according to age and physiological state. The numbers of follicles at puberty are related allometrically to body weight, and the early age of menopause in our species may have arisen adventitiously as longevity evolved beyond expectations based on body weight.

New techniques have been employed to investigate the control of ovarian follicle recruitment, growth /death and morphogenesis of the antrum. Mathematical models indicate that the profile of follicle stages in mice of different ages and genotypes is mainly influenced by death ("atresia") rates which are highest in small and large follicles. The granulosa epithelium, which originates from a small number of clones, controls the formation of the antrum. Antral fluid appears to consist of local metabolites and plasma constituents; its formation probably involves more than one mechanism. Organotypic cultures may provide the best prospect of furthering investigation of morphogenesis of the Graafian follicle and of the cellular basis of follicle dynamics.
SECTION I

EXPERIMENTAL ANALYSIS OF THE AGEING REPRODUCTIVE SYSTEM

The effects of age on the reproductive system were matters of interest long before the scientific approach began and in developed agricultural societies, the fertility of animals has always been a major indicator of family wealth. Before the advent of modern automation the numbers of children available to prepare and harvest the land was a crucial factor in rural economy. But marital fertility was not purely an economic problem, it was also a social stigma. Many examples could be cited, perhaps those of Sarah and Elizabeth in the Bible are most familiar.

In recent times fertility have changed, especially in modern Western and rapidly developing societies. The necessity to control family size by urbanization have led increasing numbers of people, married or otherwise, to choose childlessness for life. The development of highly effective contraceptives and the increasing acceptability of sterilization have made real choices possible whereas in the past it was often only the menopause which brought a welcome ending to a long succession of pregnancies.

Nevertheless, for most people the desire to procreate is a natural and strong urge and childlessness can be a source of considerable, and often unrecognised, suffering. Fortunately, the successful clinical management of infertility has been developing rapidly, bringing hope to many who would otherwise be excluded from biological parenthood.

Maternal age is a major factor affecting the prospect and outcome of pregnancy. Menopause is its most dramatic expression, marking the end of the reproductive lifespan in women (33). The germ cells lost by menopausal age (median age about 50) cannot be replaced but this natural barrier to fertility can be artificially breached, at least in some cases of premature
Introduction

The effects of age on the reproductive system were matters of interest long before the scientific era. For nomadic and undeveloped agricultural societies the fertility of animals has always been a major indicator of family wealth. Before the advent of farm automation the numbers of children available to prepare and harvest the land was a crucial factor in rural economy. But marital sterility was not purely an economic problem, it was also a social stigma. Many examples could be cited; perhaps those of Sarah and Elizabeth in the Bible are most familiar.

Attitudes to infertility have changed, especially in modern Western countries and in some rapidly developing societies. The necessity to control world population growth and the breaking of the link between family size and family wealth by urbanization have led increasing numbers of people, married or otherwise, to choose childlessness for life. The development of highly effective contraceptives and the increasing acceptability of sterilization have made real choices possible whereas in the past it was often only the menopause which brought a welcome ending to a long succession of pregnancies. Nevertheless, for most people the desire to procreate is a natural and strong urge and childlessness can be a source of considerable, and often under-recognised, suffering. Fortunately, the successful clinical management of infertility has been developing rapidly, bringing hope to many who would otherwise be excluded from biological parenthood.

Maternal age is a major factor affecting the prospect and outcome of pregnancy. Menopause is its most dramatic expression, marking the end of the reproductive lifespan in women (53). The germ cells lost by menopausal age (median age about 50) cannot be replaced but this natural barrier to fertility can be artificially breached, at least in some cases of premature...
menopause, by combining embryo donation with a regimen of exogenous gestational hormones (Lutjen et al., 1984). The loss of fertility does not usually occur abruptly at menopause: there is a continuous decline beginning a decade or more earlier. The causes of this lost reproductive potential are not well-understood and present some of the most obstinate problems for clinical management.

In the past two decades we have witnessed a major increase in research activity towards understanding the causes of senescence of the reproductive system. Much of this activity has taken place in the United States as a spin-off of the growth in gerontology with the changing population age distribution. It may seem paradoxical that a branch of reproductive biology should benefit from ageing research since few elderly people have any ambition for parenthood! The explanation lies in the peculiar advantages that the reproductive system offers for experimental ageing research. The relatively early onset of reproductive senescence implies that changes may be observed in this system at a time when pathological lesions are still relatively infrequent and before the onset of widespread senile degeneration. Moreover, it is possible to experimentally isolate primary and secondary factors since the reproductive system is organized hierarchically - involving the reproductive tract, gonads, pituitary gland and central nervous system. The possible involvement of the brain in ageing of the reproductive system is yet another reason for increasing attention on this subject because the central nervous system is of prime importance in gerontology. Much recent work has therefore focussed on central effects of hormones and neurotransmitters. Rats and mice have proved convenient models for exploring general biological principles of ageing because their reproductive physiology is well-understood, they are short-lived and relatively inexpensive. However, different reproductive strategies have evolved in man and it is important to obtain more information about non-human primates.
Despite many advantages the gerontology of reproduction suffers from most of the limitations of experimental design inherent in ageing research. Chief among these problems is experimental control. Most studies have been based on cross-sectional experimental designs which may be criticized on the grounds that cohorts of different ages have had differing seasonal, dietary and other influences which cannot be entirely controlled even under the best of modern husbandry regimes. Longitudinal studies are one answer to this problem because a single cohort is studied throughout life, e.g. for ovarian cycle history. However, this design is not appropriate for many experiments which require that animals are killed either to obtain tissues/embryos or when a measurement cannot be repeated, e.g. stereotactic manipulation of the brain (9).

Some obstacles to the advancement of the subject are professional rather than scientific. Ageing studies are slower-yielding than many other areas of biology, a fact which may deter students. Furthermore, they may be less attractive because they do not yet rest on a central paradigm in the sense that, for example, molecular genetics is grounded in our understanding of the DNA molecule and genetic code. There are many reasons why our understanding of the biology of ageing is relatively undeveloped but the fact that it is pioneering territory is the attraction for some investigators.

The following sub-sections describe investigations of the female reproductive system in mammals carried out over a period of about fifteen years. They comprise a programme to determine the balance of factors responsible for impaired fertility and fecundity. The first studies involved analysis of oocytes and embryos because epidemiological surveys have shown a major effect of maternal age on chromosomal anomalies. As work progressed it became apparent that the maternal environment could not be ignored: it has a major influence over pregnancy outcome, at least in older rodents. Later on,
increasing attention was paid to the control of the ovulatory cycle because the process of reproductive ageing was proving to be multifactorial and dependent on physiological state earlier in life.

This section and the following one are not comprehensive reviews; they are concise narratives to supplement the published works. Thus, citations are inevitably biased towards the author's publications (numbered parenthetically); other publications are quoted either to provide historical background or continuity to arguments or to indicate useful works of reference. Many investigators have contributed to the development of the fields described and due credit is given in my published reviews.

(ii) Gametes and embryos

One of the most important and intractable problems associated with maternal ageing is the deteriorating quality of oocytes. The rising incidence of Down's syndrome (exponential after age 30) is a matter of particular significance yet, despite considerable research effort, the aetiology has not been determined (Bond & Chandley, 1983). There is evidence that trisomy of other autosomes and some sex chromosomes also rises with age. Since these anomalies are widely believed to be due to chromosomal non-disjunction at one of the meiotic divisions (mainly Metaphase I), an equal incidence of monosomy and trisomy would be expected at conception. The fact that the former are rare at all maternal ages probably reflects an inferior developmental potential. Human gametes are heir to many defects and hazards during development, judging by the high incidence of abnormal gametes and fetal deaths compared with most laboratory and domestic animals that have been investigated. While it is important to characterize the complete range of anomalies most attention has been given to autosomal trisomy because some
conceptuses survive and all are affected by some phenotypic disturbance. Many hypotheses have been proposed to explain age-related trisomy. According to one view, the higher perinatal incidence is due to "relaxed selection" against anomalous fetuses. This hypothesis has been refuted by critical statistical analysis of the epidemiological data and by the demonstration of more trisomic conceptions in older animals. In 1973 two independent studies reported an increased incidence of aneuploidy in ageing mice (1; Yamamoto et al., 1973b). Since the frequency of triploidy was independent of age, it appears that the causal factors for this second major class of anomalies are different.

Human cytogenetics have demonstrated the apportionment of responsibility for aneuploidy between the male and female gametes and the stage of maturation at which it arises, but cannot explain the cause(s) of non-disjunction. Experimental studies are required to test hypothetical causes. According to the "production line" hypothesis, anomalies are predetermined during oogenesis as a result of a putative developmental gradient which adversely affects the last formed oocytes, which are ovulated towards the end of life (Henderson & Edwards, 1968; 11). This was based on the reduction in chiasma frequency and increase of unpaired univalent chromosomes during ageing. Several attempts have been made to test this hypothesis, both in the author's laboratory and elsewhere, but they have been unsuccessful because radiosensitivity of murine oocytes prohibits satisfactory tracing of follicle lineages using tritiated thymidine. It has been suggested that defective chiasmate relationships might predispose older oocytes to cumulative radiation damage from environmental and other sources (Yamamoto et al., 1973a) but the experimental evidence has been questioned (2) and subsequent research has failed to support this claim (Tease, 1982).
A number of investigators have suggested that a changing hormonal balance during ageing is responsible for many chromosomal defects in oocytes. This theory is attractive because it can explain trisomy in very young mothers whose ovarian cycles may also be irregular, and it gives hope of alleviation by hormonal manipulation. The observation that oestrous cycles become elongated towards the end of the reproductive lifespan (see (iv)) has led to a suspicion that follicle over-ripening may affect oocyte quality. More abnormal embryos were found when ovulation in rats was delayed by pentobarbital anaesthesia (Butcher, 1975), but subsequent studies using models which avoid anaesthetics were unable to confirm a significant increase in aneuploidy in mice (29).

If changes in the hormonal regulation of the ovaries are responsible for aneuploidy it might be expected that the timing of the age-dependent rise in aneuploidy and ovarian cycle irregularities would occur pari passu. In support of this prediction, unilateral ovariectomy of young mice resulted in a shorter reproductive lifespan with a precocious rise in aneuploidy (28; 50). These results lead to the conclusion that the biological age of the reproductive system is of greater significance for the pathology of oocytes than the chronological age.

Two major difficulties confronting investigators are, first, the relatively low incidence of chromosomal anomalies in animals and, second, the limited ability to verify the karyotype of individual preimplantation embryos because of paucity of metaphase cells. There is, furthermore, the limitation that conventional cytogenetics only demonstrates chromosomes: cellular apparatus responsible for their position and traction are removed. A new approach has been developed in partnership with Drs. Ursula Eichenlaub-Ritter and Ann Chandley in which meiotic spindle and chromosomes were analysed in the same preparation. The chromatin and cytoskeleton of oocytes were
preserved while other cellular constituents were removed by detergents. The tubulin of the spindle and the DNA were stained using differential fluorochromes. The timing and dynamics of spindle-chromosome interactions were revealed and changes in the size of spindle and misalignment of chromosomes were found in oocytes undergoing post-ovulatory ageing in the oviduct (39). In oocytes of older mice and in unilaterally ovariectomized animals chromosomes were less well-ordered on the Metaphase II spindle and the long axis of the spindle was reduced in length (47). These results strengthen the suspicion that it is the spindle which is defective in oocytes prone to chromosomal non-disjunction and they confirm the detrimental effects of unilateral ovariectomy.

(iii) Pregnancy maintenance

The balance between the contributions of embryonic and maternal factors to infertility in mid- and late life varies between species. This balance has been elegantly demonstrated by reciprocally transferring embryos between animals of different ages (Adams, 1970). Embryos from ageing rabbits and hamsters were less viable in young hosts than those in young-young combinations. The embryonic factor was less significant in mice than the age of the maternal environment (Talbert & Krohn, 1966), this surprising difference being confirmed by experiments in which the contralateral uterine horn served as an internal control (3).

It is generally recognised that most oocytes in older mice are fertilized normally and reach the uterus at the expected time. Most conceptuses die at or after implantation, according to age and genotype. In 10-11 month old C57BL/6J mice, a widely used model, most deaths occurred at or soon after mid-gestation (day 10). The frequency and distribution of fetal deaths along
the uterine horns indicated that random local factors were operating, but resorption of the entire litter involved a different, but unknown, aetiology (18). Uterine arterial stenosis occurs in multiparous rats (Wexler, 1964) but experimental studies are required to determine whether reduced vascular perfusion of placentae contributes to the infertility of older animals. Fetal growth retardation leading to resorption (Holinka et al., 1979) can be accounted for by the metabolic demands exceeding the capacity of the transport system in these animals, but it can also be due to chromosomal defects.

Among the hundreds of pregnancies examined during the course of these and other studies only one was ectopic, and this occurred in a young rat (20). For this improbable event to occur the embryo had to first escape from the ovarian bursa and then implant (in this case on the omentum). Generally speaking, ectopic pregnancies are much more common in humans than animals. In 1787, John Hunter reported that the numbers of piglets farrowed throughout life by a unilaterally ovariectomized sow was approximately half that of an intact control. This finding has been repeated for several species of laboratory rodents and should be verified for swine. In contrast to the sow, rat and mouse blastocysts rarely undergo transuterine migration, implying that the additional embryos produced by compensatory ovulation in the remaining ovary become crowded within the ipsilateral uterine horn. While a different explanation appears to be necessary for the sow (e.g. increased abnormal oocytes) the excess fetal death in the functional horn of mice has led workers to suggest that the uterus becomes prematurely senescent (Biggers et al., 1962). This potentially valuable model for investigating uterine ageing and parity has been explored further by transferring embryos from young donor mice to both the parous and barren horns of pseudopregnant mice which were unilaterally ovariectomized at the beginning of reproductive
life (7; 16). The poor rates of embryonic survival in the parous horn compared with intact animals of the same age confirmed the hypothesis, but the exceptionally poor viability in the barren horn and in nulliparous horns of intact mice was a surprising result. Early studies had already shown that fertility is impaired when breeding is delayed in rats (Asdell et al., 1941) but these new results indicated that local uterine factors are involved since both horns are exposed to the same systemic conditions. Nevertheless, there are grounds for suspecting that repeated cycles of oestrogen stimulation contribute to infertility. Elongated cycles and anovulation associated with persistent Graafian follicles ("persistent oestrus") leads to cystic glandular hyperplasia of the uterus and other pathological conditions such as pituitary tumorigenesis. When animals were bilaterally ovariectomized early in adulthood and restored to cyclicity by inserting ovarian grafts under the renal capsule the uterine capacity to maintain the pregnancy of transferred embryos was greater than that of animals that had retained their own ovaries (27). Whether the beneficial effects of removing ovaries was due to the absence of regular oestrous cycles or to avoidance of persistent oestrous remains to be shown.

The uterus undergoes characteristic cytological changes during ageing and as a result of pregnancy (34). The multiparous uterine horns of rodents accumulate macrophages containing lipofuscin granules, and in unilaterally ovariectomized animals the pigmentation occurs predominantly in the functional horn, marking sites of placental scars (16). In contrast, the numbers of uterine mast cells rise as a result of ageing rather than of parity. There is not yet, however, any evidence to indicate whether these cellular changes affect the success of pregnancy. Scar tissue, which accumulates with each successive pregnancy, could interfere with implantations which occur at random sites. Apart from a change in size, the human uterus does not show such lasting cytological changes after parturition.
(17; 34). Membrane-bound lipid droplets accumulate in the smooth muscle cells of this organ continuously during ageing (12). These may be precursors of lipofuscin because they appeared to be lysosomal complexes containing fluorescent products of lipid peroxidation; they do not occur, as has been suggested, only in toxaemic patients (Haust et al., 1977).

The numbers of oocytes shed each cycle in polytocous species do not decline until after the onset of diminished uterine capacity for pregnancy in mid-life (6). While the numbers of corpora lutea are well-maintained it is necessary to determine whether luteal dysfunction exists because gestational steroids from these bodies are obligatory throughout pregnancy in rodents. Before sensitive radioimmunoassays became available for measuring steroid hormones in murine blood, luteal function had to be measured indirectly. The volume of luteal tissue was found to be reduced in older CBA mice as a result of smaller cells (4). Supplementary progesterone improved the rates of implantation but not those of fetal survival: supplementary oestrogen or luteotrophic hormones (prolactin or chorionic gonadotrophin) were ineffective (6). When serum progesterone levels were measured on days 4 and 8 of pregnancy in young and ageing mice they were found to be similar, suggesting that the beneficial effects of hormone treatment were due to changes in target tissue sensitivity rather than luteal insufficiency (15). Fetal loss would be expected to reduce progesterone levels by removing placental gonadotrophin and could, potentially, affect the remaining litter mates. However, when litter sizes in young animals were controlled by embryo transfer the levels were unaffected when two or more fetuses were present (35). The specific uterine uptake of tritiated oestrogen in ovariectomized young adult and ageing CBA mice did not differ, which could imply that the concentrations of receptors had not diminished (8). Since that study, receptor assays have been refined and, on the basis of substantial investigations of the major target tissues for oestrogen, it has been
concluded that age differences in receptor dynamics exist, most notably in
the hypothalamus (Nelson et al., 1987). The effects of ageing on the
progesterone receptor have not been studied as intensively.

If oestrogen receptors were diminished then the responsiveness of uterine
cells to oestrogen would be affected, but surprisingly few deficits have been
found. Normal increases in protein and RNA and in mitotic activity occurred
in the uteri of ageing mice, although the production of prostaglandins by the
rat uterus during incubation was reduced (30). Prostaglandins are
physiological regulators with many important roles in reproduction (Poyser,
1981). The reduced production of PGE-2 and thromboxane following priming with
steroids was particularly marked and, in view of the significance of
prostaglandins for embryo implantation, further study of their production,
metabolism and physiological effects at the site of the implanting embryo are
warranted.

Pregnancy wastage is frequently associated with physiological transitions
such as implantation and the switch from dependence on the yolk sac to the
chorio-allantoic placenta at mid-pregnancy in rodents. Parturition is another
crucial transition, but dystocia was rarely encountered in ageing animals
except in small litters when pups were abnormally large. Nevertheless, it
would be interesting to investigate the responsiveness of the aged intrapartum uterus to oxytocin and prostaglandins. Stress is a potentially
important cause of complications at parturition in humans and extends the
interbirth interval in rats. Recent studies suggest that this response is
mediated by endogenous opiates and morphine administration, whether by
subcutaneous or intra-cerebroventricular route, interrupts parturition by a
mechanism that is sensitive to naloxone and involves suppression of oxytocin
release (32, 36). These findings are currently being prepared for publication
in full (Russell et al.).
(iv) The ovarian clock

The ovary is in more than one sense a biological timepiece. Besides the more-or-less regular rhythm of ovulation there is the steady depletion of the store of oocytes throughout life, which is known only to be moderated by hypophysectomy (11) and by dietary restriction (31). The strategy that has evolved in mammals and birds and some other taxa is not however universal among vertebrates. The following Table shows that in some animals oogenesis continues throughout life, enabling individuals to increase their fecundity as they grow.

<table>
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<td>(i) Eutherians</td>
<td>Amphibians</td>
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<td>(ii) Metatherians</td>
<td>Osteichthyes (most)</td>
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<td>(iii) Monotremes</td>
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<td>Birds</td>
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<td>Chondrichthyes (?)</td>
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A fixed population of oocytes, represented by primordial follicles, is highly significant for ovarian ageing because its size and dynamics will determine the time of onset of sterility. The size of this store at puberty varies allometrically with body size rather than with ovulation rate across a wide range of mammals (11). Longevity of species also varies with body weight and it is assumed that long-lived species have larger follicle endowments for correspondingly longer reproductive lives. The numbers of primordial follicles at a given age will also depend on the rate of follicle death and recruitment and, on the basis of limited data, it appears that the half-life
of the follicle store varies as a function of body weight/longevity (41). Interestingly, the follicle store in human ovaries at puberty is commensurate with body weight, but it becomes depleted by mid-life (41; 44). The evolution of human menopause is a controversial subject but, since human longevity is 3-4 times greater than predicted by body weight, these findings suggest that it has occurred adventitiously with the evolution of an extended lifespan. Accelerated disappearance of small follicles in the last years of reproductive life may further advance the onset of sterility in our species (Richardson et al., 1987; 53).

Maintenance of a species-characteristic ovulation rate throughout most of the lifespan despite waning of the primordial follicle store is a remarkable characteristic of ovarian function and indicates that recruitment is a controlled process. Much histological evidence suggests that this process involves a reduction in deaths of growing follicles rather than an increased rate of initiation of growth (22). This conclusion appears to be supported by the observation that ovarian senescence is only marginally advanced by unilateral ovariectomy (28; 50) and that the capacity for superovulation by exogenous gonadotrophins wanes with age (33).

While the association between the timing of follicle depletion and menopause in humans suggests a cause and effect relationship (53) the situation in laboratory rodents is more complex; their value as models of ovarian ageing has been discussed recently by Finch & Gosden (37). In most strains of mice and rats small numbers of follicles remain at the end of life although in a few the ovaries become sterile by mid-life. A comparative study of inbred mice by Jones & Krohn (1961) showed that early sterility in CBA strain was neither due to a smaller follicle endowment nor to more ovulations. When a mathematical model for follicle dynamics was fitted to the data, the early sterility of this strain could be accounted for by a high
death rate at primordial stages throughout life (23). An important goal for the future is elucidation of the causes of follicle death in animal and human ovaries. The CBA mouse appears to be a useful model for human ovarian ageing in several respects (14; 37); it is noteworthy that the male of this strain also undergoes gonadal senescence relatively early in life (21).

In view of early sterility in CBA mice it is not surprising that Krohn (1962) found that old ovaries could not reinitiate oestrous cycles when transplanted to young ovariectomized hosts. When comparable studies were conducted with rats, the results indicated that hypothalamic rather than ovarian or pituitary function determines the ending of their ovarian cyclicity (Aschheim, 1964-65). Subsequently, the neuroendocrine basis of this condition has been extensively investigated and, while this remains poorly understood, several general conclusions have emerged. There is a failure of the hormonal stimulus for ovulation even though follicles continue to attain Graafian maturity by continuous and asynchronous recruitment from preantral stages (11; 25). This situation leads to persistent oestrus and, consequently, pathological changes in the uterus, mammary and pituitary glands. These ovaries can ovulate following either electrical stimulation of the brain to release endogenous gonadotrophins (9) or injection of luteinizing hormone (LH) or LH-releasing hormone (LH-RH) (RGG, unpublished). In earlier studies, synthetic LH-RH was found to stimulate ovulation in young mice (5) and in rats its effects were shown to involve both pituitary and ovarian sites of action (24).

Failure of gonadal steroids to release LH by a positive feedback mechanism is responsible for anovulation in ageing rats and, to varying extents, in mice. This has been demonstrated in ovariectomized rodents by a number of laboratories, as has the impaired secretion of the hormone under basal conditions (10; reviewed by Finch et al., 1984; Nelson & Felicio, 1987). By
contrast, secretion of follicle-stimulating hormone (FSH) is unimpaired. While prolactin has been shown to have anti-gonadotrophic actions, this is not invariably so (19), and the elevated level in old rats is an effect rather than a cause of anovulation (13). The timing of positive feedback failure evidently depends on the history of ovarian function and, in particular, on the secretion of oestrogen. These conclusions rest on (a) the greater ability to restore normal ovulatory cycles in ageing animals that have been ovariectomized for a long period than in those treated acutely (26) and (b) the ability of exogenous oestrogen to bring about an anovulatory syndrome similar to that of ageing (Finch et al., 1984).

(v) Conclusion

The studies outlined above have helped to provide a groundwork for understanding the character and sequence of age changes in one physiological system. As experimental work has progressed the explanation for reproductive ageing becomes more complex, but some general principles are emerging.

While ageing is measured chronologically for lack of alternative parameters, it is clear that biological time can be uncoupled to some extent from chronological time. The timing of age changes is not inviolate; it can be altered in the reproductive system by experimental manipulation of endocrine function. This principle has been demonstrated by examples from all the major expressions of reproductive senescence, namely, the deterioration of oocytes, hypothalamic control of ovulation, uterine support of pregnancy and the pathology of various organs. The reproductive system appears to be well-suited to exploration of endocrine interactions during ageing because the hierarchical organization of reproductive function facilitates experimental control and manipulation. In virtually all the age changes
discussed the ovary assumes central importance because it is responsible for the maturation of oocytes and has profound influences over reproductive organs through secretion, notably of oestrogen. Nevertheless, it must be remembered that ageing changes are multifactorial and vary with genotype. The future direction of research is likely to change as cytological and physiological studies of the types described provide the framework and opportunity for tackling fundamental problems of the cell biology of ageing.
SECTION II

DEVELOPMENTAL BIOLOGY OF OVARIAN FOLLICLES
Knowledge of ovarian function is crucial to the understanding of the biology of ageing of the reproductive system. The capacity to produce oocytes and hormones is determined early in life when a finite reserve of small follicles is formed. The studies of reproductive senescence described above led to a recognition of the importance of factors controlling the recruitment and death of follicles at different stages and ages. Attention on the population biology of follicles is leading, in turn, to greater interest in the developmental biology of follicles as units, although this recent work is only partially represented in this thesis.

This Section describes the groundwork of a research programme for ovarian follicle biology and is an introduction to current studies of growth and differentiation of granulosa and theca cells and the morphogenesis of the Graafian follicle. Much of the work described is concerned with the development of models, both analytical and experimental, towards describing the behaviour of follicles collectively or as independently functioning units or for comparative animal physiology and anatomy. While present trends in this research emphasize the value of techniques in molecular and cellular biology, much worthwhile information has been gained by combining histological methods with suitable quantitative methods of analysis. The starting point for these studies is ovarian morphology from which a number of fundamental questions emerge.

(ii) Morphology

Ovarian follicles develop through a succession of stages which are remarkably similar in eutherian mammals. They can be broadly classified as (i)
Figure 1.
primordial (non-growing or "resting"), (ii) growing preantral and (iii) antrum-containing (Graafian) stages (Fig. 1). These stages have sometimes been called primary, secondary and tertiary follicles but this classification has not been followed rigidly by all authors and for this reason will not be adopted here. Follicles also undergo two morphogenetic transitions: first, when the primordial follicle begins to grow and, later, when the multilaminar follicle forms an antrum.

Although Graafian follicles are similar in form in most eutherian mammals they differ substantially in size. It is not surprising that the diameters of mature follicles in animals with such diverse body sizes as shrews and elephants vary accordingly, but it is important to establish whether this variation can be modelled mathematically for predicting follicular sizes and for guiding hypotheses about physiological relationships. Most of the variation in size is due to the accumulation of extracellular fluid in the antrum; ovulation occurs in the absence of this structure only in a few small tropical insectivores (e.g. Tenrec ecaudatus). Parkes (1932) obtained evidence that Graafian sizes vary with body weight but the data were scanty. Recently, the sizes have been shown to follow an allometric scaling relationship to body weight in a series of twenty-two species ranging from shrews to cattle (40). The general allometric relationship is:

$$Y = aM^b$$

where M is young adult body weight, a is the proportionality constant and b is the allometric exponent. The variance was reduced when the sum of the dimensions (Y: follicle diameter, surface area or volume) for a set of preovulatory follicles was used rather than individual follicle measurements. Since ovulation rates vary from one in many primates and ungulates to several hundred in the plains viscacha it is not surprising that they affect mature follicle size and, hence, ovarian volume. Whereas the numbers of small follicles are correlated with body weight, as discussed in the previous
Section, the species typical ovulation rate is not.

The finding that the surface area of a set of Graafian follicles is hypoallometrically related to body weight was somewhat unexpected (40). On the grounds that this secretory interface might be scaled according to the body distribution volume for hormonal products (approximately proportional to body volume/mass), an isometric function was expected. Isometric relationships are mainly found in transporting systems (respiratory, cardiovascular) and the significance of this finding for ovarian physiology remains to be shown. In contrast to follicles (and to spermatozoa), the sizes of oocytes, whether in primordial or mature follicles, tended to be similar in a wide range of species.

Graafian follicles contain three cell types: the oocyte, the granulosa and the theca cell (including theca interna and externa) (Fig. 1). Many studies have been based upon the isolation of one or another cell type in tissue culture but this is an artificial situation because normal follicle development requires communication between constituent cell types. This unity was recognised a century ago by a pioneer of reproductive biology who noted "Ohne Ei kein Follikel". The relationship between the granulosa and theca layers is less intimate than that between the granulosa and oocyte, which are physiologically coupled by gap junctions. Despite the presence of a basement membrane, the interface is crucial for exchange of dissolved gases and metabolites and regulatory molecules, such as the inductive factors postulated more than forty years ago (Hisaw, 1947). Such putative factors may have parallels with other tissues where epithelial-mesenchymal interactions are well-established (Cunha et al., 1983) and can now be tested by tissue recombination experiments using isolated preantral follicles (Roy & Greenwald, 1985; 42).
While the biology of the Graafian follicle is fairly well-known, that of primordial follicles is relatively underexplored despite their significance as the store from which all future ovulations are recruited. The primordial follicle of the mouse consists of a small oocyte which is partially or completely enveloped by a single layer of squamous pregranulosa cells which give rise to the 50,000 granulosa cells of the mature follicle over a period of about a month. The number of clonal precursors of granulosa cells in Graafian follicles has been estimated on the principle that only one X chromosome is active in female cells and by using binomial probability theory. The two alleles, A and B, of the X-linked marker phosphoglycerate kinase-1 were chosen as suitable markers because they can be distinguished by electrophoretic mobility. Analysis of the proportions of the two phenotypes indicated an average of five precursor cells exist (corresponding to the numbers of cells observed in primordial stages), although monoclonal types were found occasionally (48). The proportions of a given phenotype were highly correlated in the mural and cumulus cell sub-populations, indicating that considerable mixing of the lineages had occurred at preantral stages. These findings suggest that differentiation of sub-populations of granulosa cells is independent of their clonal origin and studies have begun to map the distribution of clonal descendents at successive stages of growth.

The majority of follicles in most species are uniovular, but binovular and polyovular follicles are occasionally observed. In a histological survey of mammalian ovaries a particularly high incidence of follicles with two or more oocytes was observed in the domestic bitch; this provided an opportunity of studying an otherwise rare phenomenon (43). The incidence of polyovular growing follicles was 14% in bitches aged 1-2 years and varied inversely with the numbers of oocytes per follicle (up to 14 have been found). Although these aberrant follicles may arise by chance association during folliculogenesis it is inappropriate to test this hypothesis by fitting the
data to Poisson distributions because follicle growth and death during the first year of life might have influenced the distribution of the various types. Polyovular follicles may disappear from the ovary differentially since they are much less frequent in older bitches (5%). Most polyovular follicles appear to be capable of completing preantral follicle development in young ovaries but few, if any, developed successfully in bitches aged 7-11 years old.

(iii) Follicular dynamics

Changes in time taken for follicles to develop from stage to stage at different maternal ages may indicate the actions of controlling mechanisms involving endocrine/paracrine/autocrine factors. Early attempts to measure follicle growth were based on macroscopic measurements of Graafian follicle diameters, dye marking or microscopic analysis of diameters or mitotic indices. These methods give a restricted impression of follicle growth rates. The ability to label follicle constituents (e.g. granulosa cell nuclei or the zona pellucida) and to subsequently trace the time course of development, was a major advance (e.g. Pedersen, 1970). Results indicated that the rate of growth at successive stages, from unilaminar to Graafian, was age-dependent and increased at each stage. They suffered from the serious limitation, however, of taking no account of circadian rhythms, nor did they provide estimates of follicle death rates.

Mathematical models have been devised as alternatives to these methods. Two models, based on different principles and providing different information, have been established in recent years (Faddy, 1976; Read et al., 1981). In a series of studies in collaboration with Dr. Malcolm Faddy, compartmental models have been used to analyse the dynamics of mouse
follicles during ageing and under various conditions. The methods can, in principle, be applied to any other mammalian or avian ovary for which data are available. The model requires follicle numbers at different stages of growth and at known ages after birth. It assumes that the initial follicle population is fixed at birth and that follicles move successively from stage to stage (I-V), but may leave by death. This death/migration model is based on the assumption that the mean numbers present at each stage \( \lambda_i(t) \), \( i = 1, 2, 3, 4, 5 \) is given by the solution of the differential equations where \( v_i \) is the migration rate and \( \mu_i \) is the death rate at stage \( i \). Then:

\[
\frac{d\lambda_i(t)}{dt} = v_{i-1}(t)\lambda_{i-1}(t) - (v_i(t) + \mu_i(t))\lambda_i(t)
\]

The fit of the model to the data obtained from three inbred strains and an F1 hybrid was found to be improved by using age-dependent parameters with a step transition(s) occurring close to pubertal age (Faddy et al., 1976; 23; 49). The parameters differed strikingly between strains and across this phase transition. They were broadly consistent with results of earlier labelling methods but further demonstrated that follicle death rates are age- and stage-specific. Variations in death rates appear to be at least as important as growth rates for influencing the profile of stages present at a given age and for determining the age of sterility. The models constructed for a range of ages and endocrine states, including ageing, hypophysectomy, unilateral ovariectomy and ovulation inhibition by progesterone, show that the rate of growth at intermediate follicle stages varies according to whether recruitment towards ovulation is occurring (23; 50; 51). Experimental studies are required to determine whether this effect is based upon fluctuations in pituitary gonadotrophin stimulation, which is doubtful, or intraovarian factors. The factors modulating preantral follicle growth initiation and maintenance are almost entirely unknown.
(iv) The follicular antrum

While not universal, the antrum is widespread in mammalian follicles. Much attention has been given to the composition of the fluid in the antrum because it is an indicator of the environment bathing the developing granulosa cells and oocyte. A large body of data shows that it resembles plasma, subject to the modulating influences of local metabolism and of the follicle epithelium which acts like a leaky molecular sieve (Edwards, 1974; McNatty, 1978; 45). The antrum begins to form when the preantral follicle has accumulated 2000-3000 granulosa cells, irrespective of body size or mature Graafian diameter (40). Since the granulosa layer is avascular, oxygen is expected to be rarefied towards the centre of the follicle, and the Graafian form may even have evolved to combat hypoxia (38).

The mechanism by which water is transported into the antrum is controversial. Transudation of plasma has been a popular hypothesis but it fails to account for the control of the rate of extracellular water accumulation and its distribution to produce the exquisite Graafian form. Any hypothesis should also take into account the differential expansion of the protoantral cavity and the Call-Exner bodies, which are part of the extracellular space in follicles of some species (52). Since neither measurements of hydrostatic pressures nor osmotic pressures across the follicle wall have provided evidence which account for net inward movement of water, it has been suggested that mural granulosa cells actively transport water coupled to solute, as occurs in many other epithelia (45). This hypothesis was been tested in pig follicles using an adapted Ussing chamber to measure the follicle wall potential difference (PD) and short-circuit current (SCC), indicators of the activity and polarity of ion transport mechanisms (46). The results were comparable with those obtained by impaling murine follicles on glass microelectrodes (McCaig, 1985) in showing that the
The follicular antrum

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PD and SCC were small or undetectable. These results do not necessarily deny that polarised ion transport across the epithelium occurs because the current may have leaked along low resistance pathways. On grounds of electrophysiological recordings and molecular markers, the follicle epithelium is characterized as "leaky".

(v) Perspectives

The ovarian follicle has been a subject of research interest for more than a century but I anticipate that present knowledge is merely a prologue to exciting new chapters about its developmental biology. At one time the follicle was chiefly of interest to steroid biochemists and reproductive physiologists, but in the future it could become an important model for the study of fundamental biological problems which are now commanding attention by cell and molecular biologists. The justification for this claim is, first, that the follicle consists of several cell types which are organised as a unit, it evidently maintains a coordinated developmental course by intercellular communication; second, it is a prime example of an epithelium which is hormone-dependent; third, it undergoes a well-defined pattern of growth with definite steps of cytodifferentiation and morphogenesis. Thus, the follicle may be said to encapsulate many of the major issues in biology today.

Moreover, there are grounds for thinking that the ovarian follicle represents a particularly tractable model system for experimental study. Current work in our laboratory and elsewhere is demonstrating how readily small follicles can be obtained and cultured.

Preantral follicles are abundant in young ovaries but, unlike the more intensively studied Graafian follicle, they are not large enough to be
isolated by dissection. However, large numbers of follicles across the entire size range can be liberated from the stroma by gentle enzyme treatment and pipetting (Roy and Greenwald, 1985; 42). Preantral follicles, like preimplantation embryos, are ideal subjects for culture because, being avascular, no artificial diffusion gradient is introduced, as is the case with organ culture. But when cultured in plastic dishes follicles lose their three-dimensional integrity; granulosa cells adhere to the substrate and flatten out to form a monolayer, liberating the oocyte. This problem can be overcome by suspending follicles in collagen gels (prepared from rat tail tendons) where they can develop from uni- to multi-laminar stages (42; Torrance et al., in preparation). Antrum formation has not yet been observed by this method in vitro but when gel cultures are transplanted under the renal capsule of host animals follicles undergo complete Graafian morphogenesis to become secretory and ovulable (C. Torrance and R.G. Gosden, unpublished).

Further refinement of these methods is needed, especially to separate follicles according to their stage and size. Nevertheless, progress is now being made towards defining the conditions required for follicles to begin and maintain growth throughout preantral stages. Beyond these immediate objectives lie opportunities for investigating many of the hitherto inaccessible problems of follicle development.


Hunter, J. (1787) An experiment to determine the effect of extirpating one ovarium upon the number of young produced. Phil. Trans. Roy. Soc. 77B, 233-239.


LIST OF PRINCIPAL PUBLICATIONS

(RGG)

Research Papers and Review Articles


50) Gosden, R.G., Telfer, E., Faddy, M.J. & Brook, J.D. Ovarian cyclicity and follicular recruitment in unilaterally ovariectomized mice.

51) Telfer, E., Gosden, R.G. & Faddy, M.J. Impact of exogenous progesterone on ovarian follicular dynamics and function in mice.

52) Gosden, R.G., Brown, N. & Grant, K. The character and significance of Call-Exner bodies in Graafian follicles of the rabbit ovary.

Monograph

HROMOSOMAL ANOMALIES OF PRE-IMPLANTATION MOUSE EMBRYOS IN RELATION TO MATERNAL AGE

R. G. GOSDEN

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(Received 30th January 1973)

Summary. A total of 168 air-dried chromosome preparations of pre-implantation mouse embryos were examined. The embryos were taken from CBA/1L.Tb female mice of 3 to 12 months of age. The majority of all age groups were found to be diploid. The incidence of diploid embryos was independent of, and that of other hyperdiploid embryos dependent on, maternal age.

It is well known that increasing maternal age in animals is associated with an increased percentage of non-disjunction errors. It has been reported (Curtis et al., 1971) that the incidence of non-disjunction errors is substantially due to the conception of diploid embryos, due to the fact that it is known that the incidence of non-disjunction errors is lower in males. Hence, if the incidence of non-disjunction errors is lower in males, it would be expected that the incidence of non-disjunction errors in the female would be higher. However, it has been reported (Curtis et al., 1971) that the incidence of non-disjunction errors in the female is lower than in the male. Therefore, it is possible that the incidence of non-disjunction errors is lower in females than in males.

It is also possible that the incidence of non-disjunction errors is lower in females than in males. However, it has been reported (Curtis et al., 1971) that the incidence of non-disjunction errors is lower in females than in males. Therefore, it is possible that the incidence of non-disjunction errors is lower in females than in males.

PUBLICATIONS

(RGG)
CHROMOSOMAL ANOMALIES OF PREIMPLANTATION MOUSE EMBRYOS IN RELATION TO MATERNAL AGE

R. G. GOSDEN

Physiological Laboratory, Downing Street, Cambridge CB2 3EG

(Received 30th January 1973)

Summary. A total of 168 air-dried chromosome preparations of preimplantation mouse embryos were examined. The embryos were taken from CBA/H-T6 female mice of 1 to 12 months of age. The majority in all age groups were found to be diploid. The incidence of triploid embryos was independent of, and that of other hyperdiploid embryos dependent on, maternal age.

It is well known that increasing maternal age in animals is associated with reduced fertility. In man, there is a corresponding increase in the proportion of certain trisomic offspring, notably those with Down’s syndrome, born to older mothers (Roberts, 1970). Finn (1962) reported that the diminished fecundity of old female mice is mainly due to the resorption of implanted embryos, but it has not yet been conclusively shown whether it is the embryo or the uterine environment which is deficient. Studies of spontaneous human abortuses have demonstrated that this reproductive failure is often associated with chromosomally abnormal conceptuses (Carr, 1972). Accordingly, in this study the incidence of chromosomally abnormal embryos from ageing female mice has been determined before postimplantation losses occur.

Inbred mice of the CBA/H-T6 strain were chosen for this study because of their short reproductive life-span (10 to 12 months in our colony). Female mice of more than 8 months old were regarded as an old group because the mean litter size declines steadily after this age (Text-fig. 1). This decline has been found to be a result of an increased failure of implantation as well as a failure of subsequent development (R. G. Gosden, in preparation). Forty-four female mice aged 1 to 12 months were mated with young fertile males of the same strain. Ovulation was induced in immature females with 3 i.u. PMSG followed by 1 i.u. HCG 44 hr later. Mice were injected with 1 μg Colcemid (Giba)/g body weight 3½ days after coitus to arrest mitosis. The animals were killed approximately 2 hr later. The uterine cornua were each dissected free, blotted and flushed with M199 medium containing 10% inactivated fetal calf serum, 5 to 8% phosphate buffer (pH 7·1) and 100 i.u. penicillin/ml. The oviducts were also flushed with medium if the yield of ova fell much below the expected number judging from CL of pregnancy counts. The ova were counted and scored for their developmental stage. Chromosome preparations of morulae and
blastocysts were made by an air-drying technique (Tarkowski, 1966), and then were later stained with lactic acetic orcein as described by Vickers (1967). Each preparation was examined with \( \times 1000 \) phase-contrast microscopy and chromosomes were drawn, photographed and counted as 'blinds'. No attempt was made to identify deleted or supplementary chromosomes or the sex of the embryos. The number of nuclei for each embryo was noted; metaphase plates were counted as one nucleus.

A total of 168 out of 332 preparations yielded chromosome spreads which could be counted precisely or approximately (\( \pm 2 \) chromosomes). The percentage recovery of morulae and blastocysts/number of CL of pregnancy was high for both adult groups (76% and 74% for young and old groups, respectively) which suggested that preimplantation losses were small. The results of the study (Table 1) showed that the majority of embryos, even from the older females, had a normal diploid complement (\( 2n = 40 \) chromosomes). The age-related decline in litter size cannot therefore be primarily due to visible chromosomal disorders.

In those preparations where precise counts of chromosome number were possible (one to five metaphase plates/embryo were counted), they were tabulated according to whether they were diploid, hyperdiploid or polyploid (Table 1). Embryos which had hypodiploid complements or overlapping chromosomes, that precise counts could not be made with confidence, were included in a separate category if they had \( 2n \pm 2 \) chromosomes. The ploidy of these groups could be determined.
The incidence of all hyperdiploids and polyploids was non-significantly different between age groups. The incidence of triploidy did not differ significantly between the groups and, overall, it was 4-2%. This result may suggest, if the claim of Vickers (1969) that delayed fertilization causes increased triploidy is well founded, that there is no delayed fertilization in the aged mouse. Two tetraploid embryos were recovered, one from an immature and the other from an 8-month-old female. One trisomic embryo occurred in the immature group (3-4%), which is perhaps not surprising because ovulation had been artificially induced. Embryos with 41 or 42 chromosomes made up 10-5% of embryos from old mothers, but there were none found in the 2- to 7-month-old adults. The differences between age groups in the incidence of

<table>
<thead>
<tr>
<th>Maternal age (months)</th>
<th>No. of females</th>
<th>Diploid (2n = 40)</th>
<th>Diploid* (±2)</th>
<th>Hyperdiploid and polyploid</th>
<th>Total no. of embryos</th>
<th>% anomalous embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>28</td>
<td>15</td>
<td>1 - 2 - 1</td>
<td>47</td>
<td>8-5%</td>
</tr>
<tr>
<td>(immature)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 to 7 (young adult)</td>
<td>20</td>
<td>39</td>
<td>21</td>
<td></td>
<td>63</td>
<td>4-8%</td>
</tr>
<tr>
<td>8 to 12</td>
<td>16</td>
<td>34</td>
<td>17</td>
<td>3* 1* 2*</td>
<td>58</td>
<td>12-1%</td>
</tr>
</tbody>
</table>

* Significantly different when compared to 2 to 7 month group (P = 0-0545) and to 1 to 7 month group (P = 0-0495).
† Some overlapping of chromosomes made precise counts impossible.
‡ Not significantly different when compared to younger groups.

these embryos reached statistical significance by Fisher's exact test of probability (P=0-0345 when comparing old with young adult group; P=0-0495 when comparing old with all others). The mean number of nuclei (and therefore the number of cells) in embryos from young adults (26-6) and old adults (24-2) was significantly different (F, 123 = 4-085, P < 0-05). This difference may be attributed either to a later time of ovulation or to a retarded rate of cleavage in the older females.

The results of the reciprocal embryo transplantation experiments of Talbert & Krohn (1966) are in general agreement with the results of this study. They found no reduced viability of embryos from older female mice compared to young controls, but there was a small increase in the proportion of morphologically abnormal ova from old mothers. Recently, Yamamoto, Endo & Watanabe (1973) reported that the proportion of aneuploid embryos in mice was dependent on maternal age, whereas the proportion of triploid embryos was not. These findings seem to be in agreement with those of the present study although it is not possible to tell from the data of Yamamoto et al. (1973) how many anomalous embryos may have been lost by the time of study (10½ days post coitum). Henderson & Edwards (1968) reported an age-related increase in the incidence of univalents and decrease in chiasma frequency in mouse oocytes at diakinesis. They proposed that the probability of an abnormal egg
being shed at ovulation was greater in the older mother and that this parameter was laid down at oogenesis. While the results reported here do not suggest that chromosomal anomalies are the major cause of infertility in older mothers, a hypothesis of Henderson & Edwards (1968) is a possible explanation for the occurrence of those trisomic embryos which are found. It seems unlikely that any of these abnormal embryos would have survived to term in view of the failure of Goodlin (1965) to find any evidence of increased aneuploidy in newborn mice with maternal age.

I would like to thank Dr R. G. Edwards, Dr R. L. Gardner and Dr M. Kaufman for helpful advice and discussion. This study was carried out during the tenure of a Medical Research Council Scholarship; working expenses were defrayed by a grant from the Ford Foundation to Professor C. R. Austin.

REFERENCES


Effects of Low-dose X-irradiation on Chromosomal Non-disjunction in Aged Mice

The factors responsible for the increasing incidence of offspring with Down’s syndrome in older women are a continuing subject of debate. The demonstration of a greater frequency of aneuploid embryos in old mice1,2 may open the way for further experimental studies of the causes of some types of chromosomally abnormal conceptions.

Clinical evidence suggests that the effect of maternal X-irradiation on the incidence of mongoloid children is a relatively small one3. Yamamoto et al.4 have presented results which, they claim, demonstrate that the bivalents of aged mouse oocytes are more susceptible to environmental injury than those of young oocytes. There is a decline in chiasma frequency and a corresponding increase in univalent frequency with age in mouse oocytes at diakinesis5,6. Yamamoto et al.4 suggest that a deficiency of chiasmata could have predisposed aged oocytes to chromosomal damage during the long dictyate stage of meiosis. They argue from their results that, whereas there was no significant effect of low-dose X-irradiation on the ova of young mice, there were significantly more chromosomally abnormal ova produced in old mice compared to young mice after irradiation. They emphasise the further increase in aneuploidy in old treated mice compared to old controls (P=0.0337 and 0.0600 for one and two-tailed tests, respectively). From all this they deduce (wrongly in our opinion) that the age of the mouse significantly affects the susceptibility of oocytes to irradiation damage. It should perhaps be mentioned that in comparing such small incidences, relatively large samples are required if significant differences are to be detected.

Table 1 Effect of low-dose X-irradiation on chromosome complement of 10.5-d-old mouse foetuses

<table>
<thead>
<tr>
<th>Group</th>
<th>Total No. foetuses</th>
<th>No. aneuploid foetuses</th>
<th>% aneuploid foetuses (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young non-irradiated</td>
<td>149</td>
<td>2</td>
<td>1.34</td>
</tr>
<tr>
<td>Young irradiated</td>
<td>111</td>
<td>4</td>
<td>3.60</td>
</tr>
<tr>
<td>Old non-irradiated</td>
<td>156</td>
<td>10</td>
<td>6.41</td>
</tr>
<tr>
<td>Old irradiated</td>
<td>43</td>
<td>7</td>
<td>16.28</td>
</tr>
</tbody>
</table>

* From the results of Yamamoto et al.4.

We have re-examined the data of Yamamoto et al.4 (Table 1) for evidence of interaction between age and X-irradiation. The differential effect of irradiation on ova from young and old mice was tested by comparing the appropriate function of the percentages with its standard error7. The value obtained was (16.28 - 6.41 - 3.60 + 1.34) = 7.61, with a standard error of 6.29, certainly a non-significant deviation from zero. This finding was confirmed by partitioning the three degrees of freedom of the contingency table into an age, irradiation and interactive effect. The latter comparison was found to be non-significant.

Both these statistical procedures, aimed at testing the conclusions of Yamamoto et al., use linear differences in the percentages, which at such low incidences could prove quite misleading. A more meaningful method would be to examine the proportionate increases apparently due to irradiation, resulting in this example in the very similar ratios of 16.28/6.41 = 2.54, and 3.60/1.34 = 2.69 for old and young mice respectively. We have concluded therefore that these results do not show evidence of an interrelation of maternal age and radiation treatment.

Aneuploid embryos found in this study included uniform monosomes and trisomics as well as mosaics. Uniform aneuploids probably arise through chromosomal non-disjunction at meiosis I or, less likely, meiosis II, whereas mosaicism is produced by two cell lines developing in a single zygote. In the analysis of Yamamoto et al.4 these anomalies were grouped together, a fact which makes their conclusions even more questionable. If the mosaic embryos are excluded the incidence of aneuploid embryos found in their data is still higher in old (2.5%) compared to young (0) non-irradiated females (P=0.0672). A closer examination of the results of Yamamoto et al.4 strongly suggests that the data from their earlier published results4 have been used as controls for the current irradiation studies. Such a procedure seems highly questionable in view of possible differences in environmental conditions.

In view of these objections we consider that the differential effect of X-irradiation on young and old oocytes to be not proven.

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7 Cox, D. R., Analysis of Binary Data (Methuen, 1970).
SURVIVAL OF TRANSFERRED C57BI MOUSE EMBRYOS:
EFFECTS OF AGE OF DONOR AND RECIPIENT*

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The technique of ovum transfer was advocated by Krohn1 to be an effective way of examining the effects of ova and maternal environment on the declining fertility of aging females. Experiments have shown the maternal environment to be a principal cause of this decline in the mouse,2,3 hamster,4 and rabbit.5,6 Also, the survival rate of embryos from older animals was found to be reduced in the hamster and rabbit but not in the mouse.3 This discrepancy may have been due to differences in experimental design and, therefore, seemed to warrant further investigation.

In the present study, the experiment of Talbert and Krohn3 has been modified and repeated. An internal control was used; ova from both young and old donors were transferred to opposite uterine cornua of the same host. This controlled differences in host response and in the experimenter's dexterity.

The C57Bl inbred female mouse was used because its decline with age in reproductive potential has been well characterized. Franks and Payne7 found most C57Bl female mice to be sterile by 12 months of age and all to be sterile by 16 months.

MATERIALS AND METHODS

Young virgin (2 to 4 months) and old multiparous (10 to 16 months) C57Bl female mice (from Animal Suppliers, London, Ltd.) were divided randomly and equally into donor and recipient groups.

Donor females were mated with fertile C57Bl males. Young recipients were allowed to mate and deliver one litter before being used in the study. Young and old recipient females were mated with vasectomized F1 hybrid males (C57Bl x A2G) which were proven sterile at the beginning and end of the study. Paired animals were checked daily for the presence of a vaginal plug. The day this was found constituted day 1 of pregnancy or pseudopregnancy.

At 3½ days post coitum, donor females were killed by cervical dislocation and the uteri were removed. The contents of each cornu were flushed out with M199 medium (Flow Laboratories, Ltd.) containing 10% inactivated fetal calf serum, 5% to 8% phosphate buffer (pH 7.1), and 100 IU/ml penicillin. The number of corpora lutea of pregnancy was recorded. What the number of ova (used synonymously here with "morula" and "blastocyst") recovered was low compared to the number of corpora lutea of pregnancy; the oviducts were examined for more ova. The ova were scored for their developmental stage and transferred to fresh medium at room temperature to await transfer less than 2 hours later.

Ova were transferred to pseudopreg
nant recipients 2½ days after coitus; this time was found to be optimal for conception.\textsuperscript{8} Recipients were anesthetized with tribromoethanol solution (Avertin, Winthrop Laboratories, New York), and the ovary and the tubal end of the uterine cornu on each side were exteriorized in turn through a dorsolateral incision in the abdominal musculature. The uterotubal junction was gripped with watchmakers’ forceps under the stereomicroscope (x 6), and the tubal end of the cornu was punctured with a mounted needle. The ova were then deposited in the lumen with a transfer pipette. After the operation the pipettes were checked for any retained ova. The transfer pipettes used were similar to those described by Gardner.\textsuperscript{9} The maximum volume of medium transferred was 1 x 10\textsuperscript{-4} ml. Transfer was bilateral; one to four ova from young donors were transferred to one cornu (chosen at random) and one to four ova from old donors were transferred to the other. All animals received between four and eight ova. All ova with intact zonae and with more than about eight blastomeres were used, regardless of their morphologic appearance.

In a pilot experiment, ova were transferred unilaterally to 13 young C57Bl hosts. Ten of these became pregnant with implantation sites occurring only on the side of transfer. It seemed, therefore, unlikely that transuterine migration would have occurred to any significant extent in the main experiment and it has, moreover, rarely been reported in the mouse.\textsuperscript{10,11} Laparotomy of recipients was performed on day 9 of pregnancy and the number of implants in each cornu was recorded. The animals were killed and the fetuses removed and counted on day 19. The live fetuses recovered were fixed in Bouin’s fluid and stored in 70% alcohol. They were examined externally for abnormalities, snap frozen in 2-methylbutane in liquid nitrogen and sectioned (about 1 mm) with a safety razor blade. The procedure used was a modification of Wilson’s technique.\textsuperscript{12} The sections were examined under the stereomicroscope for developmental abnormalities.

The results were analyzed statistically by the Chi-squared test.

**RESULTS**

The examination at 3½ days post coitum (Table 1) showed a high proportion of corpora lutea of pregnancy in young and old donors; this was represented by morulae and blastocysts in the uterus (91% and 63% in young and old donors, respectively). The higher proportion of blastocysts in the young compared to that in the old donors was statistically significant (P < 0.05), suggesting a reduced rate of development in old females. Ova were found in the oviducts of three old donors, but in none of the young animals; hence, the rate of ovum transport may also become impaired with age. The number of corpora lutea of pregnancy remained high in the old group.

Two of sixteen fetuses developing from eggs of old donors and then transferred to young recipients were abnormal. The abnormalities were unilateral anophthal-

<table>
<thead>
<tr>
<th>Maternal age (months)</th>
<th>No. of animals</th>
<th>Total no. of blastocysts recovered</th>
<th>Total no. of morulae recovered</th>
<th>% of blastocysts</th>
<th>Mean no. of embryos/pregnancy</th>
<th>Mean no. of CLP/pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-4</td>
<td>20</td>
<td>93</td>
<td>35</td>
<td>73</td>
<td>6.4</td>
<td>7.1</td>
</tr>
<tr>
<td>10-16</td>
<td>17</td>
<td>54</td>
<td>36</td>
<td>60\textsuperscript{b}</td>
<td>5.3</td>
<td>8.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Corpora lutea of pregnancy.

\textsuperscript{b}Significantly different (P < 0.05).
evidence studies on the around principally was ones for the old. But clear, the effect also transferred to young donors. Of the 18 embryos and meningoencephalocele in one fetus and partial cleft palate in the other. Of the 18 examined, all fetuses from young donors were normal. The occurrence of the two abnormal fetuses from old donors is interesting, although no statistical comparisons can be made with such small numbers.

The percentage survivals of ova to day 19 in young and old recipients (Table 2) were significantly different ($P < 0.001$). A comparison of the survival of all ova transferred to young or old recipients was also significant ($P < 0.001$). However, the effect of donor age on survival in young or old recipients was not significant. Also, the number of ova transferred did not affect the survival rate (angular transformation of survival rates). Therefore, the maternal age of the recipient appears to be the main determinant of embryo survival.

**DISCUSSION**

The reason for the impaired development of embryos in old females is not clear, but our findings confirm previous ones for the mouse and hamster.

Reproductive failure in old recipients was principally caused by losses at or around the time of implantation. Other studies on the aging mouse have found some evidence of this.

The high rate of failure of implanted embryos to survive to term in young recipients was surprising; it may have been due to the midterm operation.

The maternal environment would appear to be the chief factor limiting reproductive potential in the aging C57BL mouse. This conclusion agrees with the findings of others in mice, hamster, and rabbits. Moreover, our results confirm the finding of Talbert and Krichholt that ova from young and old donors survive equally well under similar uterine conditions.

In conclusion, there seems to be a difference in the balance of factors affecting embryo survival as related to maternal age between the mouse on the one hand and the hamster and rabbit on the other.

**SUMMARY**

The technique of ovum transfer was used to separate the effects of ova and maternal environment on the inheritance of old inbred mice. The results show that ova from old donors have the same potential for development as ova from young donors. The age of the recipient had a significant effect on embryonic survival. There was some evidence of reduced development of preimplantation embryos in old females.
Acknowledgments. I wish to thank Drs. R. G. Edwards and R. L. Gardner for helpful discussion during this study and Drs. C. E. Adams and M. H. Johnson for reading the manuscript.

REFERENCES
Corpus luteum adequacy in the ageing pregnant mouse*

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*Department of Biological Laboratory, Cambridge, United Kingdom

A decline in litter size with increasing maternal age has been described in polytocous laboratory animals (reviewed by Talbert, 1968). The rate of decline in the mouse appears to be genetically determined (Thung, Boot and Mühlbock, 1956) and independent of the age of the male (Finn, 1964). Our colony of CBA/H-T6 mice the maximum litter size occurs at four months of age, then litter size declines to zero by 10-12 months. Old females may still ovulate and mate after delivery of the last litter.

An analysis of the numbers of corpora lutea of pregnancy (CLP) on day 4 showed that the percentage ovulation rate remained high in all age groups examined (2-12 months). There were increasing numbers of CLP with age in multiparous, but not in nulliparous mice of similar age. The maintenance rate of CLP in old mice has been noted (MacDowell and Lord, 1925; Biggers, Finn and McLaren, 1962). A histological examination of the uterus of pregnant mice showed that the presence of CLP on day 4 revealed the presence of some CLP which had not been ovulated (corpora lutea etica). The incidence was, however, low (range 0.5-1.5/animal) and so the number of CLP can be estimated as approximately equal to the number of ova counted.

The reduction in litter size with maternal age has been found to be due to an increasing failure of conceptuses to survive to term (Finn, 1962). The presence of morulae and blastocysts in the uteri of mated old CBA/H-T6 and CBA mice on day 4 of pregnancy and the absence of implants at mid-term autopsy indicates that reproductive failure in these mice occurs mainly around the time of implantation. Heterochronic embryo transplantation experiments have shown that embryos from young and old mice can survive equally well under similar uterine conditions (Talbert and Krohn, 1966; Gosden, 1974). The maternal environment, therefore, seems to be the major cause of the decline in fertility with age. However, it is not known whether this decline is due to a direct effect of the uterus or to an indirect effect of failing ovarian support of pregnancy.

The effects of supplemented progesterone on the pregnancies of young and old mice have been studied. Mated virgin CBA/H-T6 and multiparous CBA mice were injected subcutaneously with progesterone or oil on days 2-9 of pregnancy. They were killed on day 10. The uteri were dissected out and cleared by the technique of Orsini (1962). The numbers of live and resorbing embryos were counted under the stereomicroscope. The results (Table I) showed that progesterone significantly increased the probability of an aged pregnant mouse having implantation sites. There was some evidence that the numbers of implants/pregnancy were increased in animals treated with progesterone ($P < 0.05$), although this result has not been confirmed in later experiments. Progesterone treatment...
TABLE I The effect of exogenous progesterone on the implantation rates in young and old mated mice. Examine mid-term

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Age (mth)</th>
<th>mg progesterone per day</th>
<th>No. of animals</th>
<th>No. with 1 or more implants</th>
<th>Proportion with implants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin CBA/H-T6</td>
<td>10-11</td>
<td>0</td>
<td>9</td>
<td>2</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25</td>
<td>10</td>
<td>6</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50</td>
<td>10</td>
<td>9</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.00</td>
<td>10</td>
<td>9</td>
<td>0.90*</td>
</tr>
<tr>
<td>Virgin CBA/H-T6</td>
<td>2-3</td>
<td>0</td>
<td>10</td>
<td>8</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.00</td>
<td>10</td>
<td>9</td>
<td>0.90</td>
</tr>
<tr>
<td>Multiparous CBA</td>
<td>11-12</td>
<td>0</td>
<td>11</td>
<td>4</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.00</td>
<td>10</td>
<td>9</td>
<td>0.90**</td>
</tr>
</tbody>
</table>

* Significantly different proportions (P = 0.004).
** Significantly different proportions (P = 0.017).

did not affect the subsequent survival of implanted embryos to day 10. Preliminary results in our laboratory indicated that estradiol benzoate treatment from day 4 does not increase the implantation rate in senile mice. Thus, a lack of progesterone in the ageing pregnant mouse is indicated. Harman and Talbert (1970) have reported that the morphology of CLP on day 7 is associated with the success of implantation in ageing female mice. However, they could not determine whether these degenerate CLP were a cause or a result of embryonic loss. The histological appearance of CLP on day 4 in the young and old CBA/H-T6 females studied was similar. An indication of CLP activity may be given by changes in gland and lutein cell sizes. The volumes of day 4 CLP were measured by two methods for a series of sections from ageing mice. Every fifth section was examined. For the first method (A) camera lucida drawings were made of the CLP outlines and the paper shapes were cut out and weighed to give a relative measurement of CLP volume. Measurements of the three maximum diameters of CLP at right angles to each other were also made (Method B) (Bassett, 1949). The CLP volume was obtained by calculating the volume of an ellipsoid using the formula \(4/3\piabc\) where \(a\), \(b\), and \(c\) were the three radii. Both the relative and absolute volumes calculated were inversely correlated with maternal age (Fig. 1). The lutein cell densities of these CLP were measured with a microscope grid by counting the number of cell nuclei in areas of the glands chosen at random.

The cell density scores were found to be inversely correlated with CLP volumes (Fig. 2), indicating that the cells are smaller in smaller CLP. Together these results suggest a reduced effectiveness of progesterone in the pregnancies of older mice.

Several studies have found no evidence for a deficient CLP function. Finn (1963) did not improve embryonic survival by increasing the CLP: implantation ratio by ligating one oviduct in old pregnant mice.

![Fig. 1](image-url)  
Maternal age of CBA/H-T6 mice and relative volume of CLP (day 4). Regression of \(Y_A\) on \(X\), \(Y_A = 2.55 - 0.195X\), \(r = -0.559\) (\(P < 0.05\)). Regression of \(Y_B\) on \(X\), \(Y_B = 2.0 - 0.0063X\), \(r = -0.559\) (\(P < 0.05\)).
Summary

The decline of litter size with age in mice is described and some of the possible causes of this infertility are examined. The results of experiments to test the luteal support of pregnancy in ageing mice are presented.

References


INDUCTION OF OVULATION IN MICE BY SYNTHETIC LUTEINIZING HORMONE RELEASING HORMONE

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(Received 21 February 1974)

Recently a single hypothalamic hormone, termed luteinizing hormone releasing hormone (LH-RH), capable of releasing luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary has been chemically characterized and synthesized (Matsuo, Arimura, Nair & Schally, 1971; Matsuo, Baba, Nair, Arimura & Schally, 1971) and has been shown to be biologically active in several species (Schally, Arimura & Kastin, 1973). Initial attempts to demonstrate LH-RH action in 4-day cyclic mice treated with sodium barbitone in pro-oestrus were unsuccessful because barbitone did not consistently block ovulation (R. G. Gosden, unpublished results). In this report we demonstrate the action of LH-RH in adult female mice as shown by the induction of ovulation and the increase of serum LH.

Young virgin (8-14 weeks) CELP mice (Carworth Europe) were injected with 3 i.u. pregnant mare serum gonadotrophin (Intervet) i.p. at 16.00-17.00 h without reference to the stage of the oestrous cycle. Approximately 44 h later they were given either human chorionic gonadotrophin (HCG) 3 i.u.) or synthetic LH-RH (75, 150 or 100 ng) s.c.; control animals received only the saline vehicle. Animals were autopsied the following morning. The ampullae were dissected, then the ova were counted after liberating them from adhering cumulus cells with 0.05% bovine hyaluronidase (Koch-Light).

Synthetic LH-RH increased the proportion of animals ovulating (Table 1) but the maximum was 85% whereas with HCG treatment every female ovulated. A proportion (35%) of females in the group receiving saline ovulated. The number of eggs ovulated did not differ significantly between treatment groups.

Another group of young adult mice were tested for their pituitary reactivity to exogenous LH-RH (Ramirez & McCann, 1963). Six weeks after bilateral ovariectomy the mice received two s.c. injections of 1 mg progesterone + 10 µg oestradiol-17β benzoate (Intervet) 24 h apart. Forty-eight hours after the second injection either saline or synthetic LH-RH (1, 10 or 100 ng) was injected into the tail vein. The animals were killed by decapitation 15 min later and a blood sample was collected from the trunk and stored as serum at -20°C.

Serum LH levels were measured by a radioimmunoassay based upon a competition between 125I-labelled rat LH and mouse serum LH for binding sites on NIAMD-anti-rat LH-S1. Previous experiments have established that NIAMD-anti-rat LH-S1 and mouse gonadotrophin cross-react (Beamer, Murr & Geschwind, 1972). Sera were assayed in duplicate samples of 0.1 ml. The LH values were expressed in terms of NIAMD-rat LH-RP1 on the standard curve, and subsequently converted to their equivalent concentrations in terms of the NIH-LH-S1 standard.
Table 1. Induction of ovulation by synthetic luteinizing hormone releasing hormone (LH-RH) in mice primed with pregnant mare serum gonadotrophin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Animals that ovulated</th>
<th>No.</th>
<th>Proportion (mean ± s.e.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>20</td>
<td>7</td>
<td>0-35</td>
<td></td>
</tr>
<tr>
<td>75 ng LH-RH</td>
<td>20</td>
<td>9</td>
<td>0-45</td>
<td></td>
</tr>
<tr>
<td>150 ng LH-RH</td>
<td>20</td>
<td>14</td>
<td>0-70</td>
<td></td>
</tr>
<tr>
<td>300 ng LH-RH</td>
<td>20</td>
<td>17</td>
<td>0-85*</td>
<td></td>
</tr>
<tr>
<td>3:1:1, Human chorionic gonadotrophin</td>
<td>6</td>
<td>6</td>
<td>1-00</td>
<td></td>
</tr>
</tbody>
</table>

Proportions of animals ovulating were heterogeneous ($x^2 = 12.871, P < 0.006$).

* Significantly different from saline controls ($x^2 = 8.438$, Yates' correction, $P < 0.005$).

In ovariectomized, oestrogen–progesterone-primed mice injected with saline, LH levels were low and treatment with 1 ng LH-RH did not raise them significantly. However, treatment with 10 or 100 ng LH-RH did increase serum LH concentrations significantly (Table 2).

Table 2. Release of luteinizing hormone (LH) by synthetic luteinizing hormone releasing hormone (LH-RH) in ovariectomized mice primed with oestradiol-17β bromide and progesterone

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Serum LH levels 15 min after injection (ng/ml, mean ± s.e.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>9</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>1 ng LH-RH</td>
<td>10</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>10 ng LH-RH</td>
<td>10</td>
<td>14.3 ± 5.0</td>
</tr>
<tr>
<td>100 ng LH-RH</td>
<td>10</td>
<td>53.2 ± 10.1</td>
</tr>
</tbody>
</table>

* NIH-LH-S1 equivalents.

† Significantly higher LH levels than saline controls ($t = 2.288, P < 0.05$).

‡ Significantly higher LH levels than saline controls ($t = 4.757, P < 0.001$).

The linear relationship between the log dose and the response (weighted) was significant ($F_{1,3} = 40.8, P < 0.01, \text{slope} = 2.4 ± 0.9$) but the quadratic relationship was not significant.

Synthetic LH-RH has been shown to induce ovulation and increase serum levels in mice which indicates a pituitary effect of this hypothalamic hormone, confirms its lack of species specificity (Schally et al. 1973).

We thank Dr A. V. Schally for the gift of synthetic LH-RH. The radioimmunoassay kit was provided by the NIAMD Endocrinology Study Section. Financial support from the MRC (R. G. G.), the Wellcome Foundation (C. R.) and the Ford Foundation is gratefully acknowledged.

REFERENCES

OVARIAN SUPPORT OF PREGNANCY IN AGEING INBRED MICE

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(Received 29th May 1974)

Summary. The principal cause of reduced fertility in 10- to 12-month-old female CBA/H-T6 and CBA mice was found to be loss of embryos at the time of, or soon after, implantation. Treatment with exogenous progesterone, but not with oestradiol benzoate, increased the number of old females having implantation sites but did not increase either the average number of implantations/female or postimplantation survival to Day 10. When bovine prolactin or HCG was administered to pregnant old mice, the implantation rate was not increased. It was concluded that the function of the CL of pregnancy in old mice may have been impaired because the lutein cells were failing to respond adequately to the luteotrophic stimulus.

INTRODUCTION

The average litter size of female mice declines with increasing age and breeding ceases well before the end of the life-span (Talbert, 1968). This reduced fertility of old mice cannot be attributed to a decline in ovulation rate since the number of CL of pregnancy remain high (Biggers, Finn & McLaren, 1962; Harman & Talbert, 1970). Embryonic losses caused by failure of fertilization and pre-implantation development in ageing CBA/H-T6 mice appear to be small (Gosden, 1973a), suggesting that most of the prenatal mortality occurs at or soon after the time of implantation. It has been shown that these losses are not primarily due to defects of the ova (Talbert & Krohn, 1966; Gosden, 1973a, 1974a). Pregnancy failure may be caused by insufficient luteal activity for there is an association between degenerative changes in the CL of old mice on Day 7 of pregnancy and the failure of implantation (Harman & Talbert, 1970).

In the present study, the effects of exogenous gonadal and gonadotrophic hormones on the pregnancies of ageing mice were examined to determine whether the ovarian function is impaired in old age and, if so, in what way it is impaired.

MATERIALS AND METHODS

Inbred CBA/H-T6 and CBA mice from the laboratory colony were used. Females of various ages (see below) were mated by fertile males and were placed in individual cages on the day a copulatory plug was found (Day 1).

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Hormones were administered subcutaneously in 0.05 ml arachis oil (progesterone and oestrogen) or saline (gonadotrophin) at 12.00 hours. Females were killed on Day 7 or Day 10 of pregnancy.

The uteri were dissected and examined. Horns lacking visible implantation sites were flushed with isotonic saline before fixation and the flushings were examined for ova under a stereomicroscope. All uteri were trimmed free of mesoenteries, fixed, bleached, dehydrated and cleared in benzyl benzoate for counting and examination of the implanted embryos (Orsini, 1962). When required, the two maximum diameters of the decidual capsules in the cleared uteri were measured at right angles to each other under a stereomicroscope with a fitted eye-piece micrometer. The mean radius (r) was calculated and the spherical volume of the decidual capsules was derived from the formula

\[ V = \frac{4}{3} \pi r^3. \]

The ovaries of some females were removed and the CL were identified and counted under a stereomicroscope. Some ovaries were subsequently prepared histologically as serial sections after fixation in Bouin’s fluid, embedding in paraffin wax, sectioning at 7 \( \mu \)m and staining with Mayer's haemalum and eosin.

Experimental results were evaluated statistically by Fisher's exact test of probability, analysis of variance and Student's t test.

**Experiment 1**

Thirty-four CBA/H-T6 female mice, of different ages and mostly multiparous except for the youngest animals, were removed from the breeding colony and killed on Day 4 of pregnancy. Eight 10- to 12-month-old primiparous females were killed at the same stage of pregnancy and the numbers of CL were compared.

**Experiment 2**

Mice of three types (see Table 1) were mated by males of their own strain and were randomly allocated to one of four groups in which they received 0.0, 0.25, 0.50 or 1.00 mg progesterone (Koch-Light)/day from Day 2 to Day 9. The animals were killed on Day 10 and the numbers of live and resorbing embryos were determined.

**Experiment 3**

The effects of oestrogen and progesterone were studied using a 2 x 2 factorial design (Table 2). Virgin CBA mice (10 to 11 months old), which had mated with F1 hybrid (C57BL x A2G) males, received one of the following treatments: (1) 1 mg progesterone/day from Day 2 to Day 9; (2) 0.05 \( \mu \)g oestradiol-17\( \beta \) benzoate (Koch-Light)/day from Day 4 to Day 9; (3) oestrogen and progesterone as in (1) and (2) above; (4) the vehicle alone. Autopsy was on Day 10.

**Experiment 4**

Virgin CBA mice (10 to 11 months old) were mated by F1 hybrid (C57BL x A2G) males and were injected morning and evening from Day 1 to Day 9 with (1) 5 i.u. bovine prolactin (NIH-P-B3), (2) 5 i.u. HCG (Organon), or (3) saline only, or were treated with 1 mg progesterone/day from Day 2.
Young females (2 to 3 months old) were also injected with saline only. The animals were killed on Day 7, and the number and size of the implantation sites was recorded.

RESULTS

Experiment 1

The number of CL in the parous mice increased until the animals were about 9 months old (Text-fig. 1; \( r = 0.422, P < 0.01 \), slope = 0.384). The old primigravid females had the same number of CL as did young females, but fewer than did multiparae of similar age (Text-fig. 1). In the ovaries of one-third of the ageing CBA mice, but not in those of young animals, oocytes had been retained within histologically normal CL (corpora lutea accessoria). This could have lead to bias in estimating the ovulation rate from macroscopic CL counts but the effect was probably small, since only one to three of these were present in individual animals.

Experiment 2

Many of the old mice, whether primi- or multigravidae, had no visible implantation sites on Day 10 (Table 1). The proportion with implantations was significantly increased by treatment with 0.5 mg or more of progesterone, and progesterone treatment increased the numbers of implantation sites/pregnancy in the old primigravid females in this experiment \((P < 0.05)\) but not in those
of Exps 3 and 4. There were more resorbing embryos in the older females but survival to Day 40 was not affected by treatment with progesterone.

No free blastocysts were found in the uterine flushings of these 10-day pregnant mice without implantation sites but two blastocysts, each containing more than seventy-two nuclei, were recovered at Day 9 from an old mouse in a pilot experiment.

**Experiment 3**

The effects of oestrogen and progesterone are shown in Table 2. Progesterone was found to have a significant effect but the oestrogen effect was not significant.

**Table 1.** The effect of progesterone on implantation in mice of different ages and reproductive history

<table>
<thead>
<tr>
<th>Type of mouse</th>
<th>Progesterone dose (mg/day)</th>
<th>No. of mice</th>
<th>No. pregnant* (%)</th>
<th>No. of implantations/pregnancy (Mean±S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA/H-T6</td>
<td>0</td>
<td>9</td>
<td>2 (22)</td>
<td>1.0±0.0 (20)</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>10</td>
<td>6 (60)</td>
<td>2.5±1.1 (32)</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>10</td>
<td>9 (90)†</td>
<td>2.6±0.6 (26)</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>10</td>
<td>9 (90)†</td>
<td>2.9±0.7 (36)</td>
</tr>
<tr>
<td>CBA/H-T6</td>
<td>0</td>
<td>10</td>
<td>8 (80)</td>
<td>3.3±0.4 (44)</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>10</td>
<td>9 (90)†</td>
<td>5.6±0.9 (56)</td>
</tr>
<tr>
<td>CBA</td>
<td>&gt;1</td>
<td>11</td>
<td>4 (36)</td>
<td>1.8±1.0 (20)</td>
</tr>
</tbody>
</table>

* At Day 10.
† Significantly different from control, P = 0.0049.
‡ Significantly different from control, P = 0.017.

**Table 2.** Effects of exogenous ovarian hormones on implantation in old mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>No. of pregnant (%)</th>
<th>No. of implantations/pregnancy (Mean±S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil (control)</td>
<td>12</td>
<td>9 (75)</td>
<td>8.1±0.9</td>
</tr>
<tr>
<td>Progesterone</td>
<td>11</td>
<td>11 (100)*</td>
<td>7.4±0.7</td>
</tr>
<tr>
<td>Progesterone + oestradiol benzoate</td>
<td>11</td>
<td>9 (82)*</td>
<td>6.9±0.9</td>
</tr>
<tr>
<td>Oestradiol benzoate</td>
<td>12</td>
<td>7 (58)</td>
<td>5.3±1.1</td>
</tr>
</tbody>
</table>

* P = 0.049 when compared to animals not treated with progesterone.

**Experiment 4**

Exogenous prolactin and HCG, unlike exogenous progesterone, did not increase the proportion of old females bearing implantations (Table 3). The volume of the decidual capsules on Day 7 was smaller in old mice than in young mice (P<0.001) and was not affected by hormone treatment. In contrast to the results for the CBA/H-T6 mice (Text-fig. 1), young CBA mice had significantly more CL than did the old primigravidae.

In these experiments, the use of either inbred or hybrid males made no apparent difference to the results.
Table 3. The effect of exogenous gonadotrophins on implantation in old mice

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>No. pregnant (%)</th>
<th>No. of implantations/pregnancy (Mean ± S.E.)</th>
<th>No. of CL/pregnancy (Mean ± S.E.)</th>
<th>Vol. of decidual capsules in mm³ (Mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 to 12</td>
<td>Saline</td>
<td>12</td>
<td>7 (58)</td>
<td>3.0 ± 0.5</td>
<td></td>
<td>2.93 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>12</td>
<td>12 (100)*</td>
<td>2.4 ± 0.4</td>
<td></td>
<td>2.53 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>Prolactin</td>
<td>12</td>
<td>7 (58)</td>
<td>2.9 ± 0.6</td>
<td></td>
<td>2.74 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>Human chorionic gonadotrophin</td>
<td>12</td>
<td>5 (42)</td>
<td>1.8 ± 0.2</td>
<td></td>
<td>2.37 ± 0.36</td>
</tr>
<tr>
<td>2 to 3</td>
<td>Saline</td>
<td>10</td>
<td>10 (100)</td>
<td>8.8 ± 0.3</td>
<td></td>
<td>5.30 ± 0.29</td>
</tr>
</tbody>
</table>

* Significantly different from old controls, P = 0.019.
† Significantly different (*47 = 5.83, P < 0.001).
‡ At Day 7.
DISCUSSION

The maternal factors that are responsible for the increased prenatal mortality of embryos in ageing mice are still unclear. The gametogenic function of the ovary is maintained for some time after the onset of infertility in senescent mice, although there is a well-documented increase in ovulation failure due to the failure of follicle rupture (Thung, 1958; Jones & Krohn, 1961). The qualitative changes that have been observed in the CL of old mice on Day 1 indicate that a progesterone deficiency may cause pregnancy failure in the animals (Harman & Talbert, 1970), although such changes could be a result of pregnancy loss (Talbert, 1971), and the histological assessment of luteal function is not reliable (Finn, 1970). The effect of exogenous progesterone found in the present experiments, and the earlier finding that older CBA/H mice have smaller lutein cells and, consequently, smaller CL on Day 4 (Gosden, 1974a), support the observations of Harman & Talbert (1970) and indicate that CL function may be deficient before the onset of implantation. The lack of a salutary effect of exogenous oestrogen on implantation in old mice suggests that the effect of progesterone was not due to contamination by oestrogen.

The smaller number of implantations in mice treated with 0·05 µg oestradiol benzoate suggests that oestrogen treatment may actually be detrimental.

Several workers have found no evidence of impaired luteal function in aged animals. Embryonic survival was not increased in pregnant old mice by ligating one oviduct and improving the CL:embryo ratio (Finn, 1963) or by giving orthotopic ovarian grafts from young mice (Krohn, 1966). Measurements of plasma progestins in the hamster (Blaha, 1971) and in the rabbit (Spilman, Larson, Concannon & Foote, 1972) have shown that hormone levels are similar in young and old animals, except when there were no viable fetuses left in pregnancy (Spilman et al., 1972), and supplementary progesterone and oestrogen did not increase embryonic survival in old rabbits (Larson, Spilman, Dunn & Foote, 1973). There is, however, some evidence of luteal deficiency in older women. Basal body temperature measurements have shown that the hyperthermic phase of the menstrual cycle is less marked and less regular in length in women over 39 and under 25 years of age (Collett, Wertenberger & Fiske, 1954; Döring, 1969).

Sub-optimal levels of circulating progesterone in mouse pregnancy could cause the increased pregnancy loss characteristic of old age by failing to prepare the endometrium adequately for implantation and by failing to maintain the decidua. The present results are not incompatible, however, with a situation in which progesterone levels are normal but the binding of progesterone is impaired. Plasma progesterone must now be measured in ageing mice to examine luteal function by a more direct method.

Implantation also fails to occur in immature and ovariectomized pregnant mice and can be induced by treatment with exogenous progesterone (Smithburg & Runner, 1956; McLaren, 1971). The parallel with old mice may not extend any further, however, since embryos are not usually present in the uterine flushings of old mice at mid-pregnancy and exogenous luteotrophic hormones will not restore a sufficient level of ovarian activity to induce implantation in
they will in immature animals (Tessaro & Runner, 1956). If progesterone secretion is in fact reduced, the lack of effect of luteotrophic hormones in pregnant old mice suggests that the lutein cells may be refractory to gonadotropic stimulation. It has been suggested that the establishment of fewer placentae in senescent female hamsters might result in a reduced output of placental luteotrophin which would lead, ultimately, to postimplantation failure (Thorneycroft & Soderwall, 1968). This hypothesis seems unlikely to be correct for mice since daily supplementation with prolactin from Day 1 to Day 18 of pregnancy in aged C57BL mice did not affect the survival of embryos (Gosden, 1973b). The dose of prolactin used was known to make the uterus of young oestrous mice sensitive to decidualizing stimuli (R. G. Gosden, unpublished results), and to restore fertility to pituitary dwarf mice (Bartke, 1966). Bindon & Lamond (1969) also found that 10 i.u. HCG/day effectively maintained pregnancy in hypophysectomized mice. The negative results in the present experiments may, however, have been due to production of anti-hormones after administration of heterologous hormones. It is also possible that a combination of luteinizing and luteotrophic hormones would have been more effective than either given separately (Tessaro & Runner, 1956; Browning, Larke & White, 1962). Alternatively, FSH may be necessary for stimulation of the CL (Choudary & Greenwald, 1969). Further experiments are needed to test these possibilities.

Extraovarian factors are almost certainly also operating to cause the age-dependent loss of reproductive capacity. These factors may include intrinsic changes of the uterus because the decidual cell reaction is diminished with age in mice and hamsters and is not restored even when supplementary hormones are given (Finn, 1966; Blaha, 1967). The smaller size of decidual capsules in the old mice used in this study confirms these earlier observations. The present results have shown that postimplantation losses in senescent mice are not reduced by treatment with progesterone and are an indication of uterine deficiency in such mice.

ACKNOWLEDGMENTS

I wish to thank Dr R. G. Edwards for his advice and interest throughout this study, Professor C. R. Austin for critically reading the manuscript and Mrs Cilla Fuller for help with the manuscript. The prolactin was generously donated by the National Institute of Arthritis and Metabolic Diseases' Endocrinology Study Section. Financial support from the Medical Research Council and the Ford Foundation is gratefully acknowledged.

REFERENCES


The female CBA mouse promptly compensates for UNI-OVX by ovulating twice as many ova/ovary. Litter size of UNI-OVX mice breeding ad libitum, although initially similar to intact animals, becomes prematurely smaller primarily because of high intra-uterine mortality. This reproductive failure may result from doubling the number of conceptuses in the functional uterine horn and consequent exhaustion of uterine function. This was tested by controlling quantity and quality of ova by ovum transfer technique and assessment of fetal survival at term. The proportion of ova surviving unilateral transfer was similar in the unspayed (21/39) and spayed uterine horn (26/48) of young UNI-OVX nulliparae and only slightly lower than in intact mice (50/82). Transuterine migration of ova did not occur. Older recipients, whether primi- or multi-parous, had fewer ova surviving to term and an associated increased incidence of placental moles. A significantly smaller proportion of ova survived in multiparous UNI-OVX mice (13/115) than in intact controls (16/35); the non-parous horn of UNI-OVX animals was, if anything, less effective at supporting conceptuses than the parous horn although premature signs of senescence were found only in the latter (auto-fluorescent pigments). Thus, uterine adaptability to embryo overloading is lost with excessive breeding. In bicornuate uteri this parity influence may extend to the opposite non-parous horn. (Supported by the MRC and The Ford Foundation)
UPTAKE AND METABOLISM IN VIVO OF TRITIATED OESTRADIOL-17β IN TISSUES OF AGEING FEMALE MICE

R. G. GOSDEN
UPTAKE AND METABOLISM IN VIVO OF TRITIATED OESTRADIOL-17\(^\beta\) IN TISSUES OF AGEING FEMALE MICE

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(Revised manuscript received 29 July 1975)

SUMMARY

The uptake in vivo of tritiated oestradiol-17\(^\beta\) has been compared in young and aged ovariectomized CBA/H-T6 mice by examining the levels of radioactivity in tissues 1 h after injection. The specificity of oestradiol uptake was demonstrated by previous treatment of some animals with either diethylstilboestrol or progesterone. The levels of radioactivity in whole tissue extracts were similar in both age groups for the uterus, hypothalamus, cerebrum and serum, but the uptake in the pituitary gland was significantly lower in old mice. There was no evidence of altered hormone metabolism with age.

INTRODUCTION

Changes in end organ sensitivity to hormonal stimulation are at least as potentially important as changes in circulating hormone levels in causing abnormal organ function. Such changes have been documented for the action of a number of hormones upon ageing tissues (Bellamy, 1967; Gusseck, 1972; Roth & Adelman, 1975) and they may partly explain the increased ovulation failure and pregnancy failure in reproductively senescent rodents where alterations in uterine, pituitary or hypothalamic sensitivity to gonadal steroids may be involved (Talbert, 1968). All oestrogen target tissues, including the aforementioned ones, are known to possess a macromolecular component in the cytosol with a high affinity for oestradiol; the formation of a receptor–oestradiol complex and its subsequent migration to the nucleus is believed to be an essential step for oestrogen action (O’Malley & Means, 1974). In the present study the capacity of tissues of inbred mice to take up oestradiol in vivo has been examined for evidence of changes with age in hormone–receptor interaction.

MATERIALS AND METHODS

Young (3–4 month) and old (14–17 month) virgin female CBA/H-T6 mice from the laboratory colony were used. Female mice of this strain become sterile at about 1 year of age although their longevity exceeds 2 years. Animals with leucocytic vaginal smears were bilaterally ovariectomized and primed 1 week later with 1 \(\mu\)g oestradiol-17\(^\beta\) benzoate (Koch–Light) injected s.c. in arachis oil to synchronize the time of their last exposure to oestrogen. They were injected with tritiated oestradiol (\(^{3}H\)oestradiol) 2 weeks later. [2,4,6,7-\(^{3}H\)oestradiol-17\(^\beta\) (Radiochemical Centre, Amersham) of high specific activity (316 mCi/mg) was dissolved in propane-1,2-diol and this was diluted with nine parts of 0-9 % saline on the day of injection.

Experiment 1

The specificity of [\(^{3}H\)]oestradiol uptake was examined in tissues by pre-treating the mice with either unlabelled diethylstilboestrol (DES) or progesterone (Koch–Light), purified by
recrystallization. Twenty young and 14 old animals were allocated at random to groups which received DES (1, 10 or 100 µg), progesterone (100 µg) or the oil vehicle i.p. 3 h before a s.c. injection of 0·05 µg [3H]oestradiol/25 g body weight.

Experiment 2

A single injection of 0·013, 0·025, 0·050 or 0·100 µg [3H]oestradiol/25 g body weight was given s.c. to 23 young and 22 old mice to compare the uptake in vivo of oestradiol.

The animals were decapitated 1 h after receiving the radioisotope and blood was collected from the trunk region. The uterus, cerebrum, hypothalamus and pituitary gland were dissected and specimens were trimmed, blotted free of surface blood, weighed and stored at -15 °C. The tissues were homogenized in acetone–ethanol by the method of Kariel and Villee (1967). Scintillation fluid (12 ml of 4 g 2,5-diphenyloxazole/litre of toluene) was pipetted into each vial after evaporation of the solvent and the vials were counted with an ICN Tracerlab scintillation counter. The specific activity was determined for each sample (in d.p.m./mg wet wt of tissue) by correcting for tissue weight, tissue background and quenching by the external standard channels ratio method.

The percentage oestradiol in the tissues was determined by evaporating the toluene from the vials after the counting was completed and then separating the labelled substances in 100% ethanol by Sephadex LH-20 chromatography (Emment, Collink and Sommerville, 1972). The radioactivity recovered in the oestradiol fraction was expressed as a percentage of the 'total' counts after correcting for background activity. Pilot studies showed that the presence of scintillator in the samples did not affect the chromatography of the oestradiol.

RESULTS

Relatively high levels of radioactivity were found in the extracts of the oestrogen target tissues compared with non-target tissues of mice injected with [3H]oestradiol. Less than 5% of the total radioactivity remained in the protein residues after extraction, except in the case of serum (about 50%).

Table 1. Effect of progesterone and diethylstilboestrol (DES) pre-treatment on the uptake of [3H]oestradiol-17β in young and old ovariectomized mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Age of mice*</th>
<th>100 µg</th>
<th>1 µg</th>
<th>10 µg</th>
<th>100 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Progesterone</td>
<td>DES</td>
<td>DES</td>
<td>DES</td>
</tr>
<tr>
<td>Uterus</td>
<td>Young</td>
<td>0·852 ± 0·464</td>
<td>1·174 ± 0·192</td>
<td>0·210 ± 0·195</td>
<td>0·037 ± 0·014</td>
</tr>
<tr>
<td>Pituitary</td>
<td>Young</td>
<td>1·440 ± 0·673</td>
<td>0·490 ± 0·242</td>
<td>0·154 ± 0·060</td>
<td>0·043 ± 0·005</td>
</tr>
<tr>
<td></td>
<td>Old</td>
<td>0·414 ± 0·115</td>
<td>0·072 ± 0·108</td>
<td>0·041 ± 0·015</td>
<td></td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>Young</td>
<td>1·192 ± 0·467</td>
<td>0·683 ± 0·163</td>
<td>0·471 ± 0·085</td>
<td>0·356 ± 0·137</td>
</tr>
<tr>
<td></td>
<td>Old</td>
<td>0·682 ± 0·091</td>
<td>0·402 ± 0·159</td>
<td>0·424 ± 0·064</td>
<td></td>
</tr>
<tr>
<td>Cerebrum</td>
<td>Young</td>
<td>0·896 ± 0·318</td>
<td>0·958 ± 0·291</td>
<td>0·917 ± 0·195</td>
<td>0·896 ± 0·258</td>
</tr>
<tr>
<td></td>
<td>Old</td>
<td>1·095 ± 0·145</td>
<td>1·024 ± 0·453</td>
<td>1·171 ± 0·306</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>Young</td>
<td>1·027 ± 0·621</td>
<td>1·095 ± 0·476</td>
<td>1·055 ± 0·519</td>
<td>0·945 ± 0·385</td>
</tr>
<tr>
<td></td>
<td>Old</td>
<td>1·062 ± 0·492</td>
<td>1·161 ± 0·595</td>
<td>0·825 ± 0·400</td>
<td></td>
</tr>
</tbody>
</table>

* Three or four animals/group.
† d.p.m./mg wet weight of tissue or d.p.m./pituitary gland in (a) pre-treated mice (progesterone or DES) and (b) oil-injected controls.

Experiment 1

The levels of uptake of [3H]oestradiol in the uterus, pituitary gland and hypothalamus were inhibited by previous exposure to DES but progesterone had no effect (Table 1). Young
mice given excess DES (100 μg) had only 3.7%, 4.3% and 35.6%, respectively, of the radioactivity in oil-injected controls for these three tissues and similar values were obtained for old animals. The levels of radioactivity in the cerebrum and serum were not affected by either treatment.

Experiment 2

The radioactivity/oestradiol relationships in the young and old animals were compared by pooled regression analyses. The relationship did not differ between age groups either in the cerebrum or serum (Fig. 1, $F_{1,40} = 0.320$ for cerebrum; $F_{1,40} = 0.027$ for serum) or in the two target tissues, the hypothalamus and uterus (Fig. 1, $F_{1,39} = 0.207$; Fig. 2,
The uptake in the pituitary gland was, however, reduced in old compared with young mice both when whole glands were compared ($F_{1,40} = 3.24, P > 0.05$, respectively). The uptake in the pituitary gland was, however, reduced in old compared with young mice both when whole glands were compared ($F_{1,40} = 3.96, P < 0.05$) or when unit weight of tissue was compared (Fig. 3, $F_{1,40} = 3.96, P < 0.001$).

The proportions of unmetabolized oestradiol in the tissue extracts varied between 30% and 90% according to the tissue type (order: serum < cerebrum < hypothalamus < pituitary gland < uterus) and tissues with the highest specific activities tended to have the highest proportion of oestradiol, serum being an exception. The proportions of oestradiol differed between age groups for a given tissue and dose of hormone.

The proportions of unmetabolized oestradiol in the tissue extracts varied between 30% and 90% according to the tissue type (order: serum < cerebrum < hypothalamus < pituitary gland < uterus) and tissues with the highest specific activities tended to have the highest proportion of oestradiol, serum being an exception. The proportions of oestradiol differed between age groups for a given tissue and dose of hormone.

**DISCUSSION**

The present results confirm the well-established fact that oestrogen target tissues possess a specific mechanism of limited capacity for taking up and concentrating oestradiol from the blood. It is believed that binding of oestradiol to proteins in the cytosol is an obligatory step for eliciting a biological response. There is evidence of a direct relationship between the quantity of hormone bound and end-organ response but only a few studies have examined the uptake or binding of steroid hormones in relation to age. Indeed, during the senescent phase of life (Roth & Adelman, 1975). This information is of interest because of the possible relation between hormone binding and altered adaptive responses to hormonal stimulation which is a characteristic of ageing animals.

Studies of rabbits and rats, reported since the present study began, have led to somewhat different conclusions than to the present ones, although it is difficult to compare where differences in methodology and relative age of animals exist. The levels of uptake of oestradiol and progesterone were found to be lower in old rabbit uterus than young controls (Larson, Spilman & Foote, 1972); similar results were obtained for uptake of oestradiol in uterine, pituitary and hypothalamic tissues of aged rats (Peng & Peng, 1973). Furthermore, the lower concentration of receptor oestradiol-17β in aged rat cerebral hemispheres (Kanungo, Patnaik & Koul, 1975) and observed decrease with age in oestradiol-induced acetylcholinesterase in this tissue (Mans.
Oestradiol uptake in ageing mice

suggests a cause-and-effect relationship. The results indicate that in tissues of these species, and in the case of the mouse pituitary gland, there is a decreasing concentration or affinity of oestradiol receptors in senescent females.

Reduced hormonal stimulation of oestrogen target tissues as a result of decreased uptake from the circulation could be of importance for neuroendocrine function and embryo implantation and development. However, there was no significant alteration in the uptake or metabolism of oestradiol-17β by tissues of reproductively senescent mice, with the exception of the pituitary gland. These results minimize the significance of factors affecting the transport of oestradiol to the cell as well as the uptake mechanism itself and may point to changes at the level of genome expression in the ageing uterus.

The author thanks Drs R. G. Edwards, A. Tait and R. E. Zigmond for help and advice during this study and Mr D. E. Walters for computing the pooled regression analyses. The study was carried out during the tenure of a M.R.C. Scholarship and working expenses were defrayed by a grant from The Ford Foundation to Professor C. R. Austin.

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Luteinizing Hormone Requirements for Ovulation in the Pentobarbital-Treated Proestrous Rat

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ABSTRACT. The LH requirements for ovulation in the pentobarbital-blocked proestrous CD rat have been studied by increasing serum gonadotropin levels through electrical stimulation of the brain and subsequently comparing the effects of timed hypophysectomy on ovulation and serum LH concentrations. The arcuate nucleus (ARC) or the medial preoptic area (POA) was stimulated unilaterally for 45 min with matched pairs of biphasic rectangular pulses through a coaxial platinum electrode. Serum LH was significantly elevated above basal values at the end of stimulation, but not in sham-stimulated controls. The results of both hormone measurement and hypophysectomy showed that the pituitary continued to release LH after extrinsic stimulation of the hypothalamus had ceased. Animals did not ovulate if they had been hypophysectomized at the end of the 45 min stimulation whereas nearly all ovulated if hypophysectomy was delayed for an additional 20 min. Some evidence suggested that the pituitary could be removed earlier without affecting ovulation if the rate of LH release was increased. The minimum peak LH concentration measured in rats that subsequently ovulated fully was 187 ng/ml, substantially lower than concentrations ordinarily attained in the spontaneous proestrous surge. When serum LH was insufficiently high to cause follicle rupture, there was nevertheless the resumption of meiosis and luteinization of the large ovarian follicles. Attempts were made to restore ovulability in animals presumed to have released a subovulatory quota of gonadotropin. Ovulation was obtained when such animals, prepared by hypophysectomy after the 45 min stimulation, had been bilaterally nephrectomized prior to stimulation. However, multiple injections of progesterone after hypophysectomy were without effect. The results are discussed in relation to variables that affect minimum requirements of LH for ovulation. (Endocrinology 99: 1046, 1976)

The release of ovulation-inducing hormone on the afternoon of proestrus in the rat is evoked by neural events that can be blocked by either atropine sulfate or pentobarbital (1). Injection of atropine or hypophysectomy at progressive times during the critical period for pituitary activation resulted in a progressive decline in the number of animals with blocked ovulation (2,3). These studies suggested that the neurogenous stimulus and the release of an "ovulatory quota" of gonadotropic hormone were concurrent and, from the percentages of animals blocked and partially blocked, it was estimated that the duration of these processes was approximately 30 min. In the rabbit, an interval of at least an hour between copulation and hypophysectomy is required for an ovulatory quota of gonadotropin to be released (4). A number of observations have indicated that much more than the minimum quantity of hormone required for ovulation is commonly released in the rat during the spontaneous surge at proestrus (1).

The purpose of the present study was to determine more precisely the gonadotropin requirements for ovulation in the 4-day cyclic rat. Ovulation-inducing hormone was considered to be LH for the sake of simplicity. The method adopted was to raise serum LH levels by hypothalamic stimulation of animals whose spontaneous surge was suppressed by pentobarbital. We then examined the effects of hypophysectomy and other treatments upon ovulation and levels of circulating LH.

Electrical, rather than electrochemical,
stimulation of the hypothalamus was chosen in order to avoid producing gas or an irritative lesion at the site of the electrode tip. Several groups of investigators have employed this technique (5–8), and experience in our laboratory has shown that it is necessary for electrical stimulation to continue for more than 30 min to elicit ovulation uniformly in rats (9).

Materials and Methods

Adult virgin Charles River CD rats were maintained after receipt in air-conditioned quarters with the lights on from 0500–1900 h daily. They were provided with Purina Laboratory Chow and water ad libitum. Only animals that had presented at least two consecutive 4-day estrous cycles were selected for study.

Proestrous animals were given pentobarbital (10) shortly before 1400 h to block the spontaneous surge of LH. Anesthetized animals, in the stereotaxic apparatus with the incisor bar 6 mm below the ear bars, received unilateral electrical stimulation of either the arcuate nucleus (ARC) or the medial preoptic area (POA) with a coaxial bipolar platinum electrode. Stimulation was by a train of matched biphasic pairs of rectangular pulses of 1 msec duration, applied for 30 sec out of every minute for 45 min and at a frequency of 30 Hz. The pulses were delivered by two Grass stimulators and were monitored continuously with a calibrated oscilloscope. The current of the positive and negative pulses was balanced with a precision microammeter. Detailed descriptions of the apparatus and the method of electrical stimulation have appeared before (5,9).

Effects on the reproductive tract were assessed on the morning following stimulation. Vaginal smears prepared at that time were confirmed in every case. The rats were deeply anesthetized with pentobarbital, and the ova ries and ampullae of the oviducts were dissected and examined for evidence of ovulation. Tubal ova were counted where present; nine or more being considered as representing full ovulation. The ovaries of animals that failed to ovulate were prepared for serial histologic sections. Heads were perfused with 10% formalin in normal saline and the position of the electrode tip was determined by examination of the fixed brain either directly under low power magnification (ARC) or after histologic processing (POA). Animals with electrostimulations deviating substantially from the intended location were excluded from the study.

A. Effects of electrical stimulation on serum levels

A cannula was inserted via the external iliac vein into the right atrium of some of the rats by the injection of pentobarbital and immediately prior to stereotaxis. Blood was withdrawn for serum LH determination at five 45 min intervals beginning at the onset of stimulation. A volume of only 1.5 ml of blood was removed and half of the volume removed was replaced each time with heparinized saline (25). The stimulus intensity delivered to the two regions was different because of known differences in sensitivity (5). The stimulus intensities were approximately the minimum necessary to elicit full ovulation in all animals with 500 μA and 670 μA peak-to-peak for ARC, POA, respectively. Control rats received the same treatment as above except that the current was not turned on.

B. Effect of time of hypophysectomy on ovulation and LH levels

Another series of rats received electrical stimulation of the ARC and POA as before, but were subsequently hypophysectomized at intervals (see Table 1) via the transauricular

<table>
<thead>
<tr>
<th>Site of stimulation</th>
<th>Time of hypophysectomy after the onset of stimulation (min)</th>
<th>No. of animals</th>
<th>Ovulating %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arcuate nucleus</td>
<td>45</td>
<td>6</td>
<td>0*</td>
</tr>
<tr>
<td>nucleus</td>
<td>55</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Preoptic area</td>
<td>65</td>
<td>7</td>
<td>7*</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>45 (sham hypox)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>7</td>
<td>0*</td>
</tr>
</tbody>
</table>

* Values having the same superscript are statistically different (P < 0.01).

† Mean ± SE.
survival analysis, and we are grateful for the collaboration of other authors who provided additional data and insights. We hope that this review will stimulate further research in this exciting area of study.

The authors declare no conflicts of interest.

References


Fig. 1. Serum LH changes with time in proestrus rats receiving unilateral electrical stimulation at the ARC nucleus (5 rats) or POA (7 rats) during the critical period. SE is represented by the vertical lines above or below the means. LH levels of comparable animals receiving either sham ARC or sham POA stimulation (4 animals/group) were below the dotted line (40 ng/ml). The standard used in the LH determinations was LEV 1213A (0.018 x NIH-LH-S1).

approach with a specially designed ear bar device (David Kopf Instruments). Hypophysectomy was completed within 2 to 3 min, and was only rarely accompanied by significant hemorrhage. To control for non-specific effects of hypophysectomy, the needle for withdrawing the pituitary was inserted to the depth of the sella in some rats without damaging the gland or its vasculature. Animals of an additional group subjected to POA-stimulation and hypophysectomy were cannulated shortly before stereotaxis and blood samples were collected at intervals for the measurement of LH. On the morning following stimulation, the animals were checked for ovulation and electrode location as before. Animals that were incompletely hypophysectomized, as judged by stereomicroscopic examination of the sella at autopsy, were excluded from the results.

C. Attempts to produce ovulation in animals having a presumptive subovulatory quantity of serum LH

The results of (B) showed that animals did not ovulate if hypophysectomized 45 min after the onset of stimulation. The following experiments tested whether the ability to ovulate could be restored in such animals:

1. Rats were bilaterally nephrectomized or sham-nephrectomized under pentobarbital shortly before the POA was stimulated. They were hypophysectomized at 45 or 65 min after the onset of stimulation. In addition, another group of animals receiving these treatments were cannulated and serial blood samples were collected.

2. Another group of rats receiving POA stimulation and hypophysectomy at 45 or 65 min were subsequently treated with progesterone (5 or 10 mg/injection) or the sesame oil vehicle alone. Three SC injections were given beginning immediately after hypophysectomy and at two successive 60 min intervals later.

Animals from both experiments were examined on the morning after stimulation for ovulation, electrode position and residual pituitary fragments.

LH radioimmunoassay

Serum LH concentrations were determined by the ovine-ovine double antibody method of Niswender et al. (11). Determinations were made in duplicate with rare exception and the averages were expressed in terms of the rat pituitary reference preparation LER 1213A (biologic activity: 0.018 x NIH-LH-S1).

Statistics

The differences between the proportions of animals ovulating were determined statistically by the Chi-squared test with Yates' correction for 2 x 2 contingency tables. Group means were compared by Student's t test.

Results

A. Effects of electrical stimulation on serum LH concentrations

Unilateral electrical stimulation of either the ARC nucleus or the POA during the proestrus critical period caused a comparable and significant elevation of serum LH as well as an ovulatory response in each case. Average serum LH levels markedly increased from basal values (<40 ng/ml) by the end of the 45 min period of stimulation. Mean levels had not declined significantly by 90 min, but fell markedly by 135 min and were approaching baseline at 180 min (Fig. 1). There was no measurable increase of serum LH in sham-stimulated rats. They neither ovulated nor showed histologic evidence of subovulatory LH stimulation in their ovaries.
There was considerable individual variation among stimulated rats in both the time that peak LH concentrations were reached and in the absolute values attained (Figs. 2, 3). Many animals continued to release LH after extrinsic stimulation had ceased and approximately half of them had higher LH levels at 90 min than at 45 min. These individual differences in LH release were present in both ARC and POA stimulated animals and were not related to the length of the interval between induction of anesthesia and stimulation. There was no clear relationship between numbers of ova and peak LH levels in ovulating animals; full ovulation occurred in animals presenting maximal LH concentrations as low as 187 ng/ml. No correlation was noted between the amounts of LH detected and location of electrodes within either the ARC or the POA. The electrode positions were similar to those described by Everett et al. (5).

B. Effect of time of hypophysectomy on LH levels and ovulation

Further evidence of continued release of pituitary LH after stimulation of the ARC or POA had ceased was provided by the results of hypophysectomy (Table 1). Ovulation failed to occur when animals receiving standard brain stimulus were hypophysectomized immediately at the end of stimulation (45 min). However, histologic examination of the ovaries disclosed in most animals varying degrees of sub-ovulatory stimulation of the graafian follicles, ranging from resumption of meiosis with little or no luteinization to uniform luteinization through the follicle walls. In any one pair of ovaries these changes were not uniform and some large follicles were appreciably affected. When the pituitary was removed 20 min in the end of stimulation, ovulation occurred on the next morning in almost every animal. Hypophysectomy at an intermediate time (55 min) resulted in half of the animals ovulating and smaller numbers of ova per ovulation. Many of the rats hypophysectomized at 65 min had contracted uteri on the following morning, whereas those with unilateral removal of the pituitary always retained some degree of uterine ballooning.

The concentrations of circulating LH in rats hypophysectomized at the end of 45 min of stimulation and those in rats receiving the stimulation without subsequent hypophysectomy are compared in Fig. 4. Pressed as percentages of the mean values. Serum LH levels declined more rapidly in the hypophysectomized group than in the controls. The rate of hormone clearance in the

Fig. 2. Individual serum LH levels in proestrous rats receiving ARC stimulation during the critical period.

Fig. 3. Individual serum LH levels in proestrous rats receiving POA stimulation during the critical period.
It should be pointed out that three cannulated animals, including one that was sham-nephrectomized, ovulated in spite of hypophysectomy at 45 min. Whether this result was due to the effects of the additional surgery or bleeding cannot now be determined, but it seems significant that these rats had the highest LH levels for their groups.

2. In experiments performed to determine the effects of progesterone administration after brain stimulation and hypophysectomy, only one animal of five ovulated following hypophysectomy at 45 min and subsequent progesterone treatment, and that one produced only a single ovum. When similar treatment was given to two animals hypophysectomized at 65 min, both animals ovulated, one producing 8 and the other, 2 ova.

Discussion

The effects of electrical stimulation of the POA on serum LH levels and ovulation in the pentobarbital-blocked proestrous rat have been described previously by Cramer and Barraclough (7). They found that the concentrations of circulating LH increased after the extrinsic stimulus had been withdrawn and the highest mean plasma LH values were not attained until 100 min after the onset of stimulation. The present results confirm the continual release of gonadotropin from the intact pituitary after stimulation of the POA or ARC had ceased, both by measurement of circulating LH and by

C. Attempts to produce ovulation in animals having an otherwise sub-ovulatory discharge of LH

1. Nephrectomy prior to brain stimulation overcame the ovulation-blocking effect of hypophysectomy at 45 min. Six of seven nephrectomized rats ovulated, whereas none of the sham-operated controls did so (Table 2). Although peak concentrations of serum LH at the end of stimulation did not differ between nephrectomized rats and controls, subsequent values were significantly higher in the former group (*P < 0.01); remaining above 300 ng/ml for the remainder of the sampling period (Fig. 5). This difference was apparently the result of a difference in the rate of clearance of LH from circulation. The average half-life of serum LH in animals after hypophysectomy at the 45 min interval was about 32 min, but in nephrectomized rats this was estimated, by extrapolation, to be increased approximately sevenfold to about 210 min.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of hypophysectomy after stimulation began (min)</th>
<th>No. of animals</th>
<th>Ova/ ovulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-nephrectomy</td>
<td>45</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Nephrectomy</td>
<td>45</td>
<td>7</td>
<td>6*</td>
</tr>
<tr>
<td>Nephrectomy</td>
<td>65</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

* Proportions significantly different (*P < 0.02).  
† Mean ± SE.
LH REQUIREMENTS FOR OVULATION

demonstrating the failure of ovulation in animals hypophysectomized immediately after stimulation. The time course for LH release, as indicated by circulating hormone concentrations, was apparently similar with either site of stimulation.

The magnitude of peak serum LH values and the time when peak values were attained varied considerably between individuals in spite of a carefully controlled and monitored electrical stimulus. The fact that LH values of some individuals had declined 45 min after the end of stimulation resulted in an apparent downward trend of the average levels during that interval, although the change was not statistically significant. The reasons for the post-stimulation rise in other animals and the individual variability in response are not understood, but they could result from differences in sensitivity of the hypothalamus and/or the pituitary at the time of stimulation. Continued release of LH may be due to residual activity of LHRH already in the pituitary or to further discharge of hypothalamic LHRH, which in turn may be due to continued activity of hypothalamic neurons.

The absence of any measurable increase of serum LH concentrations in sham-stimulated rats may be ascribed to minimal damage from the mere insertion and withdrawal of the electrode. There have been reports of gonadotropin release and even ovulation following the placement of a needle or blade into certain hypothalamic areas (12,13,14).

The pituitary had to remain in situ for at least 65 min for ovulation to occur in animals receiving the standard electrical stimulus. This is considerably longer than the 30 min estimated to be necessary for the release of an ovulatory quota of gonadotropin in spontaneously ovulating rats (3) and in animals receiving massive electrochemical stimulation of the POA (15). These differences are greater than might be expected on the basis of animal strain differences alone and it is noteworthy that animals receiving a more moderate electrochemical stimulus required that the pituitary remain in place for at least 40 min for ovulation to occur. More recently, a tentative relationship has been described between electrochemical stimuli, the entities of LH released and, hence, the ovulatory response (16,17,18). Furthermore it has been shown that quantities of serum LH initially below those of the spontaneous LH were effective in evoking ovulation in the present study, the lowest concentration of serum LH in stimulated rats that ovulated was 187 ng/ml. This is considerably lower than the LH levels generally found during the spontaneous surge of gonadotropins in this strain (17), and is presumably a result of only partial activation of the hypothalamic apparatus involved in the release of ovulation-inducing hormone. Everett et al. (15) reported that the circulating LH levels reach 90-150 ng equivalent of LHRH per ml for ovulation to take place in electrochemically stimulated CD rats. However, the excess of circulating LH above the pituitary requirements may be important for development of fully competent corpora lutea or for other functions. Quantities of LH below the requirements for follicle growth were sufficient to reinitiate meiosis, cause luteinization of the large follicle. This is not a new observation and indicates that different LH-sensitive components of the ovary may have quite

![Fig. 5. Serum LH changes in proestrous CD rats nephrectomized (- - ) or sham-nephrectomized (---) min after the onset of POA stimulation. For each group. Means ± SE are given.](image-url)
different threshold requirements for hormonal stimulation (19).

There is considerable evidence that LH alone, without other pituitary hormones, can cause follicle rupture in the rat (20,21). However, electrical stimulation of the hypothalamus has been shown to release FSH as well as LH (8). The contribution of FSH or other pituitary hormones to the process of ovulation thus cannot be completely excluded, but the correlation of LH concentration with ovulation and the parallel effects of hypophysectomy on ovulation and on serum LH levels are consistent with LH being the primary pituitary factor for ovulation.

The slower disappearance rate of serum LH in nephrectomized rats is in close agreement with the findings of Gay (22) who used gonadectomized animals. The most probable explanation for the effect of nephrectomy in reducing the minimum time that the pituitary has to remain in place in order to bring about ovulation is simply a reduced clearance rate. Other studies have described how alterations of the time that the hormone persists in the circulation can affect its biological activity (23,24,25). The present results show that peak concentration of LH is not the only factor to be considered in the induction of ovulation. Sham-nephrectomized animals failed to ovulate even though their highest LH concentrations were not significantly different from those of nephrectomized rats. Thus, it appears that the occurrence of ovulation is determined not only by a certain minimal concentration of LH in the ovarian circulation, but also by the length of time that this concentration is maintained.

Exogenous progesterone did not restore ovulation to animals that received a subovulatory quota of gonadotropin. The injection protocol and the doses employed were comparable to those that Takahashi et al. (26) claimed responsible for that particular effect in animals hypophysectomized during the spontaneous gonadotropin surge. Perhaps our differing results may have been due to differences in endocrine conditions or in ovarian sensitivity at the time of hypophysectomy in the two experimental situations.

The term "ovulatory quota of gonadotropin" refers to the minimum quantity of hormone required to induce ovulation in a given population of rats under a given set of conditions. Alterations of the biological activity or metabolism of the hormone as well as alterations of the ovarian sensitivity or circulation will naturally affect the "quota" of hormone required. Proestrus is the optimal time for follicles to receive an ovulation-inducing stimulus and it is therefore not surprising that the "quota" is larger at other stages of the estrous cycle (15) and in persistent estrus (unpublished observations).

Acknowledgments

The authors wish to express appreciation for technical assistance to Ann Bell, Lee Dellingor, Rosario Pasaro Dionisio, Douglas Guthrie, and Carol Schnake. We are also grateful to Dr. Leo E. Reichert, Jr. for gifts of purified ovine LH (LER 1056-CS) and the rat gonadotropin reference preparation (LER 1213A), and to Dr. Gordon Niswender for rabbit antiovine LH serum #15.

References

PITUITARY FUNCTION IN REPRODUCTIVELY SENESCENT FEMALE RATS

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Abstract—Ageing female rats subjected to a standard lighting schedule (L:D = 14:10) frequently enter a state of persistent vaginal cornification (PE) in which follicular development occurs without ovulation. The function of the pituitary/hypothalamic complex in these animals was compared with that of young cyclic rats by measuring levels of circulating luteinizing (LH) and follicle stimulating hormone (FSH) by radioimmunoassay after ovariectomy and after treatment with gonadal steroids. Plasma LH levels were similar in old PE rats to those of young cyclic animals on the morning of pro-oestrus. Both the post-ovariectomy increase of LH and the oestrogen–progesterone evoked surge of LH were significantly reduced in the PE group. However, the plasma FSH concentrations were higher in PE rats than cyclic controls at pro-oestrus and they attained similar values after ovariectomy in young and old groups. These results indicated that the capacity of the pituitary gland of ageing PE rats to secrete LH was impaired whereas FSH secretion was not reduced.

INTRODUCTION

Ageing female rats characteristically have irregular oestrous cycles, with periods of persistent vaginal cornification (“persistent oestrous”) and spontaneous pseudopregnancy (Bloch, 1961; Aschheim, 1965a; Huang and Meites, 1975). These expressions of changes of the pituitary-hypothalamic-ovarian axis appear at a time when reproductive capacity is waning and generally precede the final anoestrous state which presumably marks the cessation of significant endocrine activity by the ovary (Bloch, 1961).

When the ovaries of old acyclic rats are grafted into young hosts their cyclic hormonal activity is restored but young ovaries grafted into old hosts do not produce cyclic vaginal stimulation (Aschheim, 1965a; Zeilmaker, 1969; Peng and Huang, 1972). Moreover, the ovaries of PE rats may be induced to ovulate by LH administration (Aschheim, 1965b; unpublished results). It was inferred therefore, that age-related changes of the pituitary gland or its hypothalamic control, rather than of the ovary, are responsible for the failure of cyclicity in ageing rats. Since defective pituitary function may have been due to an altered response of the hypothalamus and/or pituitary gland to feedback control by gonadal steroids, we have measured the levels of circulating luteinizing hormone (LH) and follicle stimulating hormone (FSH) in ageing persistent oestrous (PE) rats when ovarian feedback inhibition was removed and after treatment with ovarian steroids. The results for PE rats have been compared with those for young animals at the most physiologically comparable time of the oestrous cycle, namely the morning of pro-oestrus.

MATERIALS AND METHODS

Young virgin (2-4 month) and retired breeder (10-14 month) female CFY rats (Carworth), derived from Sprague-Dawley stock, were housed in a temperature controlled and artificially lighted room (14 hr photoperiod). Laboratory animal food pellets and water were provided ad libitum. Young animals exhibiting two or more successive 4-day oestrous cycles and old animals having at least 14 days of persistent vaginal cornification were chosen for study.

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Fig. 1. Ovary of a 10 month old persistently oestrous rat. Large antral follicles and an ovarian cyst (OC) are present, but signs of recent ovulation are absent. Haemalum and eosin (×24).
TABLE 2. PLASMA LH LEVELS OF OVARIECTOMIZED RATS OF DIFFERENT AGES TREATED WITH OESTROGEN AND, SUBSEQUENTLY, WITH EITHER OIL OR PROGESTERONE

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Post-ovariectomy</th>
<th>After oestrogen</th>
<th>Oestrogen-treated ovariectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma LH ng/ml, Mean ± S.E. (no.)</td>
<td>Control (oil)</td>
<td>Progesterone</td>
</tr>
<tr>
<td>2-4</td>
<td>28.1 ± 4.7 (19)</td>
<td>7.7 ± 1.4 (19)</td>
<td>8.2 ± 4.8 (4)</td>
</tr>
<tr>
<td>10-14</td>
<td>9.0 ± 4.6 (20)*</td>
<td>3.5 ± 0.6 (16)</td>
<td>14.0 ± 4.2 (3)</td>
</tr>
</tbody>
</table>

*Significantly lower than values in young animals (P < 0.01).
†Significantly lower than in young animals (P < 0.025).

Treatment of ovariectomized animals with oestradiol caused a decline in LH values as measured 72 hr later. When the differences in initial hormone values were statistically adjusted for, the effect of oestradiol on plasma LH was found to be not significantly different between the age groups. The administration of progesterone to oestradiol-ovariectomized rats resulted in a marked increase in plasma LH to values which were significantly greater in the young than in the old animals (P < 0.025).

DISCUSSION

The persistent-oestrus condition, which may arise under conditions other than ageing (Everett, 1964), appears to be due to the failure of the pituitary gland to spontaneously release an ovulatory quota of gonadotrophins at pro-oestrus because ovaries of PE rats can ovulate under appropriate hormonal conditions. The present study has shown that the ageing PE rat has an impaired capacity to release pituitary LH compared to young animals after (1) removal of inhibitory feedback from the gonads and (2) a steroid treatment protocol which facilitates a surge of LH in ovariectomized rats. On the other hand, plasma FSH levels in old ovariectomized rats were not decreased compared to young controls, the differences observed prior to ovariectomy possibly being due to the degree of pituitary inhibition by ovarian oestrogen. In drawing these conclusions, we have made the assumption that differences in gonadotrophin concentrations reflected differences in hormone release rather than in hormone clearance from the circulation. Our finding that LH secretion in PE rats was impaired under conditions which would be expected to increase plasma hormone levels is in accord with results for much older animals (Pi et al., 1973; Shaar et al., 1975) and with the presumed failure of these rats to spontaneously release a surge of LH at pro-oestrus. It should be pointed out, however, that pituitary/hypothalamic failure may not precede the onset of ovarian insensitivity in all species; ovarian failure is generally believed to precipitate the menopause in man.

It is not known whether the primary lesion responsible for spontaneous PE lies in the pituitary or the hypothalamus. A recent study has shown a decreased reactivity of the pituitary gland to synthetic LH-releasing hormone in ageing rats (Watkins et al., 1975), but the gland is still capable of releasing sufficient gonadotrophin to elicit ovulation when these animals are treated with progesterone, nor-adrenaline, L-DOPA or electrical/electrochemical stimulation of the brain (Clemens et al., 1969; Quadri et al., 1973; Wuttke and Meites, 1973; unpublished results). These data indicate that PE results from defective neuro-hormonal control of the pituitary gland by the hypothalamus. It is not yet clear why the LH : FSH ratio was different in PE rats to that of young cyclic animals for both gonadotrophins are thought to be controlled by the same hypothalamic releasing hormone (Schally et al., 1973).
According to Dilman (1971), hypothalamic dysfunction would result from a progressive age-related elevation of the threshold to feedback control by gonadal steroids, which in turn influence LH release either positively or negatively. The reduced magnitude of surge levels of LH found in old compared to young ovariectomized rats after treatment with steroids is consistent with this hypothesis. There was no evidence obtained in this study to suggest a decreased pituitary/hypothalamic sensitivity to feedback inhibition of LH by oestrogen, but adequate testing of this hypothesis will require measurements of ovarian steroid secretion in young and ageing rats and a comparison of the curves of plasma LH suppression by oestrogen.

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REFERENCES

Normal and abnormal follicular growth in mouse, rat and human ovaries


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We wish to present an analysis of the data gained by a group of workers studying various aspects of ovarian activity. Our work has been concerned primarily with two aspects of the development of follicles: the initiation and control of follicular growth, and differentiation and steroidogenesis in follicles approaching ovulation. The scope of our contributions spans the lifetime of the female from the early stages of growth in the fetal ovary to the final stages of follicular development in ageing females and includes both animal and clinical studies. The results are given in two main sections: follicular growth, and normal and abnormal differentiation and steroidogenesis in follicles approaching ovulation. Several aspects of follicular growth are considered in the discussion.

Follicular growth

Is there a regularity in the formation and utilization of follicles?

A full understanding of follicular growth in the adult ovary cannot be gained until the factors governing the establishment of follicles in the immature ovary are clarified. Plentiful evidence has shown how follicles are formed in gradients in the fetal or neonatal ovary; small follicles containing an oocyte in dictyotene are identified in some areas of the cortical cords, whereas oocytes still in early prophase of meiosis or oogonia in mitosis can be found in other areas. We suspect that this sequential formation of follicles is reflected in their future utilization in the adult female as they begin growth at various times during the lifespan. Such regularity in the formation and utilization of follicles was suggested by the results of studies on chiasma frequency in oocytes and spermatocytes taken from mice of different ages (Henderson & Edwards, 1968). A steady decline was found in the number of chiasmata in oocytes, and chiasma position became located towards the ends of the chromosomes as the female aged (Text-fig. 1). Since chiasmata are formed in the fetal ovary, this observation implied that oocytes with few chiasmata were conserved until the end of the reproductive life of the female. An objection to this conclusion is that chiasma position may become terminalized during the prolonged dictyotene stage, although the existence of this process has been questioned (Henderson, 1970). There was no decline in the number of chiasmata in spermatocytes with increasing age in males.

An alternative method was needed to confirm that certain oocytes were conserved until the end of the reproductive life of the female. One approach is to study the recombination frequency of linked marker genes in offspring of ageing parents. The position of chiasmata and the position of recombination on the chromosome are believed to be closely associated, hence the reduction noted in chiasma frequency in oocytes with increasing maternal age should be associated with fewer recombinants in offspring of ageing female mice. A group of linked marker genes on chromosome 2 of the mouse was therefore used to study recombination frequency with increasing parental age, by using backcrosses to heterozygous females or heterozygous males to test for recombination in mothers and fathers respectively (Wallace, MacSwiney & Edwards, 1976). In the strain of mouse studied, a great
length of chromosome No. 2 was covered by linked marker genes which served to identify recombinants occurring along most of the chromosome:

\[
\begin{array}{cccc}
Sd & fi & w & a' \\
20 & 15 & 3 & 11 & 22
\end{array}
\]

The breeding programme was analysed to find out if differences existed between ageing males and ageing females as indicated by the studies on chiasma frequency. The analysis of recombination frequency indicates that some conclusions on chiasma frequency may be confirmed, tentatively. Analysis of offspring from ageing females showed that the number of recombinants declines with increasing age, although the trend was not statistically significant (Table 1). Unfortunately, females become infertile at those advanced ages at which more data are needed.

Table 1. Recombinations in the offspring of ageing male and female mice (data from Wallace et al., 1977)

<table>
<thead>
<tr>
<th>Age of parent (months)</th>
<th>No. of offspring</th>
<th>Mean no. of recombinants per offspring</th>
<th>Proportion of offspring with no recombinants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3</td>
<td>150</td>
<td>0.86</td>
<td>0.50</td>
</tr>
<tr>
<td>3-4</td>
<td>130</td>
<td>0.91</td>
<td>0.40</td>
</tr>
<tr>
<td>4-6</td>
<td>255</td>
<td>0.78</td>
<td>0.69</td>
</tr>
<tr>
<td>6-8</td>
<td>129</td>
<td>0.88</td>
<td>0.40</td>
</tr>
<tr>
<td>8-10</td>
<td>484</td>
<td>0.79</td>
<td>0.69</td>
</tr>
<tr>
<td>≥10</td>
<td>18</td>
<td>0.92</td>
<td>0.40</td>
</tr>
<tr>
<td>Father</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3</td>
<td>183</td>
<td>0.81</td>
<td>0.51</td>
</tr>
<tr>
<td>3-4</td>
<td>464</td>
<td>0.81</td>
<td>0.51</td>
</tr>
<tr>
<td>≥10</td>
<td>216</td>
<td>0.86</td>
<td>0.51</td>
</tr>
</tbody>
</table>

There was no significant heterogeneity between groups (heterogeneity \( \chi^2 \) \( P > 0.05 \)) except in offspring with no recombinants compared with the age of mother \( (P < 0.05) \).
difficulties in obtaining large numbers of offspring in such a mating programme. One parameter was significant: many offspring were found to have no detectable recombinants, and there was a significant heterogeneity $\chi^2$ in the distribution of these offspring with age of the mother (Table 1), although this heterogeneity was evidently not due to a linear trend with age. The absence of recombinants in the offspring implies that recombination had occurred at the end of the chromosome, i.e. outside the region covered by the marker genes (Text-fig. 2), or that a double recombination had occurred between two of the marker genes and was undetectable. Terminal recombinations would be expected from the observations on the position of chiasmata in oocytes, for many of them were also found in a terminal position in oocytes of ageing females. The two methods of analysis may therefore be giving similar conclusions.

Establishing such a linkage analysis over the lifetime involves a long-term programme, and the stimulation arising through the decline in natural fertility at about 12 months will have to be overcome by transferring blastocysts from ageing mothers into young recipients. This method should provide data from those mothers of greatest interest to us, namely those aged 1½ or 2 years, when the number of recombinations should be very low if our hypothesis is correct. It is interesting to note that no trend or heterogeneity was found in analyses carried out on the offspring of ageing males (Table 1).

**Dynamics of follicular growth throughout life in intact and hypophyssectomized mice**

Detailed quantitative analyses have been carried out on populations of oocytes and follicles at different stages of development in mice of all ages, from neonates to the aged adult. The data were initially obtained during studies on the effects of increasing age on the number of oocytes and follicles in normal and hypophyssectomized females (Jones & Krohn, 1961a, b). Ovaries from 54 normal virgin mice aged 0–560 days and 12 hypophyssectomized mice aged 52–505 days were sectioned serially and follicles were counted and classified into groups which have been described elsewhere (Zuckerman, 1951). Follicles were included in the count if the section passed through the nucleolus of the oocyte and, since the difference between 1:5 and 1:10 samples was statistically insignificant, every 10th section was scanned and the observed estimate for each follicle group was multiplied by 10. The accuracy of this estimation method will clearly decrease with increasing size of follicle and when more precise determination of numbers of large follicles was required every section was examined. Oocytes still in the pre-antral stage and lacking a nucleoluar marker were included if the section was judged to contain half, or more, of the nucleus. A correction factor to allow for over-counting (Abercrombie, 1946) was applied to the estimates of primordial follicles except for old ovaries in which the total number of follicles is low and every section was scanned. An attempt was made to distinguish between normal and atretic follicles by histological criteria. The appearance of the nucleus, cytoplasm and membranes of the oocyte, and of the surrounding granulosa cells was taken into account. Such subjective diagnosis of atresia in large follicles was relatively straightforward but became more uncertain with decrease in size.

These data have been used to test models of follicular utilization (Faddy, Jones & Edwards, 1976). Analysing follicle turnover in relation to time spent in different developmental stages is not easy, even though the pulse labelling of DNA has given invaluable information on mitosis in follicle
cells and on follicular growth (Pedersen, 1972). Designing a mathematical model which can be run against data on ovarian contents is useful because the whole spectrum of activity in follicles begins their growth, dying and leaving or entering various phases in development must be quantified and explained.

In mice, utilization from the pool of primordial follicles begins soon after birth, Group II follicles being found at 3 days, and Group III with two layers of granulosa cells at 5 days (P. I., Fig. 1). Follicles thus migrate forwards through these and other groups towards ovulation, although many are at various stages of development. Since there is no new formation of oocytes after birth, a mathematical model of the "death/migration" type would be appropriate (Faddy, 1976). In this model (Text-fig. 3), the number of follicles present in each group at any age is represented by independent Poisson cones whose means can be compared at various ages to gain information on follicle turnover. The external characteristics that must be determined are the migration (v) and death (μ) rates of each type of follicle.

Text-fig. 3. A death/migration model of follicular dynamics. The stages of development in terms of layers of granulosa cells are shown, together with a comparison of the classification groups used by Pedersen and Peters (1968). μ is death in Group V+ and those follicles proceeding to ovulation. (From Faddy et al., 1976.)

In the initial analyses, migration and death rates were held to be constant for each follicle type throughout the lifespan, but the predicted curves did not fit the observed data. Some age dependence was then built into these rates. We had no clues from the data to such an age dependency and assumed that each transition rate of each follicle type was one constant between the ages of 0 and 30 days in immature females, and another constant after 30 days. Nineteen parameters had thus to be estimated, the initial mean size of the pool (Group I) and a migration and death rate for each subsequent follicle group before and after 30 days (as shown in Text-fig. 4). The estimates of utilized...
Growth and differentiation of ovarian follicles

Text-fig. 5. Predictions from the ovarian model (solid lines) in relation to the observed values (+) of the number of follicles in mice (a) intact from Day 0, (b) hypophysectomized after 30 days. Follicle groups are indicated. (From Faddy et at., 1976.)
Some medium-sized follicles with up to two layers of granulosa cells appear to spend a longer time in these developmental stages than others of similar size, particularly in females aged more than 255-3 days of age.

### Table 2. Mean numbers of follicles (from fitted model) leaving and entering the five groups at specific intervals throughout life in (a) normal Strong A females (mean size of Group I at age 0 days = 10,504) and (b) Strong A females hypophysectomized at 40 days of age. (Reproduced by permission from Faddy et al., 1976.)

<table>
<thead>
<tr>
<th>Age-span (days)</th>
<th>Groups^*</th>
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<th>III→IV</th>
<th>IV→V</th>
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(b)

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^* Arrows indicate the mean numbers of follicles leaving (e.g. I→) the group by growth and death (e.g. I→II) and entering (e.g. II→III) the group by growth and death from Group V in post-pubertal intact females and entering (e.g. III→IV) the group by growth.\n
21 days; the follicles accumulated in these stages, together with newly recruited follicles from the pool, are then slowly reduced throughout remaining life (Faddy et al., 1976). An excess of medium-sized follicles is thus always available for growth into later stages in all but the oldest mice (Table 2, Text-fig. 5), allowing the species-specific ovulation rate to be maintained and providing the follicular system with a degree of flexibility. Oscillations in the numbers of medium and large follicles subside after 100 days, and by 350 days follicle turnover becomes sluggish, perhaps because few remain.

Hypophysectomy during early maturity has little apparent influence on the initiation of growth or on the incidence of death of follicles from the pool, the rates for both parameters being only slightly reduced compared to those in intact mature females (Table 2, Text-fig. 5). For example, the ovaries of a normal female aged 60 days contain 3917 preantral follicles; for a female hypophysectomized at 40 days of age, the corresponding number at 60 days is 4065 (from Table 2). However, as the post-operative interval increases the cumulative effects of slightly reduced growth and death rates, taken in conjunction with the resultant larger pool size at any age, become increasingly apparent. At 510 days of age, the number of primordial follicles in intact female mice is about 260 and the corresponding figure for a female hypophysectomized at 40 days and killed 470 days later is 630.

In contrast, the effect of hypophysectomy on large follicles is clear, for within 20 days after operation they are severely reduced in number. In the absence of large follicles slightly fewer follicles leave the pool per unit time as described above, and the turnover of medium-sized follicles increases (Text-fig. 5). Follicles with two layers of granulosa cells (Group III) seem particularly vulnerable to the effects of hypophysectomy. The rate of growth into this category increases, which might indicate that large follicles exert suppressive effects on their growth in intact females, but in the absence of gonadotrophins very few are able to continue growth transition into the three-layered stage and the majority die as indicated by the increased death rate (Table 2, Text-fig. 5). A small number of follicles which survive to the three-layered stage can continue to grow spontaneously to the preantral or, occasionally, to a very early antral stage.

Resumption of follicular growth in hypophysectomized rats

Hypophysectomized rats are being used to study the resumption of follicular growth, especially in relation to the effects of gonadotrophins and steroids. Follicular changes were followed by simple morphometric methods: the number of follicles or the total of follicle profiles per unit of sectioned ovary were counted to obtain frequency distributions of the sizes of follicle profiles and estimates of their numerical, area or volume densities in the sections (Text-fig. 6). Measurements on equatorial and tangential follicle profiles were made on projected images from randomly chosen independent sections. Profile diameters were measured directly and areas and densities were estimated by a point counting method. The volume density was estimated from the area density according to the Principle of Delesse (Welbel, 1973). By 21 days after hypophysectomy, large antral stages have disappeared and the area density of follicle profiles is reduced to approximately 20% of that found in intact cyclic rats. Follicles of diameter < 175 μm were by far the most frequent, with numerical densities remaining similar to those found before hypophysectomy (Text-fig. 6). Follicles of diameter < 60 μm were not measured. Developing follicles with diameters up to 400 μm were rarely found, after hypophysectomy, and the population consisted mainly of non-growing primordial follicles and those preantral stages which are either insensitive or weakly sensitive to gonadotrophin depletion. The total number of growing preantral follicles does not change markedly, although fewer of the large preantral stages are present. The remaining multilaminar follicles are not undergoing obvious atresia. These observations indicate either that normal growth transition between early follicle stages is not maintained or that initiation and migration between stages is continued, but that an increase in atresia afflicts the later preantral stages. The latter suggestion appears to be correct, because the presence of mitotic figures in granulosa cells in multilaminar follicles implies that growth is continued, although the rates of transition may be abnormal.

The hypophysectomized rat can also be used to study the effect of gonadotrophin injections on changes in follicle growth. Injections of FSH (Armour Standard), HCG (Primogonyl; Schering) or both were used to study the changing follicle profiles a few days after the injection. Daily injections of
0.4 mg FSH induced a proportionate increase in the first 24 h in the numbers and numerical density of profiles in the classes of follicles, with mean diameters of 125, 250 and 375 μm (Text-fig 6). On the next 24 h, a slight upward shift occurred in the size distribution of follicles, apparently at the expense of the smaller size classes. During the next 48 h, substantial growth was noted with the formation of many medium and large follicles, some of ovulatory dimensions. The decreased numerical density of the smaller classes after 48 h could be related to the expansion of large follicles and the possible exaggeration of sectional areas. These changes in the numerical densities of the different follicle classes imply an increased transition of growing follicles. The increased rate of transition is probably a result of reduced follicular atresia and enhanced proliferation, but might also include a general initiation of growth in primordial follicles. The suggestion of reduced atresia would support the prediction that FSH salvages growing follicles which would otherwise die (Faddy et al., 1976), which would be contrary to the observed effects of FSH on rat ovaries in vitro (Pavlic, 1963) and in hypophysectomized rats (Harman, Louvet & Rossé, 1975). It would appear from these latter studies that FSH stimulates follicular growth, the proportion of atretic follicles is increased.

The growth of all classes of follicles in hypophysectomized rats could have been influenced by changes in the amount of circulating steroids in response to the injection of gonadotrophins. Here, the injections of FSH (0.4 mg Armour Standard daily for 4 days) had not significantly increased serum levels of several steroids above the basal levels found in control hypophysectomized animals (Table 3); this lack of a steroidogenic response to FSH is in contrast to other studies using intact rats and a short period between hypophysectomy and hormone treatment (Nuti, McShan & Metz, 1974; Grimek, Nuti, Nuti & McShan, 1976). FSH thus stimulated the growth of follicles at
absence of an increase in steroidogenesis. Four daily injections of 2 i.u. HCG stimulated steroidogenesis (Table 3), indicating that hypophysectomy had not completely destroyed the steroid-synthesizing capacity of the ovary. Oestradiol-17β was found in high amounts, implying that follicles still retained a distinct capacity to synthesize this steroid 21 days after hypophysectomy. Progesterone secretion rose only slightly, perhaps because the persistent corpus luteum in the ovaries underwent functional regression in the post-operative period. The most marked effect of HCG was to stimulate production of high levels of androgens, although these may be partly of adrenal origin.

Table 3. Steroids (mean ± S.E.M.) in the serum of intact and hypophysectomized rats treated in various ways (unpublished results of S. T. H. Chan and R. E. Gore-Langton)

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<th>Animals and treatment</th>
<th>No. of animals</th>
<th>Oestradiol-17β</th>
<th>Oestrone</th>
<th>4-Androstenedione</th>
<th>Testosterone</th>
<th>Progesterone</th>
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<tr>
<td>Intact, no further treatment</td>
<td>5</td>
<td>171 ± 31</td>
<td>144 ± 50</td>
<td>464 ± 137</td>
<td>698 ± 93</td>
<td>22,030 ± 6720</td>
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<tr>
<td>Hypophysectomized, 0-5 ml saline daily for 4 days</td>
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<td>28 ± 12</td>
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<td>24 ± 11</td>
<td>66 ± 26</td>
<td>107 ± 13</td>
<td>128 ± 23</td>
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<tr>
<td>Hypophysectomized, 2 i.u. HCG daily for 4 days</td>
<td>5</td>
<td>176 ± 46</td>
<td>268 ± 43</td>
<td>2153 ± 597</td>
<td>2584 ± 158</td>
<td>6250 ± 790</td>
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* Assayed by the methods described by Abraham (1974a).
† Injections were given s.c. and blood was collected from the dorsal aorta 96 h after the start of treatment.

FSH and LH could act synergistically to promote a greater growth of follicles. The combined effects of FSH and HCG are therefore being studied. Within 24 h of the injection of both gonadotrophins (0-4 mg FSH, 10 i.u. HCG), a few large Graafian follicles had formed (Text-fig. 6), unlike the effects of FSH or HCG alone. When the combined treatment with FSH and HCG was preceded by FSH alone for 24-72 h, the follicle profile 24 h later resembled that obtained with FSH given alone over a similar period. HCG may therefore enhance the growth responses of follicles progressing through antrum formation, but not of later stages.

Normal and abnormal differentiation and steroidogenesis in follicles approaching ovulation

Timing of human ovulation

The timing of ovulation must be closely controlled for studies on changes in steroidogenesis in preovulatory follicles. There is little chance of analysing preovulatory human follicles during the menstrual cycle by identifying the LH surge, because the time needed to assay LH is greater than the interval between the surge and ovulation. It is also difficult to organize operations within a few minutes of obtaining the results of an assay. Most observations on human preovulatory follicles during the natural cycle have been made by chance or by hopefully timing operations in mid-cycle.

We have therefore induced ovulation in women by injection of HCG at different endocrinological situations (Edwards & Steptoe, 1975). Some patients were given HCG just before the surge of LH was expected during the middle part of the natural cycle; this method resembles natural ovulation very closely since follicular growth would occur under the control of endogenous hormones. The second method was to use human menopausal gonadotrophin (HMG) to stimulate follicular growth and then invoke ovulation with HCG. Thirdly, clomiphene was given to stimulate follicular growth and HCG to induce ovulation. Laparoscopy was used to examine and assess the ovaries at various times...
after the injection of HCG. Oocytes and granulosa cells were aspirated from many follicles and also used to determine the stage of follicular growth by analysis of nuclear material and chromosomes in oocytes or the structure and secretory nature of the granulosa cells.

Our results (Table 4) show that ovulation in women begins approximately 37 h after the injection of HCG (Edwards & Steptoe, 1975). It may occur at a similar interval after the initial surge during mid-cycle (Ferin, Thomas & Johansson, 1973). Some follicles were actually choriocytic rupture at approximately 37 h after HCG, the disruption of the stigma being seen during injection. Fresh CL were noticed in several patients. The best time to study human preovulatory follicles, therefore, is approximately 32–33 h after the injection of HCG, at a time when the follicular granulosa cells and oocytes can be easily aspirated. Non-ovulatory and perhaps atretic follicles may also be present in the ovary at this time, permitting a comparison between the patterns of react in these and the preovulatory follicles.

<table>
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<th>Time after HCG (h)</th>
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<td>29–31</td>
<td>59</td>
<td>1</td>
</tr>
<tr>
<td>32–35</td>
<td>58</td>
<td>2??</td>
</tr>
<tr>
<td>35–37</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>37–38*</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>40–44*</td>
<td>9</td>
<td>6</td>
</tr>
</tbody>
</table>

* Including some data from Dr A. Lopata.

Follicular steroids in human preovulatory follicles

The concentrations of various steroids have been determined in fluids aspirated from follicles 32 h after an injection of HCG (Fowler, Chan, Edwards, Steptoe & Walters, 1974). Studies were carried out on patients given 5000 i.u. HCG just before the surge of LH secretion mid-cycle. The endocrinological situation in the ovary would resemble that occurring in the natural cycle, except for differences such as those arising through the longer half-life of HCG compared with that of LH. The follicular and luteal phases of at least one earlier cycle had been normal in each patient by daily assays of oestrogens, pregnanediol and LH on 24 h collections of urine. Analyses were carried out on urine samples taken during the treatment cycle, the gonadotrophic given when the levels of urinary hormones indicated that the surge of LH was imminent. A laparoscopy was performed 32–33 h after HCG injection and the size and morphology of the ovary were assessed and recorded. Each Graafian follicle was aspirated individually. Oocytes were found in many aspirates, and the follicle was classified as preovulatory if its oocyte possessed chromosomes in meiosis-I or was fertilized within a few hours, and non-ovulatory if the oocyte was atretic. Most aspects of the aspirate were also used to classify the follicles. Large numbers of granulosa cells, including those surrounding the oocyte (Pl. 1, Fig. 2), and the presence of a fluid in addition to normal follicular fluid indicated that the follicle was preovulatory. In ovulatory follicles, few granulosa cells were recovered, the oocyte was surrounded by corona and few cumulus cells, and the fluid contained none of the viscous material (Edwards & Steptoe, 1975).

The concentrations of steroids were measured in the fluids with the antisera and radioimmunoassays extensively described by Abraham (1974a). There was a wide range of steroid concentration in the follicular fluids (Table 5) and a large variation in the levels of steroid hormones in different follicles from the same patient. Inspection of the results suggested that follicles tended to fall into two groups. Some contained large amounts of progesterone, 17α-hydroxyprogesterone and androstenediol-17β and low levels of androstenedione, 17α-hydroxyprogrenolone, dehydroepiandrosterone, androgens, and relatively high amounts of the other steroids. Cluster analyses were therefore carried out.
data using the method described by Ward (1963). In a cluster analysis, all the available data on steroid levels in different follicles are compared, and follicles of close similarity are grouped in a manner which minimizes their within-group variance. Small amounts of fluid only were available for assays from some follicles, and the cluster analyses were therefore performed in two groups. First, data on 4 steroids (progesterone, pregnenolone, 17α-hydroxyprogrenolone and dehydroepiandrosterone) were analysed since these steroids were measured in almost all follicles. A second analysis was carried out using assays of 8 steroids, all of which had been analysed in 9 follicles.

Table 5. Ranges of concentrations of steroids in fluids of human follicles near the time of ovulation after an injection of HCG in mid-cycle (data from Fowler et al., 1977)

<table>
<thead>
<tr>
<th>Steroid</th>
<th>No. of follicles</th>
<th>Range (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>17</td>
<td>1-18,000</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>11</td>
<td>27-1800</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>11</td>
<td>9-3600</td>
</tr>
<tr>
<td>Oestrone</td>
<td>11</td>
<td>2-122</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>11</td>
<td>3-2160</td>
</tr>
<tr>
<td>17α-Hydroxyprogrenolone</td>
<td>17</td>
<td>3-450</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>15</td>
<td>1-380</td>
</tr>
<tr>
<td>Testosterone</td>
<td>11</td>
<td>1-94</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>15</td>
<td>48-1000</td>
</tr>
</tbody>
</table>
Table 6. Mean steroid concentrations (log conc. ng/ml) in preovulatory and non-ovulatory follicles (see text) as ovulation approaches in women given HCG in mid-cycle (data from Fowler et al., 1977)

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Preovulatory</th>
<th>Non-ovulatory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>8.5</td>
<td>3.7**</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>6.8</td>
<td>4.2*</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>7.1</td>
<td>3.6**</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>6.3</td>
<td>4.9**</td>
</tr>
<tr>
<td>Oestrone</td>
<td>3.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>3.3</td>
<td>7.2**</td>
</tr>
<tr>
<td>17α-Hydroxypregnenolone</td>
<td>1.7</td>
<td>4.7**</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>0.9</td>
<td>4.9**</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1.9</td>
<td>3.8**</td>
</tr>
</tbody>
</table>

* P < 0.02; ** P < 0.01 by a Randomization Test.

Text-fig. 8. Dendograms showing cluster analyses of steroids in human follicular fluid aspirated 12 h after HCG injection alone in mid-cycle (a, b) and 32 h after the HCG injection following HMG or clomiphene treatment (c). The steroids analysed were: (a) progesterone, 17α-hydroxyprogesterone, pregnenolone, 17α-hydroxypregnenolone, androstenedione, testosterone, oestradiol-17β, oestrone, (b) progesterone, pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone; (c) progesterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone, androstenedione, testosterone, oestradiol-17β, oestrone. (Dendograms (a) and (b) from Fowler et al., 1977.)
The results of these analyses (Text-fig. 8) indicated clearly that each follicle could be placed into one of the two clusters. Combining the two clusters into a single group caused a very large increase in the within-group variance, revealing the existence of two distinct clusters of follicles. Clustering on the basis of steroid concentrations was entirely consistent with the grouping of follicles as either preovulatory (Cluster I) or non-ovulatory (Cluster II) at the time of laparoscopy. The mean concentrations of steroids in the two groups of follicles differed significantly (Table 6).

One follicle was exceptional. The oocyte had undergone maturation and was fertilized in vitro, but the follicle had not luteinized as judged by observations at laparoscopy. Steroid analyses also showed that the follicle was non-ovulatory. Oocyte maturation and luteinization can occur independently under some conditions, and maturation of the oocyte without luteinization has also been observed in old rats and in atretic follicles of animals, as discussed later. This human follicle was evidently not atretic because it contained levels of steroids similar to those of non-ovulatory follicles: Oocyte maturation may therefore be stimulated more easily than luteinization in human follicles.

Similar studies have been carried out on follicles of patients given HMG and HCG and included one patient given clomiphene followed by HCG (Edwards & Steptoe, 1975). Follicular growth would have been stimulated by the injections of HMG which were given on alternate days from Day 3 until Day 9 and ovulation induced by the HCG given on Day 11.

Large numbers of Graafian follicles are present in these patients when laparoscopy is performed 32 h after the injection of HCG. Analysis of the steroids in follicular fluids revealed that the relationship between steroidogenesis and the development of follicles was more complex than when HCG was given during the natural cycle. The dendograms of the cluster analysis (Text-fig. 8) showed that the follicles did not fall into two groups; the within-group variance increased in a fairly regular manner as clustering proceeded. Most of the large preovulatory follicles contained high concentrations of progesterone, 17α-hydroxyprogesterone and oestradiol-17β, but other follicles appeared to be intermediate between non-ovulatory and preovulatory follicles. Large numbers of follicles were stimulated and at laparoscopy appeared to be in different stages of development, hence the complex steroidogenenic pattern.

These observations show that conclusions about the nature of the luteal phase following the use of HMG and HCG must be made cautiously. Since follicles in various stages of development are present after the injection of HCG, some of them may continue to develop after the preovulatory follicles have ruptured. Fluctuations in plasma or urinary hormones during the luteal stage may therefore reflect a series of subsequent ovulations and luteinizations rather than the activity of those follicles which ruptured 37 h after the injection of HCG. The relatively long life of HCG compared with that of LH might also encourage the continued luteinization of some follicles.

Fluctuations in ovarian $\Delta^2\text{-}3\beta$-hydroxysteroid dehydrogenase and $\Delta^5$-isomerase activity of rats during the oestrous cycle

There have been many studies on the nature of the control of ovarian steroidogenesis by gonadotrophins. Changes in the activity of certain enzymes involved in steroidogenesis may be one cause of the fluctuation in hormone secretion during the oestrous or menstrual cycle, as shown by the following study.

The enzymes $\Delta^2\text{-}3\beta$-hydroxysteroid dehydrogenase (3β-HSD) and $\Delta^5$-isomerase convert $\Delta^2\text{-}3\beta$-hydroxysteroids to $\Delta^4$ ketosteroids in mammalian tissue (Samuels, Helreich, Lasater & Reich, 1951). The histochemical technique of Wattenberg (1958) for the visualization of 3β-HSD activity has been used to demonstrate this enzyme in a number of steroidogenic tissues in mammals. In the rat ovary, 3β-HSD activity is seen in the granulosa cells of large follicles and in the theca interna (Pupkin, Bratt, Weisz, Lloyd & Balogh, 1966; Motta & Bourneva, 1970). In contrast, no 3β-HSD activity is seen in sheep granulosa cells at any stage of the cycle (Hay & Moor, 1975) and the ultrastructure of granulosa cells of the rhesus monkey provides no evidence to suggest that these cells are involved in steroid hormone production (Amin, Richart & Brinson, 1976).

With an in-vitro technique we have measured 3β-HSD activity in rat ovarian tissue throughout the oestrous cycle and related these changes to fluctuating levels of steroid hormones in the peripheral circulation (Readhead & Abraham, 1977).
Text-fig. 9. Pathways of steroid metabolism in the rat ovary. 1, \( \Delta^2 \)-3\( \beta \)-ol-dehydrogenase \( \Delta^2 \)-isomerase; 2, \( C_{21} \)-steroid 17-hydroxylase; 3, \( C_{17} \)-\( 20 \)-desmolase; 4, 17\( \beta \)-hydroxysteroid dehydrogenase. (From Rees & Abraham, 1977.)
Groups of rats with regular 4-day cycles were used during the morning on each day of the cycle with an additional group at 18.00 h on the day of pro-oestrus, i.e. after the LH surge had begun. Ovarian homogenates were divided and incubated with four different Δ⁴-hydroxysteroid substrates (Text-fig. 9). After incubation for 2 h the expected Δ⁴-ketosteroid product of 3β-HSD activity was measured by radioimmunoassay (Abraham, 1974a). The activity of 3β-HSD was expressed as the percentage of the Δ⁴ hydroxysteroid substrate which was converted to the corresponding ketosteroid product. Arterial blood was withdrawn from each rat and 9 different steroid hormones were measured in the serum by radioimmunoassay (Abraham, 1974a). LH and FSH were also measured in these serum samples by Dr A. F. Parlow (Daane & Parlow, 1971).

The variations in 3β-HSD activity during the oestrous cycle with the different substrates are shown in Text-fig. 10. The activity was greatest during the afternoon of pro-oestrus, the increase occurring at the same time as serum levels of LH rose from a mean of 47·6 ng/ml at 11.30 h to 500 ng/ml at 18.00 h. FSH values also showed a slight increase during the same period.

**Follicular development in women with polycystic ovaries**

Follicular development can often become abnormal through pathological or physiological causes. Many examples of pathological development occur in women and the group of related disorders collectively known as the polycystic ovarian syndrome or the Stein–Leventhal syndrome is a well-known example. The steroids in fluids aspirated from the follicles of these patients have been measured by Giorgi (1963) and Short (1964). The levels of steroids in these follicles (Table 7) resemble closely those of the non-ovulatory follicles analysed in the present study. They differ from those of growing follicles examined just before the LH surge, which contain large amounts of oestradiol-17β (McNatty, Hunter, McNeilly & Sawers, 1975), and from the preovulatory follicles analysed in the present work. Follicles accumulating in polycystic ovaries are thus non-growing and non-luteinized, and this is confirmed by evidence showing that oocytes taken from these follicles require approximately 36 h to mature in vitro, i.e. the full period of oocyte maturation (R. G. Edwards, unpublished).

A syndrome with similar effects has now been reported and named the monocystic syndrome (Delahunt et al., 1975); one large follicle is evidently arrested in development, and its output of steroids apparently resembles those found in the polycystic syndrome.

**Follicular development in rats in persistent oestrus**

The cystic ovary in rodents has sometimes been proposed as a suitable model of these human disorders. The condition, which is termed persistent oestrus, arises spontaneously as a result of ageing and may also be induced experimentally by exposure to constant illumination and neonatal treatment.
with steroids (Everett, 1964). The hall-mark of this condition is follicular development and ovulation, and this is attributed to a primary disturbance of the hypothalamic/pituitary-gonadotrophin secretion. However, the consequences of this condition for ovarian function, growth and steroidogenesis are not well understood.

The ovaries of persistent oestrous rats contain follicles of all sizes, but lack recently ovulated follicles (Pl. 2, Fig. 3). The remarkable arrest in development and accumulation of small vesicular follicles found in polycystic human ovaries does not occur in persistent oestrous rats, or in polycystic human ovaries. In rats, large Graafian follicles containing an oocyte with a germinal vesicle are characteristically present and are presumably the main source of the oestrogens responsible for the chronic stimulation of the reproductive tract. Follicle replacement could be regulated by feedback actions of pituitary hormones on intra-ovarian factors. Circulating levels of LH and FSH in rats in persistent oestrus were compared with those in rats with a 4-day cycle on the morning of pro-oestrus, when LH surge (Table 8). Levels of LH were similar in both groups, but the amount of FSH significantly higher in the persistent oestrous rats, perhaps through differences in pituitary supraphysiological ovarian steroids. There is no apparent deficiency of gonadotrophins to support follicle growth in persistent-oestrous rats: peripheral concentrations of unconjugated oestrogens are variable in the different groups, but do not exceed values attained during the oestrous cycle (Naftalin, Brown-Grant et al. 1972; R. G. Gosden, unpublished).

Table 7. Steroid concentrations (pg/ml) in the follicular fluids of women with the polycystic ovary syndrome (Stein–Leventhal syndrome) and in non-ovulatory and pre-ovulatory follicles after ovulation approaches in women given HCG in mid-cycle

<table>
<thead>
<tr>
<th>Follicle type</th>
<th>Progesterone</th>
<th>17α-Hydroxyprogesterone</th>
<th>Androstenedione</th>
<th>Oestradiol (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycystic*</td>
<td>70–130</td>
<td>&lt;23–130</td>
<td>&lt;28–1000</td>
<td>&lt;15–85</td>
</tr>
<tr>
<td>Normal*</td>
<td>90–3100</td>
<td>23–850</td>
<td>10–780</td>
<td>40–2500</td>
</tr>
<tr>
<td>Non-ovulatory†</td>
<td>103</td>
<td>101</td>
<td>1438</td>
<td>35</td>
</tr>
<tr>
<td>Pre-ovulatory†</td>
<td>7019</td>
<td>1071</td>
<td>46</td>
<td>1520</td>
</tr>
</tbody>
</table>

* Data from Short (1964) and Giorgi (1963).
† Present data.

Table 8. Circulating levels (mean ± S.E.M.) of LH and FSH in young cyclic Sprague-Dawley rats on the morning of pro-oestrus and in ageing rats in persistent oestrus (R. G. Gosden, unpublished).

<table>
<thead>
<tr>
<th>Rats</th>
<th>No. of animals</th>
<th>Plasma LH (ng/ml)</th>
<th>Plasma FSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young and cyclic</td>
<td>14</td>
<td>38 ± 3</td>
<td>223 ± 23*</td>
</tr>
<tr>
<td>Old, in persistent oestrus</td>
<td>13</td>
<td>40 ± 4</td>
<td>303 ± 23*</td>
</tr>
</tbody>
</table>

The hormone values are the NIAMDD-rat-LH-RP1 and NIAMDD-rat-FSH-RP1 antiserums.
* Significantly different (*P* < 0.02).

In our experience, the large follicles of rats in persistent oestrus induced spontaneous ovulation have a reduced sensitivity to hormonal stimulation. Some ovaries occurred when rats were exposed to large quantities of LH, either provided exogenously or by electrical stimulation of the brain, but the CL were smaller than in cyclic animals treated in the same way (R. G. Gosden, unpublished). Furthermore, quantities of LH sufficient to induce ovulation in cyclic animals resulted in the formation of a CL and oocytes of persistent oestrous rats: the oocytes became surrounded by a cumulus mass, but ovulation and luteinization failed (Pl. 2, Fig. 4). The cessation of follicle growth was the likely result of diminished sensitivity to gonadotrophins and may mark the onset of degeneration of follicles in rats. Cellular degeneration could be augmented by compression atrophy from surrounding follicular fluid. Another type of cyst containing only a simple membrane is frequently seen in these ovaries, and is probably a relic of a former follicle. Further growth of these cysts appears limited and they may regress when the follicle cells disappear.
Fig. 1. Section of an ovary from a 5-day-old female mouse showing the primordial follicles and growing follicles with one or two layers of granulosa cells. x380.

Fig. 2. A living preovulatory oocyte aspirated from a human Graafian follicle 32 h after an injection of HCG and photographed in culture. The depth of the preparation is shown by the diffuse appearance of the oocyte and its corona radiata, and by the masses of cumulus cells which extend to the edge of the illustration and beyond. Non-ovulatory oocytes possess small cumulus masses closely associated with the corona radiata. (R. G. Edwards & P. C. Steptoe, unpublished.)
Fig. 3. Ovary of a 9-month-old rat which spontaneously entered 'persistent oestrus', showing the live follicles and the lack of CL.

Fig. 4. Section through a cystic follicle of a rat in persistent oestrus showing maturation of the oocyte and secretory cumulus but no concomitant luteinization.
Factors in the initiation of follicular growth

Although the mechanisms involved in the initiation of follicular growth are unknown, several principles appear to govern the loss of oocytes from the non-growing pool. In mice, the use of a semilogarithmic transformation of the data implied that a constant proportion of oocytes is lost per unit interval of time with increasing age (Jones & Krohn, 1961a). For example, from the data on mice of strain A, the mean proportional reduction in oocytes from the total population over each 100-day interval by growth, ovulation and death is the same, irrespective of age (Table 9). The principle of a constant proportion of loss appears to hold true for all the strains investigated with each strain having a characteristic rate of reduction in numbers of oocytes. A similar conclusion was drawn for strain A from predictions from the model (see p. 241) during maturity (Faddy et al., 1976).

Table 9. Relationship between the total number of oocytes and the probability of oocyte loss in virgin A-strain mice (data from Jones & Krohn, 1961a) and rats (data from Mandl & Zuckerman, 1951)

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Estimated total oocyte population</th>
<th>No. of oocytes lost /100-day interval</th>
<th>Proportion of oocytes lost /100-day interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>00</td>
<td>8531</td>
<td>4381</td>
<td>0.514</td>
</tr>
<tr>
<td>100</td>
<td>4410</td>
<td>2132</td>
<td>0.514</td>
</tr>
<tr>
<td>200</td>
<td>9887.7</td>
<td>1036.3</td>
<td>0.514</td>
</tr>
<tr>
<td>300</td>
<td>4775.3</td>
<td>504.2</td>
<td>0.514</td>
</tr>
<tr>
<td>400</td>
<td>232.3</td>
<td>245.2</td>
<td>0.514</td>
</tr>
<tr>
<td>Rats†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>4064</td>
<td>1140</td>
<td>0.281</td>
</tr>
<tr>
<td>200</td>
<td>2924</td>
<td>514</td>
<td>0.176</td>
</tr>
<tr>
<td>300</td>
<td>2410</td>
<td>311</td>
<td>0.129</td>
</tr>
<tr>
<td>400</td>
<td>1888</td>
<td>211</td>
<td>0.101</td>
</tr>
<tr>
<td>500</td>
<td>1724</td>
<td>154</td>
<td>0.082</td>
</tr>
</tbody>
</table>

* Calculations were made from the regression equation:
  \[ \log y = -4.481 - 0.0013 (x - 143.704) \]

† Calculations were made from the regression equation:
  \[ \log y = 4.561 - 0.476 \log x \]

Analysis of the numbers of follicles actually growing indicates that the fraction of growing: non-growing oocytes in Bagg strain mice increased as the size of the pool decreased, either as a result of age or of treatment with dimethylbenzamine (Krarupe, Pedersen & Faber, 1969). A similar dependence on pool size of the proportion beginning to grow has been demonstrated by reducing the pool in neonatal mice by treatment with androgen (Peters, Byskov & Faber, 1973). Such evidence implies that there is an increasing likelihood of a follicle leaving the pool by growth as the female ages. Observations from our fitted model support some of these conclusions. Calculations based on the model show that the ratio of developing: small follicles increases up to the age of 100 days, but thereafter remains constant despite the continued reduction in size of the non-growing component (unpublished observations).

When taken together, the indications of a constant proportion of follicle loss from all causes (A, CBA and RIII strains) and an increasing probability of growth initiation (Bagg strain) imply that the loss of oocytes by degeneration decreases with age as the pool is depleted. A decreasing fraction of atretic oocytes was reported with increasing age in strain A mice (Jones & Krohn, 1961a), although not in other strains, and any generalizations would be inappropriate. Conclusions from our fitted model show that loss by death was very high between 0 and 30 days and much less in older females.
These relationships in mice suggest that the pool of non-growing follicles might be composed of a resting population possessing a distribution of probabilities for utilization and specifically for initiation. These parameters may be strain-specific. Changes in the numbers of follicles in different stages of development according to age might reflect the effects of age, selective depletion, or from the pool by growth or death and the action of intra- or extra-ovarian factors. Such differences display species variation, the particular relationship between growth initiation and pool size may be incidental rather than causal. For instance, the data of Mandl & Zuckerman (1951) and recently on oocyte loss in rats showed that the relationship between the total number of oocytes and age is represented linearly by a double logarithmic transformation, i.e. a function different from that in mice. Use of this double logarithmic transformation implies that the proportion of oocytes in the total population per unit time interval decreases with increasing age (Table 9). If this rule in the rat is valid, it contrasts with the constant proportionate loss found in mice, but more data are needed to confirm that such species differences do exist.

Asynchrony during meiotic prophase and the sequential formation of follicles in the neonatal ovary may provide a basis for understanding how a distribution of probabilities for initiation of growth might arise. If oocytes in late-forming follicles have fewer chiasmata, as smaller follicles are conserved until the end of reproductive life, the implication is that these follicles lower intrinsic probability for the initiation of growth. The small follicles with the higher a probability of growth would thus be the first to be depleted from the pool by initiation, and oocytes ovulated would be those with the highest chiasma frequency. A progressive decrease in chiasma frequency would occur as pool size was reduced. We have no idea at present of the biological causes leading to such postulated differences in the probability of follicular growth. One approach would be to find out if other species show a distribution of chiasma frequency within the mouse, and the rat will be a suitable species to examine since meiotic prophase is better conserved in oocytes in the fetal rat (Beaumont & Mandl, 1965) than in the mouse. In sheep including man, the initiation of meiosis in oocytes takes place over a much longer time, perhaps as long as 2 or 3 months of fetal life, and more studies are needed on chiasma frequency in oocytes to decide if the sequential utilization of oocytes occurs in the human ovary and hence for chromosome errors in human fetuses (Fowle & Edwards, 1973). The number of follicles in the pool in human ovaries declines with age in a manner generally similar to that in mice (Baker, 1972), but more studies are needed to establish that the details of follicular growth are the same in these species.

The dynamics of follicle growth

New techniques are needed to study the growth of follicles. The timing of follicle growth, for example, is imprecise in the absence of methods for selectively labelling them at particular stages of development. The number of follicles passing through any stage at any specific age will be influenced by the time of the follicles leaving the pool, and by growth and death rates in earlier stages of development.

A model such as the death/migration model described in the present work (p. 229) describes the relationship between follicles at different stages of development from birth to senescence. It separates growth and death parameters for all follicle groups at any age. Static histological sections on numbers of follicles are transformed into a dynamic picture of the turnover of follicles at different developmental stages throughout life. All the parameters in such a model are interdependent, and alteration in one implies change in another. Each parameter can be confirmed or denied independently by different methods of analysis. Some conclusions drawn from the model agree with data obtained from methods such as tracer dilution (Pedersen, 1972), e.g. the large number of follicles leaving the pool during the first 30 days, and the lower rate of growth in follicles in groups of 30 days. Other conclusions, e.g. the further decrease in the growth of some medullary follicles and the continuing low level of death in Group 1 after 30 days (Faddy et al., 1976) are in accord.

The model has indicated a considerable regularity in follicular growth throughout the follicular cycle, and the ovarian system will be even more regular if formal proof is obtained of a sequential follicular cycle.
utilization of follicles. Interrelationships appeared to exist between the various groups of follicles, i.e. the numbers of medium-sized follicles subside as larger follicles reach a maximum, implying that there may be feedback effects from large follicles. Similar conclusions about the feedback effects of follicles have been reached by Peters et al., (1973), although they suggested that such a mechanism acted primarily by reducing the initiation of growth of follicles from the pool. Chalones may also exert feedback effects (Bullough, 1973; Clermont & Mauger, 1976). Atresia appears to assume a new significance as a regulatory device, especially during immaturity. An explosive migration of follicles from the pool is prevented during this period by a high death rate operating against Group-I follicles. Furthermore, despite the elevated levels of gonadotrophin circulating in immature female mice (Dullaart, Kent & Ryle, 1975), many follicles die during the transition from the 3- to 4-layered stage and the number available for growth to larger sizes is thereby controlled. During maturity, there is a 10-fold reduction in atresia of follicles in the pool and growth proceeds with negligible death to the 4-layered stage, after which follicle survival is influenced by the degree of gonadotrophic stimulation. The stockpiling of follicles noted in Groups II and III clearly needs further analysis; some form of selection could occur since some of these follicles appear to grow more slowly than the others and might be defective.

Using this analytical approach we can predict the numbers of different types of follicles present in the ovaries of mice at different ages and, for the first time, attempt to estimate the numbers which move into and out of each developmental group and the numbers in each group which die per unit interval of time throughout the lifespan (Faddy et al., 1976). This approach offers the opportunity for detailed studies of the influence of various treatments on follicle growth, and hypophysectomy was used as an example in the present work. Analyses are needed on the effects of oral contraceptives, especially in relation to their suppression of the development of large follicles together with any consequential effects on the turnover of medium-sized follicles. Similar studies are also needed on follicle growth in rodents given androgens neonatally, which interferes with normal ovarian development, or in female mice carrying the gene Ws which greatly reduces the size of the follicle pool (Mintz & Russell, 1957). Few primordial follicles are present in XO women (Singh & Carr, 1966), and the Ws gene may provide an animal model for this clinical condition. We may be able to use the model predictively. The parameters of follicle growth and death are currently being analysed in three more strains of mice. Any strain differences will identify those stages of follicle growth and death most susceptible to genetic variation, and could indicate the underlying physiological controls. Strain differences in levels of gonadotrophins or steroids, for example, might influence specific stage(s) of follicle growth, and follicular activity could then prove to be predictable, based on parameters such as the number of follicles in the stem population and particular rates of utilization.

Follicular growth after hypophysectomy

The analysis of follicle growth and death in mice hypophysectomized after puberty illustrated the effects of gonadotrophin withdrawal on follicles of different sizes. A considerable depression was noted in the growth of large follicles, their numbers being severely reduced within 20 days. Few follicles persisted beyond the 2-layered stage (Group III), and these follicles appeared highly sensitive to hypophysectomy. However, the rate of migration into this group from Group II increased, implying again that large follicles may exert inhibitory feedback effects on certain of the earlier stages of growth. The rates of death and migration from Group I were reduced, but these effects were slight and became increasingly apparent only after longer intervals. They were most clearly illustrated by comparing hypophysectomized and intact females at advanced ages: by 510 days of age the ovaries contained 630 and 260 primordial follicles respectively. In an intact female, about 17 primordial follicles leave the pool over a 24-h period by growth at the age of 60 days; in a female hypophysectomized at 40 days, 13 follicles begin to grow during the corresponding time interval. Failure to establish statistically significant differences between total numbers of follicles in intact and hypophysectomized rats (Ingram, 1953) may have been due to the relatively short time over which observa-
Initiation of follicular growth from the pool in mice is largely independent of gonadotrophic stimulus or of any suppressive effects of antral follicles following hypophysectomy. Our observations on hypophysectomized rats agree with this conclusion from the numbers of growing follicles in early preantral stages. Similar studies are needed in hypophysectomized at birth, when both follicle death and migration are very high and are susceptible to slight changes in levels of hormones.

FSH is an adequate stimulus for the resumption of normal follicular growth in the hypophysectomized rat, even in the absence of increased steroidogenesis, for it enhances the numbers of growing follicles by increased migration and rescues the larger preantral stages from early atresia. Hypophysectomy does not prevent the follicle cells from acquiring sensitivity to FSH, as is evident from receptors, although the continued preantral growth seen after hypophysectomy indicates that these stages are not dependent on FSH. The enhanced growth of early follicles after FSH stimulation could result from the influence of growth-promoting substances elaborated by large follicles. The production of steroids above basal levels is not essential for growth. Nevertheless, an increase in the number of oestrogen-secreting follicles is associated with the enhancement of FSH receptors in medium-sized growing follicles (Voutilainen et al., 1972; Goldenberg et al., 1973) and make them more sensitive to growth stimulus; conversely, a reduction in the number of large follicles might lower the sensitivity of early follicles to the growth stimulus. Oestrogens also prevent the early follicular arrest on FSH (Harman et al., 1975) and may thus play a role in enhancing the sensitivity to FSH and reducing atresia. In the intact animal, gonadotrophins may induce the release of steroids to potentiate the growth stimulus and therefore regulate the balance between numbers of antral follicles.

HCG alone did not increase the numbers of growing follicles, although it did stimulate growth. Oestrogens synthesized in response to HCG had evidently not influenced follicular growth despite their mitogenic effects. Nevertheless, HCG may augment the action of FSH on follicular growth through the production of oestrogen as described above, and also through the production of peptides and androgens, which may assist in the binding of gonadotrophins (Mizejewski, 1976). HCG may not have been due to methodological problems in distinguishing rapid follicular development from pre-existing large follicles. HCG also failed to promote the growth of antral follicles after priming them with FSH, which might have been due to methodological problems in distinguishing rapid follicular development from pre-existing large follicles. HCG also failed to promote the growth of antral follicles after priming them with FSH, perhaps because binding sites for HCG appeared on granulosa cells and led to the initial stages of luteinization (Zeleznik et al., 1976). HCG is also a potent growth stimulator of preantral follicles and inhibited the growth of antral follicles after priming them with FSH, perhaps because binding sites for HCG appeared on granulosa cells and led to the initial stages of luteinization (Zeleznik et al., 1976).

Our observations on follicular growth in hypophysectomized rats and mice have not been in relation to the possible inhibitory effects of large follicles, and to the stimulatory effects of FSH released from antral follicles. Clearly, there could be complicated interrelationships between follicles of different sizes at different times. This situation would not be unusual, as it has been noted to exist in other types of tissue.

**Does the oocyte or granulosa cell control the development of the follicle?**

We have outlined evidence to show that the control of follicular growth is exerted at various stages throughout the formation, utilization and luteinization of the follicle. The oocyte or granulosa cells could control follicular development, assuming that the ovarian stroma is not involved, and we will discuss briefly the relative importance of these two types of cell.

The role of the oocyte in determining the initiation of growth in follicles was stressed in the development of the oocyte and follicular diameters (Brambell, 1928), for enlargement of the oocyte is the first sign of growth of follicles in the pool followed by the proliferation of granulosa cells function as nurse cells, with many proteins being transmitted into the oocyte through the nexuses between these cells and the oocyte membrane (Zamboni, 1974). Initial enlargement of the oocyte could reflect the increasing activity of granulosa cells rather than the endogenous cell growth of the oocyte. Evidence for protein synthesis by oocytes is meagre and the ooplasm lacks storage.
somata and contains a scattered endoplasmic reticulum. RNA is synthesized at various stages of development (Wasserman & Letourneau, 1976), but evidence for the production of messenger RNA as opposed to other classes of RNA has yet to be obtained. Such observations question the role of the oocyte in initiating follicular growth.

Removal of the oocyte from its follicle is reported to result in immediate luteinization (El-Fouly, Cook, Nekola & Nalbandov, 1970), and the presence of oocytes in cultures of granulosa cells inhibits luteinization in vitro (Nekola, Stoklosowa & Nalbandov, 1971). Dictyotate oocytes may thus inhibit follicular luteinization, an influence negated by their resumption of meiosis in response to LH. However, surgical interference with Graafian follicles without removing the oocytes can also induce luteinization in several species.

Several lines of evidence indicate that the granulosa cells control follicular development. In many species, meiosis is arrested and oocytes enter dictyotene in the fetal or neonatal ovary as they are enclosed by granulosa cells during the formation of the primordial follicle; those oocytes which evade granulosa cells continue meiosis to metaphase-II and become atretic (Ohno & Smith, 1964). Proliferating granulosa cells appear to be controlled in a manner similar to that of other mitotic tissues, e.g. epithelia, haemopoietic and liver cells. Some resemblances with the haemopoietic system are very close, for example in migration of primordial germ cells and primary haemopoietic cells from their origin in the primitive streak or yolk sac (see Ritter, 1975). Follicle growth might be analysed in terms of mitotic regulation in granulosa cells, although the comparison is not strict for follicles obviously begin growth as a complete unit rather than as groups of individual cells. The terminal period of cytodifferentiation in epithelia and other systems, e.g. keratinization, could be represented by luteinization and the production of mucopolysaccharides in enlarging follicles. Chalone or other compounds influencing follicular growth remain to be identified; the initiation of growth in mouse follicles is impaired by fluid withdrawn from large bovine follicles (Peters et al., 1973), although this effect is apparently not mediated by specific proteins (Andersen, Krell, Byskov & Faber, 1976).

The endocrine activity of follicles could also indicate the importance of granulosa cells in controlling follicle growth. Our analyses of steroids in follicular fluid and observations on cultures of human granulosa cells (unpublished) indicate that these cells are involved in both aromatization and luteinization after injection of HCG. The changing levels of oestrogen during the follicular and luteal phases of the cycle could therefore reflect the activity of granulosa cells, exerted through the differentiation of gonadotrophin receptors. The relative lack of receptors in granulosa cells taken from hypophysectomized rats has been reported to prevent the full maturation of the oocyte in vitro (Erickson & Ryan, 1976).

Observations on cystic and atretic follicles also imply that the granulosa cells determine the integrity of the follicle. In rats in persistent oestrus, administration of LH causes the cells surrounding the oocyte to become secretory and the oocyte to mature, but luteinization does not occur. The corona radiata and cumulus cells thus appear to control oocyte maturation, while the granulosa cells maintain the integrity of the follicle. Oocyte maturation is well known to occur in atretic follicles as the granulosa and corona radiata cells disperse.

Changes in steroidogenesis as ovulation approaches

A surge of LH at mid-cycle or an injection of HCG induces various types of changes in the endocrine activity of preovulatory follicles. The major effect of LH on steroidogenesis involves an increased conversion of cholesterol to pregnenolone, so making pregnenolone increasingly available for further conversions (Ichii, Forchelli & Dorfman, 1963; Koritz & Hall, 1965; Hall & Young, 1968; Armstrong, Lee & Miller, 1970). Our data on the high levels of pregnenolone in the follicular fluid of preovulatory human follicles would support this contention.

Our results in rats indicate that LH also exerts another action, enhancement of the activity of \( \beta \)-steroid dehydrogenase and \( \Delta^5 \) isomerase. Each of the \( \Delta^5 \) substrates utilized was increasingly converted to its corresponding \( \Delta^4 \) product in the rat ovary after the LH surge, showing that the
activation of these enzymes is a direct result of the LH surge. Increased 3β-HSD activity can be achieved in several ways: the number of cells with 3β-HSD activity could increase, more substrate may become available, the concentration of co-factors could increase, or the amount of enzyme could rise. Significant changes in cell number are an unlikely explanation since 3β-HSD increases during the afternoon of pro-oestrus during a period of about 6h and the overall mean number of cells in the ovary presumably changes very little during this time. Substrate was not a limiting factor in the present work, because excess substrate was added to the homogenate during the experiments. The increase in 3β-HSD activity after the LH surge is more likely through an increased availability of co-factors or the induction or activation of enzymes.

Experiments are underway to test these possibilities.

Two types of follicle were present in the human ovary as ovulation approached after an injection of HCG in mid-cycle. They could be clearly identified, as shown by the complete coincidence of their classification at laparoscopy and by the cluster analysis of steroids in follicular fluid. One follicle was about to ovulate, whereas the other was still non-ovulatory. Preovulatory follicles are large and contained high concentrations of progesterone, 17α-hydroxyprogesterone and oestradiol 17β. These observations agree with earlier evidence in the human (Edwards et al., 1972; Mcintosh et al., 1975) and the rat described above showing that progesterone production begins to increase with the time. These type I or smaller and non-ovulatory, contained larger amounts of Δ4 steroids and androgens. These may be diluted in preovulatory follicles through an increase in diameter from 4 cm to 3 cm. The production of Δ4 steroids may be relatively constant during the menstrual cycle in contrast to the fluctuating production of progesterone, 17α-hydroxyprogesterone and oestradiol-17β.

This conclusion is consistent with measurements of the ovarian contribution to Δ4 steroids and androgens during the human menstrual cycle (Abraham, 1974b; Kim, Hosseinian & Dью, 1976). Alternatively, large preovulatory follicles may increasingly utilize Δ4 steroids or aromatization as ovulation approaches, so decreasing their concentration in follicular fluid.

Tentative conclusions can be drawn from our data on transformations carried out by non-ovarian cells in the human ovary in response to HCG or a surge of LH. Steroids and androgens produced by non-vascularized thecal cells may be transported more rapidly into plasma than those produced by non-vascularized granulosa cells, because steroids in follicular fluid are released only slowly into circulation (Giorgi, Addis & Colombo, 1969; Young & Short, 1970; Edwards, 1976). Differences may exist as the rate of diffusion from sheep follicles cultured in vitro appears to be.

(Seamark, Moor & McIntosh, 1974). The high levels of progesterone and oestradiol-17β in follicular fluid indicate that both hormones may be synthesized primarily by granulosa cells, and that these cells convert androgens to oestrogens in culture, in addition to synthesizing progesterone and 17α-hydroxyprogesterone (unpublished). Some of the oestradiol-17β produced in the follicle luteal phase of the cycle could therefore arise from granulosa cells. This could explain the initial neonatal and post-menopausal human ovaries lacking these cells, whereby conversions occur via the Δ4 pathway and androgens are synthesized, but conversions via the Δ5 pathway and aromatase are not found (Shenker, Polshuk & Eckstein, 1971; Payne & Jaffe, 1974). Thecal and granulosa cells may thus cooperate in steroidogenesis as initially proposed by Falck (1959). Nevertheless, cells appear histologically more active when the synthesis of oestradiol is maximal, and these cells display no ultrastructural indications of steroidogenic activity until just before ovulation (Baker, McNatty & Neal, 1975; Amin et al., 1976).

Defective follicular growth

Various anomalous forms of follicular growth and luteinization occur in mammals in vivo. Growth can be arrested at specific stages, leading to syndromes in which the accumulated follicles release androgens and other steroids as in human polycystic ovaries. Our observations would then be that these follicles are similar to the non-ovulatory Graafian follicles in human ovaries at mid-cycle.

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may have accumulated through the absence of feedback effects within the ovary from larger follicles.

The large cystic follicles containing a vesicular oocyte in rats in persistent oestrus are quite different, and have evidently progressed beyond the stage of maximal sensitivity to LH. Some follicles can still undergo ovulation in response to LH, but the response of others is restricted to secretion by corona cells and oocyte maturation. These observations are consistent with the finding that the various physiological systems in rat follicles sensitive to LH, i.e. oocyte maturation, luteinization and follicular rupture, can be stimulated independently and display different thresholds for activation (Gosden, Everett & Tyrey, 1976). The afternoon of pro-oestrus is the optimal time for rat follicles to receive an ovulation-inducing stimulus of LH, and early or late exposure to the hormone may account for such partial responses. This unusual form of atresia evidently afflicts fully grown follicles, a situation perhaps arising through the availability of large amounts of FSH during follicle growth followed by a lack of LH. A comparable situation could arise in human ovaries in the monocystic ovary syndrome, or when the ovaries are stimulated by FSH during treatment with HMG or clomiphene. One example of this type of impaired follicular reactivity was evidently found after the injection of HCG into women in mid-cycle. Abnormal follicle growth may also arise through deficiencies in the hypothalamic pituitary axis, and deficiencies in hypothalamic catecholamines involved in the release of LH have been claimed to cause persistent oestrus and other forms of ovarian failure in ageing rats (Meites & Huang, 1976). Similar situations may arise in women. More information is needed, however, on the role of factors such as prolactin in such disorders, because hyperprolactinaemia is also known to disturb ovarian activity in women.

Information on the longevity and fate of follicles in persistent oestrous rats is required: some of them appear unusually enlarged (cystic) with a thin membrane granulosa containing dividing cells, and they are comparable to the large follicles found in animals when the endogenous surge of gonadotrophins is blocked on successive days by barbiturates (Freeman, Butcher & Fugo, 1970). This appearance suggests that the existing set of large follicles continues to grow when the ovulatory stimulus is arrested, a situation evidently differing from the polycystic ovary syndrome in women. If follicles do not persist in constant oestrous rats for at least 1 day they would have to be replaced unusually rapidly to maintain the characteristic follicular constitution of the ovaries. Perhaps the existing set of large follicles is not replaced synchronously, but each follicle is replaced individually as it reaches the end of its functional lifespan. More information is therefore needed to decide whether follicle growth is continuous, the transitions between stages being similar to those in normal ovaries, or whether large follicles persist for several days or longer and influence the growth and death rates of earlier stages.

Comparison of hormone profiles in persistent-oestrous rats and in women with polycystic ovaries reveals another difference. The latter usually have elevated levels of LH with low levels of FSH (De Vane, Czekala, Judd & Yen, 1975; Rebar et al., 1976), virtually the converse of the persistent-oestrous condition. This abnormal profile in women could account for the arrest of follicle development, and the surgical reduction of the ovarian mass or other treatments applied to these patients may increase the secretion of gonadotrophins, even temporarily, and so alleviate the condition. We do not yet know whether circulating androgens are elevated in persistent-oestrous rats as in women with polycystic ovaries (De Vane et al., 1975).

The persistent-oestrous rat thus seems of limited value as a model for the polycystic ovary syndrome, but has potential significance for the study of another biological problem. The persistence of large follicles in such rats represents a period of preovulatory ageing of the oocyte distinct from other putative age changes resulting from gradients of oocyte formation in the developing ovary or from the accumulation of mutations during the prolonged dictyate phase. The normality of rat oocytes ovulated after so-called preovulatory or intrafollicular ageing has been extensively studied by Butcher (1975), who delayed ovulation by barbiturate injections or studied females with prolonged oestrous cycles. His conclusion that ‘over-ripe’ ova have reduced viability and are predisposed to chromosomal anomalies cannot be applied to persistent-oestrous rats until their rates of follicle growth and turnover have been determined. More information is also needed on follicular growth under the conditions described by Butcher (1975) before definite conclusions can be reached about the genetic consequences in oocytes.
PRIMORDIAL

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RESPONSE TO
GONADOTROPHIN

INSENSITIVE STAGE

FEEDBACK BY
FOLLICULAR

FACTORS, OESTROGENS,
CHALONES?

ATRESIA

HYPOPHYSECTOMY

SENSITIVE STAGE

POLYCYSTIC OVARIAN
SYNDROME (man)

DEPENDENT STAGE

INDOMETHACIN

OVULATION

CYSTIC
FOLLICLES

constant light (rats)
nymphomaniacs (cows)

Text-fig. 11. A representation of defective ovarian conditions, showing their relationships to the stages of follicle growth.

We wish to conclude this review with a simple plan of follicular development from the primordial follicle to ovulation, indicating when various disorders arise (Text-fig. 11). Most of the disorders indicated involve an inhibition of the growth of follicles. Inadequate gonadotrophic stimulation preovulatory follicles results in the formation of follicular cysts which then become refractory to further stimulation, presumably as the number of LH receptors declines. The Stein-Leventhal syndrome affects an earlier stage of follicle growth, and hypophysectomy virtually blocks the formation of all antral follicles. We have also indicated the deficiency of primordial follicles under certain genetic situations, and how large numbers of primordial follicles are destroyed by irradiations. A single example is given of treatments suppressing preovulatory changes in female, namely the inhibition of follicular rupture by indomethacin; other drugs are known to interfere with steroid biosynthesis at this time.

The possible influence of ovarian factors on the early stages of follicular growth is instructive. Some of these compounds, notably oestrogens, are obviously under the control of gonadotrophins which may thereby indirectly influence these early stages of growth. We have not indicated suppressive effects large Graafian follicles on their smaller neighbours, or of CL on follicles of ipsilateral ovary, since such effects remain speculative.

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Autofluorescent Particles of Human Uterine Muscle Cells

Roger G. Gosden, BSc, PhD, Hal K. Hawkins, MD, PhD, and Carole A. Gosden, BSc

Smooth muscle tissue collected from the uterine fundus of 24 patients undergoing hysterectomy was examined for chromolipoid pigments by histochemical and electron microscopic techniques. Certain cytoplasmic particles were found, mainly in smooth muscle cells, which exhibited characteristic auto fluorescence, sudanophilia, and acid phosphatase activity but did not correspond to any typical pigment described previously. These particles were present in all subjects and they tended to increase in number with age. Chemical tests on tissue lipid extracts failed to prove that vitamin A was responsible for the fluorescence. The ultrastructural appearance of the particles was somewhat variable, but most particles were rounded and of low electron density, with a lucent central space and dense bodies, probably lysosomes, at the periphery. The whole complex was enclosed by a single trilaminar membrane. (Am J Pathol 91:155-174, 1978)

It has been known for many years that some nondividing and rarely dividing cells, such as neurons and cardiac muscle, accumulate chromolipoid pigments as they age.\(^1\)\(^2\) The term "chromolipoid" is used to describe a heterogenous group of substances otherwise known as ceroid, lipofuscin, and "Abnutzungspigmente" ("wearing away" pigment) that are thought to be composed, at least in part, of oxidized fat. Similar pigments have been observed in the uteri of monkeys,\(^3\) pigs,\(^4\) rats,\(^5\)\(^6\) and mice,\(^7\) in which the quantity may be related to parity, nutritional status, or age. Occasional reports have indicated that comparable changes may occur in the human uterus\(^8\) and fallopian tube.\(^9\)

In the present study uterine smooth muscle tissue was examined for chromolipoid pigments in patients of differing age and reproductive history. Typical pigments were not identified in any of the subjects studied, but weakly auto fluorescent particles which appear to have been hitherto undescribed were observed.

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Materials and Methods

Midfundic myometrium was collected from 24 patients undergoing hysterectomy for a variety of gynecologic disorders or sterilization at Duke Hospital. The patients were of mixed age (15 to 61 years), parity (0 to 8), race, and socioeconomic group. Greatly distorted tissue specimens were discarded, but, in the case of uteri with mild focal leiomyomatosis, areas of tissue devoid of lesions were used.

Light Microscopy

Small pieces of tissue were fixed in neutral-buffered 10% formalin or formaldehyde fixative. Part of each specimen was sectioned at 7 μm with a freezing microtome; a thin part was dehydrated in alcohol and embedded in paraffin wax before being sectioned to the same thickness. Some sections were mounted directly under water or aqueous mounting medium for microscopic examination. For ultraviolet fluorescence, a Carl Zeiss photomicroscope with dark-ground illumination was used with exciter filters KP 590 (LP 595) and LP 545; barrier filters KP 560 and LP 520, and a Philips CS 200W mercury vapor lamp as the light source.

Other sections were stained with hematoxylin and eosin or subjected to histochemical tests for chromolipid pigments. Since there is no single absolutely specific test for chromolipid pigments, several tests were used, including Schmorl’s ferricyanide, Nile blue sulfate, Ziehl–Neelsen, periodic acid–Schiff. These methods were described by Perl and Tirmann method.10 was used to determine the presence of iron and hemosiderin, and subsequently demonstrated by staining with Sudan black B or Sudan III in alcoholic solution with osmium tetroxide in sym-collidine buffer. Acid phosphatase activity was demonstrated in formalin-fixed frozen sections, using the modification of Burstone’s method employing naphthol AS–TR phosphate as substrate.11 The slides were scanned by low-power light field microscopy and subsequently at higher power with oil immersion.

The relative numbers of the autofluorescent and of the sudanophilic particles (Results) were determined for each uterus by examining sections blindly and subsubjectively for differences in particle concentration (+ = low, ++ = intermediate, +++ = high). The maximum and minimum diameters of the sudanophilic particles were measured for the same specimen, using a calibrated eyepiece micrometer. Two hundred particles were measured in most specimens, and both the average particle size and frequency distributions were determined.

Electron Microscopy

Pieces of tissue with a maximum dimension of 0.5 mm were fixed by immersion in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 hour. In addition, some from some specimens was fixed in 1% osmium tetroxide in 0.1 M sym-collidine buffer, pH 7.2. Post-fixation was carried out in the 1% osmium tetroxide solution. Tissues were stored in buffer and some were then exposed to uranium acetate in veronal acetate buffer before being dehydrated in graded ethanol solutions, washed in propylene oxide, and embedded in Epon 812 according to the method of Luft.12 Thin sections were cut with a diamond knife and subsequently examined with the electron microscope.

Preliminary Analyses of Lipid Extracts of Myometrium

Freshly excised myometrium was lyophilized and stored in darkness in sealed vials containing nitrogen at −15 °C. The tissue was powdered with a porcelain pestle and mortar and subsequently homogenized with a ground-glass homogenizer in spectroscopic grade chloroform–methanol (2:1, v:v) in the proportions 1:20, w:v. The homogenates were mixed with an equal volume of deionized water and centrifuged at 1000g; the layers were separated. Fluorescence spectra of the chloroform-rich and the aqueous layers...
Table 1—Comparison of Morphologic and Histochemical Characteristics of Myometrial Particles With Typical Chromolipoid Pigments

<table>
<thead>
<tr>
<th>Property</th>
<th>Myometrial particle</th>
<th>Chromolipoid pigment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary fluorescence with ultraviolet light</td>
<td>+ (greenish hue)</td>
<td>+ (yellow-orange)</td>
</tr>
<tr>
<td>Natural pigmentation</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sudanophilia</td>
<td>+</td>
<td>+ or -</td>
</tr>
<tr>
<td>Osmiophilia</td>
<td>very weak</td>
<td>+</td>
</tr>
<tr>
<td>Solubility in chloroform-methanol</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hematoxylin and eosin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reduction of ferricyanide solution</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Acid-fastness</td>
<td>-</td>
<td>+ or -</td>
</tr>
<tr>
<td>PAS test</td>
<td>-</td>
<td>+ or -</td>
</tr>
<tr>
<td>Nile blue sulfate</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Iron reaction</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* From Strehler,1 Wolman,2 and Pearse.9

Immediately recorded with a Farrand spectrofluorometer Mark I, using quinine sulfate as a standard (1 μg/ml of 0.1N H₂SO₄).

Chemical tests were carried out on additional tissue extracts to determine whether the fluorescent material might be vitamin A. Water-washed chloroform extracts of 100 mg lyophilized uterine muscle were dried under a stream of nitrogen. The residues were taken up in 0.1 ml chloroform and mixed with an equal volume of either antimony trichloride solution (Carr-Price reagent) or trifluoroacetic acid (TFA) on a white spotting tile. Both reagents are known to produce bright blue coloration with vitamin A.13,14 Immediately after mixing further muscle extracts with one of the reagents, the maximum emission of the product was determined using a Uni Cam SP800 spectrophotometer. The previously discussed tests were also applied to similar extracts of fresh mouse liver and to synthetic trans-retinol (Sigma), 25 ng of which gave a visible product.

Results

Light Microscopy

Freshly excised specimens of uterine smooth muscle exhibited very little grossly visible pigmentation. They all contained autofluorescent and sudanophilic particles in the cytoplasm, although they were not present in every cell. The negative results obtained with several histochemical tests indicated that these particles differed from the classic chromolipoid pigments (Table 1).

The autofluorescent particles disclosed by ultraviolet fluorescence microscopy were of a greenish hue and faded within a few minutes when exposure to the light source continued. They were approximately spherical or ellipsoid and were widely distributed in the uterine smooth muscle cells and, to a lesser extent, in the walls of small caliber blood vessels. Macrophages with or without autofluorescent particles were not identified in this tissue. The autofluorescent material was not always evenly distributed within the particle but often appeared more intense at the peripheral
or polar region (Figure 1). The size and distribution of these particles paralleled those of the sudanophilic particles (Figure 2), indicating that the two tests were revealing the same structures. Of the three lipids tested, Sudan black B gave the most intense staining reaction for autofluorescent and sudanophilic material was removed from tissue which had either been immersed in chloroform-methanol for 1 hour or subjected to the paraffin-embedding procedure. This material appeared refractile in frozen sections and seemed to correspond to the acid phosphatase-positive bodies in the cytoplasm.

The concentration of autofluorescent and sudanophilic particles in uterine muscle cells was found to increase with age (See Table 2 for data on autofluorescent particles) but appeared to bear no clear relationship to other factors such as stage of menstrual cycle, reproductive history, menopause, reason for surgery, or race. The particle concentration in premenopausal women was not compared directly with the results for nonpregnant individuals because of the marked hypertrophy of muscle cells in the former group. The concentration of the sudanophilic particles paralleled those of the autofluorescent particles with the notable exception of the uterus of a 15-year-old girl, in which particulate fluorescence was absent but there was a moderate density of sudanophilic particles. There was no significant difference between uteri in average particle diameter, which was approximately 1 μm, but uteri of older patients tended to contain the larger proportion of the larger particles. Individual particles varied in size in the limit of resolution (0.25 μm) to a maximum diameter of 4 μm. The frequency distribution of sudanophilic particles from 14 specimens is shown in Text-figure 1.

Electron Microscopy

Four preparations of human uterine muscle were sufficiently preserved for electron microscopy.
The fine structure of the uterine smooth muscle cell is well known, although this has rarely been described for human tissue. Muscle cells were occasionally seen to be interconnected by attachment plaques and were individually invested by a delicate basement membrane and by a meshwork of collagen fibrils. The majority of the cytoplasmic organelles were located near the center of the spindle-shaped cells and close to the elongated nucleus. The remaining cytoplasm was filled by myofilaments which extended parallel to the long axis of the cell and were organized into numerous bundles. The mitochondria were small and ovoid. A moderate amount of rough endoplasmic reticulum was present in most cells. Muscle cells of nongravid uteri contained a poorly developed Golgi apparatus and small electron-dense Golgi vesicles. These organelles appeared notably enlarged in a gravid specimen. Centrioles were occasionally observed. Clusters of bottle-shaped vesicles, caveolae intracellulares, were packed close to the cell surface and were often connected to the plasma-lemma as focal invaginations. Cells were occasionally seen to contain cytoplasmic bodies composed of whorls of membranes, but irregular masses of electron-dense material, typical of chromolipoid pigment, were absent.

Many smooth muscle cells contained one or more bodies which exhibited characteristic features (Figures 3 through 7). These particles were large (approximately 1 μ), round or ellipsoid, and usually located in the cytoplasm adjacent to the nucleus. The bulk of their structure was occupied by homogeneous material of variable, but generally low, electron density. In some cases an irregularly shaped electron-lucent area was present within the particle, which often contained small amounts of granular material (Figure 3). Recognizable remains of cytoplasmic organelles or myelin figures were not present. Single-membrane–limited dense bodies, probably lysosomes, were frequently seen to be adjacent to the large particles and occasionally appeared to fuse with them (Figures 6 and 7). Coarsely granular, highly electron-dense material often partially sur-
rounded the large particles and may represent a stage in its formation or dissolution. When observed at higher magnification in favorable sections, the whole complex was found to be enclosed by a single membrane with the typical trilaminar structure of lipoprotein "unit" membranes (Figure 5).

These particles resembled each other, differing only in detail. The general characteristics and distribution compared closely with those of the particles already described at the light microscopic level. Similar structures were occasionally seen in endothelial cells (Figure 8).

Analyses of Lipid Extracts

Spectrofluorometric analysis disclosed significant fluorescence in the chloroform-rich phase but not in the aqueous phase of uterine mucosal extracts. The spectra recorded with an instrument range setting of 340 and with 10-nm slits are shown in Text-figure 2. The maxima of the excitation and emission spectra were approximately 365 and 435 nm, respectively, compared with 325 to 340 nm and 475 nm for pure retinol. The fluorescence was extinguished completely by exposure of the extracts in their quartz cuvettes to an intense source of ultraviolet light (Pen-Ray quartz lamp, Ultra-violet Products Inc., San Gabriel, Calif.) for 1 minute.

No visible product was formed by mixing chloroform extracts of mucosal tissue with antimony trichloride solution, whereas a faint evanescent purple was obtained with TFA reagent. This coloration (\( \lambda_{\text{max}} = 590 \text{ nm} \)) differed from the bright blue formed with either chloroform extracts of mouse liver or pure retinol (\( \lambda_{\text{max}} = 615 \text{ nm} \)) and was therefore probably not due to vitamin A.

Text-figure 2 — Fluorescence spectrums of chloroform-methanol extracts of human myometrium with excitation (EX) and emission (EM) maxima at approximately 360 nm and 435 nm, respectively. Fluorescence intensity is given in arbitrary units.
Discussion

The autofluorescent particles observed in cells of the human myometrium are a distinct morphologic entity and are sufficiently common to be regarded as a typical feature of this tissue. Their characteristic sudan staining reactions, lipid solubility, and absence of visible pigmentation and acidic groups might place them at an early stage of the development of chromolipoid pigment according to the scheme devised by Pearse. The chromolipoids have been shown to have characteristic fluorescence spectra after extraction with chloroform–methanol from various aging tissues and uteri of vitamin-E deficient rats. Spectral analysis of human myometrial extracts indicated this tissue might also contain fluorescent products of lipid peroxidation, although this fluorescence, in apparent contrast to that reported for the other tissues, was not stable to intense ultraviolet light. Particulate fluorescence of tissues is sometimes attributed to lipids either associated with membranes or in simple physical solution. The possibility that the fluorescence observed in this study might be caused by vitamin A could not be supported either by chemical tests when the TFA and Carr–Price reagents were applied to lipid extracts or to the unsaponifiable residues of myometrium or by analysis of the fluorescence spectra.

The chromolipoids represent undigestible and unextradable waste products of the cell and are commonly, although not universally, thought to develop in lysosomes. During development, their physicochemical properties change with the degree of oxidation and cross-linking, which might explain the spectrum of histochemical reactions obtained with this material. The myometrial particles described in this study increased in number with age, as do some chromolipoids, but their properties apparently did not change with age. We have obtained evidence from autopsy material that these particles have unaltered histochemical characteristics as late as the ninth decade of life, which indicates that the progressive changes expected with typical chromolipoids do not take place. The ultrastructural appearance of these particles suggested they may be involved in a continuing cellular activity rather than being merely inert storage bodies. Thus, the autofluorescent particles described here differ in several respects from typical chromolipoid pigments. There are indications that such pigments may form in the uterus under abnormal conditions. Pappenheimer and Victor identified chromolipoids on the basis of their acid-fast properties in the uterine smooth muscle of 3 women who had died with probable intestinal absorption disorders. They suggested that a deficiency of vitamin E (antioxidant) in these individuals might have caused muscle pigmentation, as is the case in the rat. The
absence of chromolipoids from our patients of variable age and parity were assumed to be enjoying an adequate diet. This suggests that these pigments only form under abnormal conditions in human uterine smooth muscle cells. Similar lipid pigments, sometimes termed “leiomyometaplasts,” are also deposited in the smooth muscle of the gastrointestinal tract under pathologic conditions.2,21–24

Chromolipoid pigments sometimes occur in the myometria of animals maintained on a standard diet. They are present in uterine smooth muscle cells and veins of pigs,4 whereas they are in cells presumed to be macrophages in the squirrel monkey (Saimiri sciurea),3 rat,6,8 and monkey uterus.7 The pigment granules in the macrophages may have either been formed de novo from ingested unsaturated lipids, which were subsequently oxidized and polymerized, or have been phagocytosed as macrophage pigment released from other cells. In the mouse, the density of the pigmented macrophages was directly related to the number of pregnancies, and it is probable that they became fixed in the tissue after their involvement in postpartum involution of the uterus.6 In women, comparable cells were not found in the fundic region, although they may be present in other areas.

Results strongly imply that the myometrial particles are a form of lysosome; however, the ultrastructural appearance of membrane-bound dense bodies and the presence of acid-phosphatase-positive bodies in frozen sections cannot be taken as final proof because the lysosomes are a polymorphic group of organelles. Chloroform–methanol-soluble fluorescence comparable to that of the myometrial particles has been observed in liver and kidney tissue25,26 and was regarded as being associated with lysosomes. Lysosomes have been described in earlier studies of human uterine muscle but their appearance was not reported to be remarkable.27,28 They are thought to be involved, at least in part, in the digestion of intracellular and extracellular material during postpartum uterine involution.29,30 Since the myometrial particles were present in both nulliparous and multiparous uteri, they probably do not constitute residual bodies from this particular lysosomal activity.

The fatty particles that were recently described in uterine muscle toxemic patients31 may be the same as those reported here for a wide range of patients. Both types of particle resemble the vacuolar structures of variable electron density that occur in vascular smooth muscle cells of experimental atherosclerosis and human atherosclerotic lesions.25–27 Structures found in atherosclerotic tissue are probably secondary lysosomes containing imbibed lipid which is incompletely digested.33 We therefore compared the concentration of particles with the tissue lipid profile.
each sample of myometrium but found no relationship for the lipids analyzed, including cholesterol and cholesterol ester. Histochemical tests for cholesterol and its esters were negative in this tissue; the particles did not appear anisotropic in polarized light, although the presence of collagen made this observation difficult. Thus, any close link between the myometrial particles and the pathologic changes of vascular smooth muscle seems doubtful.

There is insufficient information to attribute any particular significance to the myometrial particles other than their apparent lysosomal nature and relationship to aging. Further information must be obtained to determine how widely they occur in the human population and their significance in cell function.

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Acknowledgments

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[Illustrations follow]
Figure 1—Frozen section from midfundic myometrium from a 40-year-old woman (para 34) showing autofluorescent particles in smooth muscle. (Ultraviolet fluorescence, X 1300)

Figure 2—Frozen section from midfundic myometrium from a 38-year-old woman (para 8) showing sudanophilic particles in smooth muscle. Some particles appear to have an unstained central cavity. (Sudan black B, X 3250)
Figure 3—Uterine smooth muscle cell from the uterus of a 48-year-old woman who had two pregnancies and three live births. A large, rounded, rough-surfaced particle of slight electron density fills this portion of the cell. This particle and the nucleus appear to displace three filaments which course parallel to the long axis of the cell. The particle contains an irregular electron-lucent space in which small dense granules are scattered. Membrane-bound dense bodies, presumably lysosomes, are adjacent to the large particle. (Congo red, tetroxide, × 71,000)
Figure 4—A typical group of particles within a smooth muscle cell, showing wide variation in size and electron density. (Osmium tetroxide, × 47,200)
Figure 5—A large moderately electron-dense particle surrounded by a trilaminar unit membrane. Around the particle and within its membrane are accumulations of dense granular material. (Osmium tetroxide, × 58,000)
Figure 6—A typical large membrane-bound, slightly electron-dense particle which appears to be filled with a smaller particle which contains granular electron-dense material and two round dense bodies (OsO₄ tetroxide, × 49,800)
Figure 7—Higher magnification of Figure 6. The continuity of the membranes of the two particles can be seen. (Osmium tetroxide, × 175,000)
Figure 8—A cross-section of a small arteriole from human uterus, with a red blood cell in its lumen. The cell is a moderately electron-dense particle (arrow) similar to those seen in smooth muscle cells. (Original magnification 13,200)
SIGNIFICANCE OF PROLACTIN IN SPONTANEOUSLY
PERSISTENT OESTROUS RATS

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Ageing female rats maintained under standard laboratory lighting conditions (14 h light: 10 h darkness) frequently enter a state of persistent vaginal cornification because the pituitary gland fails to provide an ovulatory surge of gonadotrophic hormones. The ovaries of such animals become dominated by growing and cystic follicles as the corpora lutea disappear. The concentrations of prolactin in the pituitary gland and the circulation during persistent oestrus are known to be raised for at least part of the day (Clemens & Meites, 1971; Mallampati & Johnson, 1974; Shaar, Euker, Riegle & Meites, 1975; J. E. Beach, R. G. Gosden & J. W. Everett, unpublished observations). A possible antgonadotrophic role of prolactin in the aetiology of the persistent oestrous condition should be considered because of the known inverse relationship between the release of prolactin and gonadotrophins in a number of physiological and pathological states. More specifically, prolactin might affect pituitary secretion of luteinizing hormone (LH) by acting directly upon the gland or by affecting the output of hypothalamic luteinizing hormone releasing hormone (Grandison, Hodson, Chen, Advis, Simpkins & Meites, 1977; Muralidhar, Maneckjee & Moudgal, 1977). Prolactin might also inhibit follicular production of progesterone (McNatty, Neal & Baker, 1976), which would explain the ability of supplementary progesterone to reinstate cycles in persistent oestrous animals (Everett, 1940). If prolactin exerts either or both of these actions in the aforementioned animals, the beneficial action of L-DOPA on ovarian function could then be explained by the suppression of prolactin rather than by the stimulation of a catecholaminergic mechanism for the release of LH (Quadri, Kledzik & Meites, 1973; Linnola & Cooper, 1976).

The experimental animals comprised a group of ten retired breeder and 16 virgin rats of the Sprague–Dawley strain, aged 13–14 months and weighing 280–390 g. The animals had a minimum of 21 consecutive days of persistent vaginal cornification immediately before the experimental period. The two groups were injected subcutaneously each day for 10 or 17 days respectively, with either 2-bromo-α-ergocryptine (CB 154, Sandoz Ltd, Basel; bromocriptine) suspended in 0-9% saline or the saline vehicle alone. Experimental and control animals were selected at random and were equal in number. The daily dose of bromocriptine (4 mg/kg body weight) was chosen for its demonstrated ability to reduce the serum concentration of immunoactive prolactin in oestrogen-treated rats (Brooks & Welsch, 1974). Studies of male rats suggest that a long-term reduction in the concentration of prolactin can be achieved by this treatment (Harper, Danutra, Chandler & Griffiths, 1976; Aragona, Bohnet & Friesen, 1977). The majority of the long-term persistent oestrous animals used in these experiments had palpable milk cysts which were assumed to be due to stimulation of the mammary glands by prolactin. The efficacy of treatment with the prolactin inhibitor was therefore judged by the virtual or complete disappearance of milk cysts at autopsy.

Treatment with bromocriptine neither interrupted the vaginal smear pattern of persistent oestrous rats nor affected the morphology or weight of the ovaries and adrenal glands, although the uterine weight of the treated animals was significantly greater than that of the control rats ($t_{14} = 3.09; P < 0.01$). These results imply that hyperprolactinaemia is not mandatory for the maintenance of an anovulatory state and is probably a mere consequence
of sustained ovarian secretion of oestrogen. It is tempting, however, to suggest that
ous stimulation by one or both of these hormones may be partly responsible for the
ence of the nodules and tumours that are frequently found in the mammary glands
persistent oestrous animals.

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PITUITARY-OVARIAN RELATIONSHIPS DURING THE POST-REPRODUCTIVE PHASE OF INBRED MICE

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Abstract—Two strains of inbred mice with differences in functional ovarian lifespan have been examined for evidence of altered ovarian steroidogenesis and feedback control of the pituitary gland during the post-reproductive phase of life. The serum concentrations of unconjugated oestrogens in old anestrous animals were not detectable by radioimmunoassay (<10 pg/ml) although the histochemical demonstration of Δ^5-3β-hydroxysteroid dehydrogenase even in old CBA ovaries devoid of follicles suggested that residual steroidogenesis may occur. Changes of the follicular composition of ovaries throughout adult life and the early disappearance of follicles from one strain did not affect basal levels of immunoreactive serum luteinizing hormone (LH). Furthermore, ovariectomy of either young or old mice did not alter LH levels. The results suggested that ovaries of aged CBA mice and of post-menopausal women are comparable in terms of the early onset of senescence, residual steroidogenesis and certain pathological changes, but differ in the level of exposure to LH because of basic differences in the normal regulation of this hormone.

INTRODUCTION

The stock of gonadotrophin-sensitive follicles in human ovaries is usually depleted around the end of the fifth decade of life. This milestone in ovarian senescence results in the final cessation of menstrual cycles (menopause) and variable symptoms of oestrogen deficiency. In contrast, laboratory and domesticated animals generally have follicles remaining until the end of life and the loss of ovarian cyclicity, at least in the rat, may be secondary to a hypothalamic disorder (Jones, 1975). Among mammals, only the CBA strain of mouse is known to have a precocious but physiologically normal loss of follicle reserve which is probably the direct cause of the early onset of acyclicity in this strain (Jones and Krohn, 1961; Krohn, 1963).

It remains to be demonstrated whether the ovary of the aged CBA mouse is comparable in other ways with that of the human post-menopause. Experiments have therefore been carried out on mice to determine the steroidogenic potential of senescent ovaries and levels of circulating luteinizing hormone (LH) throughout life because decreased ovarian feedback on the hypothalamus and pituitary gland is a principal characteristic of the post-menopause (Sherman et al., 1976).

MATERIALS AND METHODS

Inbred virgin and multiparous CBA and C57 female mice from our laboratory colonies were studied in the age range 2–30 months. They were maintained in temperature controlled rooms under standard lighting conditions (14 hr photoperiod) and provided with food pellets and water ad libitum. Retired breeding stock had been neither pregnant nor lactating for at least three weeks before study. Since the oldest individuals only exhibited anoestrous vaginal smears, all other animals were killed when the smears were non-cornified to avoid the pro-oestrous increase of oestrogen and gonadotrophins. Blood was collected

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from animals killed in the morning and serum was prepared and subsequently stored at –20°C. Ovaries of young (3–6 month) and old (17 month) CBA mice were ovariectomized or sham-operated under anaesthesia 21 days before bleeding. At autopsy, the ovaries, uterine horns, adrenal and pituitary glands were dissected, blotted free of surface blood and weighed. Ovariectomized animals were checked at autopsy for the presence of ovarian remnants. The pituitary glands were stored frozen in 0.5 ml phosphate-buffered saline (pH 7.6) containing 0.1% sodium azide. Animals found at autopsy to have substantial pathological lesions, apart from ovarian cysts, were excluded from further analysis.

**Radioimmunoassay**

Pituitary glands were thawed and homogenized in buffered saline. The LH content of sera and pituitary homogenates was determined by double antibody radioimmunoassay (Monroe et al., 1968). Following LH labelled with ¹²⁵I by the Chloramine-T method was used to compete for binding sites on NIH/LH-S1 anti-rat LH-S1 with either the reference hormone preparation (NIAMDD-rat-LH-RP1) or the homogenate sample. The heterologous radioimmunoassay is thought to be reliable for measuring murine LH because (1) the standard curves for rat and mouse LH are parallel over a wide range of concentrations, (2) similar levels of LH in mice vary during the oestrous cycle in a similar way to those of the rat (Kovacs et al., 1972), (3) administration of synthetic LH-releasing hormone increases immunoreactive LH in rats of either sex (Gosden and Readhead, 1974). Aliquots of 50–200 μl of serum or gland preparation were added to a duplicate and results were expressed as equivalents of the rat standard preparation. The sensitivity of the assay was 25 ng. Mean hormone concentrations were compared statistically by Student's t test. A sufficient serum remained for the analysis of follicle stimulating hormone (FSH) concentration.

Aliquots of up to 1 ml of serum were extracted with ether and subjected to radioimmunoassay of oestradiol content (Abraham, 1974). Total unconjugated oestrogens were measured as there was no chromogenic step in the protocol. The sensitivity of this assay was 10 pg.

**Histology and histochemistry**

Ovaries of some aged mice were fixed in aqueous Bouin's fluid and processed by routine methods of paraffin-embedding, sectioning and staining. Other aged CBA ovaries together with those from young females, some of which were in early stages of pregnancy, were rapidly frozen on cold CO₂ in O.C.T. Compound (Ames Co., Indiana, U.S.A.) and stored at –20°C for less than three weeks. They were sectioned at 16 μm with a cryostat and subjected to a modified version of Wattenberg's histochemical technique for the demonstration of Δ⁴-3β-hydroxysteroid dehydrogenase (3β-HSD) (Wattenberg, 1958). Sections were washed in cold acetone and cold phosphate buffer before incubation in medium for 3 h at 37°C. The substrate was dehydroepiandrosterone (Sigma), the co-factor was nicotinamide adenine dinucleotide (NA.Boehringer) and nitro blue tetrazolium (Sigma) was the final electron acceptor. Control sections were incubated under similar conditions in either substrate-free medium or for 30 min in medium in which NAD was substituted for NAD and substrate; the latter procedure demonstrates NAD-reduction reductase (diaphorase). After incubation the sections were washed and fixed in 10% formalin to give further reaction and finally mounted in Gurr's Hydramount.

**RESULTS**

**Autopsy findings**

The weights of ovaries, uteri and pituitary glands of both strains of mice, though variable, tended to follow the pattern described by Green (1957). Organ and body weight were generally highest in animals approximately twelve months old. In contrast, the weight of adrenal glands remained relatively constant between 2 and 30 months of age. Uterine weight was significantly reduced by ovariectomy in young animals alone (p<0.01); pituitary weight of either age group was unaffected by ovariectomy (Table 1).

Ovaries of mice over 8 months old and uteri of multiparous mice contained a yellow-brown pigment. The lipochrome cells responsible for the pigmentation of the ovary were scattered throughout the organ and contained granules which exhibited a stable green-yellow fluorescence in ultraviolet light and thus resembled ceroid pigment. "Wheel" cells identified by their characteristic chromatin pattern, were occasionally observed in the interstitia of CBA ovaries more than fifteen months old (i.e. after the oocytes have disappeared). Ovarian cysts were absent from young animals though present in 80% of old mice aged 12–16 months. The cysts varied from microscopic size to more than 1 cm in diameter.
diameter and were probably of diverse origin (e.g. follicular, germinal and rete tubule epithelium).

**Serum and pituitary hormones**

Circulating levels of immunoreactive LH in CBA and C57 mice showed no systematic variation with age (Fig. 1). Occasional females had LH values exceeding 100 ng/ml serum but these values were not accompanied by pro-oestrous-like changes of the ovaries or vaginal epithelium. CBA ovaries became depleted of their follicle population at approximately 15 months but there was no indication that altered feedback relationships with regard to LH secretion had taken place in the hypothalamo-hypophysio-ovarian axis. In addition, the levels of pituitary LH were independent of age and very variable between individuals (Fig. 2); this variability could not be attributed to differences in health status or reproductive history. There was no correlation between the LH content of sera and pituitary glands.

![Fig. 1. Variation of serum LH levels with age in females of two inbred strains of mice (closed circles = CBA, open circles = C57).](image_url)
Although ovariectomy resulted in decreased uterine weight, particularly in the young females, there was no change in pituitary gland weight or serum LH concentrations in either young or old CBA mice (Table 1).

Unconjugated oestrogens in the sera of animals with anoestrous, metoestrous or dioestrous vaginal smears were undetectable (< 10 pg/ml).

**Enzyme histochemistry**

Control sections incubated without the steroidal substrate were devoid of diformazan deposits whereas sections treated for the diaphorase contained heavy deposits. The distribution of 3β-HSD in young ovaries was similar to that described by Ferguson (1968); dense diformazan accumulations were present in corpora lutea of pregnancy, interstitial tissue and theca interna (Fig. 3). Granulosa cells were stained very weakly and most of this staining was confined to the periphery of the follicle. Ovaries of 18-month old CBA mice, which were devoid of follicles and corpora lutea, contained little diformazan and this was restricted to small clumps of cells resembling the interstitial cells of younger animals (Fig. 4). These cells were present throughout the ovary, though mainly confined to the cortical region. Lipochrome cells did not contain 3β-HSD.

**DISCUSSION**

The gonadotrophin content of sera and pituitary glands of ageing laboratory animals was the subject of several earlier studies (reviewed by Talbert, 1968). The conflicting results obtained in those studies may be partly attributed to the poor sensitivity and specificity of the methods available at the time. However, certain histological observations of senescent mouse ovaries had already anticipated the present finding that basal levels of LH do not increase during the phase equivalent to the human post-menopause. "Wheat" or "deficiency" cells found in the ovaries of old mice were morphologically similar to those found in hypophysectomized animals and disappeared after treatment with equine serum.
Fig. 3. Frozen section of ovary of young mouse on Day 3 of pregnancy. Distribution of $\Delta^2$-3$\beta$-hydroxysteroid dehydrogenase (x 60).

Fig. 4. Frozen section of ovary of 18-month old CBA mouse. Distribution of $\Delta^2$-3$\beta$-hydroxysteroid dehydrogenase (dark diformazan deposits). Lighter clumps of cells in these ovaries contain natural pigmentation (lipochrome) (x 60).
PITUITARY–OVARIAN RELATIONSHIPS IN OLD MICE

gonadotrophin (Green, 1957). This observation suggested that ovaries of at least some strains might receive inadequate gonadotrophin stimulation in old age.

Pituitary function in post-menopausal women apparently contrasts with that of aged mice. During the human menopausal transition period a substantial increase in circulating levels of FSH occurs as a result of reduced suppression by ovarian oestrogen in the absence of any concomitant increase in LH levels (Sherman et al., 1976). The rhesus monkey (Macaca mulatta) experiences menopause towards the end of the natural lifespan in captivity (Van Wagenen, 1970) and recent observations show that the hormone profiles in this sub-human primate resemble those of women (Hodgen et al., 1977). Both studies emphasize the divergence of serum FSH and LH levels during the transition period but point out that serum LH levels become elevated above the normal basal levels characteristic of regularly menstruating females.

The lack of increase in circulating LH in aged mice was not caused by the maintenance of negative feedback on the hypothalamus by ovarian hormones because removal of the ovaries did not affect LH values. Parlow (1964) was unable to detect a rise in bioassayable LH in long-term ovariectomized mice and only a small increase was found in a subsequent study with the more sensitive radioimmunoassay (Kovacic and Parlow, 1972). On the other hand, FSH values were found to increase greatly over the same period. The reason for the apparent persistent inertia of the murine LH-control mechanism to changes in ovarian feedback, even in females showing a precocious decline in follicle number, is unclear and the response contrasts with that in man and rats. Young female rats of our Sprague-Dawley-derived colony have increased serum LH by four-fold and eight-fold in two and six weeks, respectively, after ovariectomy (Gosden and Bancroft, 1976). One possible, but untested, explanation for this species difference is that the adrenal gland of the mouse may act as an alternative target organ for gonadotrophins and compensates for the loss of ovarian steroidal feedback by differential suppression of LH and FSH.

Senile animals of most strains of mice have a persistently anoestrous vaginal smear which is external evidence that cyclical secretion of oestrogens has ceased. Present results have shown directly that oestrogen levels in these mice are very low. However, residual ovarian steroidogenesis may continue since 3β-HSD was found in cells of even the oldest individuals. This enzyme catalyses the conversion of Δ4-hydroxysteroids to Δ4-ketosteroids and thus governs the production of progesterone, 17α-OH-progesterone and androstenedione. Cortical stromal cells of post-menopausal human ovaries also contain 3β-HSD (Mestwerdt et al., 1975). Human ovaries have a reduced ability to secrete the classical phenolic steroids after the menopause but the production of androgens is maintained and they may be aromatized peripherally (Cooke et al., 1976). Measurement of the production rate of ovarian steroids in vivo in mice raises great technical problems but, in spite of the lack of this information, it presently appears that the steroidogenic capacity of the ovary of aged CBA mice is comparable with that of man, though subject to a differing amount of gonadotrophic stimulation.

The tendency for epithelia to proliferate in both types of ovaries might be linked with the loss of the follicles and, hence, changes of the hormonal milieu (Thung, 1961). The disappearance of follicle target tissue for steroid and pituitary hormones may result in the stimulation of other hormone-sensitive tissues and lead to the characteristic duct and cyst development of old ovaries. The questions still remain whether post-menopausal ovarian secretions have physiological significance and whether they are related causally to
the incidence of pathological changes of the genital tract. Thus far, the CBA mouse appears to be a suitable experimental model for studying some of the underlying endocrine changes of senescent human ovaries.

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Corpus luteum function in ageing inbred mice

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Summary. Impaired breeding performance of aged female mice was associated with reduced numbers of ovaulations and increased mortality of embryos. The amounts of progesterone in the sera, corpora lutea and uterine flushings of these animals were similar to those of young animals when measured by radioimmunoassay.

There is substantial evidence to show that the gametogenic potential of the ovaries of mice and some other animals outlasts the ability of the uterus to maintain conceptsus to term. The results of embryo transplantation experiments in which the age of donors and of recipients were variables showed that the latter was the major factor affecting embryo survival and that the majority of ovaa from old mice were viable in a young uterine environment. The relative contribution of extrinsic and intrinsic factors to the decreased gestational potential of the ageing uterus is not clearly established. Morphological studies and hormone supplementation studies implied that the secretory activity of the corpora lutea (CL) might be sub-optimal for uterine function. Other investigators have doubted this conclusion and implied that unknown intrinsic age changes of the uterus are responsible for most pregnancy wastage.

We have therefore measured the levels of progesterone in ageing pregnant mice in order to directly assess uterine function.

Materials and methods. Young (2–3 month) and aged (8–12 month) virgin CBA/H female mice were paired either with young albino male mice or proven sterile vasectomized animals. They were examined each day for the presence of a copulatory plug (= day 1 of pregnancy/pseudopregnancy). Mated females were allocated randomly to 3 groups which were either autopsied on day 4 or 8 of pregnancy or used for litter size determination at term. On the day of autopsy the animals were anaesthetized with ether and a blood sample was collected from the orbital sinus. They were killed by cervical dislocation before recovering from the anaesthetic. The number of uterine embryos was determined either by flushing them out with saline and counting them in a watch glass (day 4) or by counting the number of implantation swellings (day 8). The most hyperaemic set of CL was counted under the microscope. 2 CL from 1 ovary of each animal were removed by fine dissection for subsequent hormone determination, closely apposed CL being avoided. Histological preparations showed that CL isolated by this technique were intact and generally free of adhering tissue apart from occasional preantral follicles. The undissected ovaries were prepared by routine histological methods and stained with haemalum and eosin. The dissected CL were homogenized individually in ether (0°C) with trace amounts of H-progesterone added for the determination of hormone recovery. The homogenates were dried in glass vials and stored with the sera at −20°C.

A further group of animals was killed by decapitation and exsanguinated on day 4 of pseudopregnancy. The uterine horns were dissected and blotted gently with damp filter paper to remove surface blood. Individual horns were then flushed with 0.25 ml of cold sterile phosphate-buffered saline (pH 7.0). The flushings from each animal were pooled and immediately centrifuged at 3000 g for 20 min to remove cellular debris. The supernatants were stored at −70°C until the progesterone concentrations were measured.

The progesterone content of the sera, CL homogenates and uterine flushings was measured by radioimmunoassay procedures. After extraction of the samples with ether, steroids were separated on celite microcolumns using a

Table 1. Circulating and luteal progesterone levels of young and aged pregnant and pseudopregnant mice

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Day of pregnancy</th>
<th>No. of corpora lutea (CL) of pregnancy</th>
<th>Embryos (% of CL)</th>
<th>CL progesterone ng/CL</th>
<th>Serum progesterone ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–3</td>
<td>4</td>
<td>9.5 ± 0.3</td>
<td>8.3 ± 0.3 (86.3)</td>
<td>9.48 ± 0.67</td>
<td>28.02 ± 2.12</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>9.5 ± 0.5</td>
<td>9.0 ± 0.4 (97.3)</td>
<td>11.28 ± 1.14</td>
<td>29.63 ± 3.05</td>
</tr>
<tr>
<td>(pseudopregnant)</td>
<td></td>
<td>9.5 ± 0.3</td>
<td>0</td>
<td>12.14 ± 2.01</td>
<td>27.64 ± 0.84</td>
</tr>
<tr>
<td>8–12</td>
<td>4</td>
<td>5.5 ± 1.3</td>
<td>4.3 ± 1.5 (77.3)</td>
<td>11.38 ± 1.50</td>
<td>30.59 ± 6.51</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6.3 ± 0.8</td>
<td>2.4 ± 0.8 (38.6)</td>
<td>11.11 ± 1.68</td>
<td>25.27 ± 3.78</td>
</tr>
</tbody>
</table>

Means ± SEM are given. 4–7 animals/group.

Table 2. Progesterone and protein content of uterine flushings of young and aged mice on day 4 of pseudopregnancy

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Number</th>
<th>No. of CL of pseudopregnancy</th>
<th>Dry uterine weight (mg)</th>
<th>Protein/uterus (µg)</th>
<th>Progesterone/uterus (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>9</td>
<td>9.0 ± 0.4</td>
<td>15.2 ± 0.3</td>
<td>&lt; 25*</td>
<td>0.065 ± 0.016</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.2 ± 0.7</td>
<td>26.0 ± 1.3</td>
<td>&lt; 25*</td>
<td>0.053 ± 0.016</td>
</tr>
</tbody>
</table>

Means ± SEM are given. * Below the sensitivity of the protein measurement technique (25 µg).
stationary phase of ethylene glycol. The chromatographic step was omitted for plasma and CL samples because preliminary results showed there were very large amounts of progesterone in the samples. The other steroids might cross-react with the anti-sterum used (S-257 No. 2, kindly supplied by Dr G.E. Abraham). The separation of bound from free steroid was effected by dextran-coated charcoal. A standard curve was constructed from concentrations of the progesterone standard measured in triplicate. The samples were measured in duplicate and the concentrations of progesterone were determined from the standard curve using a non-linear asymptotic model which had been fitted to the curve by iterative methods. The smallest amount of progesterone that could be measured per sample was 0.04 ng. Each of the variables recorded in table 1 was analyzed as a 2-way classification with maternal age and day of pregnancy as the factors. An additional class, 'pseudopregnant', was included in the analysis of some variables. The mean values in table 2 were compared by Student's t-test.

Results and discussion. The number of live offspring born to 14 young mice ranged from 8 to 11 whereas only 2 out of 13 old mice gave birth and their litters were 1 and 3. The small litter sizes in old females cannot be fully accounted for by the observed reduction in the number of CL (table 1, p < 0.001), assuming that each CL represents an ovulation, because the number of embryos as a percentage of CL is also reduced (p < 0.01). The latter effect appears as an interaction of age and day of pregnancy (p < 0.05) and is due to a reduction in the percentage of embryos from day 4 to day 8 in old mice compared to the high percentage of embryos maintained in young animals. There was no evidence of any significant effects of maternal age upon the serum or CL progesterone levels during pregnancy or pseudopregnancy. Furthermore, the CL of young and old animals were morphologically indistinguishable in histological sections. Examination of the raw data revealed that the absence of implants on day 8 did not affect the progesterone levels in either the young pseudopregnant animals or the 2 old animals that lacked signs of implantation. Serum progesterone levels were not correlated with the number of CL and in 1 old animal 2 CL supported implantation. This result may indicate that some compensatory secretion of progesterone occurs in old mice that have reduced numbers of CL though, contrary to expectation, there was no corresponding change in the progesterone content of individual CL. These results taken together with those obtained by measurement of $\Delta^3$-3b-hydroxysteroid dehydrogenase activity in C57BL mice strongly suggest that the high incidence of prenatal mortality in aged animals is not a result of defective fetal function. A similar conclusion has been drawn in studies of reproductively senescent rabbits and hamsters. The results support the view that intrinsic age changes of the uterus are primarily responsible for decreased breeding performance prior to the onset of anovulation.

Changes in target tissue response to progesterone and other hormones might occur during ageing which could explain the beneficial effects of supplementary progesterone on implantation2. A decreased uterine response to progesterone could be due to changes in the uterine vascular supply or the uterine collagen content8 with increasing age. Additionally, there may be changes in factors affecting the concentration of progesterone binding sites in the uterus, e.g. oestrogen levels. Each of these factors can be investigated experimentally but oestrogen deserves special consideration because the numbers of Graafian follicles are reported to be diminished in aged ovaries and the numbers of CL suggests that this may be so in our data (table 1). However, we have been unable so far to detect differences in the serum levels of oestrogen in individual young and aged mice because the absolute amount of oestrogen present in each case are very small (1.4 pg/ml). The uterine luminal environment is undoubtedly critical for normal implantation and development. Uterus flushings were examined because some embryos were apparently being lost during the peri-implantation period in a rabbit there are correlated changes in the levels of progesterone and protein in the uterine flushings during pregnancy and pseudopregnancy, though the biological significance of these changes has not yet been demonstrated8. The total amounts of uterine luminal prostaglandins on day 4 of pseudopregnancy were low but similar in young and aged mice (table 2). These progesterone levels were standardized for uterine weight, even though old mice were heavier than young ones (p < 0.001), because there was evidence of an association between increased uterine weight and glandular surface area. The levels of progesterone correlated with uterine weight in both young and old mice were also low, below the sensitivity of the technique (table 2), Aitken21 has demonstrated a small transient increase in protein content of pooled samples of uterine flushings on day 5, which is the day on which implantation occurs in pregnant animals. If the relationship between levels of progesterone and of protein holds in the mouse as it does the rabbit, the results of table 2 imply that the amount of uterine protein are unaffected by ageing of the uterus.

1 Financial support was provided by the Medical Research Council (R.G.G.), and the Ford Foundation, and was received (R.E.F.) wishes to thank Lucy Cavendish College, Oxford for a Calouste Gulbenkian Research Fellowship.

2 Present address: Department of Physiology, University Medical School, Teviot Place, Edinburgh, E1B 9AG Scotland.


9 C. A. Finn, J. Reprod. Fert. 6, 205 (1963).


19 R. G. Gosden and R. E. Fowler, unpublished observations.


Effects of age and parity on the breeding potential of mice with one or two ovaries

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Summary. Although removal of one ovary from young CBA/Ca mice and CFLP mice did not reduce the overall numbers of ova shed at ovulation, the total number of live offspring delivered during the lifespan was reduced to 65% and 50%, respectively, of control values. This reduction was due to fewer and smaller litters. The capacity of intact and unilaterally ovariecetomized animals to support gestation of embryos transferred from young donors was tested to determine whether premature exhaustion of uterine function had occurred as a result of embryo overloading. An effect of breeding history on embryo survival to Day 19 of pregnancy was found after unilateral transfer, the least favourable sites for survival being the primigravid horns of ageing intact and unilaterally ovariecetomized mice (0% and 1% survival, respectively, in CBA/Ca mice). The proportion of embryos surviving in multiparous horns of the one-ova animals (24%) was greater than in horns of primigravidae and less than in horns of intact multigravidae of similar age and parity (48%). A larger proportion of ova survived in young uteri than in any of these aged horns. The results suggested that the normal decline with age in breeding potential is due to decreased uterine capacity and that the rate of loss is accelerated by both repeated embryo overloading and prolonged nulliparity, probably as a result of local factors. The decidual response was reduced in older animals, although there was no clear-cut variation with parity. Ageing uteri accumulated mast cells and macrophages, but the latter were abundant only in multiparous horns and were probably related to puerperal involutary activities. There was no evidence that these changes in cell number or response were responsible for decreased gestational potential in ageing animals.

Introduction

Removal of one ovary from young polytocous laboratory rodents leads to a prompt compensation in numbers of ova shed at oestrus by the remaining ovary (McLaren, 1966). Such ova may be fertilized normally, although they implant only in the uterine horn ipsilateral to the remaining ovary because internal migration of ova is rare or anatomically impossible in these species.

Long-term studies of the effects of unilateral ovariecetomy on fertility in rabbits and rodents have shown that when one ovary was removed in early maturity the single functioning uterine horn was initially able to carry the additional feto-placental load to term. However, the subsequent litters were reduced in size as the incidence of intrauterine mortality increased.

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Animals with a single ovary produced only about half as many offspring in toto over the breeding life-span as intact controls (Jones & Krohn, 1960; Biggers, Finn & McLaren, 1962a; Blaha, 1964; Adams, 1970). These results could not be explained by premature ova failure in animals with one ovary (Biggers, Finn, McLaren & Woolf, 1962b). The possibility therefore, that the uterus can only support a limited number of implantations during life and the breeding potential of such species may be limited primarily by uterine factors raises some interesting questions: (1) does repeated embryo overloading of the uterus mainly affect the quality of the uterine environment and, if so, is this due to an acceleration of the normal aging process; (2) what is the functional potential of the barren horn in multiparous unilaterally ovariectomized mice; and (3) what, if any, structural changes occur in relation to changing functional capacity of the uterus? The present study was an attempt to answer these questions by testing the uterine response of mice to a challenge by embryos or artificial stimuli.

**Materials and Methods**

Outbred CFLP and inbred CBA/Ca female mice were obtained at 4 weeks of age from commercial sources. They were maintained in temperature-controlled (23 ± 1°C) and enriched rooms (14 h light/day) and were provided with food pellets and water ad libitum. Randomization methods were used for allocating animals to their treatment groups and selecting the side for unilateral surgery. Surgical anaesthesia was induced with tribromoethane (Avertin: Winthrop, Surbiton-upon-Thames, U.K.).

**Long-term ovarian function**

The ovarian cycle was studied in 54 virgin CBA/Ca mice between 6 and 30 weeks old. One ovary was removed from 32 animals at 6 weeks of age, the other animals were used as sham-operated controls. Since groups of female mice tend to become acyclic in the absence of male odour, a foreign male mouse (C57BL × A, G) was kept in perforated enclosure next to the roof of the females’ cage. Oestrus cycle lengths were determined by examination of vaginal smears. The mice were killed by cervical dislocation at the first or second oestrus after surgery or at oestrus when 12, 18, 24 or 30 weeks old. The ovulation number was estimated by counting freshly ovulated ova in oviduct flushings.

**Breeding experiments.**

Pairs of female mice were each housed with a proven fertile male of the same strain. If young were removed and counted within 24 h of parturition to avoid the suckling-induced prolongation of pregnancy after re-impregnation at the post-partum oestrus, thus providing the maximum number of opportunities for conception during life. Some of the female mice had been unilaterally ovariectomized a few days before introducing them to their mates; others were sham operated. All female mice were killed and autopsied 12 weeks after the birth of the last litter when they were considered to be sterile. Sickly animals and those with substantial pathological lesions were excluded from the study. Stud males were replaced if sub-fertility was suspected or if the grounds of unexpectedly reduced breeding performance of both of the female mice.

CBA/Ca mice in an additional group were maintained as virgins until they were 32-40 weeks old when multiparous of the same strain were becoming sterile. The breeding potential of this group was then tested by pairing with males of the same strain.

The products of the first conception in 14 young intact and 13 young unilaterally ovariectomized CBA/Ca mice were studied at autopsy on Day 19 (Day 1 = day of coital plug) for numbers of hyperaemic CL and live and resorbing fetuses were counted. Fetuses were
placentae were weighed and the total fetal protein of the litter was measured (Lowry, Rosebrough, Farr & Randall, 1951).

Embryo transfer

Morulae and blastocysts were obtained by flushing the uteri of young CFLP mice on Day 4 post coitum. The animals had previously been induced to superovulate with 5 I.u. PMSG and 5 I.u. hCG (Folligon and Chorulon: Intervet, Bar Hill, Cambridge, U.K.) and mated with CFLP males. Collection and transfer of embryos was carried out with a phosphate-buffered medium containing 0.33 mm-sodium pyruvate, 5.56 mm-glucose and 3 mg BSA/ml (Whittingham, 1971).

Pseudopregnancy was established in the recipient animals by mating with vasectomized males (C57BL × A,G) and the embryos were transferred surgically to the uteri on Day 3 of pseudopregnancy. The transfers were unilateral or bilateral but the total number of embryos transferred to each female approximated to the average number of hyperaemic CL in the host group. The recipient animals differed in age, strain, reproductive history and presence of one or both ovaries (see 'Results'). Young recipients were nulliparous and 8–12 weeks old at the time of pairing. Old recipients were approximately 25 weeks old and were either multiparous (3–6 litters) (see previous section for details of husbandry) or had been maintained repeatedly pseudopregnant by pairing with sterile males. Both age and parity were closely matched for comparing the fertility of the various groups of old animals.

At autopsy on Day 19, the numbers of live and resorbing fetuses were recorded. The absence of eye pigment in surviving fetuses showed that they were derived from the transferred embryos. The numbers of CL were counted to provide an estimate of the ovulation number in breeding females.

Decidual cell reaction

The uterine response to an artificial decidualizing stimulus was studied in groups of CBA/Ca and CFLP mice maintained under similar conditions to those described above. The protocol followed that of Finn (1966). The animals were bilaterally ovariectomized and primed sequentially with oestradiol-17β benzoate (0.2 μg/100 g body wt) and progesterone (2 mg/100 g) (Koch-Light, Colnbrook, U.K.) injected s.c. in arachis oil. An injection of ‘nidatory’ oestradiol (0.05 μg/100 g) was also given. The mice were anaesthetized and the decidual stimulus was provided by transversely crushing one uterine horn in three places with a haemostat. This traumatic stimulus was chosen when strictly unilateral effects on the uterus could not be obtained by intraluminal instillation of arachis oil (0.02 ml). The uteri were dissected at autopsy and each horn was weighed separately. The number of macroscopically visible decidual swellings was noted.

Statistics

The results were assessed by analysis of variance and by the χ² test (employing Yates' correction when appropriate).

Microscopical studies

Small pieces of uterine tissue were collected at autopsy from non-pregnant animals retired from the breeding experiments. Some additional material was collected from primiparous animals during the week following parturition. The tissues were fixed in Susa, 10% neutral-buffered formalin or formal–calcium fixative. Formalin-fixed frozen sections at 10–16 μm thickness were mounted for u.v. fluorescence microscopy and hydrolytic enzyme histochemistry (acid
phosphatase (Burstone's method), β-glucuronidase and non-specific esterase). Paraffin sections (6–8 µm) were studied by the following histochemical methods: Perl's method for haematoxylin–Lake method for iron; long Ziehl–Neelsen, Schmorl's ferricyanide and Nα-dihydroxyphenyl sulphate for lipofuscin granules; Sudan black B for lipids; periodic acid–Schiff reaction (with or without diastase digestion) for lipofuscin granules and carbohydrate. The methods are described by Pearse (1968, 1972). Other 6-µm sections were stained with Gomori's paraldehyde fuchsin to demonstrate mast cells: each slide was coded before estimating the number of these cells in randomly chosen areas of 8.4 × 10⁴ µm² of endo- or myometrium in each uterine horn.

Thin slices of tissue were prepared for ultrastructural studies. They were fixed by immersion in 4% glutaraldehyde in 0.1 M-sodium cacodylate buffer (pH 7.2) for 1 h followed by osmium tetroxide in 0.1 M-s-collidine buffer (pH 7.2) for 1 h. After fixation, the slices were washed in buffer, dehydrated with ethanol, washed in propylene oxide and embedded in epoxy resin. Thin sections were cut with glass knives and examined with an AEI 6B electron microscope.

Results

Long-term ovarian function

Removal of one ovary from young virgin mice did not disturb the normal rhythm of ovulation activity, although it led to a restoration of the normal total number of ova shed at oestrus. This compensatory response was not reduced with increasing age in either the intact mice up to 30 weeks of age or in the breeding colony after as many as 7 pregnancies (Table 1 and 3). Multiparous animals of the CBA/Ca strain in particular tended to have more CL and therefore, presumably a higher ovulation number than primigravidas of the same age, while primigravidas had significantly fewer CL than either of these elderly groups (P < 0.05).

Breeding experiments

The size of the first litter in unilaterally ovariectomized CFLP mice was reduced significantly compared to intact controls (Table 1), although the difference in CBA/Ca animals only reached an acceptable level of statistical significance (P < 0.02) in a larger series of animals (N = 7). The total number of live fetuses and placental scars in young primigravid CBA/Ca mice was similar in operated animals and controls on Day 19, although there was a slight, but significant reduction in fetal wet weight and protein weight in animals accommodating the entire litter in one horn (P < 0.05). Adjacent placentae were occasionally found to be attached to each other. The so-called close pairs were more common in the overcrowded horns of animals with a singleton (P < 0.025).

Table 1. Life-time breeding performance of intact (controls) and unilaterally ovariectomized CBA/Ca and CFLP mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>No. of mice</th>
<th>Live births of first litter</th>
<th>No. of litters</th>
<th>Live births/female</th>
<th>Age at last parturition (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/Ca</td>
<td>Intact</td>
<td>14</td>
<td>5.4 ± 0.7</td>
<td>4.9 ± 0.3</td>
<td>28.1 ± 1.6</td>
<td>197 ± 10 (14-27)</td>
</tr>
<tr>
<td></td>
<td>Ovx</td>
<td>14</td>
<td>4.2 ± 0.4</td>
<td>3.9 ± 0.4*</td>
<td>18.4 ± 1.7†</td>
<td>191 ± 8 (14-26)</td>
</tr>
<tr>
<td>CFLP</td>
<td>Intact</td>
<td>24</td>
<td>12.5 ± 0.4</td>
<td>8.1 ± 0.5</td>
<td>95.9 ± 6.6</td>
<td>271 ± 13 (19-42)</td>
</tr>
<tr>
<td></td>
<td>Ovx</td>
<td>24</td>
<td>9.3 ± 0.7†</td>
<td>5.4 ± 0.4†</td>
<td>48.3 ± 4.4†</td>
<td>226 ± 12 (14-28)</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. with range in parentheses.
* P < 0.05 compared with controls.
† P < 0.001 compared with controls.
Fewer litters were produced during the course of the breeding life-span in animals with one ovary than in their controls, the reduced fertility of the CFLP mice also being associated with an earlier cessation of breeding activity (Table 1). Autopsy results showed that conception without progression to full term had occurred after the birth of the last litter in the experimental and control animals.

The contrasting patterns of breeding activity in the two groups of animals are shown by comparison of the average productivity of the functioning uterine horns in successive litters (Text-fig. 1). The number of live young/horn in intact animals increased initially and then gradually declined to a low value whereas unilaterally ovariectomized mice maintained an increased number of fetuses/horn for several litters and then abruptly stopped breeding. The high value at litter number 8 in intact CBA/Ca mice was due to a single exceptional animal. The lifetime production of live young/horn was not very different in the two groups but, on a whole animal basis, the total number produced was reduced in unilaterally ovariectomized CBA/Ca and CFLP mice to 65 and 50%, respectively, of control values. The levels of maternal morbidity and mortality in the experimental and control animals were equally low, despite the unusually intensive breeding activity.

Delayed breeding affected the size of and likelihood of having a litter. Only 2 out of 13 old primigravid CBA/Ca mice produced litters, and these contained only 1 and 3 live offspring.

Text-fig. 1. Mean (± s.e.m.) numbers of living young produced per uterine horn during the breeding life-span of mice. ■, Intact CBA/Ca (N = 14); □, unilaterally ovariectomized CBA/Ca (N = 14); ●, intact CFLP (N = 24); ○, unilaterally ovariectomized CFLP (N = 24).

Embryo transfer

Both uterine horns in young primigravid mice had the same potential for supporting implantation and development of embryos transferred within 4 weeks of unilateral ovariectomy (Table 3). The results were essentially the same when transfer was delayed 12–18 weeks after
Table 2. Survival of embryos transferred bilaterally to CBA/Ca mice differing in age, parity and presence of one or both ovaries during reproductive life

<table>
<thead>
<tr>
<th>Group</th>
<th>Side of transfer</th>
<th>No. of recipients</th>
<th>No. of embryos transferred (Day 4)</th>
<th>Survival on Day 19 (%)</th>
<th>Mean ± SE no. of CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young primigravidae</td>
<td>Both horns</td>
<td>10</td>
<td>82</td>
<td>50 (61)</td>
<td>7.6 ± 0.6</td>
</tr>
<tr>
<td>Old primigravidae</td>
<td>Both horns</td>
<td>7</td>
<td>66</td>
<td>22 (33)</td>
<td>9.7 ± 0.6</td>
</tr>
<tr>
<td>Old multigravidae</td>
<td>Both horns</td>
<td>8</td>
<td>75</td>
<td>22 (29)</td>
<td>11.4 ± 0.0</td>
</tr>
<tr>
<td>Old multigravidae uni-ovx</td>
<td>Intact side</td>
<td>9</td>
<td>44</td>
<td>13 (30)</td>
<td>11.6 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Ov x side</td>
<td>9</td>
<td>42</td>
<td>7 (17)</td>
<td>11.3 ± 0.2</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>Both sides</td>
<td>9</td>
<td>86</td>
<td>20 (23)</td>
<td>11.3 ± 0.2</td>
</tr>
</tbody>
</table>

Embryo survival was significantly affected by age of recipient (P < 0.01) but not by other variables.

Table 3. Survival of embryos transferred unilaterally to CBA/Ca and CFLP mice differing in parity and presence of one or both ovaries during reproductive life

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group (side of ovum transfer)</th>
<th>No. of recipients</th>
<th>No. of embryos transferred (Day 4)</th>
<th>Survival on Day 19 (%)</th>
<th>Mean ± SE no. of CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/Ca</td>
<td>Young primigravidae (R)</td>
<td>3</td>
<td>19</td>
<td>10 (52)</td>
<td>9.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Young primigravidae uni-ovx (intact side)</td>
<td>6</td>
<td>39</td>
<td>21 (54)</td>
<td>9.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Young primigravidae uni-ovx (ovx side)</td>
<td>8</td>
<td>48</td>
<td>26 (54)</td>
<td>9.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Old primigravidae (R)</td>
<td>5</td>
<td>43</td>
<td>0 (0)</td>
<td>Regret</td>
</tr>
<tr>
<td></td>
<td>Old multigravidae (R)</td>
<td>4</td>
<td>46</td>
<td>22 (48)</td>
<td>11.6 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Old multigravidae uni-ovx (intact side)</td>
<td>9</td>
<td>97</td>
<td>23 (24)</td>
<td>10.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Old multigravidae uni-ovx (ovx side)</td>
<td>7</td>
<td>72</td>
<td>1 (1)</td>
<td>10.4 ± 0.1</td>
</tr>
<tr>
<td>CFLP</td>
<td>Young primigravidae (R)</td>
<td>13</td>
<td>117</td>
<td>75 (64)</td>
<td>13.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Old primigravidae (R)</td>
<td>3</td>
<td>37</td>
<td>12 (32)</td>
<td>14.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Old multigravidae (R)</td>
<td>5</td>
<td>54</td>
<td>26 (48)</td>
<td>15.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Old multigravidae uni-ovx (intact side)</td>
<td>3</td>
<td>39</td>
<td>15 (38)</td>
<td>13.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Old multigravidae uni-ovx (ovx side)</td>
<td>3</td>
<td>39</td>
<td>10 (26)</td>
<td>13.5 ± 0.1</td>
</tr>
</tbody>
</table>

R = side chosen at random.

Embryo survival was significantly affected by age and parity of recipient (see text for details).

surgery: 25/63 fetuses survived on the ovariectomized side compared to 41/96 on the contralateral side. The survival of embryos in young animals with one ovary did not differ significantly from controls. Examination of the raw data did not reveal any evidence of differential fetal survival among the right and left horns or of internal migration when embryos had been introduced to only one side of the uterus (N = 94 unilateral pregnancies).

Fewer transferred embryos survived to term in the uteri of old than in young animals (Table 2 and 3, P < 0.01 by χ²). The gestational history of individual horns did not significantly affect the success of pregnancy established by bilateral transfer of embryos (Table 2). In experiments only about half as many embryos survived in the contralateral (and these previously barren) horns of multigravid with one ovary as in the ipsilateral (and multigravid) horns of the same animals. This trend was statistically significant in subsequent experiments which the entire set of embryos was transferred to a single horn (Table 3). All embryos from the aged intact primigravidae and there was only one survivor in the previously barren unilaterally ovariectomized multigravid CBA/Ca mice. This survival proportion was significantly lower than that of the multigravid horn of one-ovary mice (P < 0.001) which, in turn, was significantly lower than that for the multigravid horn of intact animals (P < 0.01). Similar trends were found after transfers to a smaller but comparable series of CFLP mice. Embryo survival was lower in old animals of this strain (P < 0.01). The uterine horns of intact primigravid
were more acceptable sites for implantation and development than were the previously barren horns of old multiparous mice \((P < 0.05)\).

Signs of embryonic resorption were common in multigravidae but absent from primigravidae of the same age. The full-term fetuses of old animals were similar in weight to those of young individuals with the same litter size. The fetal:placental weight ratios were also similar in the two groups.

Decidual cell reaction

The number of stimuli applied to the uteri of young mice corresponded closely to the number of deciduomata found at autopsy. The response of old uteri to similar treatment was severely impaired (Table 4). Weight changes of right and left horns did not differ significantly. Statistical comparisons of weight changes between individual groups were made by using values derived from the difference between the horn weight and the mean of the pooled control values. The procedure overcame the problem of non-equivalence of the two horns of previously unilaterally pregnant mice. The decidual responses had large variances and no significant differences were found when old uteri with different histories were compared. EXCEPTIONALLY, the response of the barren horn of unilaterally ovariectomized CFLP mice was less than that of the multiparous horn of similar animals \((P < 0.05)\).

Table 4. The response of ovariectomized, hormone-primed mice to decidualizing stimuli in relation to age, parity and presence of one or both ovaries during reproductive life

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Animals with 1 or more deciduomata/total animals</th>
<th>No. of deciduomata</th>
<th>Wt of uterine horn (mg)</th>
<th>(%) change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stimulated side</td>
<td>Unstimulated side</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFLP</td>
<td>Young nulliparae</td>
<td>7/7</td>
<td>2.9 ± 0.3</td>
<td>73 ± 11</td>
<td>102 ± 31</td>
</tr>
<tr>
<td></td>
<td>Old nulliparae</td>
<td>2/8*</td>
<td>2.0 ± 1.0</td>
<td>67 ± 13</td>
<td>19 ± 24</td>
</tr>
<tr>
<td></td>
<td>Old multiparae</td>
<td>2/6*</td>
<td>2.0 ± 0.0</td>
<td>84 ± 10</td>
<td>17 ± 14</td>
</tr>
<tr>
<td></td>
<td>Old multiparous,</td>
<td>3/7</td>
<td>1.7 ± 0.7</td>
<td>88 ± 12</td>
<td>31 ± 17</td>
</tr>
<tr>
<td></td>
<td>unilaterally</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ovariectomized</td>
<td>4/7</td>
<td>2.0 ± 0.6</td>
<td>60 ± 11</td>
<td>-25 ± 14§</td>
</tr>
<tr>
<td></td>
<td>Intact side stimulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ovx side stimulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA/Ca</td>
<td>Young nulliparae</td>
<td>10/10†</td>
<td>2.9 ± 0.1</td>
<td>53 ± 7</td>
<td>115 ± 27§</td>
</tr>
<tr>
<td></td>
<td>Old multiparae</td>
<td>3/10†</td>
<td>1.7 ± 0.3</td>
<td>61 ± 7</td>
<td>35 ± 15‡</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

* \(P < 0.05\) compared to young animals.
† \(P < 0.005\).
‡ \(P < 0.02\).
§ \(F_{4,30} = 4.45\) \((P < 0.01)\); each old group was significantly smaller than the young control \((P < 0.05)\).

Microscopical studies

Parous uterine horns contained large numbers of pigment cells which accounted for their yellow-brown discoloration. Pigment cells were sparse in nulliparous horns \((< 10\) per transverse section), even when the other horn in the same animal was multiparous and, consequently, heavily pigmented. The pigment appeared shortly after parturition in primiparae. Much of this material persisted in the uterine wall, the characteristic difference in pigment density between the horns of multiparous unilaterally ovariectomized mice lasting at least 58 weeks after the last litter. Most of the pigment cells were found in the muscle layers and were often present as focal accumulations on the mesometrial side.

The pigment cells were presumed to be macrophages on the basis of their fine structural appearance and presence of hydrolytic enzyme activity. The pigment granules exhibited golden-
yellow autofluorescence (Pl. 1, Fig. 1) and other histochemical characteristics of lipofuscin. The latter contained iron (Pl. 1, Fig. 2). Most macrophages seen by electron microscopy contained single nucleus and many polymorphic cytoplasmic granules, some of which resembled lipofuscin granules (Pl. 1, Fig. 3). They were notably deficient in mitochondria, Golgi apparatus and endoplasmic reticulum and, consistent with these observations, there was no clear morphological evidence of continuing phagocytosis in the majority of cells.

Mast cells were more abundant in the myometrium than in the endometrium but both types tended to accumulate them in old age (Table 5). The effect of age on mast cell density in the myometrium was highly significant ($P < 0.01$); gestational history and unilateral ovariectomy had no effect on their density.

Table 5. Mast cell density (mean ± s.e.m.) in uterine horns of CBA/Ca mice differing in age, reproductive history and presence of one or both ovaries

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Myometrium</th>
<th>Endometrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young nulliparae</td>
<td>5</td>
<td>51.6 ± 8.6</td>
<td>0.4 ± 0</td>
</tr>
<tr>
<td>Old multiparae</td>
<td>5</td>
<td>184.1 ± 23.9</td>
<td>3.6 ± 1.1</td>
</tr>
<tr>
<td>Old unilaterally ovariectomized multiparae</td>
<td>5</td>
<td>152.4 ± 18.8</td>
<td>2.4 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>140.9 ± 21.7</td>
<td>2.0 ± 1.1</td>
</tr>
</tbody>
</table>

Myometrial cell density varied significantly between groups ($F_{3,16} = 8.78$, $P < 0.01$), young uteri having lower values than other types ($P < 0.01$).

Discussion

In contrast to the present results, there is some evidence that unilateral ovariectomy in mice accelerates senescence of the remaining ovary. Thung (1961) found that the frequency of non-oestrus cycles was lower in animals with one ovary than in intact controls. This effect did not however, appear until more than 7 months after surgery of animals that were already old. It is probably not a primary ovarian effect because the rate of depletion of the follicle reserve was apparently unchanged after unilateral ovariectomy (Jones & Krohn, 1960). There is also the possibility that the quality of ova shed is further reduced in ageing mice by removal of an ovary (Henderson & Edwards, 1968). The finding that ovum survival is dependent on the age and breeding history of the recipient rather than the donor does not refute the hypothesis that minimizes the significance of ovum defects in intrauterine death in ageing mice (Talbot, Krohn, 1966; Gosden, 1974). The function of the CL appears to be maintained in old age, judging from measurements of circulating progesterone during pregnancy (Parkening, Leckie, Saxena & Chang, 1978; Gosden & Fowler, 1979) and the normal histological appearance of the CL (Biggers et al., 1962a). It is therefore highly improbable that ovarian dysfunction is primarily responsible for pregnancy failure in ageing animals with one ovary.

PLATE 1

Fig. 1. Presumptive macrophages in the uterine wall of a multiparous mouse showing intense autofluorescent lipofuscin granules. Ultraviolet fluorescence/dark-ground illumination, $x450$.

Fig. 2. Transverse section of uterine horn of a multiparous mouse approximately 3 months post partum. Numerous iron-laden macrophages are present, especially between the muscle coats of the mesometrial side. L = lumen. Perl's stain and neutral red, $x47$.

Fig. 3. Electron micrograph of a uterine macrophage from a multiparous mouse approximately 3 months post partum. The cell is replete with dense granular material ($L$ = lipofuscin granules and multivesicular bodies (M)) but is deficient in mitochondria and other membranous cytoplasmic structures. Glutaraldehyde and osmium, $x6350$. 
According to Biggers et al. (1962b), the reduced breeding potential of one-ovary mice is due to localized uterine ageing resulting from repeated feto-placental overloading of the functioning horn. The hypothesis was supported mainly by indirect evidence and assumed that compensatory ovulation is maintained with otherwise normal ovarian function and that internal migration of ova is rare. The present study corroborates both of these assumptions and provides direct evidence of premature uterine failure. The same explanation may apply to the results of comparable studies in mice, rats and rabbits, but the different effects of unilateral ovariectomy on the number of litters and age at the last parturition may be variations of a common factor, e.g. prenatal mortality (Adams, 1970). The major difference between the breeding patterns of intact and one-ovary mice is the earlier and more abrupt failure of the latter to maintain the complement of ova. During their lifetime, intact and one-ovary animals produce a similar number of living young per uterine horn which seems to imply that each horn is endowed with a ‘quota’ of implantations which would be spent during a lifetime by a relatively normal level of gestational activity.

The results of unilateral transfer of embryos provide direct proof that the uterus is less able to maintain implantations after exposure to conditions of overloading. The failure to show such an effect by bilateral transfer experiments may have been due to the younger age of the animals used or to the smaller numbers of ova injected per horn, and, hence, decreased challenge to uterine function. Density-dependent death of embryos might result from a lack of suitable implantation sites or from a decreased ability of the uterus to maintain a given feto-placental metabolic load. Reduction of the feto-placental load is beneficial for reproduction in old animals if it affords an opportunity for preferential elimination of abnormal fetuses or if it makes scarce resources available so that surviving fetuses can grow normally.

Delayed breeding did not preserve the uterus from the effects of ageing. Embryonic survival in previously barren horns of unilaterally ovariectomized multiparae was even less successful than in the opposite parous horn. Other studies have shown a detrimental effect of delayed breeding in rodents, although the embryonic contribution to infertility was not known (Asdell, Bogart & Sperling, 1941; Nishimura & Shikata, 1960). However, not all studies have shown a difference between the litter sizes of elderly primigravidae and multigravidae (Finn, 1963), although this may be due to age differences at the test breeding between the various studies. Perhaps the uterus requires stimulation by the physiological changes of pregnancy during a ‘critical period’ of mature life to optimize the long-term breeding potential. The higher rates of ovulation in multiparous than in primiparous CBA/Ca mice may be an example of a comparable process affecting the control of the ovaries. The ovulation effect might also be explained by differences in body weight (Kennedy & Kennedy, 1972).

There are many possible explanations for the reduced breeding potential. The differences in function of barren and multiparous horns of unilaterally ovariectomized mice suggest that local uterine factors are responsible for embryonic mortality since the systemic conditions were similar. Although different factors may be responsible for the detrimental effects of prolonged nulliparity and repeated overloading, the present results do not provide insight. It is possible that the different functional capacities of ageing uteri are due to a common factor, such as vascular or collagen ageing, which has different rate constants according to the local gestational history.

Embryonic death in aged animals has been attributed to vascular or collagen ageing because uterine blood flow during pregnancy (Larson & Foote, 1972) and collagen content and quality (Schaub, 1964/65) vary with age. However, direct proof for the involvement of these factors is still lacking. Collagen has sometimes been dismissed as a possible contributory factor to the decline of uterine function in unilaterally ovariectomized multiparae because both horns of these animals have a similar amount of collagen (Finn, Fitch & Harkness, 1963). The collagen hypothesis should now be reappraised because the nulliparous horn is evidently not preserved from early senescence, as has been assumed.

It is known that the decidual cell reaction is reduced in aged mice (Finn, 1966; Shapiro &
Talbert, 1974; Holinka & Finch, 1977), but the effect of breeding history has not been previously reported. The smaller weight changes resulting from decidualizing stimuli in nulliparous horn one-ovary mice compared to other groups was interesting in view of the particular gestational potential of these horns. However, firm conclusions about the functional significance of the reduction in decidual cell reaction in ageing uteri cannot be drawn from this kind of data because of considerable variability in response.

The effect of the fetal crowding in animals with one ovary can account for the great numbers of placental ‘fusions’ and, hence, perhaps for the smaller litter size of the primigravidae than would be expected with compensatory ovulation. However, the independence of placental fusions from age and parity, and the early embryonic death in elderly primigravid implies that fetal crowding cannot explain the long-term breeding data.

The relation of uterine morphology to functional potential was studied when dense pigmentation was found in the uterine horns of aged animals. Microscopy revealed marked changes in the populations of macrophages and mast cells with parity and age, respectively. Haemosiderin and lipofuscin pigments accumulating in macrophages were probably end products of intracellular digestion of material taken up by cells involved in ‘clearing-up’ operations, remodelling of the uterus after parturition (Parakkal, 1969). Pregnancy can evidently be successful in the presence of uterine pigmentation because pigment cells assume progressively with parity whereas breeding performance does not decline until after one pregnancies. It is possible that implantation or placental function might be impaired in heavily scarred uteri of multiparae. In less heavily pigmented uteri the effect of the persisting tissue would depend on whether ova preferentially implant between unfavourable sites, as occur in the hamster (Orsini, 1962).

There is tentative evidence to show that the mechanism of implantation in rats depends on the presence of mast cells (Ferrando & Nalbandov, 1968). However, there is no reason to think that the increased density of mast cells in aged uteri is other than harmless or beneficial. In course, still possible that the function of these individual cells is deficient. The age difference in mast cell density may be due to differences in hormonal environment (e.g. oestrogen) or it may be part of a generalized change in density throughout the body because the numbers of other matured cells have been found to increase with age (Ennerback & Mellblom, 1978).

Further experiments are required to show whether a causal relationship exists between the uterine changes described here and decreased organ function. The present results, however, lead to the conclusion that breeding history affects subsequent breeding potential, particularly normal level of gestational function must be optimal for long-term breed performance.

I thank Kay Grant and Noel Brown for excellent technical assistance and Davina Mel for help with the manuscript. Part of these studies was generously supported by the Med. Research Council and the Ford Foundation.

References


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Purification and Properties of Serum Albumin from Mice of Different Ages

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A substantial number of studies has been carried out to try to determine whether proteins with unusual properties accumulate in the tissues of ageing animals, especially in relation to Orgel's 'Error' in Protein Synthesis' hypothesis. However, many studies have been concerned with unlabelled proteins and many of the techniques used to examine the properties of proteins (e.g. heat denaturation kinetics) are indirect and such studies are not without considerable attendant difficulties of interpretation.

As a first step in trying to apply more rigorous techniques to studying proteins in young and old animals, albumin from the serum of 8-month-old and 21-month-old C57BL mice has been isolated and some of its properties examined using a variety of biochemical and physical techniques. Albumin preparations were pure as judged by polyacrylamide gel electrophoresis in two different buffer systems, by immuno-electrophoresis and by iso-electric focusing. Some differences were noted between the two age groups in that the immuno-electrophoresis precipitin arcs and electric focusing bands of albumin from 21-month-old animals tended to be rather more diffuse than those for the 8-month-old animals. Amino acid analysis revealed no significant difference between the two age groups and the ultraviolet spectra of the two samples were identical. Circular dichroism (CD) spectroscopy was also used to try to detect any conformational differences; again the spectra were essentially identical, both in the near and in the far ultraviolet, for each group. CD spectroscopy was also used to determine the thermal denaturation characteristics of the two samples over the temperature range 20°C to 80°C. There was little difference in the data for the two age groups at temperatures up to 50°C but at higher temperatures the albumin from 21-month-old animals appeared to be less stable than that from 8-month-old animals.

Many of the data, therefore, indicate that the samples from young and old animals have very similar properties although some of the techniques such as amino acid analysis and electrophoresis may not be sensitive enough to detect quantitatively minor changes. Some of the data, notably the thermal denaturation behaviour as followed using CD spectroscopy, do indicate differences between the two age groups. Clearly it would be interesting to see what these differences are due to and what functional significance (e.g. ligand binding) they had, if any. The data neither prove nor disprove Orgel's 'Error' Hypothesis, indeed the experiments were not designed to test it; they were solely concerned with the properties of a protein at two different ages and mechanism of any alteration has not been studied here.

Experimental and Morphological Studies of the Ageing Animal and Human Uterus

R. G. Gosden, Department of Physiology, University Medical School, Teviot Place, Edinburgh EH8 9AG, and Physiological Laboratory, Cambridge

These studies showed that many embryos delivered to the uterus of ageing mice do not implant and live to term, although they may do so if transferred to a young uterine environment. Experiments have been devised to test whether the breeding potential of the aged uterus is affected by...
previous gestational history. Pre-implantation embryos were obtained from young donor mice and transferred to pseudopregnant uterine horns of the following types of aged animal: (1) intact nulliparae, (2) intact multiparae, (3) unilaterally ovari-ectomized multiparae. Since removal of an ovary leads to compensatory ovulation and unilateral ovo-implantation, one uterine horn of animals in (3) had been previously forced to accommodate twice the normal number of embryos whereas the other horn had been maintained barren. The ability of the various groups to maintain ova to term varied: intact multiparae (48%), parous horn of one-ovary animals (24%), nulliparous horn of one-ovary and intact animals (1% and 0 respectively). These results explain the reduced breeding potential of one-ovary animals and suggest that both excessive function and prolonged lack of utilization of the uterus adversely affect long-term function.

The uterus undergoes characteristic cellular changes as a result of ageing and gestation. In mice, macrophages replete with lipofuscin and haemosiderin pigments accumulate after each parturition which leads to a characteristic yellow-brown discoloration of the uterus in old multiparae. The density of myometrial mast cells in mice increases three-fold between 8 and 88 weeks of age, but is not affected by breeding history. The most typical feature of ageing human uteri, not found in murine tissue, was the appearance of autofluorescent cytoplasmic particles in smooth muscle and other cells. These particles appeared to be a type of lysosome containing lipid as well as other material.

Changes in Mouse Colon Cell Kinetics with Age are not a Consequence of an Increasing Number of Cell Divisions

Elizabeth Hamilton, Department of Oncology, Middlesex Hospital Medical School, London W1

Cell kinetic parameters of colon crypt cells in old mice are different to those in young mice. The proliferative 'age' of young mouse crypt cells was increased by repeated irradiation, and allowing the crypts to repopulate between doses. This did not change the cell kinetics to those found in two-year-old mice. Crypt cells in the latter mice were able to repopulate the colon four times after irradiation and still function normally. Colon tumour incidence was not increased by repeated irradiations. Changes in the crypt cell kinetics with age are probably, therefore, due to humoral factors. There is no evidence of a 'proliferative' limit in colon crypt cells in vivo.

Radio-isotopic Studies of the Circulation in the Elderly

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A radio-isotopic indicator-dilution method with precordial counting has been used to measure cardiac output, blood volume, mean circulation time (MCT), pulmonary mean transit time (PMTT), and pulmonary blood volume (PBV) in elderly hospital patients. In 18 patients with a normal heart and circulation (7 men, 11 women, aged 63-86; mean 75 years) the cardiac index was 3.59±1.05 l/min/m² (about 20% above values found by others). Blood volume averaged 3.39±0.93 l/m², MCT 60±11 s, PMTT 5.4±1.0 s (all much as found by others in younger subjects). PBV averaged 296±59 ml/m²—a value identical to that found in younger subjects by others using this same method. There was no significant sex difference nor trend in any of these measurements.

The method is applicable to the study of groups of elderly subjects, both normal and with heart disease.
THE DISTRIBUTION OF FETAL MORTALITY IN AGEING C57BL/6J MICE: A STATISTICAL ANALYSIS

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INTRODUCTION

The dead fetuses of polytocous species are removed by resorption in utero since they cannot be evacuated without disturbing the living survivors. The process of resorption involves progressive autolysis and phagocytosis until the products of conception have disappeared. Fetal resorption is common in mice and especially so in middle-aged animals where it is largely responsible for the decline in litter size (Finn, 1962; Talbert, 1971). The uterus in ageing mice clearly is less capable of supporting fetal development to term as shown by transplantation of embryos (Talbert and Krohn, 1966), though there is in addition an important contribution from chromosomally aberrant zygotes (Yamamoto et al., 1973; Gosden, 1973; Fabricant and Schneider, 1978).

In the present study the occurrence of fetal resorption was analysed statistically in a large number of ageing mice. For this purpose it was necessary to regard aberrant zygotes as random “noise” because the causes of death cannot be ascertained for individuals at advanced stages of resorption. A statistical approach to the general problem of fetal resorption was adopted to help us infer whether deaths occur as a result of systemic or local causes and, hence, to construct new hypotheses for experimental testing.

MATERIALS AND METHODS

The animals used in this study were multiparous C57BL/6J mice aged 11–12 months; they were obtained from the Jackson Laboratory, Bar Harbor, ME. Young male C3HeB/FeJ mice were obtained from the same supplier. These animals formed part of a previous study (Holinka et al., 1979). Each female was placed with a male of proven fertility and the day of copulation, designated as day 1 of pregnancy, was identified by the presence of a vaginal plug.

A total of 177 mated animals were allocated randomly to groups which were autopsied on days 8–19 post-coitum. Resorbing implants were seen first on day 10 and they increased on the following three days until they represented approximately one-third of the total numbers (Fig. 1). Between days 13 and 19 of pregnancy there was no significant variation in the number or proportion of resorption sites and survivorship in a horn was independent of that in its partner. The right uterine horn carried an average

*To whom correspondence should be addressed.
Fig. 1. The height of each bar represents the mean number of live and dead implants per pregnancy in 11-12-month-old C57BL/6J mice. Solid bars indicate the contribution from resorbing implants and vertical lines mark the S.E.M.

of 4.1 implants compared with 3.4 implants in the left horn (P < 0.01), a result which closely matches the dextral bias in ovulation number in this strain (Gosden, unpublished observations).

The results obtained from animals examined on days 13–19 were combined for subsequent analyses. This sample comprised 104 animals which were carrying a total of 479 live and 295 dead implants (774 total implants). The animals were then grouped according to the size of their litter but the proportions of resorbing implants did not differ significantly between groups (Table 1).

To examine the question of whether the nine mice with whole litter resorption belonged to a functionally different sub-population the Null hypothesis was adopted that the survival of each fetus was independent and that there was an overall probability of

<table>
<thead>
<tr>
<th>Litter size</th>
<th>Total number of implants (%)</th>
<th>Total number of litters</th>
<th>Expected number* (Variance)</th>
<th>Observed number</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2 1 (33.3)</td>
<td>1</td>
<td>0.0554</td>
<td>0.0523</td>
</tr>
<tr>
<td>4</td>
<td>13 7 (35.0)</td>
<td>5</td>
<td>0.1055</td>
<td>0.1033</td>
</tr>
<tr>
<td>5</td>
<td>27 18 (40.0)</td>
<td>9</td>
<td>0.0728</td>
<td>0.0718</td>
</tr>
<tr>
<td>6</td>
<td>41 49 (54.4)</td>
<td>15</td>
<td>0.0460</td>
<td>0.0458</td>
</tr>
<tr>
<td>7</td>
<td>85 55 (39.3)</td>
<td>20</td>
<td>0.0234</td>
<td>0.0233</td>
</tr>
<tr>
<td>8</td>
<td>132 68 (34.0)</td>
<td>25</td>
<td>0.0111</td>
<td>0.0111</td>
</tr>
<tr>
<td>9</td>
<td>98 55 (35.9)</td>
<td>17</td>
<td>0.0029</td>
<td>0.0029</td>
</tr>
<tr>
<td>10</td>
<td>58 32 (35.6)</td>
<td>9</td>
<td>0.0006</td>
<td>0.0006</td>
</tr>
<tr>
<td>11</td>
<td>23 10 (30.3)</td>
<td>3</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total</td>
<td>479 295</td>
<td>104</td>
<td>0.3178</td>
<td>0.3112</td>
</tr>
</tbody>
</table>

*Derived from the binomial distribution.
the expected number of mice with whole litter resorption was calculated and compared with the observed number. Since the expected number and variance were close, the Poisson distribution was used to estimate the probability of there being nine mice or more with total resorption. This turns out to be extremely small ($p < 10^{-14}$). This indirect test assumes that the risk of resorption is the same for all litters. Variation of this risk could lead to a larger expected number of mice with total resorption without there necessarily being two populations. It would also lead to larger numbers of litters having $(X-1)$ resorbing fetuses than would be expected under the above Null hypothesis where $X$ is the litter size. The expected and observed number of mice with $(X-1)$ resorbers was therefore examined, but here there was close agreement with the Null hypothesis.

Uterine horns were grouped according to the total number of implants they were carrying (a range of 3-7 implants/horn) for an analysis of the occurrence of dead implants in relation to their order along the horn (Cochran's $Q$ test). However, there was no significant heterogeneity ($p > 0.05$).

During an examination of the raw data it was noticed that resorption sites tended to occur adjacent to each other in the same uterine horn. This possibility was evaluated by calculation of the expected number of adjacent resorbing implants and the variance for every horn and comparing it with the observed number. Three adjacent resorbers were counted as two adjacent pairs. Although resorption sites may occur in groups, this tendency did not reach a conventional level of statistical significance ($p > 0.10$).

DISCUSSION

Two general conclusions stand out from these results. Firstly, animals with whole litter mortality and those with sub-total mortality should in future be considered separately since they appear to comprise distinct sub-populations of ageing animals. The systemic factor(s) responsible for the loss of whole litters cannot yet be identified. Luteal insufficiency can be excluded as an important factor because plasma progesterone levels were not reduced by partial or total loss of conceptuses (Holinka et al., 1979 and unpublished results), a finding that can be attributed to continuing luteotrophic activity by decidual tissue (Pepe and Rothchild, 1974).

Secondly, prima facie evidence was obtained that the survivorship of individual fetuses was limited by local factors acting approximately randomly along the uterine wall. In young adult animals the growth potential and vulnerability of fetuses varies in a systematic fashion along the length of the uterine horn and probably reflects differences in the vascular perfusion of the placentas at each point along the arterial arcade (McLaren and Michie, 1960; Trasler, 1960; Beck, 1967; Barr, Jensh and Brent, 1970). In young mice made "superpregnant" by transplantation of large numbers of embryos, fetal mortality during the mid-gestational period increased with crowding above a threshold of eight implants/horn which may be a result of limited uterine surface available for implantation (McLaren and Michie, 1959). Since no comparable effect of fetal position or crowding was seen in ageing animals, other factors are presumably responsible for fetal deaths. Age-related limitations of the pool of available nutrients from the mother do not seem likely since deaths occur before pregnancy exerts its major metabolic draught. Many fetal deaths might result from accidental implantation at unfavourable localities
distributed at random along the uterine horn. At present, there is little indication of the origin or identity of these putative unfavourable sites though they may include areas which provide an inadequate vascular response to implantation and, in multiparous animals, persistent scar tissue (Gosden, 1979).

SUMMARY

Fetal death in 11–12-month-old C57BL/6J mice is first recognized on day 10 post-coitum and it increases in incidence over the subsequent three days until more than a third of all fetuses are affected. Thereafter, there is little change in the incidence of resorption sites which apparently persist until term. Analysis of 104 pregnancies between days 13–19 suggested that the loss of individual fetuses is due to local factors operating at random along the uterine wall whereas whole litter resorption results from a systemic factor, though this is not progesterone deficiency.

Acknowledgements—We thank Robin Prescott for advice and gratefully acknowledge travel grants to R.G.G. from the Carnegie Trust for the Universities of Scotland and the Wellcome Trust (U.K.) and a Population Council (NY) fellowship held by C.F.H. Working expenses were defrayed by grants to C.E.F. from the N.I.H. (AG00117, AG00446, AG07670).

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EFFECTS OF INHIBITING PROLACTIN SECRETION ON THE MAINTENANCE OF EMBRYONIC DIAPAUSE IN THE SUCKLING RAT

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SUMMARY

Treatment of rats with bromocriptine between days 5 and 8 after the post-partum mating resulted in suppression of serum prolactin levels and caused luteal regression. Although this treatment led to embryonic resorption when suckling was prevented by removing litters soon after birth, the diapausing embryos of animals nursing a litter of eight pups were unaffected by the treatment. These results suggest that the high levels of prolactin and progesterone in the circulation during lactation are not responsible for maintenance of the diapausing state.

INTRODUCTION

The gestation period of the rat may be extended considerably by concurrent lactation. This effect is due to lowered receptivity of the uterus to the nidatory stimulus and entry of blastocysts into a metabolically quiescent state. These embryos will emerge from this state and implant after treatment with oestradiol or gonadotrophic hormones which increase oestriol production (Psychyotos, 1973; Raud, 1974; Maneckjee & Moudgal, 1975).

These observations have led to the conclusion that embryonic diapause results from ovarian follicular quiescence and oestrogen deficiency during lactation. follicular quiescence has, in turn, been attributed to suppression of the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Rothchild, 1960; Raud, 1974). Comparable patterns of gonadotrophin and ovarian steroid secretion have been found in lactational anoestrus (Amenomori, Chen & Meites, 1970; Raud, 1974; Lu, Chen, Huang, Grandison, Marshall & Meites, 1976; Smith, 1978). In both states the suckling stimulus may inhibit gonadotrophin secretion by a common pathway, that is by decreasing hypothalamic luteinizing hormone releasing hormone (LH-RH) secretion.

An alternative hypothesis is that the increased circulating levels of prolactin which result from the suckling stimulus act on the ovary to inhibit follicular growth and/or steroidogenesis or act centrally to decrease FSH secretion (Smith, 1978). Since prolactin is a lutotropic hormone during the first half of pregnancy in the rat (Tomogane, Ota & Yokoyama, 1969, 1975; Morishige & Rothchild, 1974; Yoshinaga, 1974; Ford & Yoshinaga, 1975) it may also contribute to the maintenance of embryonic diapause through the action of progesterone on the uterus (Dickmann, 1973). It has, furthermore, been suggested that prolactin has a direct action on the uterus (Armstrong & King, 1971).

Since the maintenance of high levels of prolactin in the circulation during lactation depends on suckling, the occurrence of implantation after withdrawal of the nursing litter does not distinguish the two hypotheses outlined above. Prolactin secretion can, however, be inhibited by ergot alkaloids without interruption of the suckling stimulus (Tomogane et al., 1975; Smith 1978; Flint & Ensor, 1979). We have therefore used bromocriptine in the present
study to determine whether a high concentration in the circulation of prolactin and, concomitantly, of progesterone are required for the maintenance of embryonic diapause. Since suppression of prolactin secretion leads to luteal regression it was expected that supplementary progesterone would be necessary for the continuation of pregnancy if nidation occurred.

**MATERIALS AND METHODS**

The study comprised two groups of pregnant rats. Group 1 was used to establish whether pregnancy could continue during treatment with bromocriptine and, if not, whether supplementary progesterone would reverse the abortifacient action of the drug. Group 2 animals comprised the main experiment in which bromocriptine and progesterone were administered simultaneously during concurrent lactation and pregnancy to ensure that pregnancy would be supported by sufficient progesterone if embryonic diapause was interrupted (as was indicated in group 1 animals).

Virgin Sprague–Dawley female rats weighing 200–250 g and about 4 months old were maintained in temperature-controlled (23 ± 1 (range) °C) and artificially lit rooms (14h light/day from 05.00 h) and food pellets and water were available ad libitum. They were mated with males of the same strain and mated again at the next post-partum oestrus. Conception was inferred from the presence of sperm in vaginal smears (day 1 of pregnancy).

After delivery of the litter the mothers were randomly and equally allocated to one of two groups: in group I the litters were removed within 48 h of birth and in group 2 litters were adjusted to eight pups per nursing rat within the same period post partum. Each group was further divided into four sub-groups based on a factorial experimental design. Each of the sub-groups was treated with one of the following: (1) progesterone and bromocriptine; (2) progesterone and vehicle; (3) bromocriptine and vehicle; (4) both types of vehicle. Progesterone (Sigma, London) was prepared by dissolving the dose of 4 mg in 0.2 ml arachis oil containing benzyl benzoate, 10% by volume. The 1 mg dose of bromocriptine (2-ergocryptine methanesulfonate, CB-154, Sandoz Ltd, Basel, Switzerland) was dissolved in 0.1 ml ethanol to which was added 0.1 ml 0.9% saline. These doses or the equivalent volume of vehicle were injected subcutaneously at 12.00 h daily on days 5–8 inclusive of the second pregnancy. Since galactopoiesis is suppressed by bromocriptine (Flügkiger & Wagner, 1968), the litters were removed, weighed and exchanged between animals receiving those not receiving this substance. Litters were exchanged at 09.00 and at 17.30–19.00 h on each of the 4 days of treatment.

Animals were anaesthetized with ethyl carbamate (1–75 g/kg, i.p., injected as a 25% solution (w/v); BDH Ltd, Poole, Dorset) between 10.00 and 12.00 h on day 9 of pregnancy. Suckling rats were fitted with an atrial catheter for the withdrawal of blood and subsequent determination of serum concentrations of prolactin and progesterone. The volume of blood removed (1·5 ml) was replaced by an equal volume of a solution of Evan's blue dye (1% (w/v) 0.9% saline; Gurr, London). All of these steps were carried out while the animals were unconscious and they were completed by 12.30 h.

The animals were killed by cervical dislocation 15 min after injection of dye. The ovariess were inspected in situ and dissected for histology. Uterine horns were examined for implantation swellings or incipient implants (blue spots). Where evidence of implantation was absent, uterine horns were dissected and flushed with 0.9% saline solution. The flushings were then searched for unimplanted embryos with a dissecting microscope (magnification × 25) using transmitted illumination.

An additional group of eight animals with simultaneous pregnancy and lactation received similar injections to those described above but they were not killed until day 16 or 20 of pregnancy. On day 9, the litters were removed and further drug treatment ceased except in the case of some animals treated with bromocriptine plus progesterone which continued to receive 4 mg progesterone daily until autopsy.
Histology

Ovaries and uterine tissue were fixed by immersion in aqueous Bouin's fluid and prepared as paraffin-wax sections cut serially at 10 μm. The mounted sections were stained with haematoxylin and eosin. These preparations were used for morphological observation and counting of the number of medium- and large-sized ovarian follicles. In each ovary of the right side of the ovary, all the follicles above 100 μm maximum diameter (i.e., antral follicles) were measured. This was carried out by projecting an enlarged image (×40) of the sections onto a screen with a 150 mm lens (Leitz); the mean maximum diameter of each follicle was then assigned to one of the three size classes by using a simple measuring device with points 4, 8 and 16 mm apart (equivalent to 100, 200 and 400 μm).

Radioimmunoassays

Serum levels of prolactin were measured by a previously described method using reagents provided by NIAMDD, Maryland, U.S.A. (McNeilly, Sharpe, Davidson & Fraser, 1978). The results are expressed in terms of the NIAMDD reference preparation RP-1. The levels of progesterone were measured by a specific radioimmunoassay (Baird, Burger, Heavon-Jones & Scaramuzzi, 1974). Samples were analysed in a single assay having an intra-assay coefficient of variation of 7% and 9% for prolactin and progesterone respectively.

Statistics

The proportions of animals with implanted embryos and the numbers of ovarian follicles in each treatment group were analysed by Chi-squared test and ANOVA respectively. Since the form of the statistical distribution obtained from hormone measurements and weighing of litters was uncertain, the results were expressed by their median and range and analysed by non-parametric methods, namely, Wilcoxon's test and the Kruskal–Wallis method of analysis of variance of ranks.

Results

Group 1: effects of bromocriptine on pregnancy in non-lactating rats

Conspicuous implants were present in the uterine horns on day 9 of pregnancy in animals whose pups were removed soon after birth and the post-partum mating (Table 1). Implantation had evidently occurred directly or at least shortly after the normal pre-attachment period of non-lactating rats; supplementary progesterone had no effect on the numbers of implants (Table 1). Pregnancy was terminated when removal of the litter was combined with treatment with bromocriptine. The embryos had died soon after implantation in these rats because small, equally spaced patches of scar or trophoblastic tissue were found during histological examination. This abortifacient action of bromocriptine was completely overcome by simultaneous treatment with progesterone. These results indicated that progesterone would be necessary during bromocriptine treatment of group 2 animals in order to support any implanted embryos.

Table 1. Effects of treatment with bromocriptine and progesterone on implantation in rats lacking suckling stimuli

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of animals</th>
<th>With barren uteri</th>
<th>With implants</th>
<th>Implants/pregnancy (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (both vehicles)</td>
<td>9</td>
<td>1</td>
<td>8</td>
<td>15.0±0.8</td>
</tr>
<tr>
<td>Progesterone + vehicle</td>
<td>9</td>
<td>0</td>
<td>9</td>
<td>13.0±1.4</td>
</tr>
<tr>
<td>Bromocriptine + vehicle</td>
<td>10</td>
<td>10*</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Bromocriptine + progesterone</td>
<td>10</td>
<td>1</td>
<td>9</td>
<td>14.6±0.8</td>
</tr>
</tbody>
</table>

* P<0.001 compared with all other groups (χ² test).
Table 2. Effects of treatment with bromocriptine and progesterone on simultaneous pregnancy and lactation in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of animals</th>
<th>Median % change of litter weight during suckling (range)</th>
<th>Median (range) serum hormone (ng/ml)</th>
<th>No. of follicles in right ovary (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With barren uteri</td>
<td>With implants</td>
<td>With unimplanted embryos†</td>
</tr>
<tr>
<td>Control (both vehicles)</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Progesterone + vehicle</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Bromocriptine + vehicle</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Bromocriptine + progesterone</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>

*P < 0.01 compared with groups not treated with bromocriptine (Wilcoxon's test and Kruskal–Wallis analysis of variance of ranks); †P < 0.05 compared with groups not treated with bromocriptine (ANOVA). ‡ There was no significant difference between proportions of animals with unimplanted embryos in the various groups (χ²).
Implantation was prevented by concurrent lactation in vehicle-treated animals (Table 2). There was no evidence of interruption of pregnancy by treatment of nursing rats with bromocriptine; neither was there any suggestion that this treatment initiated implantation, even when the declining levels of progesterone were restored by supplementation (Table 2). That serum prolactin levels had been suppressed by bromocriptine was shown unequivocally by radioimmunoassay and the failure of litters to gain weight when suckled by bromocriptine-treated rats. The exceptional animals that either had implanted embryos or no embryos of any kind can be attributed to failure of sucking or mating respectively. The majority of animals of all groups suckling litters yielded blastocysts of normal morphological appearance in their uterine flushings. These embryos were elongated and had hatched from their zonae pellucida. Further experiments showed that they retained their viability during treatment since the expected number of implanted embryos were found on days 16 or 20 when suckling treatment had been suspended on day 9. However, implants were only obtained in animals previously treated with bromocriptine when injections of progesterone were given after day 9: all three such animals bore implants (14 or 15 in each animal).

Corpora lutea were prominent at autopsy in the ovaries of both suckling and non-suckling animals, even when bromocriptine had induced luteal cell involution. The lutein cells of bromocriptine-treated animals were shrunken and vacuolated with heteropyknotic nuclei; the neighbouring blood vessels were often congested. These cellular changes paralleled the reduction of progesterone concentration in the circulation (Table 2).

Similar populations of medium- to large-sized follicles were found in the ovaries of the various groups of suckling animals (Table 2). Despite the absence of any significant differences in total number of antral follicles, a small but significant ($P<0.05$) reduction in number of follicles in the size range 201–400 $\mu$m was found in the two groups of animals receiving bromocriptine. This difference was not, however, reflected in adjustments of numbers of follicles in the smaller or larger size categories and may be without biological significance.

**DISCUSSION**

The effectiveness of bromocriptine treatment in terminating lactation and pregnancy without lactation has been confirmed. Bromocriptine inhibited prolactin secretion and thereby suppressed milk production and caused embryonic resorption during the early phase of implantation development (Flückiger & Wagner, 1968; Morishige & Rothchild, 1974; Flint & Ensor, 1979). The drug had not interfered with the initiation of implantation as residual products of conception were seen on day 9. Since the abortifacient activity of bromocriptine was fully reversed by supplementary progesterone to replace the lost function of the corpus luteum, it was thought to be necessary also to provide replacement hormone for lactating pregnant animals.

Bromocriptine lowered serum prolactin and progesterone levels in lactating pregnant rats. Since administration of bromocriptine with or without progesterone did not interrupt embryonic diapause it is concluded that neither the raised circulating levels of prolactin nor progesterone normally found during lactation are required for delaying implantation. The hypothesis that prolactin has direct or indirect inhibitory effects on ovarian follicular growth or steroidogenesis is thus refuted because removal of the inhibition would have been expected to permit ovo-implantation since this has a small but essential requirement for oestrogen in the rat (Psychoyos, 1973). As additional evidence for this conclusion we found little change in the profile of the ovarian follicle population with declining prolactin levels. However, our conclusion that the effect of the suckling stimulus on the process of pregnancy is not exerted through the action of prolactin is in apparent conflict with several studies. In particular, the studies of Smith (1978) on lactational anoestrus and Maneckjee & Moudgal (1975) and Flint & Ensor (1980) on lactation-induced delay of implantation directly
implicate prolactin as at least part of the mechanism for the maintenance of gonadotrophin suppression.

The effects of bromocriptine treatment on embryonic viability differed according to whether or not the embryos had implanted. But in both cases there was evidence of fetal regression and lowered progesterone secretion. It is interesting that diapausing embryos apparently remained viable while progesterone levels were substantially reduced by bromocriptine treatment. There is disagreement about the need for progesterone replacement in ovariectomized rats for sustaining diapausing blastocysts (Powell, Bennett & Cochrane, 1979). Our results imply that the residual amounts of progesterone from ovaries of bromocriptine-treated rats together with the adrenal contribution (Labhsetwar, 1971) are sufficient for embryonic viability. The reduction of progesterone secretion by bromocriptine did not, however, bring about a resumption of development as a result of an altered balance of oestrogen and progesterone action on the uterus. Although the progesterone in the circulation during lactational pregnancy seems to be in excess of the requirements of a uterus carrying diapausing embryos, it may serve an essential role in priming the uterus in anticipation of the event of implantation (Martin & Finn, 1969; Dickmann, 1972). It is also likely that the progesterone requirement rises rapidly during early implantation development and this is perhaps shown by the abortifacient effect of prolactin suppression in the non-lactating pregnancies.

We therefore agree with an earlier conclusion that the suckling stimulus is the primary cause of gonadotrophin suppression during lactation in the rat (Rothchild, 1960; Ray, 1974; Lu et al. 1976), although a facilitating action of low levels of prolactin cannot be ruled out. Further studies are required to elucidate the mechanisms whereby the neural stimulus is translated into reduced gonadotrophin release; the hypothetical inhibitory effect on hypothalamic neurones containing LH-RH, first suggested by Rothchild 20 years ago, is still a plausible explanation.

The skilful assistance of Kay Grant and Rita Ghione and the generous gift of bromocriptine from the Sandoz Company are gratefully acknowledged. Part of this study was supported by a grant from the Moray Fund (University of Edinburgh).

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Spontaneous abdominal implantation in the rat with development to full term

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Summary

A fully-developed rat foetus was recovered from the peritoneal cavity of a rat, where it had formed a vascular connection to the omentum. Primary implantation on the omentum was inferred.

Whereas abdominal pregnancy has been regularly reported to be compatible with full-term development in man, it is very rare or unknown in domesticated and laboratory species. The periovarial sac and genital tract normally provide a barrier to penetration by rodent embryos, and even when this barrier is artificially breached abdominal implantation is uncommon or abortive after a few days of development (Fawcett, Wislocki & Waldo, 1947; Jollie, 1961; McLaren & Tarkowski, 1963). There is only one documented account to show that implantation in an ectopic site is not a bar to full development in the rat (Nicholas, 1934). Naturally-occurring abdominal pregnancy has been reported in other rodent species, but it is seldom possible to provide unequivocal evidence that primary extrauterine implantation had occurred rather than rupture of the genital tract with a subsequent secondary attachment to intraperitoneal membranes (Hong & Armstrong, 1978; Buckley & Caine, 1979).

During the course of post-mortem examination of Sprague-Dawley rats we noticed a large mass in the left upper abdomen of one animal. Further dissection revealed a single fully-developed foetus weighing 4.83 g. The foetus was recovered from a rat of 4 months of age which had delivered the other 15 pups of its 1st litter and had mated at the post-partum estrus. The 1st litter was suckled and grew normally until the day of autopsy, Day 11 post partum. On Day 6 post partum, drug treatment of the mother began (no further details are given here because this obviously could not have influenced the preceding pregnancy). At the autopsy, in addition to the extrauterine conceptus, the uterus was swollen with 18 normally-spaced early conceptuses (Fig. 1). There were no signs of haemorrhage or lesions or anatomical abnormality of the genital tract.

The ectopic foetus was dead, yet not macerated or mummified. Closer inspection showed that it was female and of normal anatomical appearance. It was...
surrounded by an amniotic sac filled with abundant fluid containing extravasated blood, and was attached to a placenta by a short umbilicus. The placenta, which weighed 2.50 g, was histologically normal though larger in area and thinner than is usual at full term. It was still being perfused by 2 accessory portal systems consisting of coiled dilated vessels which tapered towards their origin in the greater omentum. On the maternal side of the placenta a pale button-shaped mass (0.31 g) of coagulation necrosis was prominent and may have represented an anaemic infarction of this organ. The entire conceptus was enclosed in a wreath of tissue resembling mesentery, but histological examination showed there was no decidual-cell response of maternal stromal tissue. The conceptus was not connected with the uterus or ovary.

In man abdominal pregnancy is usually secondary to rupture of a fallopian tube (Cavanagh, 1958), although primary abdominal implantation does occur rarely (Studdiford, 1942; Pritchard & Macdonald, 1976). There were no lesions in the genital tract of the rat and the implantation site did not involve the tract, thus, apart from the late stage of development of the foetus, the present case satisfies the criteria for diagnosis of primary abdominal implantation (Studdiford, 1942). Foetal survival may have extended beyond the time when uterine siblings had been delivered, perhaps until placental perfusion became inadequate. We conclude that the occurrence of an ectopic foetus depended on the coincidence of 2 improbable events, namely, escape of an embryo from the genital tract, possibly via the foramen in the periovarial sac (Alden, 1942), and successful extrauterine implantation. As far as we are aware, this is the 1st report of full-term development of a naturally occurring ectopic foetus in the rat.

References

Spontane Bauchhohlentrichtigkeit bei der Ratte mit Entwicklung zur vollen Geburtsreife
R. G. GOSDEN & J. A. RUSSELL

Zusammenfassung
Ein voll entwickelter Rattenfötus wurde aus der Bauchhöhle einer Ratte gewonnen, wo er vaskuläre Verbindungen zum Omentum entwickelt hatte. Daraus wird auf eine primäre Implantation im Omentum geschlossen.
Structure and gametogenic potential of seminiferous tubules in ageing mice

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Summary. The gametogenic potential of young male CBA/Ca mice aged 4–5 months was compared with that of animals 20–23 months of age which had recently ceased to sire offspring. The older mice had substantially lower circulating levels of testosterone and smaller testes. Testicular and epididymal spermatozoa were reduced in number, had more abnormal forms and fewer were progressively motile. Ageing seminiferous tubules varied in their degree of functional atrophy from complete depletion of germ cells to maintenance of sperm production. They were enclosed by a thickened basal lamina and more collagenous connective tissue and occasionally allowed the penetration of lanthanum between Sertoli cells into the ad-luminal compartment. There was no evidence of autosensitization to sperm antigens.

Introduction

Ageing of the testis, like that of the ovary, involves a decreased output of sex steroids and gametes, though this does not usually result in a well-defined climacteric or transition from the reproductive to a post-reproductive phase of adult male life (Bishop, 1970; Vermeulen, Rubens & Verdonck, 1972; Nieschlag, 1979). Morphological signs of testicular ageing can be recognized relatively early in maturity, the most conspicuous one being the appearance of atrophic tubules in men and other animals (Sasano & Ichijo, 1969; Bishop, 1970).

In the present study an attempt was made to identify possible causes of tubule atrophy in the testes of ageing inbred mice by comparing their functional activity with their morphological appearance. In addition, it seemed necessary to search for signs of testicular autosensitization in these animals because this organ is susceptible to immunological injury and autoimmunity is a characteristic of ageing.

Materials and Methods

Animals. Young (4–5 months) and ageing (20–23 months) CBA/Ca male mice were obtained from the Department of Physiology colony where they were maintained at 23 ± 1°C with a photoperiod of 14 h/day and food and water ad libitum. All of these animals had previously sired offspring although animals of this colony rarely mate with or impregnate females after 20 months of age. They had been isolated from females at least 1 month before study. Each animal was killed by decapitation and trunk blood was collected and prepared as serum which was stored at −18°C for steroid radioimmunoassay (see below).

The right cauda epididymidis and adjacent third of the vas deferens were dissected free of...
adhering tissue and incubated for 10 min at 36°C in 3 ml tissue culture medium 199 containing Earle’s salts and 20 mM-Hepes buffer (Flow Laboratories, Irvine, Ayrshire). The medium was supplemented with 0.27 mM-pyruvate, 3 mg BSA (Sigma, London)/ml and 0.1 mg gentamicin (Flow Labs)/ml. The epididymis was then cut in three places and at the base of the vas to allow spermatozoa to swim out. After a further incubation of 15 min the fluid was agitated gently and samples were withdrawn for light microscope examination. The percentage of motile spermatozoa was estimated from the proportion capable of forward progression.

The left testis and epididymis were each isolated, blotted gently to remove surface moisture, weighed and minced for 20 sec in 2 ml medium (as above) to produce a cell suspension (Polytron, Lucerne). The suspensions were vortex-mixed and prepared immediately for counting with a haemocytometer. Seminal vesicles were weighed after expression of their fluid contents. Finally, a routine post-mortem examination was carried out so that diseased animals could be excluded from study.

**Light and electron microscopy.** Testes were removed from animals immediately after death and fixed overnight in aqueous Bouin’s fluid. Fixed tissues were embedded in paraffin wax sectioned at 6 μm and stained with haematoxylin and eosin. The spermatogenic activity of these organs was estimated by using the Sertoli cell number as a constant factor, and thus to obtain the “Sertoli cell ratio” (Skakkebaek & Heller, 1973). The numbers of spermatogonia, spermatocytes and spermatids in 10–20 randomly selected tubules presenting an approximately circular cross-sectional profile were counted under oil-immersion (×1000) and expressed as a ratio to the number of Sertoli cells sectioned through the nucleolus in the same tubule segment. Fine distinctions between cell stages were not made, although spermatids were classified into early and late forms which corresponded approximately to the Golgi and cap phases (1–7) and the acrosomal and maturation phases (8–12), respectively (Leblond & Clermont, 1952). In addition, the cross-sectional diameters of 50 tubules in each testis were measured with an ocular micrometer.

Fresh testicular tissue was gently teased between fine needles to allow penetration of fixative for electron microscopy. The methods of tissue preparation were similar to those of Neaves (1973). Half of the tissue fragments from each animal were immersed in fixative containing lanthanum nitrate for the study of occlusive cell junctions. Tissue prepared in the same way from 15-day-old mice served to demonstrate the appearance of the tracer in tubules which lack occluding junctions between Sertoli cells (Vitale, Fawcett & Dym, 1973).

**Estimation of serum sperm-agglutinating activity.** Blood was collected from the tail veins of conscious animals and prepared as serum. Although mainly comprising CBA/Ca mice the group also included 3 young and 5 ageing Q-strain animals. A micro-agglutination method based on that developed for the rat was used for measuring sperm-agglutinating activity (Rümke & Titus, 1970).

Sperm suspensions containing a high proportion of motile cells (>60%) were obtained from the epididymides of syngeneic male mice. They were maintained at a concentration of about 10^7 cells/ml in the medium described above. Serum samples were heated for 30 min at 56°C to remove non-specific agglutinating activity, cooled and diluted with medium. Sperm suspensions were added to the diluted sera, agitated gently and incubated at 36°C. Each tube was evaluated at 30-min intervals up to 3 h; granular suspended or precipitated material was regarded as a positive reaction. When a positive reaction was obtained at dilutions of 1:4 or 1:20 they were tested at greater dilutions up to 1:1280. Each experiment contained negative control tubes (lacking serum) and positive control tubes (containing serum from animals previously immunized with autologous mouse testis in Freund’s Complete Adjuvant).

**Immunofluorescence.** Since small numbers of plasma cells and local production of antibody might have been undetected by the above techniques, the distribution of immunoglobulin (lg) in the testis and epididymis was examined by immunofluorescence. An indirect method was used: cryostat sections cut at 6 μm were incubated with rabbit anti-mouse Ig, washed with PBS and
then incubated with FITC-conjugated swine anti-rabbit Ig (Dako, Copenhagen). The presence of non-specific fluorescence was checked by incubating some sections without the first antibody. After thorough rinsing in PBS the sections were examined by u.v. fluorescence microscopy using incident illumination.

Radioimmunoassay. Serum testosterone and 5α-dihydrotestosterone (5α-DHT) were measured by radioimmunoassay following extraction with hexane:ether (4:1, v:v) and separation by celite chromatography (Thorneycroft, Ribeiro, Stone & Tillson, 1973). An antiserum was raised to testosterone in a goat using testosterone-3-carboxymethylxime-BSA as the immunogen (Corker & Davidson, 1978). The cross-reactivity of the antiserum with 16 steroids was analysed and found to be significant only for 5α-DHT (23.9%), 11β-hydroxytestosterone (0.4%), oestradiol-17β (0.2%) and Δ4-androstenedione (0.1%). The antiserum to 5α-DHT was raised to 5α-dihydrotestosterone-3-carboxymethylxime–BSA in a rabbit and this cross-reacted with testosterone (57.0%), 5α-androstan-3β,17β diol (7.8%) and 5β-DHT (3.0%). The efficiencies of recovering known amounts of testosterone and 5α-DHT from serum were 81.0 ± 6.7% and 87.8 ± 8.5% respectively for the two steroids. The characteristics of the assays for testosterone and 5α-DHT were as follows: sensitivity (10 and 5 pg respectively), intra-assay variation (4.7 and 9.0% respectively), interassay variation (11.8 and 14.2% respectively).

Statistics. Because the form of the statistical distribution of data in the cytological studies was uncertain, the results were expressed by median values and ranges and analysed by ranking the data and applying Wilcoxon’s test.

Results

The testes of old mice were conspicuously different from those of younger animals. They were smaller, somewhat flaccid and yellow-brown in coloration and the spermatozoa recovered from them were reduced in number and quality. The median values of testicular and epididymal spermatozoa in old animals were less than 30% of those in young individuals, and substantially fewer epididymal spermatozoa of the former group were motile and many were morphologically abnormal or decapitated forms (Table 1). The serum concentrations of testosterone but not 5α-DHT were reduced in old animals (Table 1). However, there was no parallel reduction in weight of the androgen-sensitive organs, epididymis and seminal vesicles, and the latter contained an abundant secretion.

Table 1. Comparison of the weights of reproductive organs and concentrations of spermatozoa and androgens in young and aged CBA/Ca male mice

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>No. of mice</th>
<th>Testis (mg)</th>
<th>Seminal vesicles (mg)</th>
<th>No. of spermatozoa (×10⁶)</th>
<th>Epididymal sperm motility (%)</th>
<th>Serum testosterone (ng/ml)</th>
<th>Serum 5α-DHT (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Testis</td>
<td>Epididymis</td>
<td>Testis</td>
<td>Epididymis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-5</td>
<td>8</td>
<td>66</td>
<td>27</td>
<td>65</td>
<td>4.3</td>
<td>17.7</td>
<td>10.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(50-76)</td>
<td>(24-29)</td>
<td>(49-94)</td>
<td>(3.4-7.8)</td>
<td>(12.4-22.8)</td>
<td>(50-80)</td>
</tr>
<tr>
<td>22</td>
<td>8</td>
<td>33**</td>
<td>28</td>
<td>79</td>
<td>1.2*</td>
<td>4-7**</td>
<td>5*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(21-68)</td>
<td>(18-35)</td>
<td>(47-94)</td>
<td>(0.4-8.8)</td>
<td>(0.6-12-4)</td>
<td>(0.0-70)</td>
</tr>
</tbody>
</table>

Median values and ranges are given.
Values significantly different from those of young animals; * P < 0.02, ** P < 0.01.

The diminished number of testicular spermatozoa was paralleled by functional atrophy of individual tubules. Although the degree of atrophy varied greatly between segments of different tubules, serial examination suggested a greater uniformity along the length of individual tubules.
A substantial proportion of tubule segments in aged testes were depleted of germ cells (median value 22%) whereas every young tubule maintained active spermatogenesis (Pl. 1, Figs 1 and 2). The remnant of functional tubules in old animals exhibited widely varying degrees of germ cell attrition as was evidenced by their 'Sertoli cell ratio' (Table 2). The proportionate differences between 'Sertoli cell ratios' in the two age groups became progressively greater through successive stages of spermatogenesis. This result was apparently due to arrested development of spermatogonia and spermatocytes in some tubules rather than to a substantial increase in cell death, though quantitative estimates of death rates were not recorded. The reduction in spermatogenic activity was associated with reduced diameter of tubules (P < 0.01) and folding of the extracellular elements of the lamina propria (Pl. 1, Fig. 4).

Table 2. Quantitative cytological analysis of seminiferous tubules in young and aged CBA/Ca mice

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>No. of mice</th>
<th>Spermatogonia</th>
<th>Spermatocytes</th>
<th>Early spermatids</th>
<th>Late spermatids</th>
<th>Sertoli cell ratio†</th>
<th>Sertoli cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-5</td>
<td>8</td>
<td>2.4</td>
<td>4.3</td>
<td>10.3</td>
<td>6.0</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.6-3.4)</td>
<td>(3.0-5.6)</td>
<td>(7.8-11.5)</td>
<td>(9.1-13.5)</td>
<td>(7-11)</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>7</td>
<td>1.1</td>
<td>1.5*</td>
<td>2.2**</td>
<td>1.0**</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.1-3.3)</td>
<td>(0.3-9)</td>
<td>(0.13-5)</td>
<td>(0.12-9)</td>
<td>(7-11)</td>
<td></td>
</tr>
</tbody>
</table>

Values are medians and ranges.
† Total no. of germ cells of a given type divided by the total no. of Sertoli cells in the same segment of tubule. Values significantly different from those of young animals: *P < 0.05, **P < 0.01.

Whereas extensive lengths of some tubules had lost their germ cell population, Sertoli cells were always present and were not depleted by ageing (Table 2). Their cytoplasm was highly vacuolated and sometimes contained dense bodies (lipofuscin granules). Where the lanthanum tracer material had penetrated the outer shroud of myoid cells further passage between Sertoli cells of young testes was restricted by occluding junctions between adjacent cell membranes. Meiotic and haploid germ cells were segregated in the adluminal compartment. By contrast with this condition in young adult animals, the tracer penetrated freely among the primary spermatocytes of the solid cords of prepubertal animals. The great majority of tubule segments in aged mice (>90%) had sites of restricted permeability similar to those in young adults (Pl. 2, Fig. 6), but some segments with residual spermatogenesis showed a thin line of electron-opaque material extending into the interior of the tubule to surround spermatocytes and spermatids and form small pools between maturing germ cells (Pl. 3, Figs 7 and 8). The presence of lanthanum in the interior of tubules was taken to indicate permeable junctions between adjacent Sertoli cells because the alternative route of entry via the cut ends of tubules would presumably have allowed the tracer to penetrate young adult and ageing tissues similarly.

PLATE 1

Fig. 1. Intense spermatogenic activity in the testis of a 5-month-old CBA/Ca mouse. H & E, ×114.

Fig. 2. The spermatogenic activity of seminiferous tubules in 22-month-old CBA/Ca mice was much reduced; some segments were devoid of germ cells and comprised only Sertoli cells (S). Venous walls were often thickened and contained basophilic material (V). Tissue shrinkage was usually more marked in old testes. H & E, ×114.

Figs 3 and 4. The basal lamina (arrow) lying beneath Sertoli cells (S) was thick, folded and multilaminar in old seminiferous tubules (Fig. 4) compared to the appearance in young mice (Fig. 3). There were conspicuous bundles of collagen fibrils (C) between the internal and external laminas of old tubules. Glutaraldehyde and osmium, ×18 200.
Fig. 5. The fluorescent antibody technique could not reveal Ig in the testicular tubules of old mice. Weak fluorescence in the basal zone of the tubules was mainly of a non-specific nature. x390.

Fig. 6. The integrity of the blood–testis barrier in old mice may be shown by exclusion of lanthanum (electron-opaque tracer material) from the ad-luminal compartment of seminiferous tubules. In this figure, a barrier to further penetration of the tracer was presented by tight junctions (arrow) between adjacent Sertoli cells (S₁ and S₂). The spermatogonium type B shown here (G) stands on the basal lamina and lies outside the so-called blood–testis barrier. Myoid cell (M). Glutaraldehyde and osmium, x7800.
The internal basal lamina (i.e. adjacent to Sertoli cells) of aged tubules was folded, multi-laminar and substantially thicker than that in young animals (Pl. 1, Figs 3 and 4). It was also associated with bundles of collagenous connective tissue which had presumably been produced by myoid cells. These age differences were much less marked in the external lamina.

The atrophic changes in seminiferous tubules did not appear to have a specific pathological aetiology other than their relation to age. There was no evidence of obstruction of the lumen, of virus-like particles in the germinal epithelium or of an inflammatory reaction. Furthermore, there was no evidence of development of systemic or local autoimmune responses to testicular antigens. Circulating sperm-agglutinating antibodies were absent from young and old animals alike unless they had previously been sensitized by injection of testicular tissue (titre \( \geq 20 \)) (Table 3). Moreover, inflammatory infiltrates of mononuclear cells were not found in aged animals and fluorescence due to immunoglobulin was confined to the interstitial tissue and, to a much lesser extent, the basal part of the tubules (Pl. 2, Fig. 5). Large numbers of autofluorescent granules were sometimes seen within tubules but they could always be distinguished from the evanescent apple-green appearance of FITC-conjugated antibody by their golden-yellow appearance and stable emission characteristics. They were probably the lipofuscin granules described above.

Table 3. Effect of age and immunization with syngeneic testis on sperm-agglutinating antibodies in sera of male mice

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Treatment</th>
<th>No. of mice</th>
<th>No with sperm-agglutinating activity (titre ( \geq 20 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>4–5</td>
<td>—</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>20–23</td>
<td>—</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>4–5</td>
<td>FCA*</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4–5</td>
<td>FCA + testis</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

* Freund’s complete adjuvant.

Discussion

These findings endorse earlier reports that substantial changes in testicular morphology and decrements in hormone and gamete production proceed during ageing in mice (Bishop, 1970; Bronson & Desjardins, 1977). Other investigators have, however, reported that these functions are well-maintained in animals of advanced age (Finn, 1964; Eleftheriou & Lucas, 1974; Nelson, Latham & Finch, 1975). We cannot say whether the discrepancies between these studies result from differences of physiological age or of unidentified disease as is implied in the study of Nelson et al. (1975). However, the relatively early senescence of the testis during the lifespan of CBA/Ca mice corresponds to that of the ovary in this strain (Jones & Krohn, 1961).

The reserves of epididymal spermatozoa were substantially reduced in ageing animals and presumably reflected diminished testicular production. The increased numbers of immotile and

PLATE 3

Figs 7 and 8. These micrographs show the appearance of lanthanum in the minority of old seminiferous tubules in which penetration was not impeded by junctional complexes between Sertoli cells (S). The tracer substance could be seen as a thin line of opaque material extending from the basal zone of the tubule through narrow extracellular spaces (arrows) (Fig. 8). The characteristic configuration of membranes at sites of tight junctions was not usually observed in these abnormal tubules. In some tubules where the tracer had penetrated the inner compartment (arrow) maturation of spermatids (T) was apparently proceeding normally in the apical epithelium (Fig. 7). Glutaraldehyde and osmium, x7500.
morphologically abnormal spermatozoa in epididymides may also be attributed to testicular ageing since both maturation and transit time of epididymal spermatozoa are sensitive to the concentrations of androgen (Dyson & Orgebin-Crist, 1973). Accumulation of informational errors in stem cells and genetic faults during gametogenesis are likely to be additional factors of importance (Bishop, 1970).

The maintenance of the size of the litters sired by ageing male mice (Finn, 1964; Franks & Payne, 1970) may indicate that biologically significant deficits of the quantity and/or quality of epididymal sperm reserves do not occur, or occur after libido has waned. On the other hand, the frequent occurrence of insemination without subsequent impregnation in old CBA/Ca male mice may be an expression of oligospermia (R. G. Gosden, unpublished). The numbers and appearance of ejaculated spermatozoa should now be studied in these animals.

The lifespan of spermatogonial stem cells of mice appears to be limited by a randomly specified process and the numbers of these cells decrease exponentially with age (Suzuki & Withers, 1978). The present results imply that there is an additional contribution to the reduced gametogenic function of the aged testis by the failure of germ cells to progress through successive stages of maturation. Since the loss of stem cells is progressive throughout adult life but maturational failure is only found in old age, different causal factors must be at work.

During the maturation of testicular function and in advance of the full progression of spermatogonic cells through meiosis, sites of restricted permeability develop between Sertoli cells. These occluding junctions isolate spermocytes, spermatids and developing spermatozoa within an ad-luminal compartment which provides a physiologically unique environment for the development and immunological isolation of haploid germ cells (Dym & Fawcett, 1970; Vitale et al., 1973; Setchell, 1978). If penetration of aged testicular tubules by lanthanum is a physiologically significant indication of a defective blood–testis barrier, the arrested development of spermatogonia and spermocytes may be the result of an altered intra-tubular micro-environment. Consistent with this view, Neaves (1978) found lanthanum-permeable ‘tight’ junctions with reduced sperm production in the testes of vasectomized Lewis rats. However, further explanations of sperm cell attrition must be sought because the integrity of the blood–testis barrier was apparently maintained in the majority of atrophic tubule segments.

Maintenance of germ cell number and maturation in the testis is particularly sensitive to ischaemia, alterations in hormone balance and a variety of environmental factors including diet, ionizing radiation and temperature, though the mechanisms whereby they exert their control are poorly understood (Setchell, 1978). A number of these factors could play a part in senile testicular atrophy although the best documented one is the reduced production of testosterone by Leydig cells, which may be due in turn to LH deficiency (Bronson & Desjardins, 1977). Furthermore, the amount of testosterone available to the germinal epithelium may be reduced even further by thickening of the barrier to diffusion, a condition which characteristically accompanies testicular cell injury by a wide variety of agencies (Pierce & Nakane, 1969). The lamina propria may not only provide a significant barrier to the entry of hormones but also to nutrients and to the exchange of respiratory gases between the avascular tubule and the blood supply which may already be diminished (Ewing, 1967; Sasano & Ichijo, 1969).

Several potentially damaging changes appear simultaneously in ageing testes. It is not yet possible to say which is primary; besides, their individual significance may be increased when in combination. Somewhat surprisingly, we found no experimental or morphological support for an autoimmune hypothesis of testicular ageing. Except for the lack of conspicuous cellular infiltration, the morphological appearance of many atrophic tubules in aged testes resembled that of testes suffering a low-grade autoimmune orchitis. Moreover, Rümke & Titus (1970) reported low titres of naturally occurring anti-sperm antibodies in the serum of rats approximately 9–12 months old. There was no evidence of spontaneous testicular autoimmunity in ageing mice. The integrity of the blood–testis barrier in ageing mice, though probably defective in places, is presumably sufficient to prevent autosensitization even at advanced stages of testicular atrophy.
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References


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Imminent Oocyte Exhaustion and Reduced Follicular Recruitment Mark the Transition to Acyclicity in Aging C57BL/6J Mice

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ABSTRACT
Quantitative cytological analyses of aging C57BL/6J mouse ovaries revealed that the populations of primordial and growing follicles were nearly exhausted by 13–14 months, the average age of ovulatory failure. Anovulatory animals of this age had, on the average, half the follicle number of their counterparts which were still ovulating. This result suggests that follicular depletion is a factor which contributes to the loss of ovarian cyclicity during aging. However, the considerable overlap of follicle number between the two groups suggests that other, possibly extraovarian, factors also influence the loss of ovulatory function.

Although the numbers of follicles recruited for growth was much lower in ovaries from old cycling animals, the number of ova shed cyclically was generally within the range of younger individuals. The observed reduction in incidence of morphological atresia among medium-sized follicles may explain how ovulatory constancy is maintained virtually throughout the cyclical life of the ovary.

INTRODUCTION
The fact that the stock of ovarian oocytes in most mammals is laid down before birth and is progressively depleted thereafter has been established for many years. However, the relationship between this process and ovulatory failure at midlife has not been well characterized in any species. The follicular reserve in a sample of 7 perimenopausal women was less than 5% of that present at menarche (Block, 1952), although attrition is evidently not complete at the time of menopause (Costoff and Mahesh, 1975). A substantial reduction of follicular numbers also occurs in rats and mice by midlife, when ovulatory cycles generally cease, but some follicles persist long after the cessation of cyclicity (Mandl and Shelton, 1959; Jones and Krohn, 1961).

Although complete follicular exhaustion cannot therefore account for the loss of cyclicity in most animals, the question remains whether the reduction in follicular number during aging contributes to the lengthening and ultimate cessation of ovulatory cycles. The potential for maturation and ovulation of the remaining follicles in noncycling rodents presenting persistent vaginal cornification is suggested by evidence that ovarian cycles can be reactivated by exogenous treatment with progesterone (Everett, 1940), adrenergic agonists (Quadri et al., 1973) and tyrosine (Cooper and Linnoila, 1980). However, such studies have not clearly established the potential of the aging ovary to respond to normal (endogenous) endocrine signals. Despite considerable data on oocyte numbers in aging rodents (Mandl and Shelton, 1959; Jones and Krohn, 1961; Parkening et al., 1980), the size of the residual follicular store has not been analyzed in direct relation to the cycle history of individual animals. Therefore, we have examined the ovarian status during the transition to acyclicity by comparing the numbers of primordial and growing follicles in ovulatory and anovulatory subgroups of aging
mice of the C57BL/6J strain, a widely used rodent model.

MATERIALS AND METHODS

Female C37BL/6J mice were obtained as 2-month-old virgins (young) or 8-month-old retired breeders from the Jackson Laboratory (Bar Harbor, ME). They were housed singly and maintained under similar conditions to those of Holinka and Finch (1977). The stages of the estrous cycle were identified by examining Giemsa-stained smears of vaginal cells collected each day by lavage.

A daily vaginal smear record was obtained from each mouse for a continuous period of 3–8 weeks duration beginning at either 3 or 11–12 months of age. The day of autopsy was chosen at random with respect to the stage of the vaginal cycle and the animals were distributed approximately evenly between the first 4 days (proestrus=Day 1) of the cycle, regardless of its length. The time of autopsy varied between 1200–1400 h (lights on from 0730–1930 h daily). The oviducts were removed and flushed with saline for microscopical counting of ova on Days 2–4; all ovaries were searched microscopically for corpora lutea. Ovaries were removed, fixed overnight in aqueous Bouin’s fluid and subsequently prepared as serial histological sections of 7 μm thickness stained with haematoxylin and eosin. The number of oocytes in each ovary was estimated and classified according to the appearance of the granulosa cell layer (Mandl and Zuckerman, 1951): Stage I (‘primordial types,’ i.e., with 1 layer of rounded or flattened granulosa cells), Stage II (1 layer of cuboidal cells), Stage III (2 layers), Stage IV (3 layers), Stage V (≥4 layers, no antrum), Stage VI (antrum present). The nucleolus was used as a marker for follicle counting with appropriate correction factors to compensate for overcounting (Abercrombie, 1946). In those cases of atretic follicles with undetectable nucleoli, the follicles were counted at the section in which their cross-sectional area appeared largest. Every tenth section of young ovaries was examined microscopically at 400 X magnification for follicle Stages I–V, whereas every section was searched for Stage VI (antral) follicles because of the large size and reduced frequency of these types. Since fewer follicles were found in old ovaries, every fifth section was examined for Stage I follicles and all sections were examined for more advanced stages. Antral follicles were subclassified into small (Vla), large preovulatory (Vlb) and cystic (Vic) stages. The latter stage represented a terminal form of development characterized by an abnormally expanded antrum and thin granulosa cell layer. Finally, a subjective assessment was made of the microscopic appearance of growing stages: those possessing more than one pyknotic granulosa cell or having marked degeneration of the oocyte nucleus or cytoplasm were classified as atretic. Cell counting and scoring for atresia were carried out on coded slides to minimize the possibility of observer bias. However, it should be pointed out that no coding system can conceal the obvious identity of ovaries derived from widely different age groups.

RESULTS

Whereas the majority of 4- to 5-month-old mice presented regular 4- or 5-day vaginal cycles, animals aged 13–14 months presented mainly prolonged cycles (>5 days) or persistent vaginal cornification (PVC; see Nelson et al., 1981). Over 75% of the cycling aging mice had recently ovulated. The remainder (4 animals) were reallocated, along with the PVC mice, to the anovulatory subgroup when neither oviducal ova nor corpora lutea were found.

Some aging ovaries were nearly depleted of primordial follicles, yet even they sustained growing stages which, in a few animals, constituted the majority of the total follicular population. The number of nonatretic growing follicles (Stages II–VI) varied with the size of the nongrowing pool (Stage I) in a nonlinear manner (Fig. 1). An estimate of the average age at which the pool was depleted was based on the premise that the rate of depletion is exponential (Jones and Krohn, 1961; Faddy et al., in preparation). The corresponding equation in our mice was log y = 3.86–0.12x, where y was the pool size and x was the age in months. By extrapolation, half of the animals would have barren ovaries by the time they had reached their average life span of about 27 months (Kunstyr and Leuenberger, 1975; Goodrick, 1975).

The total number of ovarian follicles in young ovaries was much larger than that of old organs (Fig. 2, P<0.001 by analysis of variance). The old ovulatory subgroup had twice the number of follicles of the anovulatory subgroup (P<0.01), but there was considerable overlap between these two groups. Whereas some animals were still ovulating with <100 follicles, over half of the anovulatory mice had >100 remaining.
Although the numbers of normal growing follicles at all stages of development were dramatically reduced in aging ovaries, in cycling mice there was no corresponding reduction in the numbers of ova present in the ampullas of estrous oviducts (Fig. 3). These ovulatory mice had fewer medium-sized atretic follicles (Stages III–V) and a significantly reduced ratio of atretic/total follicles compared to either the young group (P < 0.01 for Stages III–V) or the anovulatory group of the same age (P < 0.02 for Stages IV and V). Vlc follicles were rarely seen in young ovaries, whereas they were relatively abundant in both types of old animals where they generally showed signs of terminating in atresia. The proportions of Vlb and Vlc atretic follicles were similar in both of the old subgroups and they were significantly larger than those of younger individuals (P < 0.02).

DISCUSSION

The size of the primordial follicle population in the mouse is determined at birth and is continuously depleted thereafter as a result of cellular death and recruitment into growing stages. Although the follicle stores therefore set an absolute limit on the functional ovarian lifespan, other factors may intervene to bring about ovulatory failure before these reserves are depleted. The results of this study, however, indicate that follicle depletion is of importance in the etiology of ovulatory failure in the C57BL/6J mouse. Firstly, the reserves are approaching exhaustion at an age when ovarian cycles are being lost. Secondly, the reserves at this age are 50% lower in the anovulatory as compared to the ovulatory subpopulation, indicating that the probability of acyclicity increases as follicular reserves decline. The latter argument rests on the assumption that depletion of the follicular pool is not accelerated by the anovulatory state.

These conclusions are in contrast with those drawn from studies of rats where the ovary was apparently absolved from responsibility for ovulatory failure and where evidence was obtained of impaired hypothalamic-hypophyseal function (Aschheim 1964/65; Meites et al., 1978). However, these views are not necessarily opposing since there may be considerable genetic diversity in the relative contributions of ovarian and extraovarian factors to the etiology of anovulation (Finch, 1979). For example, it is clear that the size of the follicle endowment at birth and the rates of follicle utilization are subject to considerable genetic variation among strains of mice (Jones and Krohn, 1961; Paddy et al. in preparation). Furthermore, it should be pointed out that where primary neuroendocrine changes have been emphasized, the evidence has been mainly obtained from studies of the post-cyclical state. Thus, whether these changes are primary or secondary to ovulatory failure cannot be determined.

The balance of factors responsible for the loss of cycles in C57BL/6J mice cannot be fully defined at this time. The overlapping distribution of follicle reserves in the two aging subpopulations suggests that extraovarian factors may be involved, possibly modulating the threshold number of follicles required to sustain cyclicity. An extraovarian contribution to the acyclicity of C57BL/6J mice is also suggested by evidence that the preovulatory surge of luteinizing hormone is attenuated during aging (Flurkey et
FIG. 3. This bar diagram represents the profile of growing follicles in the ovaries of young ovulatory (open bars, n=18), old ovulatory (closed bars, n=14) and old anovulatory mice (hashed bars, n=17). Follicle stage were classified in Roman numerals according to Mandl and Zuckerman (1951); the equivalent stages of the scheme devised by Pedersen and Peters (1968) are given in Arabic numerals below. Stage VI follicles were subdivided according to size (see text). The height of each bar indicates the mean number ± SEM of follicles or recently ovulated ova (i.e., enclosed in cumulus cells) per animal. The numbers of nonatretic follicles and the numbers and proportions of atretic follicles are presented separately. In addition to heterogeneity of the data with respect to the groups, the distribution of ovarian and oviductal oocytes varied within animals, the right side tending to contain the higher number (paired t test, P<0.05).

al., 1982) without a parallel reduction in preovulatory estradiol secretion (Nelson et al., 1981). However, the functional significance of this apparent hypothalamic-pituitary impairment requires further study.

Clearly, it is possible for normal ovarian function to continue when very few follicles remain. One mouse in this study ovulated six ova with only 72 follicles remaining in the ovary, and pregnancy may be established in CBA mice with less than 100 follicles present (Jones and Krohn, 1961). In the present study, the number of ova shed cyclically was remarkably similar in the two age groups, even though
the size of the follicle pool in the older group had been reduced by more than 90% and there was a corresponding reduction in number of follicles beginning their growth phase. Most of the medium-sized follicles of functioning ovaries had evidently been rescued from atresia in order to maintain an approximately normal output of ova. Protection from atresia may be the best explanation of why the proportion of healthy growing follicles increases while the reserve pool becomes progressively depleted (Krarup et al., 1969; Fig. 1), since the fraction of follicles initiating their growth per unit of time remains approximately the same throughout life in several strains (Edwards et al., 1977). Furthermore, a mechanism for maintaining ovulatory constancy which depends on control of the death rate, rather than on increased recruitment from the pool, can obviously explain why attempts to induce superovulation with exogenous gonadotropin or hemiovaricotomy are usually ineffective in aging rodents (Peppler, 1971; Collins et al., 1980).

The reduction of atresia during aging may be a common phenomenon, since it has also been reported in the aging rat (Mandl and Shelton, 1959; Peluso et al., 1979). Although in a previous extensive study of mice no relationship was found between the fraction of atretic follicles and the size of the follicle population (Jones and Krohn, 1961), this may have been due to an artifactually high incidence of atresia produced by the use of an alcoholic fixative.

The mechanism responsible for reduced atresia might depend on a reciprocal feedback relationship between plasma follicle-stimulating hormone (FSH) and an ovarian inhibitor (Hoak and Schwartz, 1980) or, alternatively, on a putative intravarian factor which is dependent on follicular density. The circulating levels of FSH are elevated during aging in another strain of C57BL/6 mouse (Parkening et al., 1980) and this hormone is capable of significantly reducing the incidence of atresia in rat ovaries (Hirschfield and Midgley, 1978). However, a comparable effect of FSH on medium-sized follicles in mouse ovaries has not yet been demonstrated. Also, the lack of a corresponding reduction of atresia in anovulatory mice is puzzling unless future experiments reveal substantial differences in FSH secretion. Perhaps the last remaining follicles become refractory to gonadotropin stimulation, in which case both follicle number and follicle sensitivity would be arbiters of the functional ovarian life span.

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Ovarian follicle dynamics in mice: a comparative study of three inbred strains and an F₁ hybrid

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SUMMARY

The rates of follicle growth and death have been estimated by a mathematical method in A, CBA, RIII and A × CBA strains of mice for which differential follicle counts were available. These rates were not uniform throughout life but were specific for the immature and mature phases of life. Significant heterogeneity of the rate estimates for particular follicle stages was also identified between strains and between intact and hypophysectomized mice, which explained the differing life-time patterns of follicular utilization in these animals.

INTRODUCTION

The development of follicles from the time of their commitment to growth until they either die or attain preovulatory maturity is a complex yet orderly process which continues throughout life. Although the mechanism of the initial trigger for growth of primordial follicles has not yet been identified, it is clear that further development is controlled by a combination of intra-ovarian factors and gonadotrophic hormones. These influence the rates of follicular utilization at different ages and under differing physiological conditions (Pedersen, 1972; Richards, 1979).

If the various parameters affecting these rates can be identified and estimated quantitatively it should then be possible to build a model which describes the dynamics of the follicle population as a whole and which may be used to predict the consequences of experimental and physiological interventions. This model of the ovary would obviously require a knowledge of the influence of genetic variation on each parameter. Such information would be of additional interest as an indicator of variables which might be involved in ovarian responses to selection pressure for ovulation rate and functional ovarian life-span.

Since the method of radioisotopic labelling of granulosa cells (Pedersen, 1972) would be excessively laborious to apply for this purpose, an alternative approach has been sought. The mathematical approach to ovarian follicle dynamics permits static data points obtained from differential follicle counting to be converted directly into estimated rates of death (atresia) and migration (growth) at specific stages (Faddy, Jones & Edwards, 1976). This method has therefore been used to identify the major expressions of genetic variation in follicular utilization in three inbred strains of mice and an F₁ hybrid of two of these strains. The data were obtained a number of years ago (Jones & Krohn, 1961a, b), but methods were not then available for carrying out analyses of follicle dynamics.

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We respectfully dedicate this paper to the memory of our late colleague, Esther C. Jones.
MATERIALS AND METHODS

The data were obtained by differential counting of follicles in serially sectioned ovaries obtained from inbred A, CBA, and RIII mice and from A × CBA hybrid mice at various ages after birth. None of the animals was pregnant at the time of study and mature individuals were killed at random stages of the oestrous cycle. Similar quantitating cytological methods were applied to tissues obtained from a heterogeneous group of 44 mice comprising the above three inbred strains in approximately equal numbers. This group differed in having been hypophysectomized at or after 28 days of age and was examined several weeks later. Full details of the experimental methods and of the raw data obtained have been published previously (Jones & Krohn, 1961a, b). In brief, these data were obtained by counting the numbers of follicles at five discrete stages of maturation throughout the life-span of the animal: the stages of follicle maturation ranged from primordial (I) to multilaminar and antral types (V+).

The data were analysed using the stochastic compartmental or death-migration model for ovarian follicle dynamics (Faddy, 1976; Faddy et al. 1976). The model describes the growth of follicles through these five stages (I–V+) by five corresponding compartments (Fig. 1, top panel) with the outflow from these compartments represented by the migration rates \( v_1(t) \) (from I to II), \( v_2(t) \) (from II to III), \( v_3(t) \) (from III to IV) and \( v_4(t) \) (from IV to V+), and by the death rates \( \mu_1(t) \) (from I), \( \mu_2(t) \) (from II), \( \mu_3(t) \) (from III), \( \mu_4(t) \) (from IV) and \( \mu_5(t) \) (indicating egress as a result of death and ovulation from V+). The model describes the process of follicular maturation throughout life in terms of the numbers of growing follicles in each of these stages in accordance with the data. No description can be given of the continuous nature of the growth process within the corresponding compartments as the data do not relate to this aspect, unlike the model and data of Read, Berry, Mariana & de Reviers (1981).

Since migration of follicles and death rates were not constant throughout life a simple form of age-dependence was built into the model in which two phases, designated phase I (immature) and phase II (mature), were identified and the rate within each phase was assumed to be constant (Faddy et al. 1976). The age corresponding to the nadir in the number of stage II follicles was chosen as the time of transition between these phases. The age at the transition was strain-specific and was approximately the time at which puberty (vaginal opening) is expected to occur in mice (Vandenbergh, 1973), although the time of puberty has not been determined in the mice used in this study. Finally, it was necessary to include the size of the follicle population at birth, when only stage I follicles are present. Thus, 19 parameters were estimated from each set of data for the normal mice but only nine parameters were required for the hypophysectomized animals since they were only studied during phase II. These estimates and their approximate standard errors were obtained by the method of maximum likelihood (Kendall & Stuart, 1967). In fact, a variance/covariance matrix describing the full sampling variation of the estimates had to be calculated but only the standard errors are quoted here. Estimates of 19 parameters were necessary since the purpose of the model is to describe follicular dynamics over the entire life-span from birth until senility in terms of the several morphological stages of follicle development. This is in contrast to the model of Read et al. (1981) who used fewer parameters but confined their attention to infancy.

RESULTS

The estimated rates of follicular migration and death together with their standard errors are presented in Fig. 1, and the concordance between the predicted profile of follicle stages throughout the life-span of mice and the experimental observations is shown in Figs 2–5. The fit of the model may be judged from these figures: the decaying curves for stage I and
Fig. 1. Estimates of the migration ($v(t)$) and death rates ($\mu(t)$) and their corresponding s.e. (in parentheses) for each of the five stages of follicular development (I-V+) among three strains of mice and an F1 hybrid. These rates were not constant throughout life and two phases were identified: immature (1) and mature (2). The transition between these phases was defined by the nadir in the population of stage II follicles and this occurred at 30 days of age in strain A, at 20 days in CBA, at 25 days in A x CBA and at 35 days in RIII. An additional combined group of the inbred strains was examined during adulthood and after hypophysectomy (HPX).
the biphasic curves for stages II–V+ were in close accord with observations, although there was a consistent underestimation of early numbers of stage I follicles. From these results it was relatively straightforward to calculate the absolute numbers of follicles moving into or out of particular compartments as a result of growth or death.

The age at which the transition between phases 1 and 2 occurred varied between 20 and
I Ovarian follicle dynamics

II

Fig. 3. Variation in numbers of follicles of differing size (stages I–V+) in mice of strain CBA from birth (day 0) until senility. The results were obtained by differential counting of follicles and the values obtained from virgin (×) and ex-breeder mice (○) were combined for the purpose of estimating the means (solid lines) with their s.E. (broken lines) predicted from the 'death-migration' model. The mean size of the follicular population at birth when only stage I types were present was 9382 ± 98.

35 days according to the profile of stage II follicles (see Figs 2–5). In phase 2 the data for virgin and multiparous animals were pooled since they appeared to correspond very closely. Consequently, revised estimates of the parameters for strain A have been presented,
Fig. 4. Variation in numbers of follicles of differing size (stages I–V+) in mice of strain A × CBA from birth (day 0) until senility. The results were obtained by differential counting of follicles and the values obtained from virgin (×) and ex-breeder mice (♀) were combined for the purpose of estimating the means (solid lines) with their s.e. (broken lines) predicted from the 'death-migration' model. The mean size of the follicular population at birth when only stage I types were present was 11,178 ± 113.

but these did not differ significantly (<2 s.e.) from those published previously for virgin mice alone (Faddy et al. 1976).

At birth all oocytes are present as stage I follicles and it is assumed that oogenesis is
Fig. 5. Variation in numbers of follicles of differing size (stages I–V+) in mice of strain RIII from birth (day 0) until senility. The results were obtained by differential counting of follicles and the values obtained from virgin (×) and ex-breeder (⊗) mice were combined for the purpose of estimating the means (solid lines) with their s.e. (broken lines) predicted from the ‘death-migration’ model. The mean size of the follicular population at birth when only stage I types were present was $13074 \pm 157$.

Complete and that no further increase in numbers can occur. The size of the follicle population at birth varied substantially between strains in the following way, expressed in order of magnitude: $\text{RIII} > \text{A} \times \text{CBA} > \text{A} > \text{CBA}$ (see legends to Figs 2–5). Some follicles moved into the stage II compartment within a few days of birth and the explosive increase in numbers which followed produced the characteristic prepubertal peak of week 2.
Recruitment of follicles into other compartments occurred progressively and resulted in peak values at correspondingly later intervals.

Of the two factors responsible for the rapid depletion of the non-growing pool during phase I, the death rate was by far the most significant. A four- to elevenfold greater number of follicles left the pool by dying than by initiating their growth. This high rate of death caused the size of the oocyte population to be reduced by approximately 50% by the end of phase I. The consequent reduction in numbers of follicles available for growth was therefore primarily responsible for the decline of the peak numbers of growing follicles towards the end of phase I.

The death rates of stage I follicles were remarkably similar in all four strains during the prepubertal phase. The migration rates of CBA, A and A × CBA mice for the transition from stage I to II were also similar to one another, but the migration rate of RIII mice was two- to threefold higher than in the other strains and hybrid mice. The follicular profile of RIII mice was exceptional in having peak numbers of stage II follicles during the first phase of life, which undoubtedly reflected the disparity in growth rates. The death rates of stages II–IV were very low in the above three strains but RIII animals were again exceptional, as evidence was obtained of follicular death at stages II and III.

The rate of migration from stage I to II was not substantially different among CBA, A and hybrid mice on either side of the phase step. This result contrasts strongly with the reduced death rate at stage I during the post-pubertal phase. The reduction exceeded in order of magnitude in all cases except CBA mice, in which it was by one-third, yet this too was statistically significant.

The effects of the transition to phase 2 on the other follicular stages were complex. However, it is safe to say that the rate of migration from stage II to III was reduced and that the death rate remained low at stages II–IV in all strains. The rate of movement from stage III to IV was unchanged (with the exception of strain A, in which it was reduced), but there was a marked acceleration of IV to V in all inbred strains, though not in the hybrid animals, in which the rate was more or less unchanged. In addition, the total rate of egress from V was increased in CBA and RIII mice. The lack of a significant result for the other two strains may be accounted for by statistical complications of deaths at stage IV. These changes in the migration and death rates in phase 2 together with the progressive reduction of the pool size were responsible for the unstable follicular profile up to about 100 days post partum or somewhat later in hybrid animals. After the post-pubertal peak of growing follicles the trend of the entire follicular profile was one of progressive loss and approaching extinction. The rate of decline in numbers was most evident in CBA ovaries, which became depleted of oocytes by about 450 days of age.

Hypophysectomy resulted in a significant decrease in the rate of egress of stage I follicles compared to intact animals. Unfortunately, the statistical necessity of amalgamating the various strains of hypophysectomized mice and thereby compounding within- and between-genotype variations reduced the amount of information that could confidently be drawn from such comparisons. However, it was clear that the rate of migration from stage II to III was significantly higher in hypophysectomized animals than in intact mice and was comparable with the rate estimate for normal immature animals. The parameter estimates after stage III were very imprecise with large standard errors because only a few follicles could evidently move beyond this stage in hypophysectomized animals. There is therefore no real basis for further comparison.

**DISCUSSION**

In a previous study of virgin A strain mice we estimated the rate of loss of follicles from each of the five follicular stages (‘compartments’) that results from either death or outflow to the next growing stage. This information enabled us to predict the behaviour of the
follicular population as a whole from the time of its formation until it was completely or substantially exhausted in old age (Faddy et al. 1976). The new results presented here, with a large set of observations, confirmed the previous results and showed that a generally comparable pattern of follicular utilization occurred in mice of different genetic constitution. Perhaps most notable of all was the general age-dependence of migration and death rates of all follicular stages, characterized here by a single transition around the expected time of puberty. The identification of the age at which this transition occurred was based on a somewhat arbitrary criterion, but this two-phase model must be an approximation to some more general behaviour and additional data could lead to a more refined model. Hence, no strict biological significance should be attached to the different ages for the phase transition.

Underlying the general patterns of follicular utilization there was significant genetic diversity of the rates of migration and death as well as of the size of the oocyte stocks at birth. The new estimates confirmed previous conclusions which were based simply on a survey of the raw data, for they showed that the size of these stocks was greatest in RIII ovaries, least in CBA ovaries and that there was an effect of heterosis in A x CBA mice (Jones & Krohn, 1961a). The factors responsible for these differences have not yet been identified.

In the original analyses of the four types of normal mice it was presumed that the loss of small follicles from the pool was a simple exponential function of time (Jones & Krohn, 1961a). It is now clear that the rate of loss is much higher during than after the prepubertal phase. Apart from the exceptionally high migration rate from stage I to II in immature RIII mice, the migration and death rates at stage I were similar among the immature animals. The eccentric behaviour of RIII animals in this and in other respects may simply be a reflection of their greater genetic disparity from the other three types of mice than existed between these three types.

The large majority of follicles that left the reserve pool in immature ovaries did so by dying rather than by entering a growth phase. Moribund primordial follicles are recognizable morphologically (Byskov & Rasmussen, 1973) but the causes of death are unknown, though there are obvious parallels with programmed cell death in other developing organs. It is unlikely that lack of trophic stimulation is responsible for deaths since plasma follicle-stimulating hormone (FSH) is abundant during the juvenile phase (Dullaart, Kent & Ryle, 1975) and early stages of follicular development may proceed independently of gonadotrophic stimulation in infantile ovaries (Anderson, Schwartz, Nequin & Ely, 1976; Peluso, Steger & Hafez, 1976; Purandare, Munshi & Rao, 1976) and after hypophysectomy (Nakano, Mizuno, Katayama & Tojo, 1975), though some contrary evidence exists (Ryle, 1970; Eshkol & Lunenfeld, 1975). It is possible that the presence of a high-affinity oestrogen-binding mechanism and the consequent reduction of the biologically active or unbound fraction of the hormone in sera of immature animals (Puig-Duran, Greenstein & MacKinnon, 1979) may have an unfavourable impact on follicular survival. But there is, as yet, no experimental proof of rescue of small follicles from atresia by oestrogen, though this effect appears to be possible at later stages of development (Harman, Louvet & Ross, 1975). Moreover, it seems unlikely that the continuing high death rate in the stage I population of adult CBA ovaries will be found to be associated with low oestrogen levels.

When mice entered the mature phase of life the death rate among stage I follicles was dramatically reduced, but only in RIII mice was this rate reduced to zero, as it is in the Bagg strain (Podersen, 1972). As a consequence of this minimal death rate in RIII ovaries the numbers of small follicles were depleted more slowly than in other strains, with the result that a substantial population remained until close to the end of life. On the other hand, the higher rate of mortality in ovaries of adult CBA mice was primarily responsible for the precocious exhaustion of the follicular store. Furthermore, it is interesting to record that a
positive correlation exists between the rate at which the store is depleted and the average age at which oestrous cycles cease in the inbred strains which were studied (Caschera, 1965; S. C. Laing & R. G. Gosden, unpublished observations), but this need not imply any causal relationship. As a consequence of the reduced death rate among stage I follicles around the time of puberty, the numbers of growing follicles were increased and resulted in a post-pubertal peak. The size of these sub-populations in their growth phase greatly exceeded that required for ovulation, but regulation of this reserve did not take place until they attained stage V+ of development. The uniformly low death rate of the intermediary stages (II–IV) was confirmed by the relative paucity of morphologically atretic types (Pedersen, 1972; R. G. Gosden, S. C. Laing, L. S. Felicio, J. F. Nelson & C. E. Finch, unpublished observations).

The transition to maturity was marked by a number of significant changes of migration rates which were stage- and strain-specific. However, there was a uniform reduction of the rate of movement from stage II to III in all groups of normal mice in contrast with the substantially higher values in adult hypophysectomized mice. Since the migration from stage II to III varied inversely with the numbers of large follicles present it is possible that this effect is mediated by an intra-ovarian cytoerin which is produced by large follicles and inhibits the recruitment of less mature forms. An inhibitory factor from the same source has been postulated to control the initial step of follicular growth (Peters, Byskov & Faber, 1973) but we have failed to find general evidence of such an effect.

Although the migration from stage III to IV was only slightly affected by age, the succeeding step from IV to V+ was definitely accelerated in phase 2 (except in A x CBA mice). The net rate of egress from V+ due to death and ovulation was also increased significantly in at least two of the strains as well as in the aforementioned Bagg strain (Pedersen, 1972). These changes might be due to an increase in mitotic activity resulting from the synergistic interactions of oestrogen and gonadotrophins (Richards, 1979). Where FSH is absent and oestrogens are deficient, as in hypophysectomized animals, full follicular development does not occur.

The numbers of follicles that ovulate at a given cycle and age can be increased by artificial selection, and when this occurs there may be a paradoxical reduction in the size of the total follicular store (Land, de Reviers, Thompson & Mauléon, 1974; Parks & Wolfe, 1977). An inverse relationship between fecundity and size of the store appears to hold for CBA, A and RIII strains when it is assumed that litter sizes indicate the numbers of ovulations in young animals (Jones & Krohn, 1961a). The basis of variation in fecundity may lie in hentable differences in concentrations of plasma hormones or, perhaps of greater importance in mice, differences in ovarian sensitivity to hormones and FSH receptor concentrations (McLaren, 1962; Land & Falconer, 1969; Wolfe, 1971; Bindon & Pennycuik, 1974; Parks & Wolfe, 1977). Unfortunately, our results throw little additional light on these phenomena. This is probably because the crucial differences of migration and death rates between strains are at the level of large follicles, at which our estimates are less precise and subject to uncontrolled variability with respect to the oestrous cycle.

The results of this study imply that the characteristic life-time profile of the follicular populations in a given strain of mouse has a complex basis in the migration and death rates, and, to a lesser degree, in their initial endowment of oocytes. Whereas growth rates appear to be overridden in the intermediary stages of development (II–IV), follicular death is the predominant activity amongst all other stages and accounts for more than 90% of the initial stock. The attrition which occurs before puberty reduces the mass migration of follicles into the growing population, while the subsequent deaths amongst large follicles presumably reflect fine control of the ovulation number. It is evident therefore that an understanding of the follicular economy of the ovary and expressions of its genetic heterogeneity depend upon further knowledge of how some follicles manage to escape death at vulnerable stages in their development.
Ovarian follicle dynamics

REFERENCES


Effects of LH-RH agonist or LH-RH immunoneutralization on pituitary and ovarian LH-RH receptors in female rats

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Summary. Hyperstimulation of pituitary function using daily injections of 50, 500 or 5000 ng LH-RH agonist for 3 weeks or hypostimulation by immunoneutralization of endogenous LH-RH led to disrupted oestrous cycles and profound changes in ovarian morphology. Despite this, the number of pituitary and ovarian LH-RH receptors remained within the range found in normally cyclic animals. Autoradiography confirmed that specific binding was located in theca, granulosa and luteal cells.

These results suggest that autoregulation of pituitary and ovarian LH-RH receptors is not of primary importance in the cyclic female rat.

Introduction

Receptors for luteinizing hormone-releasing hormone (LH-RH) are present in the anterior pituitary gland and ovary of the rat. Pituitary LH-RH receptor numbers change markedly under different physiological conditions, for example, during the oestrous cycle (Park, Saxena & Gandy, 1976; Savoy-Moore, Schwartz, Duncan & Marshall, 1980; Clayton, Solano, Garcia-Vela, Dufau & Catt, 1980; Marian, Cooper & Conn, 1981; Meidan & Koch, 1981; Reeves, Tarnavsky & Platt, 1982), lactation (Clayton et al., 1980; Reeves et al., 1982) and after ovariectomy (Reeves et al., 1982). These changes are thought to reflect exposure of the pituitary to hypothalamic LH-RH and may be important in mediating changes in pituitary responsiveness. In contrast, no marked changes in ovarian LH-RH receptors have been observed in cyclic or lactating rats (Pieper, Richards & Marshall, 1981; Reeves et al., 1982). However, LH-RH can act directly on the ovary to influence steroidogenesis (Hsueh & Erickson, 1979; Clark, 1982; Popkin, Fraser & Jonassen, 1983), oocyte maturation (Ekholm, Hillensjö & Isaksson, 1981) and ovulation (Corbin & Bex, 1981; Ekholm et al., 1981).

In the present investigation we have exposed the pituitary and ovary to very high or low levels of LH-RH, and therefore to variation in gonadotrophin stimulation, by treating cyclic female rats with daily injections of an LH-RH agonist or immunoneutralization by LH-RH antibodies. LH-RH receptor numbers were examined under these conditions which produced marked changes in ovarian morphology. In addition the distribution of labelled LH-RH in the ovary was investigated using autoradiography.
Materials and Methods

Animals. Adult female Sprague-Dawley rats (60–80 days old) were housed under conventional conditions with lights on between 05:00 and 19:00 h and were exhibiting regular oestrus cycles. Vaginal smears were taken daily to assess vaginal cytology before and during treatment.

Chronic treatment with LH-RH agonist and LH-RH antiserum. Animals were divided into 6 groups of 8. Treatment commenced at random stages of the cycle. Rats in Groups 1–3 were injected subcutaneously at 09:00 h daily for 3 weeks with 50, 500 or 5000 ng LH-RH agonist (D-Serbut6 des Gly10 LH-RH ethylamide; Hoechst AG, Frankfurt, West Germany) dissolved in 1% gelatin and 0-9% (w/v) NaCl. Rats in Group 4 were injected with 0-5 ml antiserum to LH-RH raised in sheep (Clayton, Popkin & Fraser, 1982) once every 3 days. The first antiserum injection was given i.v. and the remainder s.c. In rats at 12:00 h on the day of pro-oestrus, injection of 0-5 ml LH-RH antiserum prevented the LH surge and ovulation (R. M. Popkin & H. M. Fraser, unpublished observations). Control rats received either vehicle alone or antiserum to human serum albumin (HSA) and were killed on the day of dioestrus (Group 6) or pro-oestrus (Group 5) at 12:00 h. Since no difference was found between HSA- and vehicle-treated rats the results were pooled.

At 24 h after the final injection (or on the day of pro-oestrus or dioestrus for controls in Groups 5 and 6), rats were anaesthetized with CO2 generated from solid CO2. Blood was collected by decapitation and after centrifugation the serum was stored at −20°C before hormone assay. The adrenocortically was rapidly removed and homogenized immediately in 10 mM-Tris-HCl pH 7.4 for LH-RH agonist binding assay, an aliquot of the homogenate being stored at −20°C for gonadotrophin content and protein determination. The ovaries and distended uteri were weighed. The right ovary was taken for immediate binding assay. The left ovary was placed in Bouin’s fluid and after fixation embedded in paraffin wax. Serial sections were cut at 5 μm and stained with haematoxylin-eosin.

Radioimmunoassays. Serum concentrations and pituitary content of LH and FSH were measured as previously described (Fraser & Sandow, 1977; de Jong & Sharpe, 1977) and the results expressed in terms of the appropriate NIAMDD preparation (RP-1). Oestradiol-17β and progesterone were determined as described by Backstrom, McNeill, Leask & Baird (1982) and Scaramuzzi, Corker, Young & Baird (1975) respectively. All samples were run in single assays. All samples for individual hormones were measured in single assays with a within-assay variation (as percentage coefficient of variation) of 9%, 11%, 7% and 8% and sensitivities of 6 ng/ml, 50 ng/ml, 0-2 ng/ml and 10 pg/ml for LH, FSH, progesterone and oestradiol respectively.

LH-RH receptor assay. LH-RH receptor concentrations from individual pituitaries were assessed using previously described methods (Clayton et al., 1982; Fraser, Popkin, McNeill & Sharpe, 1982). The right ovary was subjected to the same procedure, i.e. homogenization in Tris-HCl (10 mM, pH 7-4) and the homogenate was filtered through nylon gauze. The 50 μl aliquants were used in the binding assay which was carried out on ice for 2 h. Protein determination was performed by the method of Lowry, Rosebrough, Farr & Randall (1951) using BSA as the standard. Pituitary protein content did not vary between treatment groups so the results for pituitary binding are expressed as fmol bound/pituitary. Since ovarian protein content did vary between groups, ovarian binding is expressed as fmol bound/mg protein.

Autoradiography. Two pro-oestrous rats weighing 250 g were anaesthetized at 12:00 h with ethyl carbamate (1.75 g/kg body wt, injected i.p. as a 25% solution w/v) BDH Ltd, Poole, Dorset, U.K.) and the right atrium of each was cannulated. Each rat was given a 1-ml injection of 125I-labelled LH-RH agonist (2-9 × 107 c.p.m.) alone or with a 100-fold excess of unlabelled hormone at pH 7 into the atrial cannula over a period of 2 min. The animals were killed 30 min later by perfusion with 30 ml heparinized saline and this was immediately followed by 20 ml 10% neutral buffered formal-saline solution. The ovaries and pituitary glands were dissected out and immersed in this fixative solution and were counted in a gamma spectrometer. In the animal injected with 125I-labelled LH-RH agonist ovary count was 2100 c.p.m./mg, the pituitary 3200 c.p.m./mg and the
blood count 270 000 c.p.m./ml. The corresponding values for the rat injected with 125I-labelled LH-RH agonist and unlabelled hormone were 100 c.p.m./mg and 350 and 220 000 c.p.m./ml. After fixation for 4 h the tissues were transferred to 2% glutaraldehyde (TAAB, Reading, Berks, U.K.) in 0.01 M Hepes buffer (pH 7.2) for further fixation overnight at 4°C. The tissues were then washed with distilled water, snap-frozen and sectioned at 12 µm with a cryostat. The sections were mounted on acid-cleaned slides and coated with liquid nuclear emulsion (K5, Ilford Ltd, Basildon, Essex, U.K.). Additional tissues from untreated rats were prepared similarly to control for chemographic effects (high background activity and latent image fading). The slides were exposed in light-proof boxes at 4°C for 33 days. They were then developed and fixed (D19 developer and Kodak Ltd, Liverpool, U.K.) and stained with haemalum and eosin. Each slide was examined microscopically at high power (×1000) under oil-immersion and with bright-field and dark-ground illumination.

Statistical analysis. Differences between dioestrous control and treated groups were analysed by Student's t tests.

Results

Ovarian morphology

Ovarian function was dramatically altered by long-term LH-RH agonist and LH-RH antibody treatment. Ovarian weight was increased after treatment with 500 or 50 ng LH-RH agonist and decreased after LH-RH antibody treatment (Table 1). The morphology was assessed by comparing the contribution to the overall mass of the ovaries by the various constituent structures (follicles, corpora lutea and stroma) on a scale 0–3 using coded slides from each group of animals. The greatest degree of contrast was found between animals in Group 3 (Pl. 1, Fig. 3) and the controls in Groups 5 and 6 at pro-oestrus and dioestrus (Pl. 1, Fig. 1). Growing types of follicles from unilaminar to Graafian sizes were notably lacking in Group 3 rats, and in one animal were virtually absent. These types were also less abundant in the treated animals in Groups 1 and 2, although to a smaller degree. The numbers of primordial (non-growing) follicles were counted in one approximately equatorial section of each ovary but were unaffected by the treatments (range of means 11–15 in Groups 1, 2 and 3). Small and medium-sized follicles appeared to be growing normally whereas Graafian follicles were frequently seen to be undergoing premature luteinization.

Table 1. Effect of treatment with LH-RH agonist or LH-RH antiserum for 3 weeks on serum LH, FSH, progesterone and oestradiol concentrations, pituitary content of LH and FSH and uterine and ovarian weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Serum FSH (ng/ml)</th>
<th>LH (ng/ml)</th>
<th>FSH (µg)</th>
<th>LH (µg)</th>
<th>Progesterone (ng/ml)</th>
<th>Oestradiol (pg/ml)</th>
<th>Paired ovarian wt (mg)</th>
<th>Uterine wt (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 ng agonist</td>
<td>293 ± 25b</td>
<td>145 ± 35</td>
<td>15 ± 2</td>
<td>176 ± 12</td>
<td>14 ± 2e</td>
<td>34 ± 4</td>
<td>128 ± 11a</td>
<td>328 ± 26</td>
</tr>
<tr>
<td>2</td>
<td>500 ng agonist</td>
<td>592 ± 160</td>
<td>123 ± 35</td>
<td>16 ± 1</td>
<td>182 ± 21</td>
<td>16 ± 1e</td>
<td>27 ± 4e</td>
<td>140 ± 8a</td>
<td>203 ± 13b</td>
</tr>
<tr>
<td>3</td>
<td>5000 ng agonist</td>
<td>911 ± 134c</td>
<td>125 ± 30</td>
<td>19 ± 1</td>
<td>148 ± 16e</td>
<td>9 ± 1e</td>
<td>29 ± 3e</td>
<td>92 ± 9</td>
<td>143 ± 2e</td>
</tr>
<tr>
<td>4</td>
<td>LH-RH antibody</td>
<td>204 ± 20</td>
<td>56 ± 8b</td>
<td>21 ± 2</td>
<td>232 ± 26</td>
<td>19 ± 4e</td>
<td>48 ± 48</td>
<td>52 ± 4e</td>
<td>353 ± 34</td>
</tr>
<tr>
<td>5</td>
<td>Control (pro-oestrus)</td>
<td>143 ± 9e</td>
<td>57 ± 7b</td>
<td>19 ± 1</td>
<td>218 ± 10</td>
<td>14 ± 5e</td>
<td>107 ± 10e</td>
<td>90 ± 12</td>
<td>621 ± 58e</td>
</tr>
<tr>
<td>6</td>
<td>Control (dioestrus)</td>
<td>201 ± 7</td>
<td>111 ± 17</td>
<td>19 ± 13</td>
<td>205 ± 21</td>
<td>28 ± 2</td>
<td>43 ± 5</td>
<td>90 ± 4</td>
<td>313 ± 23</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. for 8 animals/group.

Values significantly different from those of Group 6 rats: *P < 0.05, **P < 0.01, ***P < 0.001 (t tests).
without releasing the oocyte (Pl. 1, Fig. 4). These oocytes had not resumed meiosis, neither was there any mucification of the adjacent cumulus cells, suggesting that the follicles had not been exposed to a normal ovulatory stimulus despite the luteinization of the granulosa cells. Ovaries of rats in Groups 1–3 contained many lutein bodies some of which contained a structure which may have been the remnant of an oocyte that had undergone cytoplasmic cleavage (Pl. 1, Fig. 4). These bodies differed therefore from the corpora lutea of the control ovaries, although they were similar in size and in cellular morphology and eosinophilia. There was no evidence of inhibition of follicular growth in Group 4 rats, although there were no corpora lutea in half of the animals; these ovaries did contain Graafian follicles with an abnormally enlarged antrum (cystic follicles) (Pl. 1, Fig. 2).

Circulating hormone concentrations

Serum progesterone concentrations in rats in Groups 1–4 were significantly reduced when compared to those of dioestrous rats in Group 6 (Table 1). Serum oestradiol concentrations and uterine weight were lower in Groups 3 and 4 than in Group 6 rats (Table 1).

Pituitary content of FSH remained unaltered by either antibody or agonist treatment. Pituitary LH was lowered in Group 3 rats only. Serum LH was lowered in Group 4 rats relative to dioestrous controls but agonist treatment had no effect. Serum FSH was unaffected in Group 4 rats but was significantly raised in rats in Groups 1–3 (Table 1).

Pituitary and ovarian LH-RH receptor concentrations

Pituitary LH-RH receptor concentrations at pro-oestrus (Group 5 rats) were significantly higher than at dioestrus 1 (Group 6). Values in Groups 1–4 did not differ significantly from that for dioestrous control rats from Group 6 (Text-fig. 1).

Ovarian LH-RH receptor concentrations showed no significant change between pro-oestrus and dioestrus and treatment with LH-RH agonist or LH-RH antibody did not alter the number of receptors (Text-fig. 1).

Autoradiography

Silver grains over pituitary tissue were unevenly distributed amongst cells of the adenohypophysis, being primarily localized over small groups of cells (Pl. 2, Figs 5 & 6). The grains over ovarian tissue were distributed throughout thecal, granulosa and luteal tissue (Pl. 2, Figs 7& 8). In both cases labelling in the control animals was obviously much less (data not shown).

Discussion

Hyperstimulation of the pituitary with daily injections of LH-RH agonist led to a cessation of normal oestrous cycles as shown by constant dioestrous smears and decreased serum progesterone and oestradiol levels indicative of impaired ovarian function. This agrees with the findings of

PLATE 1

Histological sections of rat ovaries.

Fig. 1. Control dioestrous animal. × 30.

Fig. 2. LH-RH antiserum-treated rat showing abundance of follicles. × 30.

Fig. 3. LH-RH agonist-treated rat showing predominance of luteal tissue. × 30.

Fig. 4. LH-RH agonist-treated rat showing a trapped oocyte undergoing cleavage. × 200.
Pituitary and ovarian LH-RH receptors in rats

Text-fig. 1. Effect of daily injections of 50, 500 or 5000 ng LH-RH agonist (■ Groups 1–3) or 3 times weekly injections of LH-RH antibodies (□ Group 4) on (a) pituitary and (b) ovarian LH-RH receptors. Control animals received vehicle alone (□) and receptors were determined on the 1st day of dioestrus (Group 6) or pro-oestrus (Group 5). Values are mean ± s.e.m. with 8 animals per group. * Significantly different from value for Group 6 (P < 0.05, t test).

Johnson, Gendrich & White (1976), Cusan et al. (1979) and Maynard & Nicholson (1979). Agonist treatment resulted in multiple changes in ovarian morphology also indicative of impaired function: an inhibition of follicular growth (or an induction of atresia) and a predominance of lutein bodies, as has also been described by Sandoz (1982). The absence of any associated progesterone rise despite abundant luteal tissue could be due to desensitization of the ovary after excessive gonadotrophin stimulation (Conti, Harwood, Hsueh, Dufau & Catt, 1976) and/or to direct effects of LH-RH on the ovary, inhibiting, for example, progesterone production (Clayton, Harwood & Catt, 1979). This concept of multiple direct LH-RH effects is also indicated by studies suggesting direct actions of LH-RH on granulosa cells (Hsueh & Erickson, 1979), luteal cells (Clayton et al., 1979) and theca/interstitial cells (Magoffin, Reynolds & Erickson, 1981; Popkin et al., 1983). This picture is corroborated by the autoradiographic data showing the presence of grains in most cell types within the ovary. Although our observations are based on only 2 animals they are in complete agreement with those of the study by Seguin, Pelletier, Dube & Labrie (1982) and indicate that LH-RH binds to, and therefore has the potential to exert effects at, multiple sites within the ovary.

Immunoneutralization of LH-RH also led to a cessation of oestrous cycles and marked changes in ovarian morphology being consistent with an impaired ovulatory LH surge and resultant overmature and atretic follicles. Cystic follicles were also evident, similar to those seen in rats in persistent oestrus or when ovulation is blocked by pentobarbitone (Everett & Sawyer, 1950; Braw & Tsafriri, 1980) or after active immunization against LH-RH (Fraser & Baker, 1978). These

PLATE 2

Figs 5–8. Autoradiographic localization of 125I-labelled LH-RH binding by light (Figs 5 & 7) and dark (Figs 6 & 8) field illumination in rat pituitary cells (Figs 5 & 6) and rat ovarian tissue (Figs 7 & 8) showing grains located over granulosa (G) and thecal (T) tissue. × 300.
structures are usually associated with increased progesterone production and may contribute to the serum progesterone concentrations after antibody treatment. Despite such marked changes in ovarian morphology with the agonist and antibody treatments, no change in the number of ovarian receptors was seen. This result may be explained by the fact that LH-RH binds to most cell types within the ovary and would therefore be unlikely to be affected by changes in relative abundance of specific tissue types. Pieper et al. (1981) reported that thrice daily injections of 6 ng LH-RH into intact female rats failed to change the number of ovarian receptors, a finding that is consistent with our observations using LH-RH agonist. It is concluded, therefore, that ovarian LH-RH receptors are unlikely to be autoregulated.

Pituitary LH-RH receptors are, however, thought to be regulated by circulating gonadal hormones, prolactin and by LH-RH itself (see Clayton & Catt, 1981, for review). A number of studies in male rats have revealed the complexity of elucidating the role of LH-RH receptor autoregulation in the intact animal. Acute exposure to LH-RH appears to result in an increase in pituitary LH-RH receptor numbers (Frager, Pieper, Tonetta, Duncan & Marshall, 1981; Naess et al., 1981) whereas chronic exposure did not result in an overall change in receptor concentrations (Heber, Dodson, Stoskopf, Peterson & Swerdloff, 1982). In addition, LH-RH receptors responded biphasically with low doses of LH-RH increasing receptors and high doses reducing them (Clayton et al., 1981). The actions of LH-RH on its pituitary receptor numbers are clearly complex and involve consideration of duration of exposure as well as the dose. Intact female rats have rarely been studied, although Marchetti, Reeves, Pelletier & Labrie (1982) injected cyclic rats with 200 ng LH-RH agonist/day for 2 weeks and found pituitary receptor concentrations similar to those in untreated rats. Our results confirm and extend this finding, showing that chronic treatment of intact female rats with a range of doses of LH-RH (50, 500 or 5000 ng LH-RH agonist/day) results in pituitary LH-RH receptor concentrations within the range found in the cyclic animal.

Although we have not examined the possibility that the experimental regimes used may result in changes in the $K_D$ of LH-RH receptors, this is considered unlikely. LH-RH receptor $K_D$ appears to remain constant during the oestrous cycle (Clayton et al., 1980; Savoy-Moore et al., 1980) and during a variety of induced endocrine states (Marian et al., 1981) and indeed after a comparable study involving chronic LH-RH agonist administration (Marchetti et al., 1982).

Passive immunization of intact male rats with the same antiserum used in the present study resulted in a 25–35% reduction in pituitary LH-RH receptors (Clayton et al., 1982). The antibody does not have this effect in intact female rats, indicating that a fundamental difference may exist between the underlying mechanisms regulating pituitary LH-RH receptors in male and female rats. Chronic LH-RH agonist or LH-RH antiserum administration resulted in altered ovarian steroid function. Since there is evidence that pituitary receptor numbers can be reduced by exogenous administration of gonadal steroids (Clayton & Catt, 1981; Marchetti et al., 1982) it is likely that the continued presence of steroids, albeit at altered levels, prevents the expression of autoregulatory influences due to LH-RH alone. This can be seen in studies on gonadectomized animals in which exogenous administration of LH-RH results in marked decreases in the number of pituitary LH-RH receptors, and this phenomenon is reversed by steroid replacement therapy (Marchetti et al., 1982). In addition our data suggest that the altered serum hormone concentrations are within the physiological range found during the oestrous cycle, as seen by values for control dioestrous and pro-oestrous animals. We therefore suggest that, in the presence of near normal circulating concentrations of ovarian steroids and LH, pituitary LH-RH receptors are maintained, despite extremes of LH-RH stimulus, to concentrations within the range found in the normal cyclic animal.

In conclusion neither hypo- nor hyper-stimulation of the pituitary gonadotrophs or the ovary significantly alters the number of LH-RH receptors from the levels found during the normal rat oestrous cycle, indicating that in the female autoregulation of the LH-RH receptor is likely to be secondary to the influence of circulating hormones in the intact animal.
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References


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Graafian follicle growth and replacement in anovulatory ovaries of ageing C57BL/6J mice

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Summary. The growth of preantral follicles and replacement of large Graafian follicles was studied in reproducively senescent C57BL/6J mice immediately after the loss of oestrous cyclicity when the ovary is approaching exhaustion of its follicular reserve. Granulosa cells were labelled in vivo by injection of tritiated thymidine and were evaluated by autoradiography. At 1 h after injection, labelling was confined almost exclusively to the cumulus and neighbouring cells whereas the distribution of labelled granulosa cells in preantral follicles was approximately uniform. The proportion of labelled mural cells in Graafian follicles rose from initially low values to maximal levels 3 days later; this suggested that those follicles that were Graafian at the time of injection had been replaced by recruitment of preantral stages. The rate of growth of preantral follicles was similar in senescent anovulatory mice and in young adult animals. A simple model was constructed to illustrate how persistent vaginal cornification in ageing mice is sustained by a stream of Graafian follicles and why some of the latter did not respond fully to an ovulatory dose of hCG.

Introduction

The frequency of oestrous cycles in C57BL/6J mice begins to decline by about 9 months of age and there is a corresponding increase in persistent vaginal cornification (Nelson, Felicio, Randall, Sims & Finch, 1982). The latter condition results from the modest but sustained production of oestradiol (Nelson, Felicio, Osterburg & Finch, 1981) during the underlying anovulatory state in which follicular maturation and secretion proceed while follicles become atretic rather than ovulate. A similar condition occurs in other strains of mice and rats, and is readily induced in younger rodents by agents that inhibit ovulation by interfering with the pro-oestrous surge of gonadotrophic hormones (Everett, 1964).

The turnover of large Graafian follicles in mice with persistent vaginal cornification was studied because persistence ("overripening") of anovulatory follicles has potential effects on recruitment of smaller types of growing follicles and on ovarian steroid output. Furthermore, the oocytes contained within these anovulatory follicles might have an increased risk of fetal mortality and heteroploidy if fertilized (Butcher, 1975; Mikamo & Hamaguchi, 1975). In the present study, the turnover of large follicles and the growth rate of smaller types in anovulatory ovaries were estimated by labelling the granulosa cells with tritiated thymidine. The ability of the Graafian follicles to respond to an ovulatory dose of gonadotrophin was also determined.
Materials and Methods

Animals. C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) as young virgins (and held until 4-7 months of age) or as retired breeders (and held until 13-15 months old). Animal husbandry conditions have been described by Nelson et al. (1981). A 12 h light-12 h dark cycle began at 07:00 h, and mice were housed singly to maximize cycle regularity (Nelson et al., 1982). Vaginal smears were obtained each morning by lavaging and stained with Giemsa to determine the stages of the oestrous cycle (Nelson et al., 1982). These age groups were chosen because younger animals of this strain have a moderately high incidence of 4- and 5-day cycles, whereas most ageing mice are aycyclic and present persistent vaginal cornification.

Follicle turnover. Young mice (N = 9) that had had two successive 4-day vaginal cycles and were at pro-oestrus, oestrus or met-oestrus 1 on the day of treatment and a further group of 28 old mice that had had cornified smears for > 14 days were injected once i.p. with methyl-[3H]thymidine (2 μCi. g⁻¹; sp. act. 5 Ci.mm⁻¹; Amersham Corp., Arlington Heights, Illinois) in sterile saline a 11:00-12:00 h. All of the young mice and 5 of the old ones were killed by cervical dislocation 11 h later; the remaining old mice were sampled at 24, 48, 72, 144 or 288 h after injection. Four controls of each group were injected with the same volume of saline (9 g NaCl/l) 1 h before autopsy.

A similar group of 7 young and 3 old mice received two injections of the radioactive tracer to compare the lengths of the S-phase in granulosa cells according to the double labelling method of Pedersen (1970) which was based on principles described by Wimber & Quastler (1963). Each animal was given an initial dose of 0-4 μCi. g⁻¹, followed by a second injection of a larger dose, 5 μCi. g⁻¹, 1 h later. Mice were killed 0-75 h after the second injection.

At autopsy the ovaries were dissected out of their bursae and fixed for 2 h in aqueous Bouin's fluid. All mice were judged to be free of pathological lesions in their abdominal and thoracic cavities. Pituitary tumours and other gross lesions are rare in this strain at 15 months of age (Felicio, Nelson & Finch, 1980). Fixed tissues were prepared for paraffin-wax embedding and were sectioned serially at 7 μm. Picric acid and thymidine in the acid-soluble pool were removed by immersing the mounted sections in a saturated solution of lithium carbonate and in ice-cold 5% trichloroacetic acid, respectively. After thorough washing, the slides were coated in liquid nuclear emulsion (K2, Ilford Ltd, Basildon, Essex) at 46°C, dried and exposed in light-proof boxes at 4°C for 3-8 weeks. Control slides were exposed at the same time to detect possible or negative chemographic effects (e.g. high background activity and latent image fading). Pilot studies indicated few artefactual changes in silver grain density. Slides were developed and fixed (D19 developer and Kodafix; Kodak Ltd, Liverpool) and stained lightly with haematoxylin and eosin.

The autoradiographs were studied at high magnification with oil-immersion optics (x1000). The background density of grains was determined in experimental slides within 1 mm of the sections and above the tissue of control slides, and any having > 1 grain per 100 μm² were discarded. Cell nuclei with three or more grains were considered to be labelled, since the probability of labelling of the average nuclear area of 31 μm² by chance was then < 0.004 (by Poisson).

The labelling index (LI) in both ovaries of individual follicles, excluding primordial types, was determined in the section which passed through the nucleolus of the oocyte, and was the ratio of the number of labelled granulosa cells/total number of these cells. The numbers of cells counted per follicle section varied between 20 and 2000 according to the stage of development. Since pilot experiments showed that the LI varied within Graafian follicles, the cells were divided arbitrarily into two sub-populations for the purposes of analysis: Zone A comprised the cumulus cells and the immediately subjacent region of the membrana granulosa and Zone B comprised the remaining area of mural granulosa cells (Text-fig. 1).

The LIs in the double labelling experiment were obtained by a method similar to that of Pedersen (1970) and the results were expressed as the number of lightly labelled cells as a percentage of the total number of labelled cells. Labelled cells had 3 or more grains per nucleus and
Ovarian follicles in ageing mice

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Text-fig. 1. Diagrammatic representation of the distribution of proliferating (•) and dying (pycnotic) (x) granulosa cells in large Graafian follicles of anovulatory ageing mice. The limits of Zones A and B are shown since they defined the areas in which the labelling indices were determined.

those with 3–30 grains were considered to be lightly labelled, this being the upper limit for precise determination of the grain density.

Although follicles were sufficiently abundant in young ovaries for a random sample to be chosen for microscopical study, all follicles fulfilling the minimum criteria were analysed in old ovaries. The criteria were the presence of an intact and unwrinkled section of the whole follicle and an acceptably low background density of grains in the adjacent emulsion where it was not covering tissue. In general, only the non-atretic follicles were analysed, i.e. those with <2 pycnotic granulosa cells per section and without evidence of spontaneous maturation or degeneration of the oocyte. However, Graafian follicles at stage VIc in mice with persistent vaginal cornification are rarely devoid of pycnotic cells (Gosden, Laing, Felicio, Nelson & Finch, 1983). Therefore this stringent criterion for atresia was relaxed for these types, although any with obvious degeneration of the oocyte or atresia of the follicular antrum were not analysed.

For convenience of description, ovarian follicles were classified according to the number of granulosa cell layers and the presence of an antrum (Mandl & Zuckerman, 1951). In this classification, stages I–VI represent growth from primordial (I) to Graafian size (VI). Since stage VI includes a large range of sizes it was sub-divided: VIa (small antral types without a continuous fluid space), VIb (preovulatory antral types), VIc (large antral types which have undergone further cystic development beyond the size and appearance which is normally seen at pro-oestrus) (Gosden et al., 1983).

Follicular responsiveness to gonadotrophin. Another group of 7 mice aged 13–16 months and presenting persistently cornified smears were injected i.p. at 19:30 h with 1 or 5 i.u. hCG (Squibb, Princeton, NJ), both of which are ovulatory doses in young adult mice. A similar group of 5 controls were injected at the same time with saline (9 g NaCl/l). Mice were killed at 1300–1600 h on the following day when the oviducts were dissected to recover any freshly ovulated masses of eggs. The ovaries were prepared as histological sections stained with haematoxylin and eosin.

Results

Morphology of anovulatory ageing ovaries

Ovaries of 13–15-month-old mice with persistent vaginal cornification always contained large Graafian follicles in stages VIb and VIc; VIc follicles were only occasionally seen in ovaries of
young mice (see Gosden et al., 1983, for data). A few V1c follicles were haemorrhagic and most had some pyknotic granulosa cells, these being restricted to the mural region (1–50 per section). The old ovaries had few remaining primordial follicles in their cortex (86 ± 25, mean ± s.e.m.) but recruitment of growing follicles was unimpeded. Corpora lutea were absent but stromal tissue was abundant and contained conspicuous masses of lipochrome cells.

**Follicular responsiveness of old anovulatory mice to hCG**

All old mice injected with hCG had fresh oocytes in the ampullae, but the saline-treated controls did not ovulate. The number of ova shed per ovulating mouse was not significantly affected by the dose of hCG (1 or 5 i.u.). Overall, 4-1 ± 0-7 ova were shed (mean ± s.e.m.) which is significantly less than the numbers in comparable young mice (P < 0-01). All of the ovaries of hCG-injected mice contained some remaining Graafian follicles (generally V1c) with evidence of partial responses to hCG, such as resumption of meiotic maturation and mucification of cumulus cells. The oocytes of comparable follicles in control ovaries remained in dictyotene and without signs of follicular activation.

**Follicular turnover**

The distribution of labelled granulosa cells 1 h after injection of [3H]thymidine was approximately uniform in preantral follicles, but concentrated in the zone close to the oocyte (A) of Graafian follicles (Text-fig. 2; Pl. 1, Figs 1 & 2). This distinction was especially sharp in the largest cystic stages (V1c). The median LI for both types of large antral follicle was nearly 0-3 for Zone A, but generally much less than 0-1 in the mural cells (Zone B). The distribution of labelled nuclei varied continuously between the zones and there were gradients within each zone. Nevertheless, the distinctions between zones were sufficiently great to serve as markers for follicular turnover. Labelled cells were concentrated in the region of the cumulus oophorus of Zone A but decreased in frequency with distance from the oocyte. Labelled cells in Zone B tended to be restricted to the regions bordering the other zone and the follicular antrum, i.e. the cells that were closest to and had recently separated from the central zone of growth.

The median LI of Zone B increased with time after labelling as a result of the recruitment of follicles with a high LI and indicated that the set of Graafian follicles present at the time of labelling had been totally replaced within 3–4 days (Text-fig. 2; Pl. 1, Figs 3 & 4). Assuming that the mean rates of follicle recruitment and atresia are equivalent and constant, the LI of this zone should increase linearly with time. However, the expected increase of the LI in the 24-h sample was not obtained, though this might simply be chance variation. The LI was maximal at 72 h and significantly higher than at 1 h (P < 0-01, Wilcoxon test) and was maintained at the same level at

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**PLATE 1**

**Figs 1 and 2.** Autoradiographs of a large Graafian follicle in a 15-month-old mouse prepared 1 h before autopsy by injection with [3H]thymidine. The substantial majority of labelled granulosa cell nuclei were located within the cumulus region and the subjacent cells of the granulosa cell membrane. Serial reconstruction of this follicle showed that the labelled cells on the left side of the follicle wall were in fact located close to an extended but irregular mass of cumulus cells. Fig. 1, bright field illumination, × 104; Fig. 2, dark-ground illumination, × 104.

**Figs 3 and 4.** Autoradiographs of a large Graafian follicle in a 15-month-old mouse killed 48 h after injection of [3H]thymidine. Labelled granulosa cells are present in the wall of the follicle opposite to the cumulus region. The grain density of cumulus cells has become diluted through subsequent mitosis. The background activity over the antrum is higher than that in Fig. 1. Fig. 3, bright field illumination, × 207; Fig. 4, dark-ground illumination, × 207.
Text-fig. 2. The variation of the labelling index in granulosa cells of (a) small (VIa) and (b, c) large (VIb + c) Graafian follicles in 13–15-month-old mice that had been treated with a single injection of [³H]thymidine (at $t_0$). Each point represents one follicle and the lines are the medians. Since the labelling patterns of cells in follicles of stages VIb and VIc were heterogeneous within follicles, the LI was determined for two distinct sub-populations or zones (see Text-fig. 1); i.e. Zone A (b) and Zone B (c). The LI in Zone B was significantly higher at 48 h ($P < 0.02$), 72 h and 144 h ($P < 0.01$) than at 1 h (Wilcoxon test).

144 h. Observations of the final group (288 h) could not be interpreted because the signal to noise ratio was substantially reduced by successive cell divisions. A further prediction arising from the preceding assumptions is that follicles with a high LI at 48 and 72 h (i.e. newly recruited from the growing pool) would have fewer pyknotic cells than the remaining ones with a lower LI. Whilst there was some evidence of this, it was not confirmed statistically because of variations in the percentage of pyknotic cells. The proportion of follicles with a high LI did not vary with the size of the total population of follicles remaining.
The LI of Zone A was high at all times after the single injection of $[^3\text{H}]$thymidine, as would be expected of a relatively homogeneous population of dividing cells (Text-fig. 2). The values tended to be slightly higher at 48 and 72 h in association with an unusually high grain density over the antrum (Pl. 1, Figs 3 & 4), a phenomenon which has been attributed to release of labelled nucleoside from dying cells and subsequent re-uptake (Byskov, 1974). Although we could not determine the density of grains per nucleus in heavily labelled cells (>30 grains), it was our firm impression that the heavily labelled cells had a similar density both between and within the two zones 1 h after labelling. However, the grain density appeared to diminish more rapidly in Zone A than in Zone B in subsequent autoradiographs, and within Zone A the degree of dilution seemed to vary inversely with distance from the oocyte. Continuing growth of Zone A appeared to contribute few cells to the mural population since the LI of the latter remained very low in some of the follicles. Instead proliferation appeared to result in the lateral expansion of the cumulus oophorus, thus producing a broad platform of cells.

Text-fig. 3. The labelling of granulosa cells in the ovaries of young and ageing mice: (a) the percentages of lightly labelled cells, giving an index of S-phase length, and (b) the labelling index. Values are mean ± s.e.m. for the no. of observations indicated.

The LI of medium-sized growing follicles (II–V) in young mice seemed to vary consistently with stages of the oestrous cycle, although the differences were not statistically significant (Text-fig. 3, $P < 0.05$ by Newman–Keul’s studentized range test). The LI varied with the stage of follicular development: those of the largest preantral types (V) were similar to the values of Zone A of Graafian follicles, and significantly higher than those of smaller types (II–IV) ($P < 0.05$). The LIs of follicles in old ovaries were within the normal range of variation of young cyclic animals.

The length of the S-phase of granulosa cells in the two age groups was compared because it is possible for follicles to have a similar LI yet still grow at different rates. Differences in the proportions of lightly labelled cells in double labelling experiments provided an indication of differences of the S-phase. However, the proportions were not significantly different for follicle stages II–IV (Text-fig. 3); no estimates are provided for stage V because of the small numbers of such follicles in old ovaries.

**Discussion**

The principal conclusion of these results is that a set of Graafian follicles in ageing anovulatory mice is replaced completely in 3–4 days. This information, combined with additional data, has been
used to construct a hypothetical model to explain the growth and replacement of Graafian follicles in animals with persistent vaginal cornification.

The evidence of a short lifespan of unovulated Graafian follicles was based on the differential pattern of granulosa cell proliferation (\[^{3}H\]thymidine incorporation) which was characteristic of these follicles. Mural cells had very low levels of radioactivity whereas the activity of cumulus cells was high and comparable with that of preantral follicles in which cells were labelled more or less randomly. It follows, therefore, that the increased LI over a 3-day period after a pulse of \[^{3}H\]thymidine implies that the original set of Graafian follicles labelled at the time of injection had been replaced by follicles that were preantral at that time. The method indicates replacement of the whole population but cannot provide information about the longevity of individual follicles.

The lifespan of Graafian follicles in ageing anovulatory ovaries is similar to their potential longevity in cyclic rats and hamsters, as disclosed by barbiturate sedation to suppress the spontaneous ovulatory surge of gonadotrophins. Unovulated follicles undergo atresia spontaneously 3 or 4 days after pro-oestrus in these animals (Everett & Sawyer, 1950; Braw & Tsafriri, 1980; Terranova, 1980), although this interval is shorter in 5- compared to 4-day cycles (van der Schoot, 1980) and is slightly longer in PMSG-treated immature rats than in adults (Terranova & Ascanio, 1982).

The morphological appearance and secretory activity of these unovulated follicles has been studied to identify factors responsible for atresia. Many follicles in ageing ovaries became haemorrhagic before other signs of involution, such as extensive granulosa cell pycnosis. Accidental breaches of the integrity of the follicular wall might therefore be responsible for atresia in some follicles.

Another factor of potential significance is the changing pattern of steroid production in post-mature follicles and their diminished response to LH. Such changes may promote apoptosis rather than mitosis, thereby leading to atresia. Perhaps the most significant changes are the decreased production in vitro by explanted follicles of oestradiol and androgens and the concomitant increased production of progesterone, which might reflect impaired ability to convert progesterone to androgens (Braw & Tsafriri, 1980; Uilenbroek, Woutersen & van der Schoot, 1980; Terranova, 1981). There is evidence that a balance between the levels of oestradiol and androgens determines whether follicles continue their growth or become atretic (Harman, Louvet & Ross, 1975; Bagnell, Mills, Costoff & Mahesh, 1982). The reduced ability of unovulated follicles to produce oestradiol might partly explain why plasma hormone levels of mice with persistent vaginal cornification are less than those of pro-oestrus mice (Nelson et al., 1981) even though both types of mice have ovaries dominated by large follicles. Graafian follicles that become post-mature also show lower specific binding of gonadotrophins (Peluso, Steger & Hafez, 1977; Uilenbroek et al., 1980) and a weaker ovulation response to LH which may be attributable to decreased intra-follicular oestradiol.

These changes affecting post-mature follicles may be considered as pathological or as further stages of differentiation, but they result in follicles that are incapable of a full response to gonadotrophic stimuli. The oocyte, however, retains a normal morphological appearance until there is extensive granulosa cell pycnosis and it remains capable of responding to gonadotrophic stimulation. This suggests that the oocyte does not initiate atresia, although its developmental potential may be affected adversely by prolonged sojourn in the follicle while it remains ovulable (Butcher & Pope, 1979; Martin & Terranova, 1982). The partial responses of some follicles to hCG (e.g. meiotic maturation without ovulation and luteinization) suggest that actions of gonadotrophins that are mediated by prostaglandins are preferentially affected during atresia (see Tsafriri, Lindner, Zor & Lamprecht, 1972).

Maintenance of a set of Graafian follicles in anovulatory ageing mice is illustrated by a model in Text-fig. 4, although the model might be equally applicable to younger animals with polyfollicular ovaries and persistent vaginal cornification. Each horizontal array of follicles indicates their flow from the time of origin on the left until they disappear by atresia a few days later. Atresia and replacement of individual follicles was assumed to be asynchronous since there was no
morphological evidence of discontinuous progression of follicles, neither would this be expected where the normal synchronizing event (the FSH surge) was absent (Hoak & Schwartz, 1980). If the time taken for individual follicles to progress through these stages to atresia is approximately constant and independent of the size of the total follicular population remaining, it follows that about one-third of the Graafian stages will become atretic each day. The numbers of follicles at a given stage of development or atresia will vary since the Graafian follicle population is not an open system in a steady state because the flow rate is limited by the numbers of follicles available for recruitment (Faddy, Gosden & Edwards, 1983). Consequently, there will be an increasing probability during ageing that persistent vaginal cornification will be punctuated by intermittent and eventually chronic leucocytosis without pseudopregnancy, signifying exhaustion of large secretory follicles. Healthy C57BL/6J mice generally present persistent vaginal leucocytosis when they are 20–26 months old (Gee, Flurkey & Finch, 1983).

During the period when ovarian function is waning the rates of growth of medium-sized follicles do not change, despite the rise in levels of plasma LH and FSH reported to occur at 16 months of age (Parkening, Collins & Smith, 1980). This conclusion is drawn from the similar labelling index and percentage of lightly labelled cells in young cyclic and acyclic ageing mice and is consistent with previous studies, although Pederson (1972) and Faddy et al. (1983) did not distinguish the stages of the ovarian cycle. A change of the dynamics of growth of follicles would not be expected to result from the transition to anovulatory state per se since the longevity of large follicles does not exceed the normal length of the oestrous cycle, but it is important to discover whether the circulating levels of gonadotrophins are elevated as early as 13–15 months of age in these mice.

Earlier studies provided limited evidence of a distinction between the growth rates of mural and cumulus cells (Bullough, 1942; Pedersen, 1970) which are confirmed here. Organization of follicular growth might be compared with that of other epithelia in which a small focus of self-maintaining stem cells produces some progeny cells ('transit cells') which amplify the cellular reproductive activity and which lead, in turn, to cells with a higher probability of differentiation and a decreasing probability of self-replication (Potten, Schofield & Lajtha, 1979). Cells within the cumulus oophorus may contain a stem-type cell population which provides the focus of growth and a source of differentiating cells that migrate centrifugally. This pattern of growth may have important developmental repercussions if the follicle receives an effective ovulatory stimulus after reaching normal preovulatory ripeness (stages VIb–Vlc). The additional granulosa cells remain at one pole of the follicle and distort the normal architecture of the cumulus, perhaps leading to
impairment in the shedding of the oocyte and resulting in corpora lutea accessoria, structures that are frequently seen in ageing mouse ovaries (Jones & Krohn, 1961). On the other hand, continuing cell death amongst the mural cells without cell replacement may affect the ultimate size of the corpus luteum which is formed after delayed ovulation. Nevertheless, the potential significance of any effects of delayed ovulation on the future corpus luteum and oocyte may be overruled by intervening refractoriness of the follicle to the ovulatory stimulus.

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Restoration of ovulatory cycles by young ovarian grafts in aging mice: Potentiation by long-term ovariectomy decreases with age

(reproductive aging/ovulation/vaginal cyclicity/C57BL/6j mice)

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ABSTRACT The relative contributions of ovarian and hypothalamic-pituitary factors to the anovulatory status of aging mice were evaluated by measuring the capacity of mice to resume ovulatory cyclicity after receiving young ovaries under the renal capsule. Young grafts partially restored cyclicity if old hosts were acutely ovariectomized but almost fully restored cyclical ovulatory function if the old hosts had been ovariectomized early in adulthood. With advancing age, however, the efficacy of the grafts declined progressively in both acute and long-term ovariectomized groups. These data show that both ovarian and hypothalamic-pituitary aging contribute to the etiology of anovulation. Although chronic withdrawal from ovarian secretions retards the age of onset of hypothalamic-pituitary aging, the duration of this ameliorative effect is limited by progressive ovary-independent neuroendocrine dysfunction.

The acyclic anovulatory status of aging mammals is well-documented (1), yet the relative importance of ovarian versus hypothalamic-pituitary factors has not been established. Although steadily declining oocyte reserves clearly place an upper limit on the ovulatory life span (2), the reduced capacity of the aging hypothalamic-pituitary axis to release preovulatory surges of luteinizing hormone is also implicated in some rodent species (3-8). The possibility that this hypothalamic-pituitary dysfunction is a consequence of long-term exposure to ovarian steroids was first indicated by Aschheim’s observation in a small group of old rats that young ovarian grafts restored vaginal cyclicity only if these rats had been ovariectomized at an early age (9). Subsequent studies showed that chronic treatment of young rodents with estradiol (10) led to a premature onset of degenerative changes in the hypothalamic arcuate nucleus, a region involved in the regulation of gonadotropin secretion. However, crucial questions bearing on the etiology of ovulatory failure in rodents remain unanswered. Namely, is hypothalamic-pituitary dysfunction solely responsible for the anovulatory condition, or does primary ovarian failure contribute as well? What is the duration of the ameliorative effect of long-term ovariectomy on reproductive function? Do factors other than exposure to ovarian secretions contribute to the aging of the reproductive hypothalamic-pituitary axis?

To answer these questions, we examined the ability of young ovarian grafts to restore ovulatory cyclicity in a longitudinal study of mice at ages spanning the adult life span. We report that the anovulatory condition is a consequence of both ovarian and hypothalamic-pituitary insufficiency. Although long-term withdrawal from endogenous ovarian secretions dramatically potentiates the restoration of ovulatory cyclicity in aging animals with young ovarian grafts, this effect is transitory. Progressive neuroendocrine dysfunction, independent of ovarian secretions, sets an upper limit on the ameliorative influence of long-term ovariectomy.

MATERIALS AND METHODS

Animals and Vaginal Cyclicity. Virgin female C57BL/6j mice (The Jackson Laboratory) were kept in a limited-access aging colony (11). Mice intended for long-term ovariectomized groups were ovariectomized at age 5 months. Ovaries from 5-month-old donor mice were grafted under the renal capsule of acutely or long-term ovariectomized mice aged 17 and 25 months; donors for the 30-month-old group were 3 months old. Cycle frequency (cycles/month) and cycle length were evaluated from daily vaginal smears by a program developed for an Apple II microcomputer (11). Monthly cycle frequency was plotted as a function of age, and the integrated areas under the respective cycle frequency profiles were used to compare the effectiveness of ovarian grafts among the various experimental groups. Statistical significance of these data was determined by analysis of variance (ANOVA) followed by Duncan’s multiple-range test for comparisons among group means. Differences with P values < 0.05 were considered significant.

Ovarian Grafting. Donor and host mice were anesthetized with 2,2,2-tribromoethanol (12). One of the ovaries of the donor was gently pulled out from a dorso-lateral incision and reflected onto a piece of sterile gauze. Under a dissecting microscope, the ovarian bursa was slit and the hilum cut to release the ovary. Simultaneously, the kidney of the host was exposed by a second individual while the ovary was cut into three segments to facilitate insertion under the renal capsule. The interval between removal and transplantation was less than 1 min. Although some mice developed self-inflicted wounds at the incision sites and were culled, >75% of the animals in all treatment groups recovered completely. At all ages, >90% of the fully recovered mice with ovarian grafts exhibited vaginal cornification, an index of estrogenic support and, thus, of a functioning ovarian graft. Only fully recovered mice exhibiting vaginal cyclicity or cornification were included in the study.

Histology. Ovarian grafts were removed from some mice on the third day after vaginal proestrus, fixed in Bouin’s solution, embedded in paraffin, serially sectioned at 7 µm, and stained with hematoxylin and eosin. Sections were searched microscopically for recently formed corpora lutea (these appear more eosinophilic and with a higher nuclear/cytoplasmic ratio than previous sets of corpora lutea) and for oocytes that had been shed into the fluid-filled space between the ovarian graft and

Abbreviations: LH, luteinizing hormone; ANOVA, analysis of variance. * Present address: Centre for the Study of Reproduction, McGill University, Royal Victoria Hospital, 667 Pine Avenue West, Montreal, Quebec H3A 1A1, Canada.
the renal capsule. Total oocyte number was estimated based on counts of every 20th section.

Luteinizing Hormone (LH) Radioimmunoassay. Plasma was frozen and assayed for LH by a double antibody radioimmunoassay (rabbit anti-ovine LH) with NIH-LH-RP1 standard as described in detail (6).

RESULTS

Young (5 month) acutely ovariectomized mice bearing young (5 month) ovarian grafts served as controls (designated Y-to-Y) for the efficacy of the grafting procedure. During the initial 3-month period after grafting, the cycle frequency and cycle length of these Y-to-Y controls were indistinguishable from those of intact mice (P > 0.5, ANOVA; Fig. 1 and Table 1). Thereafter, cycle frequency declined more rapidly in grafted mice compared to intact controls, probably because of oocyte losses (about 50%) consequent to the initial ischaemia of grafting (Table 2). In 17-month-old acutely ovariectomized mice, cyclicity was maintained for 3 months after grafting at 25% of the Y-to-Y control level (Fig. 1); thereafter all the mice were acyclic. By contrast, Y-to-Y controls cycled for 4 more months. The incomplete restoration of function in 17-month-old acutely ovariectomized mice was primarily due to two factors: (i) half of the population failing to resume cycling and (ii) fewer cycles in the cycling subpopulation. In addition, the frequency of long cycles was higher in acutely ovariectomized mice (Table 1). In 17-month-old long-term ovariectomized hosts, cycle frequency during the first 3 months after grafting was 3-fold greater than in acutely ovariectomized mice (P < 0.001, ANOVA; Fig. 1). The frequency and duration of cyclicity were >90% of that of Y-to-Y controls (Fig. 1 and Table 1). In hosts aged 25 months at grafting, the efficacy of the grafts declined proportionately in both acutely ovariectomized and long-term ovariectomized groups to only 30% of their values in 17-month-old hosts (P < 0.02 and P < 0.001, respectively, ANOVA). In hosts aged 30 months at grafting, cycle frequency in long-term ovariectomized mice was restored to only 5% of that in 17-month-long-term ovariectomized mice.

The ovulatory potential of the ovarian grafts was assessed in 5-month long-term ovariectomized and 32-month long-term ovariectomized mice that had received 3-month-old ovaries 2 months earlier. All cycling mice in both groups had fresh corpora lutea with ova present in the subcapsular space 3 days after showing vaginal proestrus (Fig. 2). Moreover, ovulation rate, assessed

Table 1. Estrous cyclicity in acutely and long-term ovariectomized mice of different ages bearing young ovarian grafts

<table>
<thead>
<tr>
<th>Age at grafting, mo</th>
<th>Group*</th>
<th>n</th>
<th>% Total cycles</th>
<th>4-6 days</th>
<th>7-14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Intact</td>
<td>13</td>
<td>100</td>
<td>34.2 ± 1.0</td>
<td>89 ± 11</td>
</tr>
<tr>
<td>5</td>
<td>Acute-OVX</td>
<td>21</td>
<td>100</td>
<td>24.4 ± 1.8*</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>17</td>
<td>Acute-OVX</td>
<td>12</td>
<td>50</td>
<td>10.0 ± 2.9b</td>
<td>76 ± 24d</td>
</tr>
<tr>
<td>17</td>
<td>Long-OVX</td>
<td>22</td>
<td>95</td>
<td>20.1 ± 1.7*</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>25</td>
<td>Acute-OVX</td>
<td>11</td>
<td>18</td>
<td>7.5 ± 0.5</td>
<td>40 ± 60d</td>
</tr>
<tr>
<td>25</td>
<td>Long-OVX</td>
<td>11</td>
<td>64</td>
<td>10.3 ± 2.1*</td>
<td>73 ± 27*</td>
</tr>
<tr>
<td>30</td>
<td>Long-OVX</td>
<td>4</td>
<td>25</td>
<td>4.0</td>
<td>40 ± 60</td>
</tr>
</tbody>
</table>

* Acutely ovariectomized (acute-OVX) mice were ovariectomized immediately prior to receiving ovarian grafts. Long-term ovariectomized (long-OVX) mice were ovariectomized at age 5 months. Donor age was 6 months, except for 30-month-old hosts that received 5-month-old ovaries.

* Showing >3 cycles.

* ± SEM (values based on cycling subpopulation). The effect of the group was significant (P < 0.0005, ANOVA); a, significantly different from age-matched controls; b, significantly less than 5-month-old acute-OVX hosts; P < 0.05 by Duncan's multiple range test.

* During the first 3 months after surgery. Frequency distributions were significantly different from that of the 5-month-old intact mice (e, P < 0.05; g, P < 0.01; and e, P < 0.001) and significantly different from the age-matched long-OVX group (d, P < 0.01; f, P < 0.05; f* test).

the onset of declining cycle frequency was defined as the earliest age at which cycle frequency fell significantly below the value for the month of maximal cycling frequency (P < 0.05, Duncan's multiple range test; ref. 11). By this criterion, cycle frequency began to decline at 8 months in young mice bearing ovarian grafts—2 months earlier than in intact controls (Fig. 1).

Table 2. Comparison of follicular and ovulatory status of grafted and in situ ovaries

<table>
<thead>
<tr>
<th>Age of host at grafting*</th>
<th>Age at autopsy*</th>
<th>Primordial follicles*</th>
<th>Corpora lutea*</th>
<th>Mice with ova%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>5</td>
<td>4</td>
<td>1,385 ± 2258</td>
<td>8.2 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>4</td>
<td>610 ± 1172</td>
<td>9.0 ± 0.6</td>
</tr>
<tr>
<td>30</td>
<td>32</td>
<td>4</td>
<td>815 ± 147</td>
<td>11.0 ± 0.9</td>
</tr>
</tbody>
</table>

* Ovarian grafts were obtained from 3-month-old hosts.

* ± SEM.

* Significantly greater than hosts (P < 0.05, ANOVA).
Fig. 2. Representative examples of follicular and luteal development in intact ovaries and in ovarian grafts under the renal capsule. (H and E; ×32.) (A) Five-month-old ovary from a cycling intact mouse. Several growing follicles and corpora lutea are present. (B) Five-month-old ovarian graft in a cycling 5-month-old host, which was ovariectomized at 3 months and given ovarian grafts from a 3-month-old donor. Growing follicles and corpora lutea are present. Also visible are the underlying cortex (C) of the kidney to which the graft is attached and the renal capsule (RC), which is typically separated from the graft by a fluid-filled space (F). (C) Five-month-old graft in a cyclic 32-month-old host that was ovariectomized at age 3 months and received ovarian grafts from a 3-month-old host at 30 months. Several primary and secondary follicles are present. Recent ovulation is indicated by corpora lutea and the presence of several ova (*) in the fluid-filled space between the graft and the renal capsule (see B for symbol legend). (D) Five-month-old graft in an acyclic, 32-month-old host showing persistent vaginal cornification. The host retained its ovaries until age 30 months, when it received 3-month-old ovarian grafts. Corpora lutea are absent, although antral follicles are present (see B for symbol legend).
aging is a significant factor in the etiology of acyclicity in this strain. In old acyclic mice, similar efforts to restore cyclicity with young ovarian grafts have failed (9, 15, 16). Although this failure has been interpreted to disclaim a role for ovarian aging in the loss of cyclicity in the rat, the possibility should be considered that the different outcomes of grafting studies in rats and mice reflect experimental rather than species differences. For example, the importance of the age of the host on the success of the graft has not been taken into account previously. In this study, virtually no restoration of cyclicity was achieved when young ovaries were grafted in 25-month-old acutely ovariectomized mice, whereas partial success was achieved in 17-month-old mice. Although the ages of the rats used in earlier studies are quite disparate, most animals were over 18 months. The possibility of graft rejection also deserves consideration because animals used in earlier studies were outbred. By contrast, C57BL/6J mice have been inbred for over 40 years (17), and there was no histological evidence of graft rejection (e.g., lymphocytic infiltration) in this study.

The age-related depletion of oocyte reserves probably accounts for the implicit failure of the old ovary to support cycles. Oocyte reserves are nearly exhausted in these mice at the age of onset of acyclicity (13-16 months), and the anovulatory subpopulation of mice at this age has half the reserves of mice still ovulating (13). Although the oocyte pool is not completely depleted, the reduced pool of growing follicles may be too small to maintain the estrogenic stimulation of the hypothalamic-pituitary axis required for preovulatory surges of LH.

The failure of young ovarian grafts to completely restore cyclicity in acutely ovariectomized acyclic mice shows that extranovarian loci, presumably hypothalamic or hypophyseal, also contribute to the anovulatory state. Long-term ovariectomy followed by young ovarian grafts at 17 months permitted nearly complete restoration of ovulatory cycles and preovulatory LH surges for a duration equivalent to that of young controls. Thus, one component of the age-related loss of hypothalamic-pituitary function can be attenuated by long-term withdrawal of ovarian secretions. This dysfunction may be due to refractoriness of the hypothalamic-pituitary axis to hormonal stimulation, as suggested by the impaired preovulatory surge of LH in aging rodents with regular cycles (13-16 months) or after stimulation with exogenous steroids (7, 8). The regressive glosis of the hypothalamic arcuate nucleus of spontaneously acyclic aging rodents (18) has been offered as evidence for a lesion deafferenting the medial basal hypothalamus from the medial preoptic area and, thereby, impairing the induction of a preovulatory LH surge (19). It is noteworthy that these glial changes can also be delayed by long-term ovariectomy (18) and that the age of onset can be accelerated by chronic treatment with physiological levels of estradiol (10).

Prolactin-secreting pituitary tumors are common after 24 months in female C57BL/6J mice (20-22). Because these tumors are often estrogen-dependent (23, 24) and hyperprolactinemia can interfere with ovulatory cyclicity (25-27), the greater ability of long-term ovariectomized hosts to support cycles with ovarian grafts might be a result of reduced tumor incidence, particularly in the hosts aged 25 and 30 months. In the present study, when mice that received young ovaries at 17 months were autopsied at 25 months, tumor incidence in the long-term ovariectomized hosts was only 35% of that of the acutely ovariectomized hosts (23). However, we consider it unlikely that pituitary tumors influenced the ability of acutely or long-term ovariectomized hosts aged 17 months to initiate estrous cyclicity with young ovarian grafts. First, tumors are small and incidence is low (~20%) in 17-month-old mice (20). Second, in the present study there was no difference in the incidence or

**FIG. 3.** Plasma LH concentrations in 11- to 14-month-old acutely ovariectomized (Y to Oy) or 23- to 25-month-old long-term ovariectomized (Y to Oy) mice bearing young ovarian grafts since age 5 or 17 months, respectively. Intact mice (13-15 months) served as controls. Cycling mice (□) (n = 9-12 per treatment) were sampled 3 hr after lights were on the evening of proestrus, when LH levels are elevated (4). Acyclic mice (□) showing persistent vaginal cornification were sampled at the same time (acyclic mice from all treatment groups were pooled; n = 12). LH levels were significantly increased in cycling mice compared to acyclic mice (P < 0.005, ANOVA with logarithm-transformed data to eliminate heteroscedasticity, inequality of variances among samples), but there was no effect of treatment on the proestrus levels in cycling mice.

by the number of fresh corpora lutea, was equivalent to that of intact mice (Table 2). These data indicate that some long-term ovariectomized mice retain the potential to ovulate a full complement of ova at an age twice their normal ovulatory life span (13-16 months; refs. 11 and 13) and confirm previous observations that regular vaginal cyclicity of singly housed mice is a reliable correlate of ovulatory cyclicity (13, 14).

The efficacy of long-term ovariectomy in prolonging normal reproductive hypothalamic-pituitary function was further demonstrated by preovulatory increases of LH in old long-term ovariectomized mice that were indistinguishable from those in intact and acutely ovariectomized, middle-aged animals (Fig. 3).

**DISCUSSION**

This study establishes that both ovarian insufficiency and hypothalamic-pituitary dysfunction contribute to the anovulatory condition of aging mice. The data also show that the retarding effect of long-term ovariectomy on hypothalamic-pituitary aging is not indefinite, indicating that one or more factors in addition to exposure to ovarian secretions contribute to the aging of the reproductive hypothalamic-pituitary axis.

The ability of young ovarian grafts to partially restore cyclicity in aging acutely ovariectomized mice establishes that ovarian

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size of pituitary tumors between cycling and acyclic acutely ovariecoto\-mized hosts (unpublished data). Further studies will be needed to resolve conclusively the relative roles of pituitary pathology and primary impairment of gonadotropin secretion in the loss of ovulatory cycling potential.

The progressive decline in the effectiveness of long-term ovariec\-tomy suggests that neuroendocrine impairments independent of ovarian influence ultimately limit the extension of the ovulatory life span achievable by ovarian hormonal withdrawal. Whether extraovarian estrogens or other hormones, acting analogously to the debilitating ovarian secretions, contribute to this ultimate neuroendocrine failure remains to be determined. Studies also are needed to identify the endoge\-nous ovarian hormone(s), their duration of exposure, and the specific secretory profiles that lead to dysfunction of the re\-productive hypothalamic–pituitary axis. Elucidation of these issues may provide insight not only into the age-related disrup\-tion of the hypothalamic–pituitary–ovarian axis but also into the aging of other endocrine-dependent systems.

Note Added in Proof. Additional evidence has been obtained recently (28) for the retarding effect of long-term ovariec\-tomy on the age-related decline of the estradiol-induced LH surge in C57BL/6j mice.

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Effects of long-term ovariectomy on the potential for oestrous cyclicity and pregnancy disclosed by ovarian grafts in ageing mice

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Ageing of the reproductive system of female mice is characterized by a decreasing frequency of oestrous cycles culminating in acyclicity at midlife (Nelson, Felicio, Randall, Sims & Finch, 1982) and a concomitantly increased probability of fetal death in utero (Holinka, Tseng & Finch, 1979). The relative contributions of (a) the ageing ovary and (b) long-term ovarian secretory activity to these processes have now been assessed by evaluating the ability of young syngeneic ovaries (2-5 months) to restore normal reproductive functions after grafting beneath the renal capsules in mice of post-cyclic age.

C57BL/6J mice were ovariectomized with tribromoethanol anaesthesia or left intact when 5 months old. When ovarian grafts were given at 17 months, long-term ovariectomized mice (long-OVX) subsequently had more oestrous cycles among a larger proportion of cyclic mice than mice ovariectomized at the time of grafting (acute-OVX) (20.1 ± 1.7 cf. 10.0 ± 2.9, mean ± s.e.m. total number of cycles) ($P < 0.05$).

Primiparous CBA/Ca mice were ovariectomized as above and given ovarian grafts when 10-11 months old. When paired with males at 12-13 months, 6/19 (31.7%) of long-OVX mice mated (as assessed by vaginal plug) compared with 7/21 (33.0%) in acute-OVX controls. Blastocysts obtained from young donor animals were transferred to the uteri of these mice on day 4 post-coitum. On day 14, 28/67 (41.8%) embryos were alive in long-OVX mice whereas the corresponding proportion in controls was 8/78 (10.3%) ($P < 0.001$).

The observation that young ovarian grafts can partially restore cyclicity and fertility in mice of post-reproductive age indicates that ovarian ageing contributes to the loss of reproductive function in mice. That long-term ovariectomy potentiates the restorative ability of young grafts demonstrates that ovarian secretory activity during adulthood also plays a role in the anovulatory infertile status of the ageing mouse.

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Maternal ageing and aneuploid embryos—Evidence from the mouse that biological and not chronological age is the important influence

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Summary. Maternal ageing remains the overwhelming factor in the aetiology of human aneuploidy. Whether aberrant meiotic chromosome segregation in the oocyte relates causally to ovarian physiological ageing or to some factor dependent on the passage of chronological time, remains to be determined. The present experimental studies in the mouse indicate the former. An earlier cessation of reproductive life, brought on by unilateral ovariectomy in CBA females, resulted in the earlier onset of irregular cyclicity and an earlier rise in aneuploidy. The results could not be explained on the basis of the "production line" hypothesis. The clinical implications are that the probability of conceiving a Down foetus will be determined by distance in time from the approaching menopause, rather than by the chronological age of the woman per se.

Introduction

Numerous hypotheses have been proposed to account for the association between maternal age and the risk of conceiving a trisomic foetus. Some implicate the passage of chronological time in aneuploidy production. Thus, Alberman et al. (1972) proposed that decay of spindle components might occur over the long human female meiotic prophase. Others suggest that the raised risk of trisomy with maternal age might be due to cumulative environmental insults (Read 1982; Kline et al. 1983). Polani et al. (1960), some years ago, proposed failure of dissolution of the nucleolus in aged oocytes as the possible mechanism leading to nondisjunction in the D- and G-group acrocentric pairs.

According to the "production line" hypothesis (Henderson and Edwards 1968), trisomy is predetermined by the order of formation of oocytes during foetal development, the last-formed oocytes with fewer chiasmata and more univalent pairs being the last to be ovulated late in life. Others have proposed that it is some factor in the adult maternal environment and dependent on physiological ageing of the reproductive system which is important. Thus, delayed ovulation might lead to a higher incidence of spindle defects in oviposited eggs (Sugawara and Mikamo 1980), or to asynchrony between chiasma terminalization and chromosome alignment on the spindle (Crowley et al. 1979). The hormonal imbalance and irregular cyclicity associated with the premenopausal period (Lyon and Hawker 1973; Read 1982) could be critical.

There is an obvious contrast between the hypotheses that state that aneuploidy risk is determined before birth or that it is influenced by random environmental factors, both of which imply that anomalies appear progressively with time, and those that state that the occurrence of anomalies is linked to the physiological age of the reproductive system. These two groups of hypotheses may be distinguished if, as anticipated, advancing the process of ageing by unilateral ovariectomy early in adulthood brings a correspondingly early onset of age-related aneuploidy. This study has now been carried out with inbred CBA/Ca mice whose short reproductive lifespan and age-dependent increase of aneuploidy make an excellent model for the human female (Gosden 1973; Fabricant and Schneider 1978). Furthermore, the CBA ovary is virtually depleted of oocytes at the end of reproductive life (Faddy et al. 1983), as in the human female (Costoff and Mahesh 1975).

Materials and methods

1. Animals

Inbred CBA/Ca mice were obtained from Bantin and Kingman (Hull) at 4–5 weeks of age. When 6–8 weeks old they were allocated at random to two groups which were either unilaterally ovariectomized (uni-ovx) or sham-operated under tribromoethanol anaesthesia. The numbers of mice operated on the right and left sides were approximately equal in each group.

2. Ovarian cyclicity

The oestrous cycles of 20 uni-ovx and 21 sham-operated animals were recorded between 60 and 390 days of age. Vaginal smears were prepared from samples collected by lavage six days a week and were analysed by phase contrast microscopy. They were classified by a method based on that of Allen (1922). At the end of the period of study when at least 50% of mice in each group were acyclic, the mice were killed and their abdominal cavities were examined to verify whether ovariectomy had been complete and the extent of gross pathological lesions, if any. Diseased animals were excluded from the data analysis.

3. Recovery and cytogenetic analysis of embryos

About 400 additional virgin uni-ovx and sham-operated mice were kept in small groups in the same room as those used for studying oestrous cyclicity. They were divided into subgroups according to age (Table 1) and mated with young males of proven fertility (Swiss strain). Each morning, they were examined for the presence of a coital plug (day 1 of pregnancy).
Table 1. The recovery of 3.5-day embryos from mated CBA/Ca females

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Maternal age (days)</th>
<th>No. of animals</th>
<th>Corpora lutea</th>
<th>Mean ($A$) ± SE</th>
<th>Unfertilized eggs ($B$)</th>
<th>No. embryos recovered ($C$)</th>
<th>100 $[(A - (B + C))/A]$</th>
<th>Percent embryos analysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>ovx</td>
<td>63–91</td>
<td>29</td>
<td>261</td>
<td>(9 ± 0.25)</td>
<td>3</td>
<td>243</td>
<td>5.7</td>
<td>29.0</td>
</tr>
<tr>
<td>Sham</td>
<td>63–91</td>
<td>26</td>
<td>277</td>
<td>(10.65 ± 0.24)</td>
<td>13</td>
<td>251</td>
<td>4.7</td>
<td>27.2</td>
</tr>
<tr>
<td>ovx</td>
<td>154–182</td>
<td>33</td>
<td>295</td>
<td>(8.94 ± 0.29)</td>
<td>9</td>
<td>265</td>
<td>7.1</td>
<td>29.2</td>
</tr>
<tr>
<td>Sham</td>
<td>154–182</td>
<td>21</td>
<td>214</td>
<td>(10.19 ± 0.43)</td>
<td>10</td>
<td>187</td>
<td>7.9</td>
<td>37.1</td>
</tr>
<tr>
<td>ovx</td>
<td>245–280</td>
<td>99</td>
<td>240</td>
<td>(2.42 ± 0.16)</td>
<td>3</td>
<td>146</td>
<td>37.9</td>
<td>28.1</td>
</tr>
<tr>
<td>Sham</td>
<td>245–280</td>
<td>284</td>
<td>284</td>
<td>(4.37 ± 0.26)</td>
<td>10</td>
<td>238</td>
<td>12.7</td>
<td>24.9</td>
</tr>
<tr>
<td>Sham</td>
<td>308–350</td>
<td>66</td>
<td>190</td>
<td>(2.88 ± 0.27)</td>
<td>1</td>
<td>137</td>
<td>27.4</td>
<td>33.8</td>
</tr>
</tbody>
</table>

On day 4, they were injected with Colcemid (1 μg per g body weight, Ciba, London) and killed by cervical dislocation 3 h later. The ovaries were removed for counting numbers of hyperaemic corpora lutea under the dissecting microscope. The uterus was dissected and pre-implantation embryos and unfertilized ova were recovered by flushing with physiological saline (0.15 M NaCl). Embryos and ova were examined, and counted under the low power dissecting microscope and then transferred to a hypotonic solution of 0.8% sodium citrate for 10 min. The embryos were then placed on a clean microscope slide in a minimal volume of fluid and fixed with a few drops of methanol-acetic acid (3 : 1 v:v) (Tarkowski 1966). They were stained with Giemsa (Gurr’s) for 5 min and the numbers of chromosomes in clear, well-spread plates were determined blindly using coded slides scored at ×1000 magnification. Anomalies were verified by independent observers.

iv. Ovarian follicle counts

A further group of about 60 animals was set aside for studying the rate of utilization of primordial follicles. The ovarian cycles were monitored daily until they showed a pro-oestrous type of vaginal smear in which nucleated epithelial cells were predominant. The mice were anaesthetized at noon and the ovaries removed, fixed overnight in aqueous Bouin’s fluid, processed as serial paraffin histological sections of 7 μm thickness, and stained with haematoxylin and eosin. Every tenth section was examined after coding slides to avoid observer bias, and the numbers of primordial follicles were counted. Only those follicles in which at least 50% of the oocyte nucleus was visible were recorded so that overcounting was avoided. The numbers of primordial follicles per animal were therefore obtained by simple multiplication by ten.

Results

i. Ovarian cyclicity

The lengths of successive oestrous cycles were constructed from raw data obtained from vaginal smearing. Although the modal length of the cycles was 4 or 5 days, strings of cycles of this length were infrequent and cycles of irregular length were common even in young adult animals. The frequency of 4 or 5 day cycles in intact mice was stable until about 290 days of age when they declined significantly ($P < 0.001$). The early cycle history in uni-ovx animals was similar but the frequency of cycles started declining at 230 days. Few animals were acyclical before 310 days but the proportions rose rapidly after this time, especially among uni-ovx mice. At 330 days approximately half of the latter were acyclical but a comparable proportion of intact animals was not attained until 40 days later. Between 90 and 390 days of age intact animals had an average of 20.4 4–5 day cycles whereas the corresponding value in uni-ovx mice was 15.3, a reduction of 25%. Further, more detailed information on ovarian cyclicity in these mice will be given in a later publication.

ii. Cytogenetic analysis of embryos

The mean number of embryos obtained per animal was higher in intact than uni-ovx animals at all ages, though the difference was most marked among the oldest groups (Table 1). At 308–350 days of age, uni-ovx mice were post-reproductive although sham-operated animals were still fecund, if feebly so. The reduced number of embryos was not simply due to fertilization and early developmental errors since there was a concomitant reduction in the number of corpora lutea. When the number of embryos was expressed as a percentage of the corpora lutea, it was clear that there were additional losses due to ovulation failure or early post-ovulatory development which increased progressively with age. The age effect was potentiated by uni-ovx and at 245–280 days, the value was 39.2% compared with 16.2% in age-matched controls and which represents nearly a six-fold increase over the youngest subgroup.

Only those cells in which the chromosomes could be clearly distinguished were scored. The number of cells scored per embryo ranged from 1 to 5, but in most cases (about 80%), only one cell was analyzable per embryo. Between 24.9% and 37.1% of embryos could be analysed satisfactorily, the percentage being comparable among the various subgroups (Table 2). The majority of embryos at all ages and treatment groups were diploid (2n = 40). Trisomic embryos were not found in either subgroup at ages 63–91 days whereas monosomic embryos were present in both subgroups (11.9% and 6.1% in uni-ovx and sham-operated animals respectively, Table 2). In the intermediate age group (154–182 days), the proportion of monosomic embryos was virtually unchanged but three trisomic embryos (4.0%) were found in the uni-ovx subgroup. Thus, the overall incidence of embryos with either 39 or 41 chromosomes had increased to 17.3% in the latter whereas it was only 4.6% in sham-operated animals.

The highest incidence of aneuploidy was found in uni-ovx animals at 245–280 days (22.0%). The value in the corresponding control subgroup had increased to 12.1% and this reached 19.6% in the oldest mice (308–350 days). Trisomic embryos were recovered from both uni-ovx and intact animals in the two oldest groups. They were always less plentiful than moni-
Some though not open to the criticism of the latter that a chromosome could have been lost during preparation.

Although there were insufficient data to deduce a mathematical relationship between maternal age and the incidence of aneuploidy, when linear regression analysis was used as a first approximation the regression for both the treatment and control subgroups were significantly greater than zero (P = 0.01). The proportion of aneuploid embryos was significantly greater in uni-ovx mice at 154–182 days of age than in controls (P < 0.02 by chi-squared). The incidence of aneuploidy in the younger and older groups appeared also to be higher in the uni-ovx mice but this was not statistically significant. The raw data suggested that the effects of age and treatment applied to trisomy and monosomy alike, although the relatively lower frequency of the former type combined with the difficulties of collecting sufficient material resulted in statistics failing to reach conventional levels of significance (P = 0.05) when trisomy was analysed independently. However, when the incidences of trisomy in the two middle-aged groups were combined, it was found that the long-term effects of unilateral ovariectomy were marginally significant (P = 0.068). The incidence overall of polyploidy was much less than that of trisomy and monosomy combined (1.4% cf. 12.7%), and polyploid embryos were distributed approximately randomly with respect to age and treatment.

The interval between the day of pairing females with the stud males and of finding a coital plug was remarkably similar in the various age-groups and treatment subgroups for those females producing euploid (2n = 40) embryos. The mean values were between 2 and 3 days with the sole exception of uni-ovx animals 154–182 days old which took 3.9 days, which might be spurious high. Although there was a tendency towards extended intervals between pairing and mating in animals conceiving anomalous embryos, the results were not statistically significant, perhaps because of the limited number of observations and large variance.

### iii. Ovarian follicular stores

The data obtained from counting the numbers of primordial follicles in uni-ovx mice and in both ovaries of sham-operated controls were analysed by linear regression after logarithmic transformation, since there was abundant evidence of an exponential decline. Quantitative histological studies showed that the rate of primordial follicle utilization was not increased by unilateral ovariectomy, but the rate of their depletion was comparable to that of intact controls. The ovaries of the oldest group (48 weeks) were approaching exhaustion of their follicular population, commonly having fewer than 10 primordial follicles remaining. Further information will be given in a later publication.

### Discussion

The principal finding among our studies was the earlier increased incidence of aneuploid embryos in ageing mice after unilateral ovariectomy. This is, as far as we are aware, the first demonstration that the occurrence of age-related aneuploidy can be experimentally modified by manipulating the normal pattern of physiological ageing of the reproductive system. The findings cast doubt on the postulated significance of factors which are dependent on chronological age per se. They raise the obvious question of whether fewer aneuploid embryos would be found at a given age if the numbers of ovarian follicles could be reinforced.

A significant potentiating effect of uni-ovx on aneuploidy was found only in animals aged 154–182 days, which implies that the effects of treatment are delayed and that the peak incidence of aneuploidy is accelerated in time but not increased in magnitude compared with intact controls. The composition of the population of aneuploid embryos was contributed unequally by monosomies and trisomies in which the former predominated. Only one trisomic embryo was found in sham-operated animals up to 280 days of age whereas five were recorded in a comparable group of uni-ovx mice.

Removal of one ovary from young adults of polytocous species results in compensatory ovulation of the contralateral ovary. This is reflected in the numbers of embryos and corpora lutea present (Table 1), but the compensatory response has been shown to wane later in life, as does the gametogenic activity of intact animals, primarily as a result of a reduced number of ovarian follicles available for recruitment. Fewer corpora lutea were found to be represented by uterine embryos later in life, especially in uni-ovx animals (Table 1). This might be explained by delayed transport of embryos via the oviduct, or embryos with highly lethal conditions. It seems more probable, however, that the rupture of some preovulatory follicles was impaired and that corpora lutea accessoria had formed (Gosden 1975).

The long-term consequences of uni-ovx also include fewer subsequent oestrous cycles and an earlier onset of acyclicity, though the pattern of change to irregular cycles and thence to acyclicity is similar to that seen in intact animals. These results confirm the earlier onset of the “climacteric” in uni-ovx mice, shown first in F1(O26×DBA) mice by Thung (1961). The shortening of the reproductive lifespan cannot, however, be attributed to an increase in the rate of utilization of the store of

### Table 2. Chromosome anomalies of 3.5-day embryos in CBA/Ca mice: variation according to maternal age and unilateral ovariectomy

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Maternal age (days)</th>
<th>Total embryos analysed</th>
<th>Percent analysed</th>
<th>Chromosome analysis</th>
<th>Percent aneuploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4n 1 3 n</td>
<td></td>
</tr>
<tr>
<td>oyx</td>
<td>63–91</td>
<td>67</td>
<td>29.0</td>
<td>– – 39</td>
<td>11.9</td>
</tr>
<tr>
<td>Sham</td>
<td>63–91</td>
<td>66</td>
<td>27.2</td>
<td>– – 39</td>
<td>6.1</td>
</tr>
<tr>
<td>oyx</td>
<td>154–182</td>
<td>75</td>
<td>29.2</td>
<td>– – 38</td>
<td>17.3</td>
</tr>
<tr>
<td>Sham</td>
<td>154–182</td>
<td>65</td>
<td>37.1</td>
<td>– – 38</td>
<td>4.6</td>
</tr>
<tr>
<td>oyx</td>
<td>245–280</td>
<td>41</td>
<td>28.1</td>
<td>2 3 41</td>
<td>22.0</td>
</tr>
<tr>
<td>Sham</td>
<td>245–280</td>
<td>58</td>
<td>24.9</td>
<td>2 3 41</td>
<td>12.1</td>
</tr>
<tr>
<td>Sham</td>
<td>308–350</td>
<td>46</td>
<td>33.8</td>
<td>– – 37</td>
<td>19.6</td>
</tr>
</tbody>
</table>

* Represents embryos in which two good analysable cells were found to have 39 chromosomes.
primordial follicles since this was unchanged in our CBA mice as in mice of the outbred Schofield albino strain (Baker et al. 1980). The results suggested that the size of the follicle population limits the length of the functional ovarian lifespan in CBA mice and it seems probable, though unproven, that acyclicity results when a critical threshold number of large secretory follicles, required for maintaining the positive feedback mechanism for pituitary gonadotropin release, is obtained.

The increasing incidence of aneuploidy among uni-ovx mice cannot therefore be explained by an earlier utilization of the last remaining oocytes which, according to the production line hypothesis, are more likely to be defective (Henderson and Edwards 1968). Neither can the results be explained by any factor which is determined strictly by the chronological age of the female. They suggest rather that abnormal segregation of meiotic chromosomes is an epiphenomenon of the process of physiological ageing of the reproductive system which is manifested in the changes of the oestrous cycle patterns. This conclusion is consistent with the observation that embryopathies are more common in rats with elongated cycles (Fugo and Butter 1971), and after delayed ovulation (Butcher 1975; Mikamo and Hamaguchi 1975). It has been proposed that the duration of oestradiol stimulation is a factor in the aetiology of these anomalies (Butcher and Pope 1979).

The present results cannot, however, provide a specific hypothesis of what aspect of ovarian ageing is at fault. It is of interest, nevertheless, that the increase in aneuploidy for both uni-ovx and intact animals occurred early relative to the onset of irregularities in the oestrous cycles. Cytogenetic analysis was not carried out, however, throughout the reproductive lifespan and measurements of cycles did show a wide variation between animals despite homoygosity. The result raises the possibility that some individual females are at risk for an early cessation of regular cyclicity and could contribute to this early rise in aneuploidy.

The implications of our experimental results for human aneuploidy, and Down syndrome in particular, are worthy of consideration. First, they would imply that unilateral ovariectomy may be an additional risk factor for Down syndrome. Also, it is tempting to speculate that the probability of conceiving a Down foetus is determined primarily by the size of the remaining oocyte store and hence, the time of the approaching menopause. The “relaxed selection” hypothesis which suggests that more Down babies may be born late in life because of reduced intra-uterine selection (Erickson 1978; Aymé and Lippman-Hand 1982) would appear to receive little support at this time (Carothers 1983; Hook 1983; Warburton et al. 1983).

We would favour the view that there is a continuum of age-related aneuploidy in women, governed not by chronological, but by biological age. The biological age-related cases would then be superimposed on non age-related cases, for example those paternally derived or those due to translocation. Some young mothers of children with primary trisomy 21 might thus be found to have evidence of earlier biological ageing or to be “at risk” for irregular oestrous cyclicity. Read (1982) has already proposed that the taking of the contraceptive pill among young women could be the causal explanation for recent data trends which indicate a shift in the incidence of Down syndrome to younger age groups. He believes that the critical factor in nondisjunction both in older women and young pill-users is hormonal imbalance prior to the time of fertilization. A group of women displaying early biological ageing, and conforming well to our model, are those rare individuals with Turner syndrome (X0), who manage to achieve pregnancy during their brief reproductive span. Menopause occurs generally in such women around the late 20's (E. Magnen, personal communication), but for their young chronological age, they show a remarkably high level of foetal wastage and greatly increased risk for producing Down syndrome offspring (Reyes et al. 1976; King et al. 1978).

A logical extension of our hypothesis is that any factor, idioopathic or environmental, which depletes the oocyte population, could advance the maternal age effect for aneuploidy. It is of interest that Kline et al. (1983) have recently proposed premature oocyte ageing as the explanation for an increased incidence of trisomic abortion observed in older women who smoke. Smoking in women is known to advance the age of menopause (Jick et al. 1977; Kaufman et al. 1980).

In the past studies have been carried out by a number of authors to investigate the reproductive histories of mothers of Down syndrome babies to investigate, for example, whether they show evidence of ovarian dysfunction (Geyer 1939) or have an earlier menopause, more abortions, stillbirths or other offspring with congenital abnormalities, more pregnancy-free intervals prior to the Down syndrome birth, or more menstrual irregularities (e.g. Østre 1953; Smith and Record 1955; Coppen and Cowie 1960; Sigler et al. 1967). Equivocal answers have not been obtained, however, suggesting the need for further, and perhaps more rigorous, enquiry.

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Isochromosome 6p, a unique chromosomal abnormality in retinoblastoma: Verification by standard staining techniques, new densitometric methods, and somatic cell hybridization

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Summary. Study of chromosome rearrangements in retinoblastoma tumors revealed that all tumors contained either an unusual isochromosome and/or extra copies of chromosome 1q. Extra copies of chromosome 1q occur in many malignancies. The pattern of G-bands suggested that the isochromosome was derived from either the short arm of chromosome 6, i(6p), or the long arm of chromosome 17, i(17q). Standard staining techniques using G-, C-, Q-, and R-banding; high resolution G-banding; and density profile analysis were consistent with the characteristic isochromosome of retinoblastoma being i(6p), rather than i(17q). This conclusion was substantiated by the analysis of segregants derived from retinoblastoma X mouse hybrid cells which had been grown in bromodeoxyuridine to select for loss of chromosome 17. The unique isochromosome was not lost under these conditions confirming that it is an i(6p) rather than an i(17q). The i(6p) abnormality has not been observed frequently in other tumors, but occurs in 60% of retinoblastoma tumors. Thus, although the mutation predisposing to retinoblastoma is known to map at 13q14, somatic amplification of genes on 1q and 6p may play a role in the pathogenesis of this tumor.

Introduction

The germlinal mutation predisposing to the dominantly inherited, human tumor, retinoblastoma (RB), has been shown by family segregation studies to be linked to the enzyme esterase D at chromosome 13q14 (Sparkes et al. 1983). The occurrence of RB in patients with congenital karyotypic deletion of 13q14 (Yunis and Ramsay 1978) suggests that it is deletion of gene function in that region that predisposes to RB tumors. Induction of an RB tumor requires at least one somatic event in addition to the germ-line mutation (Knudson 1971). Analysis of chromosome abnormalities in RB tumors may provide clues to the location of the somatic events.

Most RB patients have a normal constitutional karyotype but all RB tumors studied have contained one or more chromosomal rearrangements. Deletion of chromosome 13q14 has been reported in five of six RB tumors (Balaban et al. 1982) and is mentioned in three of five RB tumors without detailed data (Yunis 1983). Benedict et al. (1983) reported one tumor with deletion of the normal chromosome 13 and retention of the chromosome carrying the germ-line mutation. Other authors, however, have found a low frequency of deletion of 13q; one of ten (Gardner et al. 1982) and none of nine RB tumors (Kusnetsova et al. 1982). We have noted the consistent presence in RB of either extra copies of chromosome 1q, an abnormality found in many other malignancies (Kovacs 1978), or a distinctive isochromosome (Gardner et al. 1982; Gallie and Phillips 1982). In early studies the isochromosome of RB was identified as isochromosome (17q) i(17q) (Balaban-Malenbaum et al. 1981; Gardner et al. 1982), which has been associated with disease progression in leukemia (Borgstrom et al. 1982). A detailed analysis of this isochromosome indicates that it is probably an isochromosome (6p) i(6p), a rearrangement rarely reported (Gallie and Phillips 1982; this conclusion is also supported by the recent studies of Kusnetsova et al. 1982). To determine whether the unique RB isochromosome was derived from 6p or 17q, we analyzed an RB tumor which appeared to have both the unique RB isochromosome and a true i(17q). The conclusion from these studies is that the RB isochromosome is i(6p) and that this abnormality is present in 60% of RB tumors.

Materials and methods

Cytogenetic studies

Cytogenetic studies were performed on 25 RB tumors derived from 23 individuals. None of the patients had a constitutional chromosome abnormality. Fourteen children had bilateral tumors and nine were unilaterally affected. Separate tumors were obtained from each eye of two of the bilaterally affected patients.

Following enucleation, fragments of tumor were placed in tubes containing culture medium (Dulbecco minimum essential medium and 15% fetal calf serum). Tumor fragments were easily dispersed by pipetting. Small samples of tumor cell suspensions (approximately 10^6 cells/ml) were incubated at 37°C in culture medium. Direct harvesting was successfully achieved following the techniques of Atkin and Baker (1979). Our protocol was modified slightly, with incubation of tumor cells for 24 h before mitotic arrest and addition of ethidium bromide (10 μg per ml) with colcemid (Ikeuchi and Sasaki).
Cytogenetic analysis of mouse oocytes after experimental induction of follicular overripening*

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Summary. Graafian follicle overripening was induced in (1) adult mice by inhibiting the ovulatory discharge of gonadotrophins with antibodies to LH-RH and (2) immature mice by injection of PMSG to promote follicular maturation before the neuroendocrine system was competent to produce an ovulatory stimulus. The numbers of follicles capable of meiotic maturation after exogenous LH were sharply reduced during the period of overripening and there was a corresponding increase in the proportion of cystically enlarged follicles, many of which were undergoing atresia. Freshly ovulated ova were collected after delaying ovulation for 2 days and prepared for cytogenetic study of metaphase chromosomes. The incidence of non-disjunction and other errors was indistinguishable from that of ova collected after spontaneous ovulation during 4- or 5-day cycles.

Introduction

Responsibility for early developmental errors has been attributed partly to delayed ovulation ('overripening') of Graafian follicles. Evidence in support of this postulated mechanism has been obtained from studies of *Xenopus laevis* (Witschi & Laguens, 1963), rats (Butcher, 1975; Mikamo & Hamaguchi, 1975) and rabbits (Bomsel-Helmreich, 1972) but there have been no comparable studies of the mouse, despite the fact that this is a preferred species for mammalian cytogenetics.

Two new experimental models have been developed for studying the effects of follicular overripening in mice: (1) inhibition of the ovulatory surge of gonadotrophins by antibodies to luteinizing hormone-releasing hormone (LH-RH), and (2) Graafian follicle development was promoted by PMSG in immature mice that were incapable of producing an endogenous stimulus for ovulation. In both methods ovulation could be induced with exogenous luteinizing hormone (LH) after a pre-determined period of overripening. Oocytes obtained in this way were analysed for errors of chromosome segregation during the first meiotic division.

Materials and Methods

Female CBA/Ca mice were obtained from the inbred colony of the Department of Physiology, University of Edinburgh, where they were maintained on a lighting schedule of 14 h light (lights on at 05:00 h) per 24 h and at 20–22°C. A pelleted diet and water were always available.

* Reprint requests to Dr R. G. Gosden.
Induction of follicular overripening

Adult mice. Virgin animals aged 3–6 months were removed from stock cages and housed singly so that a daily history could be compiled of vaginal smears taken by lavage. Smears were obtained at 10:00–12:00 h and were examined unstained by phase-contrast microscopy. Animals were used when they had exhibited at least two consecutive 4- or 5-day oestrous cycles. They were injected with 0.2 ml LH-RH antiserum obtained from a ewe (No. 27) that had been immunized with synthetic LH-RH conjugated to BSA by carbodiimide (Clarke, Fraser & McNeilly, 1978). The antiserum was directed towards the C-terminal end of the LH-RH molecule and showed no significant cross-reactivity (< 0·01%) with thyrotrophin-releasing hormone, somatostatin, oxytocin or LH-RH<sub>1–10</sub>OH, but didn't show cross-reaction with LH-RH<sub>2–10</sub> (3%) and LH-RH<sub>3–10</sub>. Each mouse received an injection i.p. at 12:00 h on the day before ovulation was expected, i.e., on the day of pro-oestrus in 4-day and of oestrus I in 5-day cycles. Control animals were injected with 0·2 ml saline (9 g NaCl/l). Those treated with antiserum were induced to ovulate by injection of 2·5 or 7·5 μg ovine LH (oLH–22/23, kindly donated by Dr S. Raiti through the NIAMDD, Bethesda, MD, U.S.A.) at 06:00 or 18:00 h on the first or second day after pro-oestrus. The animals were killed by asphyxia in 100% nitrogen at 10:00–12:00 h on the morning of ovulation for collection of oviducal ovum or 6 h after injection of LH for ovarian histology. Blood was collected from the heart immediately post mortem and prepared as serum for assaying the titre of LH-RH antibodies by the method of Clarke et al. (1978).

Immature mice. Animals aged 20 days and weighing 6·95–8·05 g received an injection i.p. at 18:00 h of 5 i.u. PMSG (100 i.u./ml; Organon, Oss, The Netherlands, courtesy of Dr B. M. Hobson). Pilot experiments showed that animals outside this weight range either failed to respond to gonadotrophin or ovulated spontaneously after treatment. Ovulation was induced in immature mice in a way similar to that used for the adult animals by injection i.p. of LH at 48, 60, 72, 84 or 96 h after PMSG. Animals were killed on the morning of ovulation or 6 h after injecting LH.

Recovery and preparation of oocytes

Freshly ovulated ova were liberated from the swollen oviducts into Medium 199 (Flow Laboratories, Irvine, U.K.) containing 1% BSA (B.D.H., Poole, U.K.) and 100 U ovine hyaluronidase/ml (Sigma, London, U.K.). After dispersal of the cumulus cells, the oocytes were counted and transferred to 0·85% sodium citrate solution for 15 min and then to clean glass slides for fixation by the method of Tarkowski (1966). Air-dried slides were stained for centromeric heterochromatin (Chandley & Fletcher, 1973) and mounted. The numbers of metaphase chromosomes and prematurely disjoined chromatids of the oocytes were counted using oil-immersion optics (× 1000). Metaphase preparations with substantially overlapping or widely dispersed chromosomes were ignored. A similar percentage of successful preparations was made of each experimental group and overall this was 41%.

Histology of ovaries

Ovaries were removed at autopsy, fixed in aqueous Bouin's fluid and prepared as serial paraffin-wax sections of 7 μm thickness stained with haematoxylin and eosin. Slides were coded and the total numbers of antral follicles were counted for each animal. Large Graafian follicles were classified according to their size and morphology: those of normal preovulatory appearance were V1b and 'cystic' types with an enlarged antrum were V1c (Gosden, Laing, Felicio, Nelson & Finch, 1983). The numbers of pycnotic granulosa cells were counted in one approximately equatorial section passing through the nucleolus to provide an indication of atresia. Finally, the sizes of some of the follicles and their oocytes were measured with an ocular micrometer and their morphological appearance was recorded.
**Results**

*Experimental production of follicular overripening*

**Adult mice.** A single injection of anti-LH-RH serum given shortly before the expected time of the preovulatory discharge of gonadotrophins inhibited ovulation in all 17 mice (Table 1) whereas 82 of 89 individuals amongst several cohorts of control animals had ovulated on the projected day of oestrus in 4- and 5-day cycles. By the 2nd day after injection, 1 animal had ovulated (2·9%) and the incidence of ovulation continued to rise thereafter until, on Day 14, all mice had ova in the oviducts. During the period of ovulation inhibition, the oestrous cycles were suppressed and replaced by persistent vaginal cornification although some leucocytes were usually present. The antibody titres were low even on the first day after injection (median 1:100) but were undetectable by Day 7 (<1:50).

**Table 1. Short-term inhibition of ovulation in 4- and 5-day cyclic mice after injection of 0·2 ml ovine antiserum to LH-RH at 12:00 h on the day of pro-oestrus**

<table>
<thead>
<tr>
<th>Interval between treatment and autopsy (days)</th>
<th>No. of animals with oviducal ova/total no. animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4-day cycle</td>
</tr>
<tr>
<td>1</td>
<td>0/8</td>
</tr>
<tr>
<td>2</td>
<td>0/9</td>
</tr>
<tr>
<td>3</td>
<td>1/9</td>
</tr>
<tr>
<td>7</td>
<td>2/5</td>
</tr>
<tr>
<td>14</td>
<td>5/5</td>
</tr>
</tbody>
</table>

The numbers and appearance of large antral follicles were studied during the first 3 days after treatment with antiserum following a 4-day cycle. During this period the size of the VIb population remained approximately constant whereas the numbers of cystic VIc type follicles increased substantially over the same period (P < 0·05 by Newman–Keul's studentized range test). The latter types were characterized by an enlarged antrum and a granulosa cell population which was exceptionally polarized towards the region of the oocyte with a mural population only 1 or 2 cell layers thick (Pl. 1, Fig. 1). The proportion of VIb follicles that were atretic increased on the days after treatment (P < 0·05) and that of VIc follicles remained close to 100% atresia.

Some animals were given an injection of LH to identify the follicles that were sensitive to ovulatory stimuli after delay of ovulation. Breakdown of the germinal vesicle (GVB) of the oocyte and mucification of the neighbouring cumulus cells in VIb and VIc follicles 6 h after injection were adopted as criteria for sensitivity. Untreated ovaries never presented follicles with this appearance except at advanced stages of atresia. The numbers of follicles with this appearance declined substantially with increasing time between injections of antiserum and LH and there was a concomitant increase in the proportion of type VIc follicles containing few pycnotic cells (< 10 per section) (Text-fig. 1a). However, not all of the follicles responding would have proceeded to ovulation since fewer ova were collected from the oviducts of a comparable group of animals that were killed after ovulation (Text-fig. 1a). A comparison of these data also shows that a substantial proportion of ovulated ova came from follicles classified as VIc.

The proportion of animals ovulating and the numbers of ova shed per animal by 5-day cyclic animals 2 days after antiserum treatment were less than half of the values of comparable animals with 4-day cycles (P < 0·005). Since very few ova could be obtained from the former group the cytogenetic data presented below are based upon treatment of 4-day cyclic mice only and the results in Text-fig. 1(a) were obtained from this type of animal.
Immature mice. Spontaneous ovulation did not occur during the week after PMSG treatment of 20-day-old mice. Injection of LH on Day 22 produced an ovulatory response in 94% of animals, although when injection was delayed until 18:00 h on Day 24 significantly fewer ova were shed per animal ($P < 0.025$).

The numbers of VIb follicles were very low on the day of PMSG treatment ($< 2/\text{animal}$) but were increased at least 5-fold on the 4 subsequent days. Follicles of type VIc were present from Day 22 and they increased in number on the 2 subsequent days. The proportions of atretic follicles with $\geq 2$ pycnotic cells/section at both of these stages increased from Day 22, and by Day 24 most antral follicles contained $> 10$ pycnotic cells.

The number of large Graafian follicles responding to LH treatment are shown in Text-fig. 1(b). The rate of decline in numbers of responsive follicles in immature mice was greater than that of adult mice and was approximately exponential from the morning of Day 23 with a population half time of 15 h. Fewer ova were shed from the ovaries of immature and adult mice than was suggested by the numbers of follicles showing GVB, and both types of animal contained an increasing proportion of VIc follicles with increasing time after PMSG or pro-oestrus, respectively.

### Cytogenetic analysis of oocytes

The majority of oocytes in adult and immature mice were haploid (Table 2; Pl. 1, Fig. 2). Only 3 oocytes (0.58% of total) contained 21 chromosomes (Pl. 1, Fig. 3), these being found in an animal with a 5-day cycle and in one animal of both sub-groups in which ovulation had been delayed experimentally for 2 days. Only 19 chromosomes were present in 30 oocytes (5.8%). The preponderance of such oocytes amongst control animals was not statistically significant and, furthermore, it was impossible to determine what proportion of them resulted from loss of a chromosome during preparation. Three oocytes (0.58%) from 4-day controls contained an unpaired chromatid in addition to the normal set of 20 chromosomes. No diploid oocytes were found. Although these experiments were not strictly designed to test whether PMSG treatment increases cytogenetic anomalies, the results obtained here provided no support of this hypothesis.
Fig. 1. Ovary of a young adult CBA/Ca mouse 2 days after ovulation was inhibited by injection of anti-LH-RH serum on the day of pro-oestrous. The 3 large ('cystic') Graafian follicles have enlarged antra and exceptionally polarized development of granulosa cells. Haematoxylin & eosin, × 50.

Fig. 2. Metaphase II preparation of a mouse oocyte with a normal haploid complement \((n = 20)\). Giemsa, × 1250.

Fig. 3. Metaphase II preparation of a mouse oocyte with 21 chromosomes \((n + 1)\). Giemsa, × 1250.
Table 2. Cytogenetic analysis of mouse oocytes obtained after experimentally delaying ovulation by 2 days (follicular overripening) compared with those ovulated spontaneously (in adult mice) or at the anticipated time of optimal follicular maturity (in immature mice)

<table>
<thead>
<tr>
<th>Age group</th>
<th>Ovarian cycle length (days)</th>
<th>Treatment group</th>
<th>No. of animals</th>
<th>No. of oocytes analysed</th>
<th>Distribution of oocytes according to the number of chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>4</td>
<td>Control</td>
<td>29</td>
<td>95</td>
<td>19 20 21</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Control</td>
<td>38</td>
<td>134</td>
<td>8 87* -</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Delayed ovulation</td>
<td>58</td>
<td>105</td>
<td>2 102 1</td>
</tr>
<tr>
<td>Immature</td>
<td>-</td>
<td>Control</td>
<td>17</td>
<td>104</td>
<td>9 95 -</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Delayed ovulation</td>
<td>66</td>
<td>76</td>
<td>3 72 1</td>
</tr>
</tbody>
</table>

* Three oocytes in this group had an additional chromatid.

Discussion

Two substantially different methods of delaying ovulation were developed for mice and they produced essentially similar results, indicating that Graafian follicles remain capable of ovulation for only a few days after becoming mature and that the probability of non-disjunction of meiotic chromosomes is not significantly increased during the period of overripening as follicles become progressively atretic. It is not known whether oocytes in follicles at more advanced stages of atresia are more vulnerable to segregational errors, but even so this would be of little biological consequence since they are unlikely to remain ovulable.

For technical reasons it was not possible to demonstrate directly by follicle marking that overripening had occurred but this assumption can be drawn from several indirect quantitative and morphological observations, some of which have been presented here. For example, there was no evidence of discrete waves of atresia of large follicles or of replacement by smaller antral stages during the period of study. Profiles of follicle stages present whilst ovulation was delayed suggested that the distributions were continuous, with progressive development of follicles towards the cystic stage (Vic) and, therefore, termination in atresia. This pattern of development may explain the rapid fall in numbers of follicles which were partly or fully responsive to LH. The period of overripening was measured from the day of pro-oestrus in cyclic mice and a maximum period of 2 days of ovulation blockade was chosen since few follicles will respond to treatment on the 3rd day. The timing of overripening in PMSG-primed mice was inevitably arbitrary and was based upon the assumption that follicles were optimally mature 2 days after injection of PMSG (Fowler & Edwards, 1957); this assumption is consistent with the declining follicular response to later injections.

The method of inducing follicular overripening in immature mice resembles that used in rabbits (Bomsel-Helmreich, 1972) and rats (Peluso, Steger & Hafez, 1977), but the use of antibodies to LH-RH for inhibiting ovulation in mice has not been described previously. The action of the antiserum presumably involves immunoneutralization of LH-RH in hypophysial portal vessels. Direct action on the ovaries is unlikely since LH-RH receptors or endogenous ligands are absent in mice (R. M. Popkin, unpublished observations), although not in rats (Clayton, Harwood & Catt, 1979). The use of an antiserum has an advantage over the conventional method of pentobarbitone-blockade of ovulation since it does not require precise knowledge of the timing of the critical period for the
neurogenic stimulus for LH-RH release, whilst the effects on circulating gonadotrophin levels are similar in these methods: namely, abolition of the preovulatory surges with minor reductions of baseline levels (Hasegawa, Miyamoto, Yazaki & Igarashi, 1981). A further advantage of the 2 models used here is that direct effects of treatment on the meiotic spindle are unlikely, whereas anaesthetic agents may have such effects (Kaufman, 1977).

The consequences of follicular overripening on the quality of oocytes are only of major interest if they develop during the short span of time whilst follicles remain ovulatory. It has been proposed that preovulatory deterioration of the oocyte was responsible for the increased incidence of chromosomal anomalies in rat embryos when ovarian cycles were extended to 6 days either spontaneously during ageing or after pentobarbitone treatment (Butcher, 1975; Mikamo & Hamaguchi, 1975). However, these data do not provide an acceptable statistical basis for showing an effect of follicular overripening on the incidence of trisomy ($P > 0.20$, by Fisher's exact test), which is consistent with the conclusions obtained here from mice. Follicular overripening probably cannot, therefore, explain the striking effect of maternal age on trisomy arising from nondisjunction at anaphase I (Bond & Chandley, 1983), although there may be other effects of overripening on oocytes, perhaps involving cytoplasmic structures and membranes, and they may be partly responsible for loss of fertility (Page, Kirkpatrick-Keller & Butcher, 1983). It remains possible that there is a very low risk of chromosomal non-disjunction as evidenced by the presence of one egg with 21 chromosomes in each of the treatment groups, but there was a similar incidence in animals with normal 5-day cycles. It has been suggested on the basis of the poor ovulatory response and early fall in circulating oestradiol levels during delayed ovulation that preovulatory follicles in 5-day cycles undergo earlier atresia and are at a more advanced stage of development than those in 4-day cycles (van der Schoot, 1980; Laing, 1982), but this hypothesis requires further study.

A few oocytes were found to have an additional chromatin which could not be explained simply as a technical artefact, but it is not clear whether this is a potential basis for trisomy. Whatever is the significance of premature segregation of chromatids, the anomaly does not seem to arise as a consequence of follicular overripening.

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References


Follicle overripening in mice


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Effects of age and steroid treatment on prostaglandin production by the rat uterus in relation to implantation

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Summary. Prostaglandin (PG) production by the uterus of ovariectomized virgin rats, with and without replacement steroid therapy, was compared in animals aged 4–5 and 13–14 months. The synthesizing capacities of the uterus for PGE-2, PGF-2α, 6-keto-PGF-1α and thromboxane (TX) B-2 (expressed as the amounts synthesized per mg protein by the homogenized tissue during a 90-min incubation) were significantly lower in the ageing uteri than in young uteri. The PG and TX synthesizing capacities of the young uteri, but not of the ageing uteri, were stimulated by sequential treatment with oestradiol and progesterone implants. Tissue concentrations in and release from the uteri of PGE-2, but not of PGF-2α, were significantly lower in ageing rats than in younger rats, and occurred irrespective of the hormonal status. Treatment with steroids did not stimulate the tissue concentrations in or release from the uterus of PGE-2 or PGF-2α in young or ageing rats. Since PGs, particularly PGE-2, produced by the rat uterus have been implicated as mediators of the implantation process, reduced production of PGE-2 by the ageing uterus may explain the lower implantation rate and the consequent reduction in fertility of ageing rats.

Introduction

Litter size in laboratory rodents is maximal at the 2nd or 3rd pregnancy and declines progressively thereafter until fertility is totally and irreversibly lost in middle age at 1–2 years of age (Ingram, Mandl & Zuckerman, 1958; Biggers, Finn & McLaren, 1962). A major cause of this infertility has been shown to be loss of uterine ability to support implantation and development of embryos (Talbert, 1977). During the phase when reproductive capacity is being lost, there is a marked reduction in the ability of the ageing uterus to respond to decidualizing stimuli, even when priming levels of oestrogen and progesterone are maintained (Finn, 1966; Maibenco & Krehbiel, 1973; Shapero & Talbert, 1974; Holinka & Finch, 1977). The impaired uterine response might be a significant cause of early implantation failure, although it has been suggested that the effect of age on decidualization induced by embryos is less severe than is indicated by artificial stimuli (Finn & Holinka, 1982). Decidualization is preceded by, and possibly dependent on, increased capillary permeability (Psychoyos, 1973), a process which has also been shown to be reduced in ageing pregnant hamsters (Parkening & Soderwall, 1973).

Several experiments have implicated prostaglandins (PGs) in the control of endometrial vascular changes associated with implantation in the rat. Indomethacin treatment delays the localized increase in capillary permeability in the endometrium (Kennedy, 1977; Phillips & Poyser, 1984).
The concentrations of PGE-2, PGF-2α and 6-oxo-PGF-1α (which reflects PGI-2 synthesis) are increased at the sites of increased capillary permeability compared with other areas of endometrium (Kennedy, 1977; Kennedy & Zamecnik, 1978). However, of these 3 PGs (i.e. PGE-2, PGF-2α and PGI-2), only PGE-2 increases capillary permeability in the uterus of rats sensitized for the implantation process (Kennedy, 1979a, b).

Decidualization induced by artificial stimuli is also inhibited or reduced by indomethacin, while PGE-2, PGF-2α, PGI-2 and 9,11-epoxymethano-PGH-2 (a thromboxane A-2 receptor agonist) each induce decidualization of the suitably primed rat uterus (Tobert, 1976; Sananes, Baulieu & Le Goascogne, 1976, 1981; Kennedy & Lukash, 1982; Miller & Morchoe, 1982a, b). Arachidonic acid, but not oleic, palmitic or ω-6-dihomo-γ-linolenic acids, applied to a suitably primed uterus also induces decidualization, an action prevented by indomethacin (Sananes et al., 1981). In addition, the intraluminal administration of sesame oil to a rat uterus sensitized for decidualization increases the endometrial concentrations of PGE and PGF within 5 min (Kennedy & Lukash, 1982). All these studies suggest that, at the time of implantation in the rat, the initial increase in capillary permeability is due to an increase solely in PGE-2 production within the endometrium, while the subsequent decidualization of the endometrium may be due to the increased local production of one or more PGs of the 2-series (including TXA-2). Defective production of these compounds by the uterus may explain the reduced responsiveness of the ageing uterus to the implantation stimulus. Consequently, PG production by the uterus has been compared in ovariectomized young adult and middle-aged rats under basal conditions, in which there was no hormone replacement, and after treatment with steroids to mimic the endocrine conditions preceding implantation, to investigate whether ageing has any significant effect on PG production by the uterus.

Materials and Methods

Virgin female Sprague–Dawley rats were housed in thermostatically controlled rooms (22 ± 1°C) with a photoperiod of 14 h beginning at 05 :00 h, and with free access to a pelleted diet and water. They were caged in small groups of 3–5 animals and studied at 4–5 months or 13–14 months of age when they weighed 230–310 g and 270–350 g respectively.

Each animal was bilaterally ovariectomized under ether anaesthesia, this being designated Day 1 of treatment. During surgery special care was taken to avoid injury to the uterus or its blood supply. Animals were then allocated randomly to the treatment or control groups according to a 2 × 2 factorial design. On Day 8, a Silastic tubing implant (Dow Corning Ltd, Reading, Berks, i.d. 0·155 cm, o.d. 0·3125 cm, length 3·0 cm) was introduced subcutaneously between the scapulae under brief ether anaesthesia. Implants were sealed at each end with silicone-rubber medical-grade adhesive A (Dow Corning) and were incubated in phosphate-buffered saline (pH 7·1) at 37°C for 12 h before insertion into the animal. Treatment groups were given implants containing crystalline oestradiol-17β (Sigma London Ltd, Poole, U.K.) whereas control animals received empty implants. On Day 11 the oestrogen implants were removed and replaced by implants of identical construction which contained crystalline progesterone (Sigma). The implants in control animals were renewed at the same time with another empty capsule. The steroid implants produced circulating hormone levels within the physiological range for early pregnancy: 50–100 pg oestradiol-17β/ml, 15–60 ng progesterone/ml (R. G. Gosden & H. M. Fraser, unpublished observations). There were no differences in circulating hormone levels between the mice in the two age groups.

Steroid-treated animals received a s.c. injection of oestradiol-17β (0·5 μg/kg) in a vehicle composed of benzyl benzoate and arachis oil (1:9, v:v) at about 12:00 h on Day 14. Control rats received the vehicle alone. On Day 15, 21–23 h after the injections, the rats were killed by a blow on the head and their uterine horns were quickly dissected out. Whole uterine horns were used as it is technically extremely difficult to obtain a pure endometrial preparation from a young ovariectomized animal. One or both uterine horns were set aside for the following experiments.
Experiment 1: production of PGs and TXB-2 by uterine homogenates. Freshly dissected tissue was homogenized in 5 ml Krebs' bicarbonate solution (for composition see Mitchell, Poyser & Wilson, 1977) and 100 μl were set aside for measuring the protein content by the method of Lowry, Rosebrough, Farr & Randall (1951). The remainder of the homogenate was incubated for 90 min at 37°C, and aerated with 5% O₂, 95% CO₂. After incubation, the pH of the homogenate was lowered to 4 and the PGs and TXB-2 were extracted by shaking 3 times with 2 volumes of ethyl acetate. The ethyl acetate fractions were combined and evaporated to dryness under reduced pressure at 45°C. Dried samples were re-dissolved in 10 ml ethyl acetate and stored at −20°C. There were 10 rats per group.

The amounts of PGs and TX formed were measured by RIA. Control experiments have shown that the recovery of PGE-2 and PGF-2α is greater than 90% (Poyser & Scott, 1980). The recoveries of 6-keto-PGF-1α and TXB-2 are 81.5 ± 2.01% and 85.7 ± 1.97% respectively. None of the results obtained were corrected for recovery.

Experiment 2: levels of PGE-2 and PGF-2α in uterine tissue. Each uterine horn or pair of horns was homogenized in 5 ml ethanol and then centrifuged at 1000 g for 5 min. The supernatant fluids were transferred to flasks for evaporation to dryness at 45°C under reduced pressure. Each extract was dissolved in 15 ml water and the PGs were extracted and stored as above. A small portion of the homogenate (100 μl) was set aside for protein estimation by the method of Lowry et al. (1951). Sufficient of each extract was available for measuring only PGE-2 and PGF-2α. There were 7 animals in the group.

Experiment 3: release of PGE-2 and PGF-2α by superfused uterine tissue. Each horn was cut open longitudinally and attached by cotton threads to the base of a 20-ml chamber at one end to an isotonic lever under a tension of 1 g at the other end. The horns were superfused with Krebs' solution, pre-aerated with 5% O₂, 95% CO₂, at a rate of 5 ml/min at 37°C. After an initial period of 60 min superfusion to allow the tissue to equilibrate in the new medium, the superfusate was collected for a 10-min period (1st collection); another 10-min collection of superfusate was made after an interval of 20 min (2nd collection). PGs were extracted and stored as above. Sufficient of each extract was available for measuring only PGE-2 and PGE-2α. There were 4 animals per group.

Radioimmunoassays. The antiseria used in these assays were raised in rabbits in the Department of Pharmacology, University of Edinburgh. PGE-2 was measured using an antibody which shows cross-reactivity with PGE-1 (100%), PGA-2 (13-6%) and PGB-2 (260%) but has low cross-reactivities with other PGs and their metabolites (Lytton & Poyser, 1982). The limit of detection was 25 pg/tube. PGF-2α was measured by an antibody which cross-reacts with PGF-1α (100%), but has low cross-reactivity with other PGs and their metabolites (Poyser & Scott, 1980). The detection limit was 25 pg/tube. Although these antibodies show cross-reactivity, the rat uterus does not synthesize significant quantities of PGE-1, PGA-2, PGB-2 or PGF-1α (Poyser & Scott, 1980). Therefore, it is probable that only PGE-2 and PGF-2α are being measured in the respective radioimmunoassays. 6-Keto-PGF-1α and TXB-2 were measured by antibodies which have low cross-reactivities with other PGs and PG metabolites (Poyser & Scott, 1980; Lytton & Poyser, 1982). The detection limits were 25 pg/tube and 30 pg/tube respectively. At least 2 volumes of each extract were assayed in duplicate.

The intra-assay coefficients of variation, calculated from the variation between duplicate results, for the PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2, radioimmunoassays were 12.6%, 11.1%, 9.7% and 6.1% respectively. The interassay coefficients of variation, calculated from the results obtained by including a known amount of the appropriate compound in each assay, were 9.2%, 8.6%, 8.2% and 8.0% respectively.

Statistics. The results (mean ± s.e.m.), expressed as amounts of PG or TX per unit weight of protein or per uterus, were analysed by two-way analysis of variance and Student's t test (Sneecor & Cochran, 1967).
Results

Experiment 1

The total amount of protein in the uterus was significantly increased \((P < 0.001)\) 1.8-fold by ageing. As the protein content was approximately 10% of the wet weight of the uterus in young and old rats, ageing therefore increased the uterine weight 1.8-fold. Steroid treatment increased significantly \((P < 0.05)\) the protein content of the uterus from young and old rats by a similar amount. There was no interactive effect of ageing and steroid treatment on uterine protein content (Table 1).

The amounts of PGs and TXB-2 produced per unit weight of protein by homogenates of the uterus from young and ageing rats varied in magnitude, as follows: 6-keto-PGF-1α > PGF-2α > PGE-2 > TXB-2 (Table 1). Production of each of these compounds was significantly lower in ageing uteri than in young uteri, and, with the exception of 6-keto-PGF-1α production, was increased by steroid treatment as indicated by the Anovar test (see Table 1). However, analysis of the data individually by Student’s \(t\) test revealed that steroid treatment significantly increased PG and TXB-2 production only in the uteri from young animals \((P < 0.01)\). Steroid treatment had no effect on PG and TXB-2 production by the old uteri. Consequently, significant interactive effects of age and treatment were revealed by Anovar for PGF-2α \((P < 0.025)\) and TXB-2 \((P < 0.01)\) using the non-transformed data, and a significant interactive effect \((P < 0.025)\) was revealed when the data for all the PGs and TXB-2 were nested and transformed to logarithms.

Table 1. Effects of age and steroid treatment on prostaglandin (PG) and thromboxane (TX) production by homogenized rat uteru

<table>
<thead>
<tr>
<th>Age group</th>
<th>Treatment group</th>
<th>PGE-2 (ng/mg protein)</th>
<th>PGF-2α (ng/mg protein)</th>
<th>6-Keto-PGF-1α (ng/mg protein)</th>
<th>TXB-2 (ng/mg protein)</th>
<th>Total uterine protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>Control</td>
<td>4.6 ± 0.4</td>
<td>6.8 ± 1.1</td>
<td>140.0 ± 2.3</td>
<td>1.9 ± 0.3</td>
<td>24.1 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>6.7 ± 0.6</td>
<td>11.1 ± 0.9</td>
<td>20.4 ± 3.0</td>
<td>3.7 ± 0.2</td>
<td>31.7 ± 5.6</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>Control</td>
<td>2.8 ± 0.2</td>
<td>5.3 ± 0.4</td>
<td>10.1 ± 1.5</td>
<td>1.7 ± 0.3</td>
<td>44.3 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>3.6 ± 0.3</td>
<td>5.5 ± 0.7</td>
<td>12.4 ± 2.0</td>
<td>2.0 ± 0.3</td>
<td>51.5 ± 2.5</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>Control</td>
<td>104.6 ± 11.8</td>
<td>144.8 ± 20.9</td>
<td>310.3 ± 51.5</td>
<td>48.6 ± 12.0</td>
<td>48.6 ± 12.0</td>
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<tr>
<td></td>
<td>Treated</td>
<td>208.0 ± 18.6</td>
<td>350.9 ± 36.9</td>
<td>659.0 ± 110.8</td>
<td>114.1 ± 61.1</td>
<td>114.1 ± 61.1</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>Control</td>
<td>119.9 ± 9.9</td>
<td>236.7 ± 35.0</td>
<td>479.2 ± 112.3</td>
<td>78.3 ± 17.5</td>
<td>78.3 ± 17.5</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>185.7 ± 18.5</td>
<td>279.2 ± 44.3</td>
<td>642.6 ± 120.2</td>
<td>99.8 ± 14.6</td>
<td>99.8 ± 14.6</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. for 10 observations.
(a) Significant effects of age and treatment for PGE-2 \((P < 0.01)\), PGF-2α \((P < 0.01)\), TXB-2 \((P < 0.001)\).
Production of 6-keto-PGF-1α was reduced with age \((P < 0.025)\) but unaffected by treatment. Uterine protein was more abundant in aged uteri \((P < 0.001)\) and after steroid treatment \((P < 0.05)\).
(b) Significant effects of treatment on production of PGE-2 \((P < 0.001)\), PGF-2α \((P < 0.01)\), 6-keto-PGF-1α \((P < 0.025)\) and TXB-2 \((P < 0.01)\).

The total amounts of PGs and TXB-2 produced per uterus were unaffected by ageing but, as the older uteri weighed approximately 1.8-fold more than the younger uteri, PG and TXB-2 production per unit weight was less in the ageing than in the younger uteri. There was a significant stimulation of PG and TXB-2 production per uterus following steroid treatment \((P < 0.025)\), and there was a significant interactive effect of age and treatment for PGE-2 \((P < 0.05)\).
Experiment 2

Uterine PGE-2 and PGF-2α levels were an order of magnitude lower than the amounts produced by the incubated homogenates in Exp. 1. The uterine levels of PGE-2 were higher than those of PGF-2α, especially in the young rats. When expressed per mg protein, the levels of PGE-2 were significantly lower in ageing uteri than in young uteri ($P < 0.001$; Table 2). PGF-2α levels showed a similar trend but the values were not significantly different. The total contents of PGE-2 and PGF-2α were not significantly different between young and old uteri, but this was due to the larger weight of the ageing uteri compensating for the lower tissue concentration. Steroid treatment did not significantly affect the levels of PGE-2 or PGF-2α in young or ageing uteri.

Table 2. Effects of age and steroid treatment on (i) levels and (ii) release of prostaglandins (PG) in rat uterus

<table>
<thead>
<tr>
<th>Age group</th>
<th>Treatment group</th>
<th>(i) Tissue concentration</th>
<th>(ii) Release</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PGE-2</td>
<td>PGE-2α</td>
<td>1st collection</td>
<td>2nd collection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ng/mg protein</td>
<td>ng/mg protein</td>
<td>1st collection</td>
<td>2nd collection</td>
</tr>
<tr>
<td>Young</td>
<td>Control</td>
<td>0.459 ± 0.070</td>
<td>0.420 ± 0.051</td>
<td>0.043 ± 0.050</td>
<td>0.046 ± 0.050</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>0.455 ± 0.067</td>
<td>0.296 ± 0.081</td>
<td>0.238 ± 0.047</td>
<td>0.100 ± 0.072</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>Control</td>
<td>0.258 ± 0.043</td>
<td>0.198 ± 0.055</td>
<td>0.215 ± 0.060</td>
<td>0.12 ± 0.015</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>0.173 ± 0.016</td>
<td>0.169 ± 0.032</td>
<td>0.122 ± 0.007</td>
<td>0.18 ± 0.076</td>
</tr>
<tr>
<td>Young</td>
<td>Control</td>
<td>11.32 ± 1.00</td>
<td>7.20 ± 1.670</td>
<td>5.04 ± 1.64</td>
<td>2.50 ± 0.38</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>14.61 ± 1.80</td>
<td>9.55 ± 2.66</td>
<td>3.84 ± 0.45</td>
<td>1.59 ± 0.38</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>Control</td>
<td>11.29 ± 2.11</td>
<td>8.69 ± 2.63</td>
<td>4.00 ± 1.16</td>
<td>4.21 ± 1.55</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>8.48 ± 1.13</td>
<td>8.12 ± 1.62</td>
<td>2.77 ± 0.30</td>
<td>3.70 ± 1.15</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. for 7 (i) and 4 (ii) observations.
Significant reduction in levels of PGE-2 ($P < 0.001$) and release of PGE-2 ($P < 0.05$) in aged uteri (a).

Experiment 3

PGE-2 release from young uteri was approximately 2-fold higher than PGF-2α release. This difference was not present in ageing uteri, due to a significant decrease ($P < 0.05$) in the release of PGE-2 but not of PGF-2α from ageing uteri compared to young uteri, when the results are expressed per mg protein (Table 2). Steroid treatment did not significantly affect PGE-2 or PGF-2α release from young or ageing uteri. However, steroid treatment did tend to suppress PGE-2 release in both groups of animals.

Discussion

The present studies on rats have shown that (i) the tissue concentration of PGE-2, but not of PGF-2α, is significantly lower in ageing uteri than in young uteri, (ii) the basal release of PGE-2, but not of PGF-2α, from ageing uteri is significantly lower than from young uteri, (iii) PG production by uterine homogenates (which is a measure of PG synthesizing capacity, since uterine tissue levels of PGs and metabolism of PGs by the rat uterus in the absence of NAD⁺ are low: Poyer & Scott,
1980; Phillips & Poyser, 1981) is significantly lower in ageing rats than in young rats, and (iv) PG synthesizing ability of the uterus is stimulated by progesterone and oestradiol treatment of young animals but not of ageing animals.

The reduced concentration in and release from ageing uteri of PGE-2 occurred irrespective of the hormonal status of the rats studied. The amounts of PGs synthesized, on a unit weight basis, by the homogenized uteri of young, ovariectomized rats treated with progesterone and oestradiol and by homogenized uteri from young, intact rats on Day 5 of pseudopregnancy or pregnancy are similar (Fenwick, Jones, Naylor & Wilson, 1977; Phillips & Poyser, 1981). The amounts of PGs synthesized per unit weight by homogenates of ageing uteri suitably primed for implantation are therefore less than the amounts normally synthesized per unit weight by uterine homogenates from young, intact rats at the time of implantation. Ageing has had, therefore, several significant effects on the uterus as regards PG synthesis and release.

Ageing rats have reduced fertility, a primary cause of which is a lower ability of the uterus to support implantation (see ‘Introduction’). Since PGE-2 of uterine origin is apparently the main mediator in causing a local increase in capillary permeability at the site of implantation in the rat uterus (Kennedy & Armstrong, 1981), the reduced tissue concentration in and reduced output from the ageing rat uterus of PGE-2 may be one of the factors responsible for the lower implantation rate in older rats.

It was surprising in the present study that, although steroid treatment of young, ovariectomized rats increased uterine PG synthesizing ability, there was no increase in tissue concentration in or release from the uterus of PGE-2 or PGF-2α following such treatment. In ovariectomized guinea-pigs, similar steroid treatment increases both uterine PG synthesizing capacity and uterine PG release, particularly of PGF-2α (Poyser, 1983a, b). The rate-limiting step in the release of PGs from tissues is regarded to be the liberation of arachidonic acid by phospholipase A2 (PLA2) from phospholipids, particularly phosphatidylcholine (see Vogt, 1978), although stimulation of the phosphatidylinositol cycle may also have a role in platelets (see Lapetina, 1983). PLA2 activity in the rat uterus is stimulated 2-5-fold after treatment with progesterone and oestradiol (Dey, Hoversland & Johnson, 1982), and 2- to 4-fold during early pregnancy just before implantation (Cox, Cheng & Dey, 1982). If the activity of PLA2 is the sole factor in controlling arachidonic acid release, in the present study one would have expected a greater release of arachidonic acid which, together with the increase in PG synthesizing ability, should have resulted in a much greater release of PGs from the uteri of young rats after steroid treatment compared to those of ovariectomized control animals. This was not the case.

Measurements of PLA2 activity in the guinea-pig uterus have shown that, although activity increases 1-5-fold between Days 7 and 16 of the cycle, the potential of the enzyme for releasing arachidonic acid is more than adequate (by at least one order of magnitude) to account for the amounts of PGs released (Downing & Poyser, 1983). It was considered, therefore, that activation of PLA2 by an appropriate stimulus is more important than the absolute activity of the enzyme in controlling arachidonic acid release in the uterus. However, once the PLA2 has been activated and arachidonic acid has been released by the application of such a stimulus, the amounts of PGs synthesized and released are dependent on the PG synthesizing capacity of the uterus as determined by the hormonal status (Poyser, 1983b). If PLA2 is not activated, only basal amounts of arachidonic acid would be released, and this would result in only basal amounts of PGs being synthesized and released irrespective of the PG synthesizing capacity of the uterus. In the present study therefore, it appears that, in contrast to the guinea-pig uterus, steroid treatment did not activate PLA2, which suggests that the presence of the blastocyst (or suitable artificial stimulus) is necessary to ‘switch on’ arachidonic acid release and PG synthesis in the rat uterus which has been suitably primed for implantation. Application of an artificial stimulus to a rat uterus which has been so primed increases the uterine tissue concentrations of PGE and PGF within 5 min (Kennedy & Lukash, 1982). The magnitude of these increases would depend on the PG synthesizing ability of the uterus. As decidualization of the uterus is apparently dependent upon a local production of PGs
(see 'Introduction'), the lack of effect of oestradiol and progesterone on the PG synthesizing ability of ageing uteri, coupled with the lower basal PG synthesizing ability of such uteri, may result in a decrease in the amounts of PGs synthesized by ageing uteri in response to a decidualizing stimulus, and hence may account for the reduction in the decidual cell reaction in the uterus of older rats. This possibility merits further study.

Reduced responsiveness of the ageing uterus to steroids, as regards the effect on PG synthesizing ability, may be the result of decreasing concentrations of uterine steroid receptors (Holinka, Nelson & Finch, 1975; Saiduaddin & Zassenhaus, 1979; Gesell & Roth, 1981), although not all studies have documented such an effect of ageing (Blaha & Leavitt, 1978; Belisle, Beaudry & Lehoux, 1982). However, there was no evidence that uterine protein production in response to treatment was reduced in ageing rats compared with young rats, suggesting that the growth-promoting effect of steroids on the uterus is not lost in the older rats. Nevertheless, it is possible that the lower PG synthesizing ability of the uterus in ovariectomized control and ovariectomized steroid-treated ageing rats may be the result of a decreased growth response of those cells concerned with synthesizing PGs at the time of implantation. In addition, since the uterus continues to grow throughout life and accumulates collagen at a disproportionate rate (Schaub, 1964/65), the increasing proportion of extracellular protein may be part of the reason why basal PG production is lower in ageing uteri than in young uteri when the results are expressed per mg protein. However, the interpretation of the reduced responsiveness to steroid treatment is not limited in this way, indicating that there is a genuine decrease in PG production by the uteri of older rats. The PG synthesizing ability of the liver and its stimulation by phenobarbital is also reduced with ageing in rats (Morita & Murota, 1980), suggesting that a decrease in PG production by tissues might be a generalized effect of ageing. Further study is required on this aspect.

Authentic PGs and TXB-2 were kindly supplied by the Upjohn Co., Kalamazoo, U.S.A.

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Effect of Dietary Restriction on Estrous Cyclicity and Follicular Reserves in Aging C57BL/6J Mice

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ABSTRACT

Restricting the food intake of female mice by alternating days of feeding and fasting delayed the age-related loss of estrous cycling potential and retarded the rate of follicular depletion, as determined after reinstatement of ad libitum (AL) feeding. During the period of food restriction (FR; 3.5–10.5 mo), food intake and body weight were about 80% of AL values. Mice were acyclic and predominantly in a state of diestrus during FR, but after reinstatement of an AL diet at 10.5 mo all FR mice resumed cycling regularly. By contrast, 80% of AL controls had become acyclic by this age, and the cycles of the remaining mice were significantly longer than those of the reinstituted FR mice. Follicular reserves of 12.5-mo-old FR mice were twice those of age-matched AL controls. Cycling performance of reinstated FR mice, measured by cycle length and the proportion of mice still cycling, was equivalent to that of AL mice when the latter were 2–5 mo younger. Ovarian age, measured by the size of the follicular reserve, was similarly retarded in FR mice. Based on these data and previous evidence that follicular depletion plays a major role in the cessation of cyclicity in this strain, we hypothesize that the delayed loss of estrous cyclicity in aging FR mice is mediated at least in part by the retarding effect of dietary restriction on the rate of follicular depletion.

INTRODUCTION

Restricting caloric intake of laboratory rodents markedly prolongs life (Young, 1979) and delays the age-related loss of many physiologic functions, including fertility in females (Ball et al., 1947; Berg, 1960; Segall and Timiras, 1976; Merry and Holehan, 1979). Although dietary restriction can delay the loss of estrous cyclicity in Sprague-Dawley rats (Merry and Holehan, 1979), it was not known whether a comparable effect occurs in other strains or species of rodents.

The purpose of this study was twofold: first, to determine whether dietary restriction initiated early in adult life would delay the loss of estrous cyclicity in aging C57BL/6J mice, and second, to determine whether the rate of declining follicular reserves showed a correlative change. Because follicular depletion seems to play an important role in the cessation of cyclicity of this strain (Felicio et al., 1983; Gosden et al., 1983), it seemed probable that a retarding effect of dietary restriction on the loss of cyclicity might be mediated by a corresponding effect on the age-related loss of follicular reserves. Previous studies showed a
retarding effect of dietary restriction on oocyte depletion in prepubertal rats (Lintern-Moore and Everitt, 1978), but whether this effect occurred in other species or persisted in older animals had not been firmly established.

**MATERIALS AND METHODS**

**Animal Husbandry**

Virgin 3-mo-old female C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in a limited-access, 23-m² room restricted to this strain. The total number of females and males in the colony during the experiment averaged 163 ± 20 and 12 ± 5, respectively. The temperature in the animal room averaged between 20° and 23°C, and the colony was maintained on a 12L:12D schedule (lights on at 0700 h). Mice were randomly assigned to food restricted (FR) and ad libitum-fed (AL) groups and were housed singly in transparent polycarbonate cages (19 × 25 cm; 15 cm high) with Beta Hardwood Chips (Northeastern Products, Warrensburg, NY). Animals had free access to tap water and food (Purina Rodent Laboratory Chow #5001), except for FR mice, whose access to food was restricted to every other day. Previous studies have shown that a similar regimen of intermittent fasting prolongs the average lifespan of this strain of mouse (Cheney et al., 1980; Talan and Ingram, 1983) as well as of rats (Goodrick et al., 1982). Because FR mice adapted to the initial every-other-day regimen (50% fasting) by overeating on feeding days relative to AL controls, and consequently approaching the food intake of AL mice (data not shown), more stringent regimens (62–67% fasting) were adopted beginning when mice reached 6 months of age (Table 1). Body weight and food intake were monitored biweekly. The difference in weight of food remaining in the cage hopper at successive weighings was taken as an index of food consumption. Although pieces of food found on the cage floor were collected and weighed, this measure of food intake is probably an overestimate since not all spillage could be accounted for. Body weight was measured on two consecutive days, first after the maximal period of fasting (24 or 48 h, depending on the food restriction regimen; see Table 1) and again 24 h after initiation of refeeding. These times were chosen to obtain the minimum and maximum weights of FR mice. AL mice were weighed at the same times, but the two consecutive measurements were averaged for each animal.

**RESULTS**

**Experimental Design**

Estrous cyclicity was monitored by daily vaginal lavage. Stages of the estrous cycle were determined from Giemsa-stained vaginal smears by one individual without knowledge of treatment group. Valid estrous cycles and cycle lengths were determined by programs written for a microcomputer (Nelson et al., 1982). Onset of acyclicity was defined as the day of the first cycle or acyclic interval >9 days in length, unless such an interval was followed by more than 3 consecutive cycles <9 days long. Although the age of cessation of cyclicity is criterion dependent, we have shown that relative differences in onset of acyclicity between groups are maintained across widely varying criteria (Felicio et al., 1984). Restriction of food intake began at 3.5 mo of age and continued until 10.5 mo, when FR mice were returned to an AL diet. Vaginal lavage continued until mice were 12.5 mo old, when they were ovariec-tomized under anesthesia (2,2,2-trichloroethylacetate; BDH Chemicals, Ville St. Laurent, Quebec) (Nelson et al., 1981). ovaries were fixed in Bouin's solution, embedded in paraffin, serially sectioned at 7 µm and stained in hematoxylin and eosin (Goslen et al., 1983). The total number of primordial follicles was estimated based on counts of every tenth section. When estimated values of follicular reserves were <150, additional sections (every second) were counted to obtain a more accurate estimate.

**Statistical Analysis**

All interval data are expressed as the mean ± standard error. Significance of differences among groups

**TABLE 1. Feeding schedules of food-restricted mice.**

<table>
<thead>
<tr>
<th>Interval</th>
<th>Sun</th>
<th>Mon</th>
<th>Tue</th>
<th>Wed</th>
<th>Thu</th>
<th>Fri</th>
<th>Sat</th>
<th>Time fasted (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/12–10/6/82</td>
<td>RM²</td>
<td>RT²</td>
<td>RM</td>
<td>RT</td>
<td>RM</td>
<td>RT</td>
<td>RM</td>
<td>50</td>
</tr>
<tr>
<td>10/7–11/7/82</td>
<td>RM</td>
<td>RT</td>
<td>RM</td>
<td>RT</td>
<td>RM</td>
<td>RT</td>
<td>RM</td>
<td>67</td>
</tr>
<tr>
<td>11/8–2/16/83</td>
<td>RM</td>
<td>—</td>
<td>RT</td>
<td>RM</td>
<td>—</td>
<td>RT</td>
<td>RM</td>
<td>62</td>
</tr>
</tbody>
</table>

¹ Food removed in the morning (RM) of this day.
² Food returned in the morning (RT) of this day.
³ Food returned in the morning and removed 8 h later (RT am or RM pm).
⁴ Food not returned (—).
was determined by t test or analysis of variance (ANOVA) followed, when appropriate, by Duncan's Multiple Range Test using the GLM procedure of the SAS statistical package (Helwig and Council, 1979).

The percentage of acyclic mice and the frequency distribution of cycle lengths were analyzed by the Chi-square and G tests for independence; Mann-Whitney U tests were employed to evaluate differences in follicular reserves (Sokal and Rohlf, 1969). Differences were considered significant at P<0.05.

RESULTS

Food Consumption and Body Weight

Average food intake of FR mice was 79 ± 2% of control values (P<0.001, t test). Although food intake on the every-other-day feeding regimen (fasting 50% of the time) was initially at 70% of control values, it increased by the third month on this regimen to 95% of AL intake. On the subsequent, more restricted regimens, food intake of FR mice never exceeded 80% of the control values. Body weight of control mice increased gradually during the course of the experiment from 21 to 25 g (Fig. 1). The weight of FR mice, whether measured after fasting or refeeding, was significantly less than that of controls (80.4 ± 4.1% or 93.5 ± 1.3% of AL weights, respectively; P<0.001, ANOVA). After FR mice were returned to an AL diet, their body weight rapidly increased to a plateau significantly greater (P<0.05, t test) than the 24-h-fed value of the last weighing obtained during FR. However, this plateau weight (24.3 ± 0.1 g) remained significantly less than that of AL controls (25.1 ± 0.1 g) (P<0.01, t test).

Estrous Cyclicity

Food restriction markedly suppressed estrous cyclicity (Figs. 2 and 3). By 5 mo of age, 77% (7/9) of FR mice were acyclic, and cycle lengths of the remainder were significantly longer than those of AL controls (P<0.005, G test). Age of onset of acyclicity in FR mice was directly proportional to their body weight (average of fed and fasted values; r=0.68, P<0.05), indicating a protective effect of body weight against the acyclic effect of FR. Over 70% of the vaginal lavages from acyclic mice were leukocytic (i.e., diestrus or metestrus-2). After the more stringent FR regimen was instituted, all FR mice became acyclic. Although the proportion of AL mice still cycling began to decline by 7 mo of age, this value remained significantly greater than that of FR mice through-out the period of FR (P<0.05, G test). By 11 mo of age 80% of the AL mice were acyclic. Contrast all 11-mo-old FR mice, which had been reinstated to an AL diet at 10.5 mo, were cycling regularly. This level of cyclicity was equivalent to that of AL mice when they were 2–5 mo younger (Fig. 2). The age-related lengthening of cycles was also attenuated in FR mice (Fig. 4). Cycle lengths of 11-mo-old AL-reinstated FR mice were equivalent to those of 6- to 9-mo-old controls. Although mice were ovariectomized before they completed their 12th month of life, 45% (4/9) had ceased cycling at the time of ovariectomy.

Follicular Reserves

The number of primordial follicles in 12.5-mo-old FR mice (2 mo after returning to an AL diet) was twice that of AL controls (P<0.05, Mann-Whitney U test; Fig. 5). Based upon the more rapid rate of follicular depletion estimated for the AL mice (see Fig. 4), it can be inferred that the number of primordial follicles present in the 12.5-mo-old FR mice was equivalent to that present 2 mo earlier in AL mice.

![FIG. 1. Effect of age and food restriction on average body weight of 10 AL-fed and 9 FR female C57BL/6J mice. Because food restriction involved intermittent feeding, mice were weighed on two consecutive days, first after the maximal period of fasting (24 or 48 h, depending on the food restriction regimen; see Table 1) and again 24 h after initiation of refedding (Restricted-Fasted and Restricted-Fed, respectively) to obtain their minimum and maximum weight during the food restriction regimen. "t", "r", and "r" refer to the times of onset of the 3 different dietary regimens (50%, 67%, and 62% fasting, respectively). "a.l." indicates the age of reinstatement of the ad libitum diet to the food-restricted mice.](image-url)
FIG. 2. Effect of age and food restriction on the percentage of the population still cycling (see Materials and Methods for determination of cycling status). Note the marked increase in the percentage cycling in the FR group at 11 mo (2 wk after they were returned to an AL diet).

DISCUSSION

Three major observations were made in this study. First, intermittent feeding (fasting 50–67% of the time) markedly suppressed estrous cyclicity of mice. Second, this dietary regimen delayed the age-related loss of capacity to cycle and the age-related increase in cycle length, as determined after reinstatement of ad libitum feeding. Third, dietary restriction resulted in a corresponding retardation of the age-related loss of primordial follicles.

FIG. 3. Effect of food restriction between 3.5 and 10.5 mo of age on cycle length. Data are based on the total number of cycles during this time interval: 36 and 279 cycles in the FR and AL groups, respectively. Effect of group on the distribution of cycle lengths is significant (P<0.005, G test).
That dietary restriction can suppress estrous cyclicity in mice has been previously reported (Lee et al., 1952). Whether this effect is the consequence of generalized caloric restriction or the reduction of some specific nutrient cannot be ascertained from the available data. Although marked caloric restriction, using a wide range of dietary regimens, suppresses cyclicity and fertility in animal models (Leathem, 1966), even small modifications of the dietary constituents of nearly isocaloric diets can have marked effects on fertility (Pryor and Bronson, 1981) and estrous cyclicity (Nelson and Felicio, 1984).

After reinstatement on an AL diet at 10.5 mo of age, all FR mice resumed cycling, and their cycle lengths were equivalent to those of their AL counterparts when the latter were 2–5 mo younger. Moreover, whereas all FR mice

![Figure 4](image_url)

**FIG. 4.** Cycle lengths in 4- to 10-mo-old AL-fed mice compared to those of FR mice at 11 mo of age, 2 wk after reinstatement of an AL diet. Ages at which the distribution of cycle lengths differ significantly between AL-fed mice and 11-mo-old, formerly FR mice are indicated with asterisks (***, P<0.01; **, P<0.05; *, P<0.1, G test). The dotted lines intercept the first age of AL-fed mice that is not distinguishable from the 11-mo-old formerly FR mice.

![Figure 5](image_url)

**FIG. 5.** Effect of age and food restriction on the primordial follicular reserves. Estimates of the rates of follicular depletion (FR: \( \log y = 3.89 - 0.11x \); AL: \( \log y = 4.07 - 0.15x \), where \( y \) is the number of primordial follicles at \( x \) months of age) are based on the evidence that follicular depletion occurs exponentially (Jones and Krohn, 1961; Faddy et al., 1983). At 12.5 mo of age, FR mice (food restricted between 3.5 and 10.5 mo) had twice the follicles of AL-fed mice (P<0.05, Mann-Whitney U test). The dotted line intercepts the estimated regression of follicular depletion for the AL-fed mice at the age when their follicular reserves would match those of 12.5-mo-old FR mice.
were cycling at this age, 80% of the AL controls had become acyclic. This result extends previous studies showing that FR mice returned to an AL diet remained fertile for a much longer duration than AL mice (Huseby et al., 1945; Ball et al., 1947). The underlying mechanisms involved in the delay of age-related infertility by food restriction remain unknown. However, the FR-mediated delays in oocyte depletion and in the loss of regular cycles may be contributing factors, since the size of the follicular reserve (Thung, 1961; Brook et al., 1984) and the regularity of estrous cyclicity (Fugo and Butcher, 1971; Barkley and Bradford, 1981) can affect fertility.

The retardation by FR of the rate of oocyte depletion may also explain the prolonged cycling potential of FR mice. In C57BL/6J mice, oocyte reserves are nearly exhausted when cyclicity ceases, and anovulatory mice have half the remaining oocytes of age-matched mice that are still ovulating (Gosden et al., 1983). Moreover, young ovaries partially restore cyclicity when grafted into old anovulatory mice (Felicio et al., 1983), and fully restore cyclicity in mice about to become acyclic (Felicio, Nelson, and Finch, unpublished data). These observations implicate ovarian aging—follicular depletion, in particular—in the cessation of cyclicity in this strain, and support the view that the FR-mediated retardation of the rate of follicular depletion contributes to the delayed loss of cycling potential in FR mice. Consistent with this hypothesis is the finding that the functional ovarian age of FR mice, as measured by the number of remaining follicles, was retarded by an amount of time similar to that of the functional cycling age of FR mice.

Although ovarian aging appears to be the primary determinant of the cycling lifespan of the C57BL/6J mouse, a modulatory role of hypothalamic-pituitary dysfunction seems probable. In both rats and mice, the size of the preovulatory LH surge begins to deteriorate well before the cessation of ovulatory cycles (Wise, 1983). That these reduced LH surges reflect a primary impairment of the hypothalamic-pituitary axis is indicated by the reduced ability of exogenous estradiol to elicit an LH surge in middle-aged rats and mice (Gray and Wexler, 1980; Gee et al., 1984). It was beyond the scope of the present study to determine whether dietary restriction delays the age-related decline of this positive feedback sensitivity to estradiol.

Neural effects of dietary restriction have not been extensively studied. Segall et al. (1978) reported that a 30-day period of tryptophan deficiency suppressed serotonin levels in brains of adolescent rats. However, generalized food restriction did not have a comparable effect, nor did it alter catecholamine levels. Food restriction delayed the age-related loss of striatal dopamine receptors in rats (Roth et al., 1984) and retarded age-associated losses of choline acetyltransferase activity in several brain regions of male rats, although other neurochemical age changes were not affected (London et al., 1983). Although the effects of dietary restriction on neural activity appear to be selective, given the pervasive influence of dietary restriction on aging processes (Young, 1979), an effect of dietary restriction on neuroendocrine mechanisms governing estrous cyclicity seems probable and deserves study.

In the present study, postpubertal dietary restriction resulted in a marked retardation of the age-related loss of primordial follicles. Food restriction can also delay the loss of follicles in prepubertal rats (Lintern-Moore and Everitt, 1978), and a similar effect was reported for adult C3H mice (Huseby et al., 1945), but this observation was not quantitative. Although FR can thus retard follicular depletion in adulthood as well as prepubertally, the mechanism(s) mediating this effect are unknown. Depletion of primordial follicles can occur via two routes: oocyte death or transition to a growing stage. Both hypophysectomy (Jones and Krohn, 1961; Faddy et al., 1983) and food restriction (Lintern-Moore and Everitt, 1978) appear to reduce the rate of transition from primordial to growing follicles. Whether there is a corresponding reduction in the spontaneous rate of cell death in hypophysectomized and FR animals has not been examined, although hypophysectomy appears to protect specifically against oocyte death induced experimentally by ionizing radiation (Beaumont, 1969).

Because both FR and hypophysectomy can retard oocyte depletion, it is of interest that FR has been proposed in another context to be the functional equivalent of hypophysectomy (Mulinos and Pomerantz, 1940). Although the effects of chronic FR on pituitary tropic hormone secretion are largely unknown, evidence indicates that FR is not the equivalent of hypophysectomy. In chronically FR male rats, neither LH nor androgen levels were reduced
(Merry and Holehan, 1981). Even during acute FR or starvation, not all circulating levels of pituitary hormones are reduced (Campbell et al., 1977; Rattner et al., 1978). Although hormone concentrations were not measured in this study, the absence of estrous cycles and the persistent leukocytosis of FR mice are consistent with low concentrations of estradiol and suppressed gonadotropins. Previous studies have shown that acute FR in pregnant mice suppresses LH but not FSH concentrations; in fact, FSH may be elevated (Rattner et al., 1978). Thus, it seems probable that at least the circulating levels of LH were reduced in the FR mice of this study. It is therefore possible that altered levels or patterns of pituitary hormones retard the rate of follicular depletion in FR mice; however, there is presently little basis for speculating as to which hormones may be involved.

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Morphine acts centrally to interrupt established parturition in rats

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Opiates powerfully inhibit stimulated oxytocin secretion (Clarke & Wright, 1984; Russell & Spears, 1984), but an effect during parturition has not been reported although naloxone increases the secretion of oxytocin in parturient rats and accelerates parturition once established (Bicknell, Dean, Dyer, Ingram, Leng, Mansfield, Marsh & Yates, 1984; Hartman, Miller, Rosella-Dampman, Emmery & Summy-Long, 1984). We have investigated the effects of morphine in primiparous Sprague-Dawley rats, observed continuously on the day of expected parturition. In experiment 1, immediately after birth of the second pup, the mother was injected subcutaneously with either vehicle (saline, 0·9 % (w/v), n = 8) or morphine sulphate (10 mg/kg, n = 12) or morphine sulphate (10 mg/kg) and naloxone hydrochloride (5 mg/kg, n = 7). The mean interval (± s.e. of mean) from injection to birth of the 9th pup was, respectively, 51·7 ± 5·0 min, 203·0 ± 34·7 min and 60·1 ± 8·4 min (P = 0·0004, Kruskal–Wallis test). In experiment 2, on days 14–16 of pregnancy, rats (under ether anaesthesia) were fitted with a cannula for injection into a lateral cerebral ventricle. Immediately after birth of the second pup these rats received via the cannula either 5 µl saline (n = 7) or 18 µg morphine sulphate in 5 µl saline (n = 7). Mean interval (± s.e. of mean) from injection to birth of the 9th pup was, respectively, 71·6 ± 16·6 min and 211·4 ± 18·7 min (P = 0·003, Wilcoxon test). Subcutaneous injection of 18 µg morphine sulphate did not prolong parturition (68·4 ± 9·6 min, n = 7). The morphine-treated rats eventually delivered viable pups. To study direct effects of morphine on the uterus, uterine horns, removed from five control rats (killed by cervical dislocation) within 24 h of parturition, were immersed in an organ bath containing de Jalon’s solution (37 °C, gassed with 95% O₂, 5% CO₂). Morphine sulphate (4·5 × 10⁻⁶ M) had no effect on spontaneous or oxytocin-stimulated (100 u/l) contractions, recorded via a strain-gauge transducer and chart recorder.

Thus morphine interrupts established parturition, in a naloxone-reversible manner and acts centrally, not on the uterus or its contents. Inhibition of oxytocin secretion may be involved.

Members of the Honours Physiology Classes of 1983 and 1984 contributed to the observations reported.

REFERENCES


Maternal Age: A Major Factor Affecting the Prospects and Outcome of Pregnancy*

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Among physiological systems, that of reproduction in females probably shows greater variation in functional activity during life than any other. In our own species, the reproductive lifespan begins at menarche and ends at menopause, a span averaging 36 years in Western countries. Reproductive potential reaches a peak value after a short period of postpubertal maturation, but steadily declines during the fourth and fifth decades of life and is lost irretrievably when ovarian cycles cease at about age 50. The effects of maternal age on fecundity and fertility are complex and multifactorial, involving a progressive depletion of the ovarian follicular store, production of defective oocytes, and reduced competence of the organs and systems required to sustain gestation successfully.

PRODUCTION OF GRAAFIAN FOLLICLES

Mice are born with an endowment of about 10,000 ovarian follicles which must serve the needs of reproduction throughout life. As a consequence of follicular involution (atresia) and recruitment for ovulation, the store of primordial follicles is progressively reduced in size, leading to sterile ovaries in some inbred strains by midlife, as in women of postmenopausal age. The numbers of follicles that become graafian and therefore potentially “ovulable” depend on the size of the primordial follicle population and on stimulation by the pituitary gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). The dynamics of ovarian follicle turnover have been estimated by constructing mathematical models using data obtained by differential follicle counting in serial histologic sections (Table 1). The results show that follicles are recruited into the growing population (Groups II+) at similar rates at all ages, but because the store from which they are drawn is not replenished, fewer are recruited with advancing age. A larger proportion of follicles is lost by atresia than by ovulation. Most of these deaths occur among primordial (Group I) follicles during prepubertal ages, but in CBA mice a substantial, although lower, death rate is present after puberty and this is responsible for the early loss of ovarian function in this strain. Thus, the incidence of atresia does

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not increase during aging; on the contrary, there is additional evidence that a larger proportion of the dwindling numbers of larger follicles are conserved in order to maintain ovulatory constancy.5,10

The numbers of follicles reaching mature stages (V+) reflect the numbers that entered their growth phase about three weeks earlier.11 That few follicles die at the intermediate stages of growth II–IV is indicated by equivalent numbers entering and leaving these categories at most ages (TABLE 1) and by the morphology of the follicular epithelium, which rarely shows signs of atresia (such as the presence of apoptotic bodies). The number of follicles that ovulate at estrus is a species characteristic and is independent of spontaneous variations in ovarian cycle length. Despite fewer follicles leaving the store, the ovulation rate (that is, the number of ova shed per cycle) rises gradually with age in parous animals.12,13 This phenomenon is paralleled in our own species by the rise in twin births, which reach a peak frequency in multiparas aged 36 years.14 Such data have been interpreted tentatively as evidence of the formation of additional dominant follicles resulting from a subtle increase in pituitary gonadotropin output before menopause. There is, however, no evidence to suggest that the rate of follicle growth between successive stages is altered during adult life, even at postcyclic ages.9,15,16

At most ages, the numbers of follicles that can be recruited for ovulation greatly exceeds the ovulatory quota. This redundancy enables the ovary to respond flexibly to altered gonadotropic stimulation. The normal quota is limited by gonadotropins through the feedback of gonadal secretions on the hypothalamo-pituitary complex. Flexibility is revealed when young ovaries superovulate after removal of the contralateral ovary or superstimulation with exogenous gonadotropins. The superovulation response to treatment with pregnant mares' serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) 48 hours later is shown for CBA mice in FIGURE 1.

TABLE 1. Mean Numbers of Follicles (from Fitted Model) in CBA Mouse Ovaries Leaving and Entering Five Groups* (Primordial to Graafian) at Specified Ages throughout Life**

<table>
<thead>
<tr>
<th>Age Span (calendar months)</th>
<th>Primordial Follicles</th>
<th>Growing Preantral Follicles</th>
<th>Large Preantral and Graafian Follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I→II</td>
<td>II→III</td>
<td>IV→V</td>
</tr>
<tr>
<td>0–1</td>
<td>5657 593</td>
<td>366 366 182 182 155</td>
<td>155 84</td>
</tr>
<tr>
<td>1–2</td>
<td>1342 348</td>
<td>185 185 204 204 209</td>
<td>209 255</td>
</tr>
<tr>
<td>2–3</td>
<td>859 223</td>
<td>224 224 212 212 210</td>
<td>210 207</td>
</tr>
<tr>
<td>3–4</td>
<td>549 143</td>
<td>203 203 214 214 215</td>
<td>215 215</td>
</tr>
<tr>
<td>4–5</td>
<td>352 91</td>
<td>164 164 190 190 194</td>
<td>194 200</td>
</tr>
<tr>
<td>5–6</td>
<td>225 58</td>
<td>125 125 155 155 159</td>
<td>159 169</td>
</tr>
<tr>
<td>6–7</td>
<td>144 37</td>
<td>96 91 119 119 123</td>
<td>123 132</td>
</tr>
<tr>
<td>7–8</td>
<td>92 24</td>
<td>64 64 87 87 91</td>
<td>91 99</td>
</tr>
<tr>
<td>8–9</td>
<td>59 15</td>
<td>45 45 62 62 65</td>
<td>65 71</td>
</tr>
<tr>
<td>9–10</td>
<td>38 10</td>
<td>31 31 44 44 46</td>
<td>46 50</td>
</tr>
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<td>10–11</td>
<td>24 6</td>
<td>21 21 30 30 31</td>
<td>31 35</td>
</tr>
<tr>
<td>11–12</td>
<td>15 4</td>
<td>14 14 21 21 21</td>
<td>21 24</td>
</tr>
<tr>
<td>12–13</td>
<td>10 3</td>
<td>9 9 14 14 14</td>
<td>14 16</td>
</tr>
</tbody>
</table>

*Follicle groups are based on the classification of Mandl and Zuckerman.1
**From Faddy et al.58
The numbers of ovulations rise abruptly from zero at about 20 days of age when ovulable follicles first emerge, and maximum responses are obtained before puberty (that is, in mice <35 days old). The numbers of ovulations subsequently fall to zero by the time mice are 13 months old, and animals showing residual responses produce few ova. In these experiments the same dose of hormones was used at all ages because of the complexity of the effects of age on the hormone distribution volume, metabolic clearance rates, and quantity of target tissue. However, since trial experiments showed that this dose produced maximum numbers of ovulations without hyperstimulation (hemorrhagic follicles or luteinized follicles) at all ages, these data are probably a fair estimate of the variation in numbers of follicles that are available. Evidently, the rate at which follicles begin to grow (Group 1 — II) does not parallel the numbers of ova shed by PMSG/hCG treatment, for while the former is an exponential function of age, the latter is linear (Fig. 1). Nevertheless, these slopes are sufficiently similar to suggest that the rate of outflow of follicles from the pool of primordial stages is the major factor limiting the ovulation response. The numbers of follicles beginning to grow during a given 4–5-day period (median cycle lengths at young ages) slightly exceed the numbers of ovulations that are possible at the same time, but this is not surprising considering that these follicles ripen several weeks later, when ovarian responses are diminished. Finally, the reduced outflow of follicles into the growing population can explain why compensatory ovulation fails to occur after unilateral ovariectomy in middle-aged rodents and why the rate of spontaneous ovulation in CBA mice declines after 300 days of age.

Thus, ovarian function is limited by the size of the follicular store which is laid

![Figure 1. Effects of age on numbers of oocytes shed by both ovaries of CBA mice following treatment with 5 IU PMSG (Organon, Oss, The Netherlands) and 5 IU hCG (Sigma, London) compared with numbers of primordial follicles entering the growing population (Group II+ follicles) per month (from fitted model).](image-url)
down prenatally and by the subsequent rate of follicle utilization and death, each of which vary according to genetic line. The size of the follicular store and of the superovulation response obtained with exogenous hormones varies markedly between individuals of similar age, even among siblings. Such differences can be considered to be differences in biological age of the reproductive system since the maximum number of ovarian cycles is partly limited by the size of the follicle store. Further study is required to clarify whether individual variation in the size and dynamics of the follicular pool late in life arises from postnatal environmental influences or are determined during oogenesis in the fetus.

QUALITY OF OOCYTES

An outstanding feature of maternal aging is the rising frequency of trisomic conceptuses in animals and man, in whom the most notable anomaly is Down's syndrome. Since aneusomic oocytes are more frequent in mice of middle age than in younger animals, the occurrence of aneuploidy in embryos and fetuses can be attributed to a primary effect of age on oocytes rather than to the alternative hypothesis that selection against defective fetuses is relaxed. Indeed, there is evidence that the survival of fetuses is more compromised in the aging uterus, which is perhaps explained by greater competition for limited resources. The cause(s) of age-dependent trisomy in man is conjectural. Whereas it has been postulated that random damage to cellular organelles accumulates during aging, most workers now suspect that internal (physiological) factors are primarily responsible for trisomy and this view is strengthened by recent experimental findings. Two contrasting physiological factors are currently receiving close attention.

According to one hypothesis, a proportion of the oocyte population is doomed to become chromosomally imbalanced during meiosis because of influences during oogenesis. The primary evidence that some oocytes are predisposed to abnormal chromosomal segregation has been drawn from the observation that while chiasma frequency is related inversely to age, a positive relationship exists between age and frequency of univalents. Henderson and Edwards were the first to report these associations, which have been confirmed in several strains and laboratories. Since chiasmata are formed prenatally, a gradient of chiasma frequently later in life implies that oocytes might be ovulated in the order in which they were formed.

So far, it has not been possible to falsify the “production line” hypothesis, although contrary evidence is accumulating. A direct test using tritiated thymidine to label oocytes has not yet proved possible because of oocyte sensitivity to radiolysis and the recombination frequency for linked genes does not decrease with maternal age. In CBA mice, which have a strong association between aneuploidy and maternal age, the median chiasma frequency decreases from 25 to 23 (Fig. 2). This small change is mainly a result of a more positively skewed distribution and the lower limit of the range remains unchanged at 20, at which level each bivalent contains a single chiasma. Not surprisingly, oocytes obtained from young ovaries grafted under the renal capsules of either young or aging hosts did not show a marked change in chiasma frequency in this study which was designed to test whether the age of the maternal environment affects the morphology of oocytes (Fig. 2). CBA mice have a lower age-specific chiasma frequency than do other strains, and the small effect of age on the distribution is insufficient to explain why trisomy is restricted to the aged individuals. The frequency of oocytes containing univalents was similar to that of our earlier findings, and in neither of our studies was maternal age a factor, in contrast to...
FIGURE 2. Chiasma frequency of isolated CBA mouse oocytes which were cultured to metaphase I as described previously. The cells were recovered by puncturing ovarian follicles from the following 4 groups of animals: young (age 2–3 months) and old (8–12 months) intact mice, and young and old mice carrying ovaries transplanted from young donors under the renal capsule one month before oocytes were recovered. This figure also shows variation in percentage of oocytes attaining metaphase in vitro. n = number of cells. (From Gosden and Speed.)
studies cited above. Aged oocytes evidently have reduced developmental potential since fewer attain metaphase I (Fig. 2) and metaphase II under in vitro conditions, and selective elimination of those with abnormal chromosomal complements may account for data showing that the incidence of aneuploidy decreases after a peak in midlife. The high rate of maturation among oocytes from young ovaries in old hosts suggests that the aging environment can be satisfactory, but it is not clear why this rate was higher than that in young—young combinations of graft and host. Further doubt has been cast on the “production line” hypothesis as a result of experiments using Chinese hamsters in which it is possible to identify chromosome groups. A positive relationship was found between maternal age and the frequencies of chiasmata, univalents, and aneuploid oocytes, but different chromosome groups were responsible for the univalents present at metaphase I and for aneuploidy at the ensuing division. These findings extend those obtained in mice, which also indicated that univalents are not necessarily responsible for aneuploidy, but are a result of a more contracted state of the chromosomes in some strains.

### TABLE 2. Effects of Maternal Age and Unilateral Ovariectomy (OVX) on the Incidence of Chromosomally Anomalous 4-Day-Old Embryos and Ovarian Function in Primiparous CBA/Ca mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Maternal Age (months)</th>
<th>No. of Embryos Analyzed</th>
<th>Percent of Aneuploid Embryos</th>
<th>No. of Corpora Lutea/Animal</th>
<th>No. of Primordial Follicles/Animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX</td>
<td>2-3</td>
<td>29</td>
<td>67</td>
<td>11.9</td>
<td>9.0 ± 0.3</td>
</tr>
<tr>
<td>Intact</td>
<td>26</td>
<td>66</td>
<td>66</td>
<td>6.1</td>
<td>10.7 ± 0.2</td>
</tr>
<tr>
<td>OVX</td>
<td>5-6</td>
<td>33</td>
<td>75</td>
<td>17.3</td>
<td>8.9 ± 0.3</td>
</tr>
<tr>
<td>Intact</td>
<td>21</td>
<td>65</td>
<td>65</td>
<td>4.6</td>
<td>10.2 ± 0.4</td>
</tr>
<tr>
<td>OVX</td>
<td>8-9.5</td>
<td>99</td>
<td>41</td>
<td>22.0</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>Intact</td>
<td>65</td>
<td>58</td>
<td>58</td>
<td>12.1</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>Intact</td>
<td>10-12</td>
<td>66</td>
<td>46</td>
<td>19.6</td>
<td>2.9 ± 0.3</td>
</tr>
</tbody>
</table>

*Ovariectomy and sham operations carried out in animals 1.5-2 months of age.
*From Brook et al. and unpublished data.
*Mean ± SEM (3-5 animals per group).

Other authors stress that maternal age-related aneuploidy is an epiphenomenon of aging of the reproductive system. According to this less discrete hypothesis, oocytes are not condemned from an early age to become defective, but may reflect deteriorating conditions within the follicular environment. Longer estrous cycles in rats are associated with reduced fertility and more morphologically abnormal embryos. There are corresponding changes in the plasma hormone profiles of elongated cycles, and these might affect the assembly of the meiotic spindle and/or traction of chromosomes. However, there is only limited evidence that hormones directly affect chromosomal segregation in oocytes, although the steroid hormone environment of oocytes is known to be critical for cytoplasmic maturation.

Further evidence that hormone-related changes during aging affect the quality of oocytes has been obtained in CBA mice by removing one ovary soon after puberty to accelerate the timing of events in reproductive system aging. Blastocysts obtained by mating these animals with young fertile males were analyzed to estimate the incidence of aneuploidy (TABLE 2). While the operation did not lead to an abrupt rise in aneuploidy, the frequency did rise earlier than in intact controls, and similar maximum frequencies were attained at different ages. The ovariectomized animals were also
characterized by an earlier decrease in production of embryos and corpora lutea and earlier cessation of cycles. Cycles ceased in half of the ovariectomized mice at 330 days of age whereas this point was not reached until 40 days later in the control group. This effect of reducing the size of the oocyte population is consistent with other data showing that CBA mice are particularly prone to univalent formation and aneuploidy, and are depleted of oocytes at an early age. Hormonal changes could explain these associations and this conjecture should now be tested more rigorously. It is less likely that aneuploidy in unilaterally ovariectomized mice is due to accelerated movement of follicles along a "production line" because the rate of disappearance of the primordial group is not altered (Table 2). Nevertheless, it would be interesting to test whether the chiasma frequency is lower in these mice compared with that of intact controls, as was predicted by the original "production line" model.

INTRAUTERINE ENVIRONMENT

That pregnancy wastage in older female mice is not entirely due to defective embryos has been shown by transplanting preimplantation embryos from young fertile matings into aging pseudopregnant hosts. Results have shown that the aging uterine environment is inferior to that of young control animals. Conceptuses may be lost at any stage of gestation between fertilization and parturition, but most deaths in inbred strains occur either at implantation or during the following week. According to some studies, the age-specific fertility rate can be modulated by the pregnancy history, fertility being inferior in elderly primigravidas and in previously superpregnant mice compared to that of animals breeding ad libitum.

A number of cellular and morphologic changes occur in the aging uterus, although their functional significance has not been demonstrated. The uterus becomes more fibrous and accumulates scar tissue at placental sites, and the epithelial lining of the uterine lumen presents an abnormal microvillous architecture. Attachment of a blastocyst to the uterine wall following zona lysis involves interdigitation of microvilli from the uterine epithelium and the trophoblast. Contact becomes more intimate as microvilli shorten and cellular adhesiveness rises. In the aged mouse uterus the density of uterine microvilli is substantially reduced and could be a cause of infertility. To test whether the paucity of microvilli in early pregnancy is due to environmental factors, the middle segments of uterine horns of inbred mice were transplanted reciprocally to orthotopic sites in hosts at different ages. The surface features of the epithelium observed in grafted organs by scanning electron microscopy faithfully mirrored the general changes that were occurring in contiguous host tissues as a result of hormonal changes. However, the grafts did not acquire the density and distribution of microvilli characteristic of the age of the host (Fig. 3). Such results are consistent with other studies showing that plasma levels of ovarian steroids are not deficient in aging animals. The uterine epithelium evidently fails to undergo normal differentiation in response to hormonal signals, despite the normal concentrations and physical characteristics of uterine steroid receptors found in some studies. When the fertility of animals carrying uterine grafts was tested, the transport of gametes and embryos was unimpaired and fertilization and implantation took place in many cases. However, it was not possible to determine graft function further since even when optimal combinations of graft and host age were used, implanted embryos failed to thrive in grafted uterine segments. This failure could be attributed to a poor vascular supply.

One of the earliest responses that the uterus makes to an implanting embryo is a
FIGURE 3. Scanning electron micrographs of the surfaces of the uterine epithelium derived from CBA mice aged (a) 6 months (magnification x 5100) and (b) 16 months (magnification x 5400) (glutaraldehyde fixation). Each tissue was part of a uterine segment that had been transplanted to the orthotopic site of a syngeneic host aged 5 months. Day 5 post coitum. (From Jones.21)
local increase in capillary permeability with hyperemia. This leads to the differentiation of the decidua from stromal cells. Both the capillary permeability changes and the decidual reaction are reduced in the aged uterus even when it is exposed to optimal combinations of estrogen and progesterone, and these factors might contribute to early postimplantation embryonic mortality. The signal from the blastocyst that elicits the normal chain of events has not yet been determined, although there is experimental evidence that prostaglandins are responsible for uterine edema, which may in turn be a trigger for decidualization. The reduced ability of the uterus in aging rats to synthesize prostaglandins (especially PGE-2), either under basal conditions after ovariectomy or in response to stimulation by ovarian steroids, might explain the observed senescence.

Fetal survival at later stages of gestation may be limited by sclerosis and stenosis of uterine blood vessels, which have been observed in multiparous rodents and in women. The uterus in young animals is well adapted to carry a full quota of embryos arising from normal ovulations, but this capacity falls faster than the change in ovulation rate during aging in most strains. There is evidence that the rate of blood flow in the pregnant uterus of aged animals is reduced, but it remains to be shown whether this change is a cause or an effect of lower demand. If fetal death is a result of placental ischemia, then the death rate might be expected to be density-dependent, implying that transfer of excess numbers of embryos (if available) may be counterproductive in older women. From the biological standpoint, fetal death can be beneficial, for not only does it dismiss abnormal fetuses, but it also may allow limited maternal resources to be concentrated for the survival and optimum growth of a few privileged offspring.

The uterus of CBA mice at postcyclic ages (>12 months) has a residual ability to sustain gestation given a source of embryos and ovarian steroids. When young ovaries were transplanted under the renal capsules of acutely ovariectomized aging mice, one-third of the hosts became receptive to males and pseudopregnant. A small proportion of embryos transferred from young donors developed successfully in these animals (Table 3). A significantly larger proportion of embryos survived in a set of hosts which were identical, apart from having been bilaterally ovariectomized 7–8 months before receiving the grafts. Thus, the aged uterus is capable of resuming pregnancy function after a prolonged period of postcastration atrophy, and a comparable result has been obtained recently in a young postmenopausal woman. It is not clear at the present time whether the potentiating effect of long-term ovariectomy in mice is due to improvement in estrous cyclicity or suspension of estrogenic stimulation of the endometrium during repeated estrous cycles.

**CONCLUSIONS**

Many factors contribute to the loss of fecundity and fertility in middle age, and only a sketch of some of the major ones is possible here. Despite the fragmentary nature of the present knowledge of aging, some general conclusions of relevance to human biology can be drawn. The maximum reproductive lifespan is limited by the size of the store of ovarian oocytes formed before birth and by the stochastic process responsible for their utilization and loss, leading to less flexible ovulation responses and, ultimately, sterility. However, the reproductive potential of laboratory animals is restricted by the intervention of other factors before this climax is reached. The reduced capacity of the uterus to initiate and maintain embryo implantation and the production of aneuploid oocytes are of prime importance in the infertility of mid- and late age. There is growing evidence that the timing of these age changes is interdepen-
TABLE 3. Effects of Long-Term Ovariectomy on the Ability of Ovarian Grafts from Syngeneic Donors to Provide Physiological Support for Pregnancy in Primiparous CBA Mice of Postreproductive Age

<table>
<thead>
<tr>
<th>Group</th>
<th>Age of Host at Ovariectomy (months)</th>
<th>Age at Time of Grafting Ovaries (months)</th>
<th>No. of Animals Grafted</th>
<th>No. of Animals Mated (%)</th>
<th>No. of Blastocysts Transferred on Day 4 post Coitum</th>
<th>No. of Blastocysts Surviving to Day 14 post Coitum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute ovariectomy</td>
<td>10-11</td>
<td>10-11</td>
<td>21</td>
<td>7(33)</td>
<td>78</td>
<td>8(10)</td>
</tr>
<tr>
<td>Long-term ovariectomy</td>
<td>3</td>
<td>10-11</td>
<td>19</td>
<td>6(32)</td>
<td>67</td>
<td>28(42)*</td>
</tr>
</tbody>
</table>

*p < 0.001.
dent and may arise from a web of physiological interactions, possibly involving hormones, and modulated by the history of reproductive function. Thus, chronological age is not an absolutely reliable guide to the timing or sequence of deterioration during the reproductive lifespan.

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51. JONES, E. C. Unpublished study.
Reproductive potential of inbred mice falls progressively after five months of age or the third litter and is lost irreversibly by 12 months. Elderly primigravidae become infertile earlier, but parity does not otherwise have a major impact on patterns of ageing. Three factors restrict fecundity and fertility in middle age, as follows.

1) Quality of oocytes. Although maturation and fertilization of oocytes are maintained in older animals, there is a marked increase in fetal aneuploidy due to rising chromosomal non-disjunction in oocytes. Earlier evidence implied anomalies are determined by chiasmate relationships during oogenesis. Alternatively, since unilateral ovariectomy accelerates age changes of ovarian cycles and fetal aneuploidy, the latter may arise from physiological (endocrine) changes during adulthood.

2) Intra-uterine environment. That fetal mortality rises with maternal age and is irrespective of (1) above is shown by poor fertility of ageing hosts of embryos from young donors. However, the uterus of animals >12 months old is still capable of supporting full development of a few implants following transplantation of ovaries and embryos.

3) Production of ova. Numbers of ovulations per cycle are stable until near the end of fertile life. But the declining pool of primordial follicles limits the flexibility of ovulation response and the possibility of superovulation.

The potential for reproduction extends from menarche to menopause, a span of about 36 years in Western women (Treloar, 1974). Biological variation in fertility during this phase is difficult to gauge. Demographic studies of "natural" fertility in historical populations (Henry, 1961; Hinde and Woods, 1984) and in contemporary groups, such as the Hutterites in North America (Eaton and Mayer, 1953), indicate that it peaks during the third decade of life and subsequently declines. The mean age at sterility precedes that at menopause by about 10 years (Gray, 1979) but conception is a continuing possibility for some individuals until shortly before the final menstrual cycle (Metcalf et al., 1981). The extent to which paternal ageing accounts for age-specific fertility patterns found among married pairs is still not clear, although experience with artificial insemination shows unequivocally that infertility in women rises from age 30 and could be an important problem in those aged above 40 (Fédération CECOS et al., 1982).

This decline in fertility is associated with a steeply rising frequency at full-term of chromosomally imperfect conceptions, particularly trisomies (Hook, 1981). The birth incidence of autosomal trisomy underestimates the underlying frequency of these conceptuses by at least an order of magnitude (Ferguson-Smith and Yates, 1984). To what degree the higher incidence of spontaneous abortion in mothers aged above 35 (Leridon, 1977; Stein et al., 1980) reflects the changing biological frequency of these anomalies is not known since women who are prone to abortion may continue to attempt to conceive when others have completed their desired family size, leading to overrepresentation of the chronically sub-fertile group (Billewicz, 1973; James, 1974).
The infertility of older women can sometimes be attributed to overt pathological changes such as endometriosis, fibromyomata and pelvic inflammation (especially salpingitis). Idiopathic infertility in this group is probably due to a combination of undetected pathogenic changes with continuous physiological age changes ("eugeric") affecting both the gametes and genital tract. In rodents, ageing of the uterus is the major factor contributing to smaller litter sizes in middle age, according to the results of embryo transfer experiments. Whilst about 50% of embryos obtained from young donors survived in hosts of the same age, the corresponding value in ageing animals was less than 15% (Talbert, 1977). In the following sections, the possible causes and consequences of uterine ageing in animals are discussed in relation to the more limited information about pregnancy wastage in women.

### Timing and Extent of Pregnancy Failure

Demographic studies have shown the incidence of clinically recognizable spontaneous abortion in mothers aged over 40 is of the order 25-30% (Leridon, 1977), but there are indications that the majority of fetal deaths are occult. In longitudinal studies of young, healthy women, about 60% of conceptuses detected by rising urinary HCG/β aborted in the first 12 weeks of gestation (Edmonds et al., 1982). Many of those were lost at very early stages. Unfortunately, it is not possible to estimate early pregnancy failure in women aged 40-50 years because of the difficulties of finding a random sample. Judging from experience with in vitro fertilization at Bourn Hall Clinic, a substantially lower proportion of embryos replaced will implant successfully in this age group compared with younger patients (Edwards and Steptoe, 1983). Although the numbers of patients involved is admittedly small at present, similar findings have been obtained in several other centres. Thus, the limited information available would suggest that conceptuses are lost at per-implantation as well as at later stages of gestation in older women. It is, therefore, important to discover the responsible factors: whether mainly a matter of a deteriorating uterine environment or of chromosomally aberrant conceptions, in which case ovum donation might be considered in some cases. At the present time, it is not possible to draw up a balance sheet for our own species, though the situation in laboratory rodents is becoming clear.

Compared with ourselves, the incidence of cytogenetic aberrations is almost an order of magnitude lower in most strains and species of animals, though the rate of trisomic conceptions has been shown to rise...
In rodents, the litter size falls steadily from peak values at about 4–5 months old and the third pregnancy, most animals becoming sterile in mid-life under laboratory conditions, at about 12–16 months (Talbert, 1977). The decreased fertility precedes an irreversible fall in ovulation and fertilization rates which are not significant until towards the end of the reproductive life phase. There are notable genetic variations in the timing of fetal wastage. The incidence of preimplantation embryonic death is particularly high in hamsters and varies among different strains of mice (Fabricant et al., 1978). In CBA mice, entire sets of embryos may fail to implant (Gosden, 1975) whereas postimplantation losses are the rule in C57BL/6J (Fig. 1). Postimplantation deaths occur sporadically along the length of each uterine horn and there are statistical reasons for suspecting that resorption of entire litters cannot be accounted for by the product of individual probabilities of mortality but is concerted by systemic factors (Gosden et al., 1981). The prenatal mortality generally presents an exaggerated

![Graph](image-url)
pattern of what is seen among younger animals (Fig. 1) with additional losses occurring close to the time of parturition, which is delayed by one day (Holinka et al., 1978).

It is a common finding, though not a universal one, that delayed breeding potentiates the age-dependent decrease in fertility (Asdell et al., 1941; Nishimura and Shikata, 1960). The infertility of elderly primigravidae might be attributable to the effects of oestrogenic stimulation resulting either from repeated oestrous cycles or from prolonged cycles which characterize middle age (Huang and Meites, 1975; Nelson et al., 1982). Experimental evidence of the second hypothesis has been obtained (Butcher and Pope, 1979; Page et al., 1983).

A detrimental effect of high parity in animals has been obtained under the special conditions of long-term unilateral ovariectomy in rodents in which a single uterine horn is repeatedly challenged to carry a double load of conceptuses during successive pregnancies. As a result of this feto-placental overload, only half the normal number of pups is delivered during the lifespan (Adams, 1970). Biggers et al. (1962) suggested that premature ageing of the functional uterine horn was responsible for the infertility. Although their hypothesis has been corroborated (Gosden, 1979), a higher incidence of aneuploid embryos is also a factor (Brook et al., 1984). The anatomical and physiological bases of uterine ageing in animals have been explored in considerable detail.

Structural Changes

Withdrawal of ovarian steroids (especially oestrogen) leads to uterine atrophy with a considerable reduction in bulk of the genital tract as a whole. Although the post-menopausal uterus retains a sensitivity to these steroids the question of whether normal physiological stimulation and pregnancy potential can be realized have rarely been investigated. Successful pregnancy is possible in a minority of CBA mice aged over one year by replacing sterile or anovulatory ovaries with young grafts (Krohn, 1962; Felicio et al., 1984). In a young woman in whom there was evidence of precocious menopause of 5 years standing, combined treatment with oestrogen and progesterone enabled the gestation to full-term of a zygote derived from her husband’s spermatozoon and a donor’s egg (Lutjen et al., 1984). Despite these exceptional records indicating that uterine atrophy can be reversed, changes in uterine structure preceding sterile ages may contribute to reduced fertility.
Extracellular Fibre

There is a steady increase in the amount of collagenous material in both the endometria and myometria of ageing rodents, probably resulting from reduced catabolism (Schaub, 1964–65; Maurer and Foote, 1972). This situation is in apparent contrast with a single study of the human uterus in which collagen did not accumulate between ages 30 and 50 (Woessner, 1963). There is considerable turnover of collagen during each pregnancy cycle but multiparous uterine horns have the same amounts as do nulliparous horns of the same age (Finn et al., 1963). The extent of fibrosis of middle-aged rodent uteri is variable, perhaps according to the history of oestrogenic stimulation (Loeb et al., 1939). It has been conjectured that fibrosis may interfere with tissue metabolism (Biggers et al., 1962), but it is also possible that the greater physical and chemical stability resulting from intermolecular covalent bonding (Kao et al., 1976) leads to intrauterine death as a result of changes in tension and compliance of the uterine wall and the parturient canal.

There are major changes in the non-cellular components of uterine arterial walls resulting from ageing and pregnancy history. Sclerosis leads progressively to the obliteration of the lumen in human (Lang and Aponte, 1967; Naeye, 1983; see Fig. 2) and rodent uteri (Gillman and Hathorn, 1959; Wexler, 1964). Arteriosclerosis advances more rapidly

FIG. 2. Myometrium of a non-pregnant uterus aged 37 showing the distribution of elastic tissue with only moderate sclerosis of arterial walls (Gomori and Light Green, × 50).
in rats experiencing the stressful effects of repeated pregnancy, when it is associated with deposition of immune complexes (Lattime and Strausser, 1977). The elastic tissue component also increases as a result of decreased resorption with litter number, but formation of new elastin is delayed in elderly primigravidae (Albert and Bhussry, 1967).

**Cell Types**

In the uteri of rodents, but not humans, macrophages accumulate as a result of "clearing up" operations post-partum (Spicer, 1960; Gosden, 1979). This scar tissue remains for an indefinite period after the last parturition and, because of its location, might interfere with subsequent placentation (Fig. 3). Its presence is indicated by yellow-brown discolouration, which is most clearly demonstrated by the contrasting

![Transverse section of uterine wall of an ex-breeder CBA mouse showing \( \beta \)-glucuronidase activity in a cluster of macrophages lying on the mesometrial side (arrow) (L: lumen) (x 75).](image-url)
FIG. 4. Human uterine muscle cell. Two membrane-bound lipid particles which lie adjacent to the nucleus displace the cytoplasmic organelles and microfilaments (glutaraldehyde and osmium, $\times 47200$).
appearance of multiparous and nulliparous uterine horns of long-term unilaterally ovariectomized mice.

The density of mast cells, which are mainly found in the myometrium, increases 3-fold during the reproductive lifespan of mice but is unaffected by pregnancy history (Spicer, 1960; Gosden, 1979). No such accumulation has yet been reported for human uteri.

**Cellular Inclusions**

The only notable inclusions which have been observed in rodent uteri are autofluorescent lipofuscin granules and haemosiderin in the aforementioned macrophages. These pigments are also present in monkeys (Graham, 1968) but were not found in human uteri one year or more after the last parturition (unpublished observations).

Human uterine smooth muscle cells accumulate lipid particles during ageing (Fig. 4). The particles differ from lipid droplets seen in other cells in having a trilaminar membrane and a complex internal structure comprising several bodies of differing electron opacity (Gosden et al., 1978). They are relatively large structures measuring 1-3 μm diameter, irrespective of age, and are not confined to toxaemic patients, as has been suggested (Haust et al., 1977). Their physical and histochemical properties suggest they are a type of lysosomal complex containing autofluorescent materials, which may be related to the putative peroxidized lipids in lipofuscin. Since some of their characteristics resemble atherosclerosis in vascular smooth muscle, the lipid composition was determined for myometria of different ages and particle densities. However, the expected rising concentrations of cholesterol, cholesterol ester and phospholipids were not observed (Table 1). On the basis of indirect results, it seems probable that the lipid is triglyceride, perhaps resembling that found in ischaemic myocardium (Bryant et al., 1958) which also contains autofluorescent lipid peroxides (Davis et al., 1984). However, it would be surprising if the myometrial particles were caused by uterine ischaemia since they were present in non-pregnant and nulliparous organs.

<table>
<thead>
<tr>
<th>Density of lipid particles</th>
<th>Patient mean age (years)</th>
<th>No.</th>
<th>FFA</th>
<th>C</th>
<th>CE</th>
<th>LPC</th>
<th>SPH</th>
<th>PC</th>
<th>LPE</th>
<th>PS</th>
<th>P1</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>27</td>
<td>5</td>
<td>17.0</td>
<td>13.6</td>
<td>1.74</td>
<td>2.00</td>
<td>7.99</td>
<td>10.6</td>
<td>6.35</td>
<td>3.98</td>
<td>1.56</td>
<td>3.29</td>
</tr>
<tr>
<td>Intermediate</td>
<td>31</td>
<td>6</td>
<td>16.2</td>
<td>14.5</td>
<td>1.21</td>
<td>2.19</td>
<td>9.31</td>
<td>9.75</td>
<td>6.59</td>
<td>4.36</td>
<td>1.36</td>
<td>2.8</td>
</tr>
<tr>
<td>High</td>
<td>45</td>
<td>8</td>
<td>17.4</td>
<td>12.9</td>
<td>1.53</td>
<td>2.24</td>
<td>6.90</td>
<td>8.31</td>
<td>3.58</td>
<td>3.49</td>
<td>1.39</td>
<td>2.7</td>
</tr>
</tbody>
</table>

These mean lipid concentrations were estimated by multidimensional thin-layer chromatography using specific microchemical assays (Bowyer and King, 1977). Gosden and Bowyer, unpublished.
Physiological Changes
Preparation of the Endometrium for Implantation

For successful implantation to occur, the developmental stages of the embryo and endometrium must be synchronized. In ageing rodents, developmental lagging of the order of 12 h has occurred by the normal time of implantation (day 5), but both components are affected and desynchronization is unlikely to be a cause of infertility (Parkening, 1979). During implantation, blastocysts are clasped by apposing uterine surfaces and microvilli protruding from trophoblast and uterine epithelial cells interdigitate to bring about greater intimacy (Nilsson, 1967; Enders, 1975). In aged mice, the lumen fails to close (Finn, 1970) and uterine microvilli, which are sensitive indicators of ovarian hormone stimulation, are sparse or abnormal (Smith, 1975; see Gosden, 1985). However, the lumen closes normally in aged hamsters (Parkening, 1979) and, also in contrast with mice, the uterine epithelia of ageing rats and post-menopausal women can respond normally to steroid hormones (Nathan et al., 1978; Craig and Jollie, 1984). That such species differences have been found for parameters of ageing need not be surprising in view of differences of biological age and hormone and pregnancy experience in the different situations.

There is an increasing frequency of abnormal menstrual cycles in the decade preceding menopause. Limited evidence suggests that mean levels of progesterone fall (Reyes et al., 1977) and, more significantly, that the frequency of luteal phases of less than 10 days long increases (Döring, 1963). In animals, there is abundant evidence showing that secretion of progesterone by the corpus luteum of pregnancy/pseudopregnancy is well-maintained (Spilman et al., 1972; Holinka et al., 1979; Parkening et al., 1978; Miller and Riegle, 1980). The earlier finding that exogenous progesterone can improve implantation rates in ageing CBA mice (Gosden, 1975) might therefore rest on a pharmacological action of countering adverse hormonal effects during abnormal conception cycles (see above). The physical characteristics and concentrations of progesterone and oestrogen receptors in ageing uteri have been determined for a number of species but conflicting results have been obtained and it is not safe to state whether individual cells are less sensitive to hormonal stimulation (Blaha and Leavitt, 1978; Hsueh et al., 1979; Gesell and Roth, 1981; Belisle et al., 1982; Strathy et al., 1982).

Increased RNA synthesis in stroma cells and oedema are the earliest recorded responses of the uterus to implanting blastocysts (Lundkvist, 1978). Extravasation of plasma constituents from superpermeable capillaries may be one stimulus for the transformation of stroma into...
decidual cells (Sorger and Soderwall, 1981; Milligan and Mirembe, 1984). Initiation and maintenance of the decidua depends on sufficient hormonal priming and a marked rise in uterine blood flow (Garris et al., 1983; Garris, 1984), both hyperaemia and decidualization requiring local production of prostaglandins (Kennedy and Armstrong, 1981; Kennedy and Lukash, 1982). In ageing rodents, the size of the decidual response has been found to be reduced in almost all studies (Finn, 1966; Blaha, 1967; Maibenco and Krehbiel, 1973; Holinka and Finch, 1977). For example, the decidual capsules measured in benzyl benzoate cleared uteri of CBA mice on day 7 were $5.3 \pm 0.3$ mm$^3$ at age 3 months in comparison with $2.9 \pm 0.2$ mm$^3$ at 10 months. That such a difference is not due to different levels of steroid hormone priming or strength of the embryonic stimuli has been demonstrated in ovariectomized mice given exogenous hormones and stimuli. The poor response is associated with a lowering of uterine prostaglandin concentrations (Table 2), of capillary permeability (Parkening and Soderwall, 1973) and of stroma cell RNA synthesis (Soriero, 1980), but a causal nexus has not yet been demonstrated. Additional circumstantial evidence of poor vascular perfusion in ageing uteri (see following section) might explain poor embryonic survival and decidualization since even transient uterine ischaemia during the peri-implantation stage is harmful (Franklin and Brent, 1964).

### Utero-placental Circulation

Many embryos that successfully implant in aged uteri do not thrive. In mice, it has been observed that most deaths occur when metabolic demands for growth are rising rapidly during the phase following

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>No.</th>
<th>Prostaglandin E$_2$</th>
<th>6-keto-prostaglandin F$_{18}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>control treated % change</td>
<td>control treated % change</td>
</tr>
<tr>
<td>4-5</td>
<td>10</td>
<td>4.6±0.4 6.7±0.6 65.3%</td>
<td>14.0±2.3 20.4±3.0 83.8%</td>
</tr>
<tr>
<td>13-14</td>
<td>10</td>
<td>2.8±0.2 3.6±0.3 36.1%</td>
<td>10.1±1.5 12.4±2.0 21.7%</td>
</tr>
</tbody>
</table>

Mean ± SEM given.

*Treatment consisted of Silastic implants containing crystalline oestradiol-17β and progesterone given sequentially to mimic physiological hormone levels required for priming and sensitizing the uterus for implantation.

From Brown et al., 1984 and unpublished observations.
organogenesis, and some developmental lagging occurs (Holinka et al., 1979). Since fetuses are wholly dependent on their placentae for oxygen, nutrients and the exchange of metabolites, it is important to consider whether this organ is adequately perfused. In rats, placental blood flow is correlated with fetal weight (Gilbert and Leturque, 1982) and average rates of uterine blood flow increase more than 20-fold during pregnancy as a result of falling resistance (Bruce, 1976). The flow rate per unit weight of uterus in old animals is only about half that of young controls at the same stage of gestation (Larson and Foote, 1972; Rahima and Soderwall, 1978). However, such results do not necessarily indicate under-perfusion but could reflect smaller metabolic demands (because of fewer or moribund fetuses) or the larger proportion of extracellular protein which would lead to underestimation of cellular perfusion.

Normally, the fetus is protected physiologically by a wide safety margin through the reserve capacity of the placenta for maintaining fetal oxygen uptake, acid-base balance and nutrient transfer (Wilkening and Meschia, 1983). Nevertheless, experimental occlusion of uterine blood vessels can cause fetal hypoxaemia, growth retardation and even death (Wigglesworth, 1964; Gilbert and Leturque, 1982; Robinson et al., 1983). It is important to recognize that the placenta does not undergo reactive hyperaemia following maternal hypoxia (Makowski et al., 1973; Wilkening and Meschia, 1983). Therefore, the possible consequences of uterine vascular stenosis on pregnancy outcome in older mothers should be considered. It is also important to consider whether production of oestrogen by the feto-placental unit and of prostaglandins could be additional factors since these hormones are potent vasodilators of the uterine vessels (Barton et al., 1974; Clark et al., 1981). Production of PGE2 and the metabolite of prostacyclin are already known to be impaired in ageing rats (Table 2). Rat conceptuses are not equally vulnerable to hypoperfusion at all stages of pregnancy, there being a higher risk at early and late implantation stages than on days 8–9 (Wigglesworth, 1964; Franklin and Brent, 1964; Bruce and Cabral, 1975).

Results of acute experimental ischaemia may not, however, represent the physiology of ageing which involves slow cumulative changes. It might be predicted that the lower placental blood flow rates at the central site in guinea pig uterine horns compared to either end (Garris, 1983) would indicate spatial heterogeneity in fetal death, but this was not found in a large series of mice (Gosden et al., 1981). Despite occasionally inconsistent findings, the balance of evidence favours the view that fetal growth retardation and death in older mothers is caused by poor uteroplacental perfusion, this being aggravated by any hypertension present or by a fall in cardiac output during ageing. Infarction, besides
other placental disorders, is an additional hazard for the older mother (Naeye, 1979; Naeye, 1983). It may be related to the presence of essential hypertension and pre-eclampsia in this group (Fox, 1978) and, when extensive villous ischaemia occurs, could explain the production of smaller babies (Selvin and Janerich, 1971; Russell et al., 1984).

The prospects of successful pregnancy outcome in most women over 35 who are enjoying a good diet and obstetric care are still reassuring. Nevertheless, in view of the possibility of lost functional reserve in the placenta, it would seem prudent to carefully attend to any hypertension present, ensure adequate rest and abstain from smoking. Avoidance of undue stress is another worthwhile precaution since catecholamines cause uterine vasoconstriction and may make matters worse (Barton et al., 1974). In the case of patients having treatment with in vitro fertilization, the replacement of more than one embryo to attempt multiple pregnancy might be counter-productive if the ageing uterus is less capable of sustaining the normal metabolic demands of gestation.

Survival of Chromosomally Abnormal Fetuses

That the rising incidence of babies presenting Down’s syndrome and other trisomies among older mothers could be due to a greater proportion of abnormal conceptuses surviving gestation rather than a higher frequency of chromosomal non-disjunction in oocytes has been suggested from time to time (Stene et al., 1981). This hypothesis has been supported on the basis of the age-specific probabilities of spontaneous abortion for a combination of trisomies (Aymé and Lippman-Hand, 1982), but this analysis has been challenged on several grounds and there are contrary findings (Carothers, 1983; Hook, 1983; Warburton et al., 1983; Ferguson-Smith and Yates, 1984). Furthermore, the hypothesis conflicts with considerable evidence showing that the age-specific incidence of trisomy in animals rises in preimplantation embryos, i.e. before intrauterine selection is likely (see Bond and Chandley, 1983); it is also opposed by limited evidence of proportionately lower survival rates for abnormal fetuses in older dams (Parsons, 1964). The latter finding is anticipated on the grounds that survival of trisomic fetuses, which are already jeopardized by phenotypic disturbances and growth retardation (Bond and Chandley, 1983), would be compromised still further by competition with normal siblings in a deteriorating uterine environment. Uterine ageing might then explain the falling frequency of human trisomies detected among mothers aged above 40 who undergo amniocentesis. Interestingly, this departure from the exponential rate
of ageing is earlier and more dramatic among trisomies 13 and 18 than trisomy 21, which is physiologically more robust (Ferguson-Smith and Yates, 1984). Some non-chromosomal congenital anomalies, such as neural tube defects, show J- or U-shaped age-frequency distributions (Record, 1961; Carter and Evans, 1973). However, further research must be undertaken to determine whether these patterns are accounted for by variable penetrance of the causal agent or differential fetal survival in utero.

Conclusions

There can be little doubt that the maternal age-dependent increase of infertility which is characteristic of most, if not all, domesticated and laboratory mammals besides man is a result of a deterioration in the quality of zygotes and of the uterine environment. Maternal changes involve pathological alteration of structure and function of the genital tract as well as continuous, physiological age changes. The balance of factors responsible for infertility probably varies in different species and at different ages. Implantation losses in rodents may involve uterine fibrosis, abnormal surface architecture of the epithelium and decreased capillary responses leading to attenuation or even absence of the decidual reaction. Growth retardation and death of implanted conceptuses with normal karyotypes could be due to ischaemia of the utero-placental circulation, for which a considerable body of circumstantial evidence exists. Whatever are the mechanisms of uterine ageing, it would seem likely that a more hostile environment would encourage earlier and, perhaps, selective elimination of abnormal conceptuses.

Acknowledgements

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References


THE AGEING UTERUS

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Discussion on the Ageing Uterus

Cleavage of Embryos in Older Mothers

Gardner: What is meant by age-related changes in the rate of cleavage? It is important to know if this is measured in vivo or in vitro, and whether changes in "rate" mean changes in the interval between successive cleavage divisions, or that actually starts later.

Gosden: Studies carried out in vivo or in vitro on the timing of ovulation and fertilization do not indicate any marked differences between young and aging animals. The overall time taken to reach a 32-cell embryo or a blastocyst is evidently slightly longer in the older animal according to some authors, and most agree there is more fragmentation of ova. I am not aware of any critical timings of each division in vitro apart from those of Peluso et al. (1983).

Gardner: Relevant knowledge could be obtained by exchanging embryos between young and old mothers. This would help to decide if the environment within the tract is changing with age or if the phenomenon is intrinsic to the embryos themselves.

Gosden: Reciprocal transfer of zygotes between young and middle-aged rats has indicated a deterioration in the oviductal environment during ageing (Page et al., 1983), but it is too early to draw up a balance sheet of causes of early embryonic losses.

Fishel: We have recently carried out a whole series of examinations on the cleavage in vitro of human embryos and their development to blastocyst when taken from mothers of increasing age. The embryos were those remaining after three others had been selected for replacement into the mother. Detailed statistical analyses have failed to reveal any effect on the rate of development or the proportion developing into blastocysts.

Gosden: There are marked species differences in the effects of age growth and survival of preimplantation embryos in vitro. Comparison between species or the use of animal models are always hazardous. Many hamster eggs fail to divide beyond the 4-cell stage (Blaha, 1964), and a high incidence of arrested cleavage has been described in rabbit embryos (Adams, 1970). In mice and rats, the results vary from strain to strain, with pre-implantation development
lagging in some strains although not impaired in a major way. Slow cleavage appears to be matched in some animals by a later onset of endometrial preparation for implantation (Parkening, 1979).

**Uterine Ageing and Reproduction**

**Johnston:** I have the impression that there is insufficient knowledge in animals as to whether uterine ageing impairs fertility in older females. Do you feel we will be justified in transferring young embryos to older mothers during human in vitro fertilization?

**Gosden:** We do not know the nature of the factors involved in uterine ageing. There are clear indications of a decline in fertility in older females of every species of mammal so far investigated, but it is not known whether the uterus or the embryo is responsible for low fertility in each species. We need to know about the quality of the embryos from older mothers, including their risk of chromosomal imbalance, and information about chromosomes is likely to come more quickly than that on uterine ageing. Perhaps ova donation would be clinically advisable in some cases, and it may well improve prognosis in older patients. I strongly suspect that uterine problems, both pathogenic and eugenic, are a very significant factor in the declining fertility with age. The problem is how to obtain clear evidence of uterine malfunction.

**Edwards:** Much of the evidence so far available on fertility and age is still at an early stage, e.g. examining the morphology or vasculature of the uterus. There is really no method of assay, except those described by Gosden where embryos from young females are transferred to older recipients and *vice versa*. This is a first approach to an assay, providing standard conditions are established such as the transfer of a specific number of embryos.

A clear-cut assay is needed, similar to those established in hormone assays, and the standardization of such strict criteria is the only way of obtaining clear evidence about ageing. Has anyone ever tried, for example, to ovariectomize females of various ages, inject standard amounts of oestrogen and progesterone, or give some other standard stimulus of some kind, and then assay the uterine response. One method would be to inject known numbers of lectin-coated particles into the uterus of females of various ages to stimulate a decidual response. This method has been described by Fishel (1984). Such methods would permit a measure of the overall response of the uterus, and they could then be refined to find out if any particular component of the response was impaired. A series of simple and rapid assays might be established, taking the whole uterus first, and then each tissue in turn. Have such tests been carried out on any animal?

**Gosden:** Many of the ageing experiments I have described incorporate the sorts of control you rightly advocate, e.g. the discharge of prostaglandins and the decidual reaction. However, there are major problems in carrying out any gerontological investigations. Attempts to measure biochemical responses have
included estimates of the trophic responses of the uterus, DNA synthesis, oxygen consumption, and others, yet there are uncertainties in the interpretation of the results. One difficulty is that the proportions of cellular and extracellular compartments of the uterus change with age, making comparisons of homogenized tissues from young and ageing animals fraught with problems of interpretation. Another problem is the risk of overlooking pathogenic changes when physiological age changes are being searched for. There is the problem of making comparisons between ageing animals which may have had subtle differences in their history or diet, causing differences in the pattern and rate of ageing of different cohorts even if genetically identical.

Surprisingly few molecular age changes have been described in the uterus, apart from the physical changes of collagen. The uterotrophic response to oestrogen, for example, is undiminished (Holinka et al., 1977). Sometimes fewer microvilli are identified, or other changes are detectable. Gerontological studies have not been very successful in pinpointing any cellular or molecular changes that could be responsible for organ ageing. That is why I think attention should be given to whole organ ageing, particularly of the blood vascular supply since the conceptus is so obviously dependent upon adequate placental perfusion.

Edwards: When older uteri are examined, especially in females where implantation fails completely, there must be a problem with the recognition of the embryo by the uterine epithelium or vice versa. Here, a model that involved recognition of trophoblast and epithelium might assist in analysing the components of implantation, even though the whole uterine system is being assessed. Different methods may be needed in different species. If the failure of recognition could be excluded, the next step for analysis might be decidualization. I would plead for standardization in such work.

Gosden: The question of a failure during very early implantation in all animals has not been examined thoroughly because such experiments are slow- yielding and expensive and sometimes frustrated by inter-cohort variation.

Biochemical Aspects of Uterine Ageing

Kloesterboer: Gosden mentioned an increase in concentration of immunoglobulins in the blood vessels of the uterus. Is the increase restricted to blood vessels or is it a general feature of all tissues in the ageing animal?

Gosden: It is restricted as far as I know to the arteries, but not only the uterine artery. It arises in others such as the aorta and iliac vessels (Lattimer and Strausser, 1977). All of the major arteries display such sclerotic changes, and might contribute to hypertension which develops in normal ageing animals. There is a tendency for blood pressure to rise during ageing even in strains of animals which are not genetically prone to hypertension.

Psychoyos: At the time of implantation, there are changes in collagen turnover due to variations in the levels of collagenase of cathepsin D. Studies with dyes which stain collagen have revealed that a compact mass of collagen bundles characterizes the ageing uterus (unpublished). One of the problems
of ageing could therefore be a lack of reduction of enzymes which dissociate the collagen, because the formation of intracellular spaces is essential for decidualization and for the sequence of events leading to implantation.

Gosden: This is probably true. Fibrosis is a major problem in some animals, especially when it gets severe. Collagenase activity has been measured and there does seem to be a reduced amount of it in older uteri (Maurer and Foote, 1972)

The physical and chemical stability of collagen also changes with age, this being a universal feature of ageing, leading to greater resistance to resorption during implantation and pregnancy.

Bell: One of the most dramatic effects in the ageing uterus is its weakening ability to decidualize as compared to the young uterus. Decidualization is the end product of a very complex period of differentiation in the rodent uterus. Since Gosden has only recorded gross weight of the uterus, are there any other studies on the patterns of histological differentiation, growth and regression in the uterus with increasing age? There may be specific stages that fail to develop normally during the integrated process of decidualization.

Gosden: There are some indications of reduced oedema as a result of a deciduogenic stimuli in the ageing uterus. These reductions have not been quantified. Nor have sequential changes been analysed, except for a reduced production of RNA by stromal cells during ageing in response to oestrogen stimulation (Soriero, 1980). It is clear that the reduced gain in uterine weight following a decidual stimulus in ageing females is related to the declining weight of the decidual response. Moreover, the volumes of conceptuses on day 7 have been assessed, before most of them died or degenerated, and it is clear that they are smaller in older females. This consistent finding that the decidual reaction is reduced in the ageing rodent has been reported for at least three species, i.e. hamster, rat and mouse, and could have marked effects on implantation and early fetal development (Talbert, 1977).

Bell: Variations in the weight of the decidua and the growth of fetuses would surely be an excellent model to examine the process of decidualization in older uteri. It would be easy to make comparisons between old and younger females in this respect.

Gosden: Yes, it might well be. There are two phenomena involved, but I am not sure whether they are accounted for by a common age change. One is the reduced size of the decidual reaction, and the second is its complete absence following hormone priming and exogenous stimulation. Even with very strong stimuli, such as traumatization by haemostat and the administration of large priming doses of oestrogen and progesterone, the uterus does not decidualize adequately.

Bell: The absence of decidualization could relate to the exhaustion of stem cells. In De Feo’s original paper (De Feo, 1967), examples were shown of massive deciduomata in response to various stimuli, but there was no evidence of any decidualization in response to stimuli after the next priming with hormones. This could have been due to stem cell deficiency.

Gosden: We have attempted to examine the situation, using the phosphoglycerate kinase isoenzyme system (unpublished). Unfortunately,
instead of producing small deciduomata, all of the older mice were completely refractory, so it has not yet been possible to answer the question of whether fewer stem cells contribute to smaller deciduoma.

Leroy: The total refractoriness of the uterus is relevant to the early work by Finn (1970). He showed that the ageing uterus would not respond with what was called the corrugation reaction of the epithelium. This reaction corresponds in fact to the interdigitation of epithelial microvilli. The uterine epithelium is believed to play an obligatory role in the induction of decidualization, and the presence of a refractory epithelium must indicate that the uterus has become incapable of conveying a decidualizing message.

References


Relationship between corpora lutea or fetal number and plasma concentrations of progesterone and testosterone in mice

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Summary. Blastocysts (1–14) were transferred unilaterally into 63 pseudopregnant mice which were killed on Day 17. Plasma progesterone concentrations were significantly \((P < 0.05)\) lower in animals with one fetus than in those with 2–5 or 9–14 fetuses. Plasma testosterone concentrations were correlated with fetal number in mice with 1–13 fetuses \((P < 0.001)\). The total placental content of chorionic gonadotrophin in 13 litters varied directly with the number in the litter \((1–6)\), and was \(1.67 \pm 0.15\) ng/placenta. The number of corpora lutea per mouse was negatively correlated with mean CL volume per mouse \((P < 0.001)\), and the number of conceptuses was positively correlated with mean CL volume per mouse \((P < 0.001)\). The effect of conceptuses on the ovary was systemic. The relationship between plasma testosterone concentration and conceptus number may be due to gonadotrophins acting on the ovary, or androgens produced by the placenta or fetus.

Introduction

The mouse placenta contains a gonadotrophin similar to human chorionic gonadotrophin \((hCG)\) (Wide & Hobson, 1977, 1978; Wide, Hobson & Wide, 1980; Rao, Pointis & Cedard, 1982). Placental CG reaches peak levels on Days 11 and 16 \textit{post coitum} (Wide & Wide, 1979), and may be partly or wholly responsible for maintaining the secretion of ovarian steroids during the second half of pregnancy, when the continuation of gestation is not dependent on the presence of the pituitary (Newton & Beck, 1939).

A quantitative relationship might exist between the number of implantations and the production of ovarian steroids, but the evidence for this is contradictory in both the mouse and the rat. Plasma progesterone and testosterone concentrations increase in the second half of pregnancy in the mouse, and plasma progesterone values are greater in mice selected for large litters than for small (Michael, Geschwind, Bradford & Stabenfeldt, 1975; Barkley, Michael, Geschwind & Bradford, 1977). In experiments using C3H mice in which the number of fetuses was experimentally adjusted to between 1 and 10, serum concentrations of progesterone were directly proportional to litter size (Soares & Talamantes, 1983). However, neither the number of corpora lutea nor that of fetuses is correlated with plasma progesterone concentrations in Rockland Swiss mouse litters varying in size from 7 to 11 (Simon, Bridges & Gandelman, 1978). In the rat there is a significant correlation between total corpus luteum weight and serum progesterone concentration on Day 16 of pregnancy, but reducing the number of conceptuses from 12 to 5 does not significantly alter the levels of progesterone (Elbaum, Bender, Brown & Keyes, 1975). However, Kato, Morishige &
Rothchild (1979) found a direct relationship between the number of conceptuses and serum progesterone concentration in rats on Day 15 of pregnancy.

Our experiments were designed to test whether the number of fetuses is related to plasma concentrations of testosterone and progesterone in mice, by independently controlling the numbers of fetuses and corpora lutea by using embryo transfer. This design also enabled us to examine whether fetuses exert a local trophic effect on the ipsilateral ovary, as has been found in rats carrying one or two experimentally implanted fetuses on Day 18 of pregnancy (Zambrana & Greenwald, 1971).

Materials and Methods

The mice were from our own closed colony of randomly bred albino, maintained under a constant schedule of 14 h light and 10 h dark. Food and water were freely available.

Fetal number was controlled by transferring blastocysts obtained from donor mice on Day 4 post coitum to hosts on the 3rd day after mating with vasectomy males. Donor mice were prepared for superovulation by i.p. injections of 5 i.u. PMSG and 5 i.u. hCG 48 h apart. Animals were then paired with males of proven fertility, and inspected for a coital plug the following morning (Day 1 of pregnancy). Blastocysts were flushed from the uterine horns into sterile Petri dishes using Medium 199 (Flow Laboratories, Irvine, U.K.) containing 20 mM-Hepes buffer, 10% fetal serum and 100 units penicillin-G/ml (Sigma, London, U.K.). Host animals were anaesthetized with 2,2,2-tribromethanol (Avertin: Winthrop, Surbiton upon Thames, U.K.), on the 3rd day after a sterile mating with a vasectomy male, and the uterus was exposed via a flank incision. Between 1 and 18 embryos were collected in a small volume of medium (< 1 ml), in an orally controlled pipette, and injected unilaterally into one uterine horn per animal, choosing alternate sides in successive mice.

The mice were anaesthetized on Day 17 of pregnancy with an injection of amylobarbitone sodium. The thorax was opened and the mouse exsanguinated via the heart. The blood was placed in heparinized tubes, centrifuged and the plasma kept for the radioimmunoassay (RIA) of progesterone and testosterone.

The number of implantation sites and fetuses was counted, and placentae and fetuses were weighed. The maternal ovaries were weighed after fixation for 24 h in Bouin's fluid. The ovaries from 14 mice with 1 fetus, 15 with 2-5 fetuses, 5 with 6-8 fetuses and 11 with 9-14 fetuses were cut at 10 µm and stained with haematoxylin and eosin. The number of corpora lutea in each ovary was counted, and their volumes measured by the method of Rowlands (1961).

Plasma from 62 mice was assayed for progesterone by the method of Scaramuzzi, Corker, Young & Baird (1975). Two 50 µl aliquants from the plasma of each animal were extracted with 2 ml petroleum ether (Analar reagent grade). The ether was evaporated to dryness under nitrogen and the residue dissolved in 1 ml phosphate buffer. Duplicate 0-1 ml portions of the extract were assayed with a specific RIA using sheep anti-progesterone antiserum 91929/9 as described by Scaramuzzi et al. (1975). There was little or no cross-reaction between this antiserum and androstenedione, cortisol, pregnenolone, testosterone, or 17-hydroxyprogesterone. There was a significant cross-reaction with progesterone, 11α- and 11β-hydroxyprogesterone and 11-ketoprogesterone.

Testosterone was assayed in plasma from 26 mice, with litters of 1-13, by the method of Collins, Mansfield, Alladina & Sommerville (1972). The testosterone antiserum (NEA-042B; New England Nuclear, Boston, MA, U.S.A.) was from a rabbit immunized with a testosterone-3-CMO-bovine serum albumin conjugate. Cross-reactivity at 50% displacement was about 50% for dihydrotestosterone and was negligible for other androgens, progesterones or oestrogens. The efficiency of the extraction method was 85% (range 75-94%). The intra-assay variation was 4-8% and the inter-assay variation about 9%. The detection limit was 0.05 nmol/l.
Table 1. Effects of variation in numbers of fetuses on fetal and placental weights, and plasma progesterone and testosterone concentrations

<table>
<thead>
<tr>
<th>No. of host mice</th>
<th>No. of fetuses per host</th>
<th>No. of blastocysts transferred to host</th>
<th>No. of implantation sites/host</th>
<th>Mean placental wt in litter (mg)</th>
<th>Mean fetal wt in litter (mg)</th>
<th>Progesterone conc. (nmol/l)</th>
<th>Testosterone conc. (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>1</td>
<td>2.41 ± 0.64</td>
<td>1.24 ± 0.11</td>
<td>179.5 ± 6.8</td>
<td>831.2 ± 40.37</td>
<td>127.9 ± 7.02a</td>
<td>0.89 ± 0.07a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.64</td>
<td>±0.11</td>
<td></td>
<td>(16)</td>
<td></td>
<td>(8)</td>
</tr>
<tr>
<td>23</td>
<td>2–5</td>
<td>5.00 ± 0.58</td>
<td>4.26 ± 0.18</td>
<td>144.3 ± 3.7</td>
<td>756.04 ± 24.04</td>
<td>164.4 ± 9.61b</td>
<td>1.38 ± 0.14b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.58</td>
<td>±0.18</td>
<td></td>
<td>(16)</td>
<td></td>
<td>(8)</td>
</tr>
<tr>
<td>12</td>
<td>6–8</td>
<td>11.00 ± 0.89</td>
<td>9.00 ± 0.52</td>
<td>124.0 ± 2.7</td>
<td>735.75 ± 33.08</td>
<td>155.03 ± 11.39</td>
<td>2.06 ± 0.23f</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.89</td>
<td>±0.52</td>
<td></td>
<td>(5)</td>
<td></td>
<td>(5)</td>
</tr>
<tr>
<td>11</td>
<td>9–14</td>
<td>14.00 ± 0.83</td>
<td>12.64 ± 0.68</td>
<td>114.0 ± 3.6</td>
<td>689.82 ± 32.78</td>
<td>173.79 ± 16.5p</td>
<td>2.39 ± 0.27f</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.83</td>
<td>±0.68</td>
<td></td>
<td>(5)</td>
<td></td>
<td>(5)</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
Figure in parentheses is the number of mice if different from that in column 1.
Analysis of variance established significant differences between the groups (progesterone, \(P<0.05\); testosterone, \(P<0.001\)), and pairs of groups were subsequently compared using error estimates from the analysis of variance: a–b, \(P<0.05\); d–e, \(P<0.05\); c–d, \(P<0.01\); d–f, \(P<0.01\); c–e, \(P<0.001\); c–f, \(P<0.001\).
The total amount of chorionic gonadotrophin in all placentae in each of the 13 litters varied directly with the number present (range 1–6) (Text-fig. 3), but the placental CG value was not related to the plasma progesterone concentration in these 13 mice (intercept = 134.46; slope = 2.33; \( r = 0.24 \), \( P > 0.4 \)).

To see whether there was any effect of the total number of CL/mouse, or of conceptuses on the mean CL volume/mouse, a multiple regression was done using the data shown in Text-fig. 4. The

![Graph showing the relationship between the average volume of corpora lutea per mouse and the number of corpora lutea per mouse.](image)

**Text-fig. 4.** The relationship between the average volume of corpora lutea per mouse and the number of corpora lutea per mouse.

**Table 2.** Multiple regression analysis showing the effect of the number of corpora lutea/mouse, and of the number of conceptuses on the mean volume of the corpora lutea/mouse (mm\(^3\)) (y)

<table>
<thead>
<tr>
<th>Term</th>
<th>Estimate</th>
<th>Standard error</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept of line on y axis</td>
<td>0.47765</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effect of number of fetuses (partial regression coefficient ( b_1 ))</td>
<td>0.00726</td>
<td>0.00152</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>Effect of no. of CL/mouse (partial regression coefficient ( b_2 ))</td>
<td>-0.01175</td>
<td>0.00187</td>
<td>( P &lt; 0.001 )</td>
</tr>
</tbody>
</table>
Hormones and fetal and CL number in mice

Text-fig. 5. The relationship between the number of corpora lutea and the mean volume of corpora lutea in each ovary of mice with unilateral implantations. Intercept = \(-0.0053\), slope = \(0.021\), \(r = 0.1747\), \(P > 0.2\).

results (Table 2) show that both factors have a small but significant correlation with mean CL volume/mouse. For example, in a mouse with 7 fetuses and 13 CL we can predict that the mean volume of the CL would be \(0.376 \text{ mm}^3\). If the number of fetuses was increased by 1 the mean volume would be \(0.383 \text{ mm}^3\), an increase of 1-9%. If, instead, the number of CL was increased to 14, the mean volume would be reduced to \(0.364 \text{ mm}^3\), a decrease of 3-1%. The multiple regression analysis was repeated after the data from two mice with 23 and 27 CL (Text-fig. 4) had been excluded, since such large numbers are unusual. The significance levels were only marginally increased, and our conclusions are unaffected.

To see whether there was any correlation between the number and mean volume of CL in each individual ovary of the mouse we plotted the difference in the number of CL on the implanted and unimplanted sides against the corresponding differences in their mean volumes. There was no correlation (Text-fig. 5). There is no evidence that within each mouse the mean CL volume is less in the ovary with the greater number of CL than in the ovary with the smaller number.

Discussion

We have shown that concentrations of plasma progesterone on Day 17 of pregnancy are independent of fetal numbers in our strain of mice when more than 1 fetus is present. In this strain the
gestation period is related to litter size: in 1246 dated mouse pregnancies 60% of litters with one fetus, but < 18% of those with 8 or more fetuses were born after the 20th day of pregnancy (Dewar, 1968). Our results cannot exclude the possibility that a correlation between litter size and progesterone levels existed earlier in pregnancy. By Day 17 progesterone levels in mice with larger litters might have already declined. The period of functional activity of the mouse corpus luteum lasts from the 8th to the 16th day of gestation, i.e. up to 3 days before parturition, and from the 18th day the corpus luteum accumulates fat and gradually shrinks (Deanesly, 1930). Soares & Talamantes (1983) showed that the level of serum progesterone correlated with litter size on Day 15 of pregnancy in C3H mice in which fetuses were destroyed on Day 7 to adjust litter sizes to groups of 1–2, 3–4 or 8–10. However Simon et al. (1978), using Rockland Swiss mice, found that neither the numbers of corpora lutea nor fetuses correlated with levels of plasma progesterone on Day 15 of pregnancy. These differences may be due to the strains of mice used.

It has been suggested that the mouse placenta synthesizes progesterone during the second half of gestation, and that at least part of this progesterone is secreted into the maternal circulation, although the contribution of the placenta to overall progesterone concentration is very low compared with that of the ovary (Pointis, Rao, Latreille, Mignot & Cedard, 1981). In the mouse, removal of the ovaries or corpora lutea leads to the termination of pregnancy (Parkes, 1928; Newton & Lits, 1938). Progesterone and oestrogen are necessary for the maintenance of gestation (Robson, 1938a, b; Jaitly, Robson, Sullivan & Wilson, 1966). Hypophysectomy after the 10th day does not interrupt pregnancy, and the trophic support of the ovary thereafter is dependent upon the presence of the placenta (Mirksaia, 1929; Selye, Collip & Thomson, 1933; Newton & Beck, 1939).

There are two placental hormones which may maintain the production of steroid by the ovary. Mouse placental lactogen is positively correlated with litter size (Markoff & Talamantes, 1981) and may play a part in the maintenance of luteal cell LH receptors as has been suggested in the rat (Gibori & Richards, 1978). The role of endogenous CG in the mouse is not known but in the rat the injection of hCG stimulates the production of both oestradiol and testosterone, although it has no effect on ovarian progesterone secretion. In vitro, hCG activates the enzymes involved in the conversion of progesterone to testosterone (Kalison & Gibori, 1983).

We have shown that the amount of CG is related to the number of fetuses in the litter, and this is undoubtedly a function of total placental mass. The amount of CG per placenta in our strain of mice at Day 17 of gestation is similar to that found by Wide & Wide (1979) in the NMRI strain. The amount of mouse CG is not related to the mean plasma progesterone concentration.

Both placental CG and plasma testosterone concentration increase with increasing litter size, and the CG may stimulate the production of testosterone by the mouse ovary. In the rat the ovaries secrete only a limited amount of testosterone in the second half of pregnancy, when the conceptuses become an important source of testosterone (Sridaran, Basuray & Gibori, 1981), and a similar situation may exist in the mouse.

Bartholomeusz & Bruce (1976) showed that mean corpus luteum weight per rat was negatively related to the number of corpora lutea per rat. We have shown the same for luteal volume in the mouse, and that there is a positive correlation between the number of conceptuses and mean CL volume, suggesting a tropic effect of the conceptuses on the corpora lutea.

In our mice there was no local effect of 1 or 2 fetuses on the weight of the ipsilateral ovary, as was found in the rat on Day 18 of pregnancy (Zambrana & Greenwald, 1971), nor was there any unilateral effect of the number of fetuses on the volume of the corpora lutea. The effect of the conceptuses on the ovaries was therefore systemic.

We thank Dr R. J. Prescott for statistical advice, Mr Christer Bengtsson, Miss Margareta Hoffstedt and Mr I. A. Swanston for technical assistance. L.W. received financial support from the Swedish Medical Research Council (grant 637).
References


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Evidence that morphine interrupts parturition in rats by inhibiting oxytocin secretion

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Morphine interrupts parturition in rats by a central action (Gosden, Humphreys, Johnston, Liddle & Russell, 1985); we have investigated in two experiments whether this action can be attributed to inhibition of oxytocin secretion. On days 14–17 of pregnancy Sprague–Dawley rats (under ether anaesthesia) were fitted with a cannula for injection into a lateral cerebral ventricle (i.c.v.); the rats were observed continuously on the day of parturition. In Expt. 1, immediately after the birth of the second pup, five rats were injected i.c.v. with 5 μl saline and six rats with 18 μg morphine sulphate in 5 μl saline; 40 min later the rats were decapitated, trunk blood was collected and plasma separated and acidified for measurement of oxytocin content by radioimmunoassay (Bicknell, Flint, Leng & Sheldrick, 1982). Mean (± s.e. of mean) oxytocin concentration in the saline-treated group was 39.3 ± 6.52 pg/ml, and in the morphine-treated group 243 ± 39.56 pg/ml (P < 0.005, Wilcoxon); the mean (± s.e. of mean) number of pups born after i.c.v. injection was, respectively, 5.6 ± 1.08 and 0.5 ± 0.22 pups (P = 0.003, Wilcoxon).

In Expt. 2, after birth of their second pups, twelve rats were injected i.c.v. with 18 μg morphine sulphate in 5 μl saline. A cannula in the jugular vein (inserted under ether anaesthesia at the time of the i.c.v. cannulation) was then connected to a 1 ml syringe containing either saline (six rats) or oxytocin in saline (Syntocinon, 0.357 μg/ml; six rats) and driven continuously by a slow infusion pump (Braun, Perfusor IV). Oxytocin was infused initially at 2 μg./min, increased, if necessary, to 5 μg./min after 60 min: the mean total infused (± s.e. of mean) was 448.5 ± 61.9 μg during 144.3 ± 8.2 min. The volume infusion rate of saline in the controls was yoked to that in the oxytocin-treated group. The mean time (± s.e. of mean) to delivery of the 9th pup in the oxytocin-infused group was 118.3 ± 10.65 min (range 97–156 min) and in the saline-infused group 318.8 ± 69.8 min (range 214–659 min), p < 0.002 (Wilcoxon). All of the oxytocin-infused rats completed parturition (mean: 148.5 ± 7.81 min after i.c.v. morphine) before any of the saline-infused rats restarted parturition (mean: 281.8 ± 62.9 min after i.c.v. morphine).

We conclude that i.c.v. morphine interrupts parturition by inhibiting oxytocin secretion.

Dr R. J. Bicknell kindly measured oxytocin concentration in plasma samples. Supported in part by the Sir Stanley and Lady Davidson Medical Research Fund.

REFERENCES

ANIMAL MODELS FOR THE HUMAN MENOPAUSE

CALEB E. FINCH and ROGER G. GOSDEN

Introduction

This review emphasizes phenomena associated with reproductive aging changes in the human female that also occur in laboratory models. The decrease and loss of fertility during midlife is a universal characteristic of aging in the human female, as well as in many shorter-lived mammalian species (Finch, 1976; Harman and Talbert, 1985). Although it is often thought that reproductive aging in female laboratory rodents (Meites, 1982) might be a fundamentally different neuroendocrine process than reproductive aging in women (Guyton, 1981), recent studies suggest that many mammals share similar phenomena of reproductive senescence, in which ovarian aging changes play a key role. We refer to more comprehensive reviews where possible: see Gosden (1985), Krohn (1962), Finch et al. (1980, 1984), Meites (1982), Nelson and Felicio (1985), and Wise (1983). For general information on animal models of aging, see Gibson et al. (1979) and Anonymous (1981).

A controversial issue in comparing aging phenomena of humans and higher primates with laboratory rodents concerns species differences in the relative contributions of the hypothalamus, pituitary, and ovary in initiating the preovulatory surge of gonadotropins and in governing fertility cycle length. A prevalent view holds that rodents differ from higher mammalian spontaneous ovulators in the greater importance for an increased hypothalamic output of gonadotrophin-releasing hormone (GnRH) during the preovulatory gonadotro-
pin surge of rodents (e.g., Fink, 1979; Leadem and Kalra, 1984; Ramirez et al., 1984). Concurrently, the rodent pituitary becomes increasingly responsive to GnRH just before the preovulatory surge (e.g., Fink, 1979; Crowder and Nett, 1984). In primates, the balance of control for the preovulatory surge appears to reside more at the ovarian and pituitary level, where elevations of E2 (estradiol) greatly increase the sensitivity of the gonadotropes to GnRH; only a permissive role of the hypothalamus for continued pulsatile release of GnRH is hypothesized (Knobil, 1980; Ramirez et al., 1984). The frequency of GnRH and gonadotropin pulses, however, has profound influences on the recruitment and development of ovarian follicles and or their secretion (Pohl et al., 1983).

Age Changes in Estrous Cycles

Cycle Irregularity

Women. The monumental longitudinal studies of Alan Treloar and associates, which now span two generations (Treloar et al., 1967; Treloar, 1974, 1981), suggest that menstrual histories can be divided into three phases according to cycle length and variability (Figure 1B). During phase I (first 5-10 years after menarche) the variability of cycle length gradually declines. Regular cycles prevail during phase II, and cycle variability decreases further during these 15-20 years. In phase III, the variability of cycles increases sharply during the approach to menopause; phase III includes the perimenopause of another analysis (Metcalf et al., 1981a). Both abnormally short and long cycles occur; yet, normal ovulation can occur within 6 months of menopause (Metcalf et al., 1981a). Because menarche and menopause can occur over a wide range of ages, the demarcation of phases can not be assigned by age alone and requires longitudinal analysis. These and other studies (Metcalf et al., 1981b) document the great extent of individual variations in menstrual cycle length and stability throughout life, and the variable ages of menarche and menopause. Although no analysis has predicted the detailed characteristics of later phases from earlier phases, long menopausal transitions are associated with slower increases of variability (Treloar, 1981).

Rodents. Phases of reproductive aging and variations similar to those observed in women were found in longitudinal records of estrous cycles in C57BL/6J mice, as determined from daily vaginal smear patterns in this laboratory (Nelson et al., 1982, Felicio et al., 1984) (Figures 1A and 2; Table I). The extent of variability in this highly inbred strain is largely unexplained, but may be due in part to intrauterine influences, such as effects from the sex of the fetal neighbors in utero (e.g., vom Saal et al., 1981; Meisel and Ward, 1981). These effects involve a puzzling hormonal interaction between fetuses of different genders. There is no evidence for exchange of placental blood between neighboring fetuses, each of which normally has an autonomous circulatory system;
Figure 1. Comparison of age changes in menstrual cycles of C57BL/6J mice (A) and estrous cycles (vaginal smears) of human (B) from longitudinal studies. Redrawn from Nelson et al. (1982) (top) and Treloar et al. (1967) (bottom).
Figure 2. Individual longitudinal records of daily vaginal smears from C57BL/6J mice (same cohort). The elevations represent relative extents of leukocytes or cornified epithelial cells in the vaginal smear. Adapted from Finch et al. (1980).

placental fusion is rare in mice and anastomosis of feto-placental vessels is rare still (vom Saal, 1983). Detection of such epigenetic effects is greatly facilitated by use of inbred rodents. The fetal neighbor effect now appears to extend beyond young adult characteristics to influence aging, since female fetuses flanked

Table I
Types of Variations in Ovulatory Cycles during Aging

<table>
<thead>
<tr>
<th></th>
<th>Humans</th>
<th>Rats and mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Shortening</td>
<td>(20–25 days) Commonly due to shortened follicular phase</td>
<td>I. Shortening: Not observed; no cycles &lt; 4 days</td>
</tr>
<tr>
<td>II. Lengthening</td>
<td>(35–100 days) Often anovulatory; commonly due to lengthened or defective luteal phase with elevated LH and FSH, but reduced or negligible progesterone</td>
<td>II. Lengthening: (6–12 days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. Strains with early onset of acyclicity (before 14 months; see Table II) commonly have an extended follicular phase, with extra days of proestrus and estrus (cornified vaginal cytology)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B. Strains with later onset of acyclicity (after 10 months; see Table II) commonly have extra days of diestrus (leukocytic vaginal cytology, but distinct from pseudopregnancy)</td>
</tr>
</tbody>
</table>
in utero by males have an earlier loss of fertility than female-flanked fetuses (vom Saal and Moyer, 1985).

As observed in women, the cycle length variations observed during the approach to acyclicity in mice include interspersed normal short (4 days) and longer cycles. However, unlike humans, very short ovulatory cycles are not reported in rats or mice, during aging or in the young; 4 days is the observed lower limit of fertility cycle length in Myomorphs, and this is the most frequent cycle length in the young under optimum conditions (Hoffmann, 1973). During aging in C57BL/6J mice, repetitive pseudopregnancy is uncommon (Nelson et al., 1982; Mobbs et al., 1984a); however, repetitive pseudopregnancy is quite common in aging rats, as discussed below.

Mouse strains differ in their age-related patterns of cyclicity (Table II) (Thung et al., 1956; Jones and Krohn, 1961a; Finch, 1978; Nelson et al., 1982); the CBA family of strains are noteworthy for a relatively early loss of fertility and cyclicity (Jones and Krohn, 1961a), which is attributed to precocious exhaustion of ovarian oocytes (see below). Although studies on reproductive aging in widely used rat models (Fischer 344; Sprague–Dawley; Long–Evans) do not suggest major genotypic differences, comparisons from the literature are easily confounded by environmental variables; detection of genotypic influences requires concurrent study in the same environment.

Hamsters. In contrast to rats and mice, the estrous cycles of the golden hamster, *Mesocricetus auratus*, are remarkably regular at most ages under optimal photoperiods. Researchers often comment that most aging hamsters do not have the lengthening of cycles usually seen in mice and rats and may not become acyclic until just before death, when pathologic lesions are very common. In one study, most 19- to 22-month-old hamsters continued to have 4-day cycles and only 10–20% had irregular cycles (Chen, 1981; Blaha, 1967); only exceptional (< 10%) mice or rats when 20 months old would continue to have 4-day cycles. Such species differences in effects of aging are not surprising in view of the major species differences between myomorphs in sex dimorphisms of hypothalamic neuroanatomy (Bleier et al., 1982) and in the absence of E₂-induced daily luteinizing hormone (LH) surges in ovariectomized, young female mice (Gee et al., 1984), whereas daily LH surges are readily induced by E₂ in ovariectomized rats and hamsters.

Gonadotropins and Steroids

Women. Both longitudinal and cross-sectional studies of women approaching menopause document a complicated range of endocrine states that parallel the variations of cycle lengthening. Decreasing cycle length can occur
Table II
Prolonged Cycles and Acyclic in Different Strains of Mouse and Rat Ranked by Age: Incidence and Vaginal Cytological Status

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Age at onset of acyclicity (month)</th>
<th>Prolonged cycles</th>
<th>Vaginal cytology of initial postcyclic state</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>DA</td>
<td>6-7</td>
<td>Yes</td>
<td></td>
<td>Everett (1939)</td>
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<tr>
<td>Rat</td>
<td>Sprague-Dawley</td>
<td>6-8</td>
<td>Yes</td>
<td></td>
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</tr>
<tr>
<td>Rat</td>
<td>R×UF1</td>
<td>8-10</td>
<td>No</td>
<td></td>
<td>Van der Schoot (1976)</td>
</tr>
<tr>
<td>Rat</td>
<td>Long Evans</td>
<td>10-11</td>
<td>No</td>
<td></td>
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</tr>
<tr>
<td>Rat</td>
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<td>12</td>
<td>Yes</td>
<td></td>
<td>R. Gosden (unpublished)</td>
</tr>
<tr>
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<td></td>
<td>Yes</td>
<td></td>
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</tr>
<tr>
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<td>Wistar</td>
<td>12-15</td>
<td>Yes</td>
<td></td>
<td>Butcher and Page (1981)</td>
</tr>
<tr>
<td>Mouse</td>
<td>C57BL/6J</td>
<td>13-16</td>
<td>Yes</td>
<td>×</td>
<td>Aschheim (1976); Nelson et al. (1981); Felicio et al. (1984)</td>
</tr>
<tr>
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<td>Long Evans</td>
<td>14</td>
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<td></td>
<td>Wilkes et al. (1979)</td>
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<tr>
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<td>14-15</td>
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<td></td>
<td>Lu et al. (1979)</td>
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<td>(DBA×CE)F1</td>
<td>12-19</td>
<td>Yes</td>
<td></td>
<td>Dickie et al. (1957)</td>
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<td>DBAf</td>
<td>15</td>
<td>Yes</td>
<td></td>
<td>Thung et al. (1956)</td>
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<tr>
<td>Rat</td>
<td>Fischer 344</td>
<td>16-18</td>
<td></td>
<td></td>
<td>Nelson, et al. (unpublished)</td>
</tr>
<tr>
<td>Mouse</td>
<td>C57BL/6Nnia</td>
<td>16-20</td>
<td>Yes</td>
<td>×</td>
<td>Parkening et al. (1980)</td>
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<td>Mouse</td>
<td>C3HfC57b/Se</td>
<td>&gt;17</td>
<td>Yes</td>
<td></td>
<td>Caschera (1959)</td>
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<tr>
<td>Rat</td>
<td>Wistar</td>
<td>18</td>
<td></td>
<td></td>
<td>J. Clemens (personal communication)</td>
</tr>
<tr>
<td>Mouse</td>
<td>RIII/Dm/Se</td>
<td>&lt;20</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Mouse</td>
<td>C57BL</td>
<td>20</td>
<td>Yes</td>
<td></td>
<td>Thung et al. (1956)</td>
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<tr>
<td>Mouse</td>
<td>020</td>
<td>20</td>
<td>Yes</td>
<td></td>
<td>Thung et al. (1956)</td>
</tr>
<tr>
<td>Mouse</td>
<td>(020×DBA)F1</td>
<td>24</td>
<td>Yes</td>
<td></td>
<td>Thung et al. (1956)</td>
</tr>
</tbody>
</table>

*From Nelson et al. (1981) and Felicio et al. (1984).*
because of a curtailed follicular phase, in association with decreased plasma E\textsubscript{2} and increased follicle-stimulating hormone (FSH). Nonetheless, blood levels of LH and progesterone (P) and the duration of the luteal phase in shortened cycles may be normal and ovulation may still occur (Sherman and Korenman, 1975; Sherman et al., 1976; Metcalf et al., 1981).

There is a general trend for elevated FSH long before cycles cease; FSH rises to the postmenopausal range in the last menstrual cycles of many middle-aged women (Metcalf et al., 1981a); this is consistent with the hypothesis that circulating levels of the putative hormone folliculostatin decrease as the pool of growing follicles shrinks (Sherman et al., 1976). Prolonged cycles, lasting 50 days or more, are often anovulatory and associated with high plasma LH and FSH, but with low E\textsubscript{2} and P (Sherman and Korenman, 1975; Sherman et al., 1976; Metcalf et al., 1981b). Follicular maturation appears to continue even with elevated gonadotropins (Metcalf et al., 1981b). The next cycle may be of normal length, with apparent reinstatement of follicular growth (Sherman and Korenman, 1975). The LH surge in climacteric women has not been analyzed in detail, but sufficient LH is clearly secreted for ovulation (Sherman and Korenman, 1975). Even after menopause, normal surges of LH and FSH can be induced by E\textsubscript{2} and P (Odell and Swerdloff, 1968). In contrast, the induced LH surges of aging rodents can be markedly impaired.

**Primates.** Endocrine changes in aging chimpanzees (*Pan troglodytes*, Pongidae) and rhesus monkeys (*Macaca mulatta*, Circopitidae) demonstrate clear similarities to menopause, including increased cycle length and variability, decreased circulating E\textsubscript{2}, and increased FSH and LH (Gould et al., 1981; Dierschke et al., 1983). Ovulation in rhesus monkeys ceases by about 31 years (Collins et al., 1983) and probably does so after 40 years in chimpanzees (Gould et al., 1981).

**Rodents.** Aging mice and rats can show a truncated LH surge on proestrus of a 4-day cycle (Gray et al., 1980; Miller and Riegle, 1980; Flurkey et al., 1982; Cooper et al., 1983; see Nass et al., 1984, for an exception). Reduced proestrus surges of LH and P also occur during lengthened cycles and may indicate a reduced sensitivity of the hypothalamus–pituitary unit to E\textsubscript{2} (Wise, 1983; Mobbs et al., 1984b).

Effects of aging on plasma E\textsubscript{2} suggest two different phenomena: aging rats with prolonged cycles tend to have additional days of elevated (proestrouslike) plasma E\textsubscript{2} before ovulation (e.g., Page and Butcher, 1982; Nass et al., 1984). However, in C57BL/6J mice the lengthened cycles are associated with a slower rise of plasma E\textsubscript{2} (Figure 3). The slower rise of E\textsubscript{2} with lengthening cycles in C57BL/6J mice might be a model for the lengthened follicular phase in some cycles of perimenopausal women (Sherman and Korenman, 1975).
Pregnancy and Aging

Fertility

Women. Although decreased fertility of women with increasing age is widely acknowledged, confounding results from reduced coital frequency or male infertility are difficult to resolve. However, a French study clearly documented reduced fertility with age (live births) in a population of 2193 women, whose husbands were totally sterile and who received artificial insemination with donor semen (Federation CECOS, 1982). Although the causes of decreased fertility are complex, the frequency of ovulation probably does not change enough before 45 years to account for the loss of fertility, since ovulatory cycles continue to occur until just before menopause (Sherman and Korenman, 1975). Thus, the decreased fertility with age can be hypothesized to result in large part from defective oocytes or from uterine deficiencies, as found in aging rodents.

Primates. Menopause in laboratory primates is probably also preceded by declining fertility, as indicated by limited data on rhesus monkeys (van Wageningen, 1972) and chimpanzees (Flint, 1976).

Rodents. The decline of fertility with age is documented in great detail for laboratory rodents (Harman and Talbert, 1985). The initial stages of procreation seem relatively intact, including the normal incidence of insemination even in age groups with a big loss of fertility (Holinka and Finch, 1981). As is
the case of humans, ovulatory cycles yielding normal or close-to-normal clutches of eggs continue until just before cycle cessation, despite the imminent exhaustion of the ovary at the onset of cyclicity in several mouse strains (e.g., Jones and Krohn, 1961a; Gosden et al., 1983a). However, rats may have larger follicular reserves at acyclicity (Sopelak and Butcher, 1982a; Mandl and Zuckerman, 1951). Many studies show a sharp decline in viable embryos, shortly after fertilization. Depending upon maternal age, there can be a major sevenfold decrease in the number of implantation sites just after implantation, as well as increases of resorption in established implantation sites in 13- to 16-month-old hamsters (Connors et al., 1972). Alternatively, in 11- to 12-month-old C57BL/6J mice the number of implantation sites is only slightly reduced from normal, whereas the fetal resorption increases by twofold (Holinka et al., 1979a). Defective ova are generally considered to be the major factor in declining fertility of rodents and may be a consequence of delayed ovulation in association with lengthened cycles (Page and Butcher, 1982; Fugo and Butcher, 1971). There is also a large literature documenting uterine age-related impairments in the experimentally-induced decidual response of virgin and multiparous mice (Holinka and Finch, 1977; Finch and Holinka, 1982). Although uterine growth during implantation in old mice is not obviously impaired (Finch and Holinka, 1982), uterine scars from implantation can accumulate during successive pregnancies and may limit the number of implantations that the uterus can sustain (Gosden, 1979). A major candidate for decreased embryo viability with maternal age in mice seems to be defective ova and fetal aneuploidy (see below).

Another source of reduced fertility in aging rodents is the prolongation of gestation, or delay of parturition, as observed in 12-month-old C57BL/6J mice, which have average delays of 2 days (range 0–4 days) (Holinka et al., 1978). This delayed parturition is associated with a slower preparturitional decrease of circulatory progesterone (Holinka et al., 1978, 1979a), slower elevations of circulating estradiol (Holinka et al., 1979b), and a great increase of stillbirths, which are rare in younger mothers (Holinka et al., 1978). The prolonged gestation was not a consequence of delayed implantation in the older mice or of retarded growth of the fetus (Holinka et al., 1979a). However, about 50% of fetuses in 12-month-old mice lagged by one day before optic cups were visible in the gross (Holinka et al., 1979a). Detailed studies are needed to characterize the factors in the fetal mortality as a function of developmental stage.

In contrast to rodents, the incidence of prolonged pregnancy in humans (>45 weeks) decreases with maternal age (Beischer et al., 1969a). Nonetheless, the aging rodent may serve as a useful model for the fetal distress syndrome observed in labor after prolonged gestation (Beischer et al., 1969b) and the greater incidence of postterm neurologic problems in the neonate (Field et al., 1977).
Birth Defects

Increases of birth defects with maternal age in humans result predominantly from chromosomal aneuploidy, such as Down’s syndrome (trisomy of chromosome 21). However, nonchromosomal developmental anomalies also occur. The frequency of clinically significant chromosomal abnormalities among newborn infants in the U.S. population rises from 0.2% for mothers younger than 30 years old to 5.4% at 45 years (Hook, 1981; see also Chapter 8 by Hook in this volume). The age distribution of several types of aneuploidies is J-shaped, with a steeply ascending limb above age 30; similar patterns occur in other human populations (Ferguson-Smith, 1983).

The incidence of aneuploidy at birth is a small proportion of those conceived. Among survivors the trisomies are numerically dominant. Monosomies are far less frequent at full term than trisomies. Studies of mice show that deletion of an autosome leads to embryonic death at an earlier stage in gestation than when the same chromosome is overrepresented in trisomic karyotypes (Gropp, 1976). The viability of conceptuses with aneuploidy of the sex chromosomes is greater than for the autosomes, although aneuploidy always confers some phenotypic disturbances. In contrast to 47-XXX and 47-XXY conceptuses, the proportion of 45-XO fetuses surviving to term is diminished and does not show the expected rise with maternal age (Court Brown et al., 1969; Hook, 1981). In the future, human oocytes obtained from in vitro fertilization programs may help research on the etiology of aneuploidy, which has hitherto been confined to epidemiological studies in man. Nevertheless, animal models will continue to have a major role in the study of these phenomena, despite the lower incidence of fetal aneuploidy at most maternal ages (Bond and Chandley, 1983).

Maternal age-dependent aneuploidy is better documented in mice than in any other species (Table III). The variable estimates of age-specific frequency partly reflect technical difficulties and small sample sizes. The genotype also influences the susceptibility to aneuploidy (Fabricant and Schneider, 1978; Kram and Schneider, 1978). The higher incidence of aneuploidy among older mice (Fabricant and Schneider, 1978; Maudlin and Fraser, 1978; Tease, 1982) contradicts the hypothesis proposed for our own species that selection against defective fetuses becomes less stringent during middle age because of a compromising uterine environment. Indeed, competition by growing fetuses may even increase under these conditions, leading to differential elimination of aneuploidies (Parsons, 1964); this opposing hypothesis can now be tested by constructing mouse embryos with aneuploid karyotypes (Gropp et al., 1974) with transfer to hosts of different ages.

A number of hypotheses for the age-related increase of aneuploid embryos are summarized (Table IV). Trisomy was once widely thought to arise from an accumulation of accidental damage to the cellular apparatus responsible for the meiotic spindle during the prolonged diplotene stage. However, there is no par-
Table III
Variation in the Incidence of Aneuploid Embryos in Mice According to Maternal Age and Strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age of embryo (days postcoitum)</th>
<th>Percent aneuploidy (maternal age, months)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/Ca</td>
<td>4</td>
<td>5.3% (2-6); 12.1% (8-9); 19.6% (10-12)</td>
<td>Brook et al. (1984)</td>
</tr>
<tr>
<td>CBA</td>
<td>10-14</td>
<td>0% (2-5); 13.6% (7-10); 0% (&gt;10)</td>
<td>Fabricant and Schneider (1978)</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>10-14</td>
<td>0% (2-10)</td>
<td>Fabricant and Schneider (1978)</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>10-14</td>
<td>0% (2-5); 9.0% (7-10); 5.5% (&lt;10)</td>
<td>Fabricant and Schneider (1978)</td>
</tr>
<tr>
<td>CH/H3J</td>
<td>10-14</td>
<td>0% (2-10); 8.0% (&lt;10)</td>
<td>Fabricant and Schneider (1978)</td>
</tr>
<tr>
<td>CBA/H-T6</td>
<td>4</td>
<td>0% (2-7); 21.0% (8-12)</td>
<td>Gosden (1973)</td>
</tr>
<tr>
<td>TO</td>
<td>1 (fertilized in vitro)</td>
<td>3.3% (2-4); 7.5% (8-10)</td>
<td>Maudlin and Fraser (1978)</td>
</tr>
<tr>
<td>CBA</td>
<td>9.5-12.5</td>
<td>0% (2-6); 7.4% (&gt;10)</td>
<td>Max (1977)</td>
</tr>
<tr>
<td>F</td>
<td>9-10</td>
<td>0.7% (1.5-2); 1.2% (9-12)</td>
<td>Speed and Chandley (1981)</td>
</tr>
<tr>
<td>H</td>
<td>1</td>
<td>0.4% (2-4); 3.1% (11-13)</td>
<td>Tease (1982)</td>
</tr>
<tr>
<td>CF</td>
<td>10.5</td>
<td>1.3% (3-5); 4.3% (11-13); 12.8% (14-16)</td>
<td>Yamamoto et al. (1973)</td>
</tr>
</tbody>
</table>

*Cases where reliable estimates of monosomy could not be obtained, the incidence of aneuploidy was estimated by doubling values for trisomy. This procedure assumes that univalent or nondisjoined chromosomes migrate randomly to either pole of the meiotic spindle.*

Moreover, aging oocytes are not hypersensitive to mutagens, as predicted by this hypothesis (Speed and Chandley, 1981; Tease, 1982; Golbus, 1983). Although environmental factors may account for temporal and geographical variations in trisomy (Collman and Stoller, 1962; Evans et al., 1978), many suspect that physiological (hormonal) changes underlie the age effect.

One hypothesis proposes that a proportion of oocytes are doomed to chromosomal nondisjunction as a result of prenatal factors during oogenesis. This is based on the inverse relationship between chiasma frequency and age in mouse oocytes at metaphase I and the correspondingly higher incidence of univalent

Table IV
Factors Proposed to Increase Risk of Aneuploidy among Older Mothers

1. Oocytes shed late in life, with defective chiasmata relationships determined during oogenesis
2. Persistent nucleoli associated with acrocentric chromosomes
3. Overripeness of oocytes; delayed ovulation
4. Physiological changes of the aging maternal environment
5. Relaxed selection in utero against aneuploid fetuses
6. Environmental agents (ionizing radiation, mutagenic chemicals, viruses)
7. Autoimmune changes
pairs that might segregate randomly at the ensuing anaphase (Henderson and Edwards, 1968; Luthardt et al., 1973; Polani and Jagiello, 1976; Speed, 1977). Henderson and Edwards (1968) postulated that, since chiasmata are formed prenatally, a "production line" might exist in which the first oocytes to be formed are the first ovulated. However, a gradient of anomalies is not obvious in cytological studies of oogenesis (Speed and Chandley, 1983), nor is there a correlation between chromosome groups responsible for univalents and those involved in nondisjunction at the following division (Sugawara and Mikamo, 1983).

Alternatively, aneuploidy may not be predetermined during oogenesis, but may arise from physiological age changes in follicular oocytes involving changes in the timing of hormone-dependent steps (Butcher, 1975; Crowley et al., 1979). Such hypotheses are consistent with data in humans (Jacobs and Hassold, 1980; Mikkelsen et al., 1980) and mice (De Boer and van der Hoeven, 1980) showing that aneuploidy arises chiefly, but not exclusively, during the first meiotic division of the egg, i.e., before ovulation. It has been suggested that lengthening of the follicular phase of the human menstrual cycle is a risk factor for pregnancy loss and birth defects (Hertig, 1967). While major practical obstacles prevent rigorous testing for hypothetical effects of follicular overripening in humans, rodent oocytes can be obtained readily from Graafian follicles after delaying ovulation by blocking the gonadotropin surge with barbiturates (Butcher, 1975) or with an antiserum to GnRH (Laing et al., 1984). Such treatments significantly increase the risk of triploid or mosaic karyotypes, but effects on the incidence of meiotic nondisjunction are still unresolved. Delayed fertilization has also been proposed as a factor in maternal age-related birth defects (German, 1968), but appear to be ruled out by evidence mentioned above, showing that nondisjunction mainly occurs during meiosis I. The risk of aneuploidy may also be affected by physiological age changes of the ovarian-hypophyseal axis. Removal of one ovary early in adulthood precociously increases the incidence of morphologically abnormal and aneuploid embryos, in association with an earlier onset of irregular cycles and acyclicity (Sopelak and Butcher, 1982b; Brook et al., 1984). The explanation of these findings is still unclear, because high levels of gonadotropins may not account for aneuploidy (Hansmann and El-Nahass, 1979). However, such results may explain why mouse strains that lose oocytes precociously (e.g., CBA) also have a high incidence of trisomy and why the incidence of trisomic offspring is much higher than expected in Turner's syndrome patients, who sometimes are fecund (but rarely fertile) before their unusually early menopause (Reyes et al., 1976; King et al., 1978).

Two other hypotheses concerning autoimmune changes and persistent nucleoli (Table IV) are not well supported. Persistent nucleoli associated with chromosomal bivalents could increase the risk of nondisjunction, but this would
not explain why most (perhaps all) chromosomes can be responsible for aneu-
pyloidy. Claims that autoimmune processes heighten the risk of aneuploidy were
not supported by animal experiments (reviewed in Kram and Schneider, 1978).

Ovarian Aging: Rodents, Primates, and Humans

Follicular Attrition

Since oogenesis occurs exclusively at pre- or perinatal stages in most mam-
mals, ovarian follicles are lost irreversibly. The follicular store in each newborn
infant must serve the needs of reproduction throughout life, and numbers about
10,000 in mice (Jones and Krohn, 1961a) and 2 million in women (Block, 1953;
Baker, 1963). Most oocytes are wasted; less than 0.1% are ovulated in humans.
Age changes in the follicular store during the adult lifespan of women and mice
are very similar when scaled to the lifespan (Figure 4). There is relatively little
direct data on the follicle store at menopause (Nelson and Felicio, 1985). How-

![Graph of oocyte loss as a function of age in A strain mice (top) and humans (bottom). Redrawn from Jones and Krohn (1961a) (top) and Block (1952) (bottom).]
ever, some studies suggest that sporadic follicular growth and formation of corpora lutea continue after menopause (Novak, 1970; Costoff and Mahesh, 1975).

The combined processes of recruitment into the growing population and death ("atresia") account for the steady loss of the primordial follicle store, which asymptotically approaches exhaustion in late midlife in most mammals. The rate of this decline therefore imposes an upper limit on the functional lifespan of the ovary that can be quantitatively modeled (Nelson and Felicio, 1985). In immature ovaries, more follicles die each day than are recruited, but this situation reverses after puberty in most mouse strains (Faddy et al., 1983). CBA mice become sterile by about one year because primordial follicles continue to die in relatively large numbers at adult ages. In CBA mice, as in C57Bl/6J, few follicles remain after the last ovarian cycle (Gosden et al., 1983a), but oocyte depletion occurs earlier in CBA mice compared to most strains (Papadaki et al., 1979; Faddy et al., 1983). Thus several mouse strains provide models for the condition found in human and some subhuman primate ovaries after menopause (Costoff and Mahesh, 1975; Graham et al., 1979; Gould et al., 1981).

Primates can now be added to the list of mammals whose ovary is essentially depleted of follicles during aging, as shown in rhesus monkeys (Collins et al., 1983; Dierschke et al., 1983) and chimpanzees (Gould et al., 1981).

Although some follicles may be eliminated because of defects that arise during meiosis or from mutations, the excess of potential germ cells at young ages may serve the reproductive strategy of providing flexible ovarian responses (see below). While this strategy is widespread in nature, some species have different patterns in the timing of elimination of excess germ cells. A striking example of species differences in the plains viscacha (Lagostomus maximus). This hystricomorph rodent sheds 200–800 ova at each estrus, while many others fail to be released from luteinizing follicles (Weir, 1971); yet of this relatively huge clutch of ova, only seven are fertilized and only two of these survive to full term. Probably, premature ovarian failure is avoided in this species by shifting germ cell elimination from the primordial to secondary oocyte stage.

The importance of the follicle store in limiting the numbers of ovarian cycles is evident from clinical case histories of precocious menopause, in which biopsies show that the ovaries are frequently devoid of follicles. Early ovarian exhaustion leads to significant sexual and metabolic disorders, which vary in severity according to age. Precocious menopause occurs sporadically in the population and for a variety of reasons (Table V), but it is difficult to study. Therefore, suitable animal models should be sought. Precocious menopause may be genetically inherited from either parent (Mattison et al., 1984), but it is not clear whether this is due to a smaller endowment of oocytes, as occurs in some mutant mice (Mintz and Russell, 1957; McCoshen, 1982), or a higher rate of postnatal follicular death, as in CBA mice (Faddy et al., 1983).
Table V

<table>
<thead>
<tr>
<th>Etiologies of Precocious Ovarian Failure in Women (Precocious Menopause)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Genetic predisposition with diploid or aneuploid karyotypes</td>
</tr>
<tr>
<td>2. Autoimmune destruction of ovarian tissues</td>
</tr>
<tr>
<td>3. Other disease effects, e.g., tuberculosis, mumps, oophoritis, metabolic disorders (17-α-hydroxylase deficiency)</td>
</tr>
<tr>
<td>4. Cytotoxic drugs</td>
</tr>
<tr>
<td>5. Ionizing radiation</td>
</tr>
<tr>
<td>6. Partial oophorectomy</td>
</tr>
</tbody>
</table>

Aneuploid karyotypes are a common cause of germ cell hypoplasia and streak gonads in humans, e.g., in Turner’s syndrome (McDonough et al., 1977). Further understanding of germ cell hypoplasia might be gained from XO mice; although these animals are more fertile than XO women, they have fewer oocytes and earlier loss of fertility (Burgoyne and Baker, 1981). Premature ovarian failure often has an immunological basis and may be associated with hypoplasia of the thymus gland, as in ataxia telangiectasia (Miller and Chatten, 1967), or with autoimmune disorders, particularly Addison’s disease (Tulandi and Kinch, 1981). The importance of thymic-ovarian interactions during ovarian development in neonates is clearly demonstrated in mice (Michael et al., 1981). Circulating antibodies against gonadotropin receptors occasionally are found but are not common (Tang and Faimen, 1983). Ovaries, like many other glands, can be damaged by mumps virus, but mumps oophoritis is considered rare (Morrison et al., 1975). Nonetheless, a recent epidemiologic survey suggests a slightly earlier menopause (not significant) with a strong inverse correlation of age at menarche and menopause in those with childhood mumps (Cramer et al., 1983). Besides these effects of disease, the ovaries can be damaged by a wide range of toxic environmental agents, including mutagens present in industrial pollution and tobacco smoke (Mattison and Thorgeirsson, 1978) and ionizing radiations (Baker, 1971). It is potentially important in this regard that menopause may occur earlier in cigarette smokers (Jick et al., 1977). These agents can usefully be tested on rodent ovaries, providing that attention is paid to species differences in metabolism and timing of developmental stages of oocytes.

Follicular Kinetics

Ovarian follicular growth continues at most ages or physiological conditions throughout most of postnatal life, even after the loss of cycles in mice with persistent vaginal cornification (Gosden et al., 1983b). Primordial follicles enter the growing pool at a rate that is approximately constant at all ages; subsequent follicular development is interrupted only by atresia or ovu-
lation. The rate of outflow from the primordial store is independent of the size of the Graafian population and does not depend on ovarian or pituitary hormones, though hypophysectomy retards it (Jones and Krohn, 1961b; Faddy et al., 1977). Thus, small follicles are not conserved by repeated pregnancy and lactation (Shelton, 1959; Jones and Krohn, 1961a). These general characteristics of mammalian ovaries have far-reaching implications for ovarian function in middle age, because they imply that the numbers of follicles available for ovulation reflect the numbers of follicles beginning to grow a few weeks earlier and, hence are limited by the remaining follicular store. The number of potential follicles recruited substantially exceeds the ovulation quota needed in the young and provides the possibility of rapidly increasing the ovulation rate if the contralateral ovary fails or is removed, or in response to supplementary gonadotropins. However, there is a critical size of the follicular pool at which all available follicles are being recruited; from this time onward superovulation is not possible (Peppier, 1971; Gosden, 1985). Further attrition of follicles is responsible for a lowering of the numbers of ova that are shed at estrus just before acyclicity; thus, irregular cycles at this time may reflect the stochastic nature of follicular growth initiation. Some mice become acyclical with several hundred follicles remaining, whereas other continue to produce progressively smaller clutches at increasingly irregular intervals until monovular cycles are produced: this suggests that several different factors, including neuroendocrine, can cause acyclicity in inbred mice (Gosden et al., 1983a; Nelson and Felicio, 1985). Nevertheless, the functional lifespan of the ovary is clearly determined by the follicular stores rather than by chronologic age per se, whether or not there is a prolonged period of anovulatory cycles with vaginal cornification.

The growth kinetics of preantral and small antral follicles appear to follow similar patterns in rodents and women, though the transit time between successive stages are much longer in the latter (Pedersen, 1972; Gougeon, 1982). In these species, the ratio of large/small follicles rises during aging (Block, 1952; Gosden et al., 1983a). Therefore, since the follicular growth and transit times are similar at all adult ages (Pedersen, 1972; Gosden et al., 1983b), the survivorship of follicles must be increased to maintain ovulatory constancy as the follicular store dwindles. This hypothesis accounts for the smaller proportion of atretic follicles in aged ovaries by rescue through FSH. However, the ovulation rate is only approximately constant throughout life and can vary considerably. In man, the frequency of dizygotic twinning (and presumably double ovulations) rises with parity and age (Bulmer, 1970); rodents have a corresponding rise with parity (Kennedy and Kennedy, 1972). Since these changes are opposed by the dwindling numbers of follicles available for recruitment, extrinsic factors must be responsible. These phenomena could involve subtle changes in the output of GnRH by the hypothalamus and/or changes in gonadotrope responsiveness. Further investigation is required.
ANIMAL MODELS FOR THE HUMAN MENOPAUSE

Postcycling States

Humans

The predominant characteristics of menopause are major decreases of blood $E_2$ and $P$ with elevations of LH and FSH to castrate levels (Sherman et al., 1976). Similar endocrine states eventually occur at advanced ages in some laboratory rodent strains described below.

Rodents

Persistent Vaginal Cornification. The most common initial acyclic state in aging laboratory rats and mice is associated with the anovulatory, polyfollicular ovary, which secretes moderate amounts of $E_2$, at about the average blood levels in cycling young rodents (Lu et al., 1979; Felicio et al., 1980). The vaginal cytology characteristically has cornified epithelia (Table II), with thick vaginal smears, and is variously known as persistent vaginal cornification, persistent estrus, or constant estrus. Blood LH, progesterone, and 20-α-hydroxyprogesterone are usually low, whereas FSH may be moderately elevated (Huang et al., 1976; Lu et al., 1979; Flurkey et al., 1982; Nelson et al., 1982). Blood testosterone and androstenedione are usually low (Lu et al., 1979). A characteristic of this state of some strains is the sustained (but modest) elevations of circulating $E$ and low $P$. Unopposed estrogenic stimulation often causes a stromal and cystic glandular hyperplasia of the uterus (Christy et al., 1951; Dickie et al., 1957; Malinin and Malinin, 1972; Cosgrove et al., 1978). This glandular hyperplasia may be similar in some regards to the endometrial hyperplasia that is common in premenopausal women, which is also a feature of the dysfunctional uterine bleeding (DUB) syndrome (Schroder, 1954; Fraser and Baird, 1974; Van Look et al., 1977). The DUB syndrome in humans may be associated with elevated levels of FSH and LH, but its major characteristic is the sustained stimulation of estrogen target cells by moderately elevated $E_2$ and low $P$, as in the rodent syndrome. The rodent syndromes of persistent vaginal cornification should be distinguished from the polycystic ovarian syndrome (the Stein–Levinthal syndrome) of humans, since, in the latter, large pulses of LH are released without much FSH; also, in particular contrast to the aging rodent, androgens are commonly elevated in the human disorder (Goldzieher, 1981). During persistent vaginal cornification in rats, testosterone and androstenedione are at average values for young cycling rats; however, androgens can become elevated in very old rats with enlarged and hemorrhagic pituitaries (Lu et al., 1979).

Repetitive Pseudopregnancy. Particularly in laboratory rats, persistent vaginal cornification is followed by strings of pseudopregnancies with marked
elevations of circulating prolactin and P (Lu et al., 1979; Huang et al., 1976; Aschheim, 1976; Everett, 1980). Pseudopregnancy is much rarer in normal aging C57BL/6J mice, but it appears if middle aged mice are given young ovary transplants (Mobbs et al., 1984b). A major cause is the prevalence of pituitary tumors (lactotrope adenomas) in aging rats (reviewed in Duchen and Schurr, 1976; Clayton et al., 1984), which can cause hyperprolactinemia (Huang et al., 1976) and which may stimulate the corpora lutea of the ovary and the mammary glands, leading to galactorrhea and tumors in hyperprolactinemic rats. Suppression of prolactin by bromocriptine in repetitively pseudopregnant rats can return them to persistent vaginal cornification (Everett, 1980). There is no clear analogue of these phenomena during menopause in most women, since prolactin levels generally remain low (Yamaji et al., 1976; Govoni et al., 1983). However, slight elevations were observed in normal 70-year-old women in one population (Govoni et al., 1983) and could imply disturbed neuroendocrine control of prolactin during aging, for which there is substantial evidence in aging rats (Gudelsky et al., 1981; Reymond and Porter, 1981). It is of interest that hypothalamic defects are implicated in some prolactin secreting tumors of adult humans (Van Loon, 1978; Tucker et al., 1980).

Anestrus. Eventually, secretion of E$_2$ and P by the ovary fall to castrate values at the end of persistent vaginal cornification or repetitive pseudopregnancy in both rats and mice; then, persistent anestrus ensues (Lu et al., 1979; Gee et al., 1983). The ovary of old persistent anestrus rodents is probably devoid of follicles (an inference only, for which data remain to be obtained) and vaginal cytology becomes atrophic as at menopause (see below); these changes are associated with thin leukocytic vaginal smears. However, the postcastration elevations of LH and FSH expected from reduced ovarian E$_2$ secretion are only observed under special circumstances in aging rodents. In the absence of lactotrophic pituitary adenomas and other gross pathologic lesions, the healthy anestrus subpopulations of 2-year-old C57BL/6J and C57BL/6NNia* mice have dramatic elevations of LH, close to postovariectomy levels (Figure 5) (Gee et al., 1983; Parkening et al., 1980; Collins et al., 1979). The C57BL/6 family of mouse strains may be particularly favorable models for some aspects of menopause, because the incidence of prolactinemia (Flurkey et al., 1982; Parkening et al., 1980) is so much lower than in most aging rats, even if tumors are present (Nelson et al., 1980). The failure to observe elevations of LH and FSH in old

*Two strains widely used in current aging studies are the C57BL/6J and C57BL/6NNia. C57BL/6J mice are, by definition, derived exclusively and obtained directly from the Jackson Laboratory (Bar Harbor, ME) where they have been inbred since 1937 (Staats, 1976). The C57BL/6N strain was derived from C57BL/6J mice at the National Institutes of Health, Laboratory Animal Genetic Center (Bethesda MD). The resulting C57BL/6N mice were sent in 1975 to Charles River Laboratories (Wilmington, MA) to establish a breeding stock for an aging colony initially contracted by the National Institute of Aging (Bethesda, MD), and are designated C57BL/6NNia. The C57BL/6NNia may not be strictly identical with C57BL/6J or C57BL/6N genotypes.
animal models for the human menopause

Figure 5. Effects of age on basal LH levels of intact C57BL/6J mice (top) and on the E2-induced LH surge in ovariectomized C57BL/6J mice (bottom). Data redrawn from Gee et al. (1983) (top) and Mobbs et al. (1984a) (bottom).

anestrus rats (Huang et al., 1976) is most likely a consequence of prolactinemia and pituitary tumors since prolactin elevations inhibit LH secretion (Cheung, 1983). CBA mice also provide a model for relatively early depletion of oocytes, acyclicity, and genital tract atrophy (see below).

Potential Neuroendocrine Variables in Human Menopause and Rodent Neuroendocrine Aging

Most strains of laboratory rats and mice manifest progressive neuroendocrine impairments during the approach to acyclicity at 12–16 months, which include diminished spontaneous LH surges at proestrus, diminished E2-induced LH surges (Figure 5) and pulsatile LH in ovariectomized rodents, and altered responses of hypothalamic catecholamine metabolism to steroids (Finch et al., 1980, 1984; Estes and Simpkins, 1982; Meites, 1982; Wise, 1983; Mobbs et al., 1984b). Despite this extensive evidence, the causal relationship of altered gonadotropin output to the lengthening and loss of cycles is far from proven, e.g., the amount of LH released at proestrus greatly exceeds that required for ovulation (Turgeon, 1979).

Intriguing questions arise about the sources of variability in cycle lengthening, especially because effects of age on distribution of cycle frequency is so similar in humans and mice (Figure 1), species which appear to have different pacemakers governing the ovulatory surge of gonadotrophins (see above). A
stochastic hypothesis is suggested by the dwindling size of the pool of growing follicles (see above): As the follicular pool shrinks, fluctuations in numbers of the large follicles that produce E₂ could influence the length of subsequent cycles; fewer growing follicles could require more time to reach the circulating E₂ threshold for triggering the preovulatory surge of gonadotropins (Nelson et al., 1981). Alternatively, a reduced frequency of the pulsatile release of LH and FSH during aging, as documented for LH in rats that were ovariectomized for two weeks (Estes and Simpkins, 1982), could reduce the recruitment of follicles. The frequency of GnRH pulses in monkeys has a major influence on follicular development: slight reductions in GnRH pulse frequency cause anovulatory cycles with smaller peak plasma E₂ (Pohl et al., 1983). If aging influences GnRH pulses in women, as it does in rats (Estes and Simpkins, 1982), then age-related alterations in central mechanisms controlling GnRH could contribute to cycle irregularity in premenopausal women, even though the primary cause of menopause would remain ovarian. It is hoped that detailed data on LH and FSH pulses will become available for premenopausal women. Other factors in irregular cycles might involve increased stress responses and psychological depressions, whose incidence increases with age, since cortisol can inhibit the pituitary response to GnRH (Padmanabhan et al., 1983). Modest plasma corticosterone elevations are common in aging rats (Landfield et al., 1978; Sapolsky et al., 1983).

The most certain neural component of menopause is the hot flash, which is discussed below.

Pathophysiology of Menopause

Hot Flashes

Hot flashes are well characterized as an “organic,” neurological disturbance of low E₂ after menopause and involve a coordinate thermogenic output in synchrony with episodes of LH secretion (Judd, 1983; see also Chapter 13 by Judd in this volume). Similar hot flashes also occur in recently ovariectomized adult rhesus monkeys (Dierschke, 1982). Laboratory monkeys should thus be very useful in studying ovariprival aspects of the menopause.

Uterine and Vaginal Atrophy

Uterine atrophy is a common consequence of decreased E₂ after menopause. Similar atrophic cellular changes in the uterus, cervix, and vagina were observed in 11-month-old CBA mice (Papadaki et al., 1979), a strain with early ovarian exhaustion (see above), and can also be simulated in young rodents by ovariectomy.

Striking individual differences are reported in vaginal atrophy after men-
opopause. Some women do not experience vaginal atrophy, even in the absence of steroid therapy (Masukawa, 1960; Novak et al., 1965; Lin and So-Bosita, 1972, 1973; Lieblum et al., 1983). Diet may contribute to individual differences since postmenopausal vegetarians had lower urinary estrogens than their carnivorous controls (Armstrong et al., 1981). Sexual activity in women 6 years after menopause was correlated with reduced vaginal atrophy and higher levels of both androgens and LH; however circulating E2 and estrone were not correlated with vaginal atrophy (Lieblum et al., 1983). Genotype may also influence response to decreased E2, since mouse strains show important differences in their vaginal cell sensitivity to E2 (Trentin, 1950; Claringbold and Biggers, 1955; Drasher, 1955).

Receptors for E2 are being characterized in the postmenopausal uterus. There were no differences in the E2 cytosol-binding characteristics of uteri from pre- and postmenopausal women (not steroid treated) if endogenous proteases were inhibited (Pellika et al., 1983). Moreover, in vitro autoradiography detected abundant E2-binding sites in the postmenopausal vagina (Gould et al., 1983). The maintenance of E2 receptors is obviously crucial for the response of these target cells in estrogen-replacement therapy. In rodents, studies show significant loss of E2 binding sites in the aging uterus (Papadaki et al., 1979; Roth and Hess, 1982), but it is unclear whether the loss is due to a decrease of E2-binding sites per cell, or to a loss of E2-binding cells.

**Osteoporosis**

The view prevailing since the classic study of Albright, et al. (1941) holds that deficiencies in estrogens are a major cause of postmenopausal osteoporosis. Many studies support this view, including the recent findings that postmenopausal women with hip fractures had lower free plasma E2 and testosterone than nonfractured (not E2 treated) controls (Davidson et al., 1982) and that the favorable effects of E2 therapy on postmenopausal bone loss is dose related, e.g., in metacarpal cortex (Horsman et al., 1983). However, it must be stressed that loss of bone mineral content in adults of both sexes is a universal feature of aging (Garn et al., 1967; Avioli, 1982). Decreases of bone mineral are progressive from the fourth decade in normal women (Riggs et al., 1981; Marcus et al., 1983; Parfitt et al., 1983), which is 5–10 years before significant decreases in circulating E2. Thus, osteoporosis in women is not precipitated only by sex steroid loss at menopause. Many factors may be involved in the “normal” osteoporosis of aging, including alterations of mineral intake, physical activity, adrenal steroids, parathyroid hormone, calcitonin, and growth hormone (see reviews by Urist, 1962; Avioli, 1982; Raisz, 1982; Zerwekh et al., 1983; Armbrecht, 1984). The potential, multifactorial etiology of osteoporosis poses major difficulties in determining why osteoporosis leads to vertebral collapse and crush fractures in some older women and men, but not in others. One
source of difficulty in identifying possible common denominators of age-related osteoporosis is the limited genetic uniformity in most of the noninbred animal models studied.

The deficiencies of plasma P that often precede menopause may also contribute to osteoporosis. In ovariectomized young rats (Aitken et al., 1972) and in postmenopausal women (Nordin et al., 1981; Mandel et al., 1982), synthetic progestins were at least as effective as estrogens in ameliorating bone changes, as judged by mineral content or mineral metabolism respectively.

Osteoporosis occurs with age in C57BL/6J female mice, as indicated by decreased cortical thickness of the femur by 26 months and decreased Ca\textsuperscript{45} retention (Sha et al., 1967; Silberberg and Silberberg, 1962). Decreased thickness of the femur and spinal vertebrae was detected in (C57BL/6J × DBA/2) F\textsubscript{1} hybrids, at 6 vs. 18 months (Krishna Rao and Draper, 1969). Osteoporosis also occurs during aging in other mouse strains (Silberberg and Silberberg, 1941; Krishna Rao and Draper, 1969). The premature onset of tone loss by castration of female rats at 3 months (Aitken et al., 1972; Wronski et al., 1985) or male rats at 12 months (Wink and Felts, 1980) suggests that osteoporosis in old female mice, at least in part, is related to changes in ovarian activity. Moreover, treatment of 11-month-old CBA mice with E\textsubscript{2} for 3 months tended to increase the amount of cortical bone in the femur (Papadaki et al., 1979).

Investigation of the timing of osteoporosis in relation to the sequential decreases of plasma, E\textsubscript{2}, and progesterone during reproductive senescence as C57BL/6J mice pass from persistent vaginal cornification to anestrus (Nelson et al., 1982; Gee et al., 1983) could provide a valuable animal model for this major debilitating condition. Plasma testosterone should also be characterized during aging because of some association of hip fractures in postmenopausal women with low plasma testosterone (Davidson et al., 1982).

**Summary**

This review was intended to emphasize major features of menopause for which animal models are plausible. The extent of information on homologous changes of aging varies widely; it is much less for osteoporosis than for oocyte depletion or fertility cycle irregularities. Nonetheless, we conclude that laboratory animal models are now available to study most aspects of the human menopause. The types of individual variations of human aging can probably be found in the different rodent genotypes and primate species, for which detailed information should become available in the future.

**ACKNOWLEDGMENTS**

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Oxygen concentration gradient across the ovarian follicular epithelium: model, predictions and implications

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A mathematical model has been devised for predicting the oxygen concentration gradient across the epithelium of ovarian follicles at pre-antral stages. Most dissolved oxygen entering the follicle by diffusion is evidently consumed in the outer layer of cells; little reaches the oocyte. Even when the rate of consumption or the diffusion coefficient of oxygen was adjusted by an order of magnitude to favor oxygen penetration, the concentration gradient into the follicle remained steep. On the basis of measurements of ovine granulosa cell respiration in vitro, the model predicts that a large pre-antral follicle with a radius of 0.15 mm consumes oxygen at the rate of 0.22 nmol min⁻¹.

Introduction

The ovarian oocyte grows and matures within a follicular epithelium which provides physical and nutritional support besides exerting controlling influences over it (Moor, 1983). During follicular growth the epithelium evolves from being a single squamous layer to become a multi-layered, pseudo-stratified structure, which finally undergoes Graafian morphogenesis. Thus, during pre-antral follicle development, the oocyte becomes progressively isolated from the supply of dissolved oxygen which enters the follicle by diffusion from capillaries in the theca layer. Mitochondria act as oxygen sinks and, consequently, raised metabolic activity or additional cell layers will further rarefy the oxygen concentration at the follicular core.

Knowledge of the physical conditions in which oocytes and other follicular cells are maturing in vivo is essential for understanding their metabolism, and optimization of culture conditions depends on it. It is possible, in principle, to measure directly the gradient of tissue oxygenation across the follicular epithelium, for example, microelectrodes to measure the gas tension or fluorescence to indicate the redox state of NAD⁺/NADH. In practice, however, such methods cannot be applied to small follicles, and even in Graafian follicles, from which fluid can be drawn for analysis, reliable results are difficult to obtain under strictly physiological conditions. Alternatively, the gradient can be estimated indirectly using a well-established model (Krogh, 1919; Warburg, 1923). Assuming steady-state thermodynamics, the change in the flux of oxygen in a single dimension can be expressed as:

\[ \frac{\partial C}{\partial r} = \frac{D}{r} \frac{\partial^2 C}{\partial r^2} \]

where \( C \) is the oxygen concentration, \( D \) is the diffusion coefficient, and \( r \) is the radial distance from the follicle center.

The results in Figure 1 show that the oxygen concentration at the surface as a result of diffusion of follicles and their growth can be measured, it is more convenient to determine the oxygen concentration at any point \( Y \) equal to the initial value of \( Y_0 \). This parameter for pre-antral follicles in vivo is to appear to be acute while the concentration at the surface is less than that at the incident point of the follicle.

Materials and methods

Model of diffusion in a sphere

To modify the preceding model:

For radial symmetry, this reduces to

where \( r \) is the distance from the center of the follicle.

Then

\[ \frac{\partial Y}{\partial r} = \frac{n}{r Y} \frac{\partial Y}{\partial r} \]

The results in Figure 1 show that the oxygen consumption is effectively constant during pre-antral follicle development. In the present model, \( C \) assumes a constant value greater than zero. Since no dissolved oxygen is present in the follicle, \( C = 0 \) if \( Y_0 = 0 \). For radial symmetry, this reduces to

Then, where \( A \) and \( B \) are constants

\[ Y = Y_0 \frac{n}{r} \]

\[ B \text{ must be zero since } Y_0 \text{ is constant.} \]

The condition \( r = a \), where \( a \) is the radius of the follicle, results in

This solution is valid for all \( r < a \)

If, however, \( \lambda > 1 \) then \( Y_0 \) is zero.

For values of \( r > a \), \( C \) is to be determined numerically.

\[ Y = Y_0 \left( 1 - \frac{r}{a} \right) \]

\[ A \text{ and } B \text{ are related by } A = B r \]

\[ \lambda \text{ is the ratio of the radial distance of the follicle center to the radius of the follicle.} \]

Then, where \( A \) and \( B \) are constants

\[ Y = Y_0 \left( 1 - \frac{r}{a} \right) \]

\[ B \text{ must be zero since } Y_0 \text{ is constant.} \]

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\[ Y = Y_0 \left( 1 - \frac{r}{a} \right) \]
Oxygen concentration gradient across the ovarian follicular epithelium: model, predictions and implications

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\[
\frac{D_o d^2 Y_o}{dx^2} = C
\]

The equation states that the rate of consumption in a differential volume equals the change in the rate of concentration across the surface as a result of diffusion. Since the geometry and dimensions of follicles and the values of \( D_o \) and \( C \) are either known or can be measured, it is possible to estimate the oxygen concentration at any point across the epithelium when given the initial value of \( Y_o \). This paper describes a model and its application to pre-antral follicles in which the limitations of diffusion would appear to be acute whilst there are fewer theoretical assumptions and variables than in modelling the more complex situation in Graafian follicles.

Materials and methods

Model of diffusion in a spherical follicle

To modify the preceding model for three dimensions \((x, y, z)\), \( \frac{\partial^2 Y_o}{\partial x^2} \) is replaced by \( \frac{1}{r} \frac{d^2 (r Y_o)}{dr^2} \).

For radial symmetry, this reduces to:

\[
\frac{1}{r} \frac{d}{dr} \left( r \frac{dY_o}{dr} \right) = \frac{D_o}{A}
\]

where \( r \) is the distance from the centre of the follicle.

Then

\[
\frac{d}{dr} \left( r Y_o \right) = \frac{D_o}{A} + A
\]

\[
Y_o = \frac{C r^2 + A r + B}{6D_o}
\]

Then, where \( A \) and \( B \) are constants of integration, dividing by \( r \),

\[
Y_o = \frac{C r^2 + A r + B}{6D_o}
\]

\( B \) must be zero since \( Y_o \) is bounded as \( r \) goes to zero and \( A \) is determined from the condition \( Y_o(r) \), where \( r \) is the radius of the follicle. Then

\[
Y_o = Y_o(a) - \frac{Ca^2}{6D_o} \left( 1 - \frac{r^2}{a^2} \right)
\]

This solution is valid for all \( 0 \leq r \leq a \) provided that \( Y_o(a) \geq 0 \). This requires

\[
\lambda = \frac{Ca^2}{6D_o Y_o(a)} \leq 1
\]

If, however, \( \lambda \geq 1 \) then \( Y_o \) reaches zero at a value \( r = r^* \) where

\[
r^* = a \sqrt{1 - \frac{1}{\lambda}}
\]

For values of \( r < r^* \), \( C \) is taken to be 0 and \( Y_o = 0 \) so that

\[
Y_o = Y_o(a) - \lambda(1 - \frac{r^2}{a^2}) \quad r \geq r^*
\]

\[
= 0 \quad r^* \leq r
\]

If this is the case, then the total oxygen consumption per follicle, \( C^f \), is given by

\[
C^f = 4\pi \int r^2 Cdr = \frac{4}{3} \pi (a^3 - r^3)C
\]

\[
= \frac{4}{3} \pi a^3 \left( 1 - (1 - \frac{1}{\lambda})^{3/2} \right) C
\]
Table I. Rates of oxygen consumption by granulosa cells isolated from ovine follicles <2 mm diameter: effect of stimulation with PMSG

<table>
<thead>
<tr>
<th>Treatment</th>
<th>nM mg protein$^{-1}$ min$^{-1}$</th>
<th>nM mm$^{-3}$ cells min$^{-1}$</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control medium</td>
<td>12.43 ± 2.22</td>
<td>2.18 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>PMSO medium</td>
<td>16.26 ± 2.38</td>
<td>2.85 ± 0.42</td>
<td>30.7</td>
</tr>
</tbody>
</table>

*Mean ± SEM. Differences between treatment and control groups were statistically significant (paired t-test, $P = 0.009$, 2-tailed).

If $\lambda$ is large the following approximation holds

$$C^* = \frac{4}{3} \pi \alpha \left[ 1 + \left( 1 - \frac{3}{2} \right) \frac{c}{C} \right] = \frac{2\pi a^2 c}{\lambda} = 12\pi Y_o a D_o \alpha$$

**Estimation of the parameters**

**Follicular radius** ($a$). Sheep ovaries were prepared as serial histological sections cut at 8 μm and stained with haematoxylin and eosin. Radii of the largest solid (pre-antral) follicles were measured in equatorial sections using a micrometer.

**Diffusion coefficient of oxygen** ($D_o$). A published value of 0.7 × 10$^{-7}$ cm$^2$ s$^{-1}$ (Jones and Kennedy, 1982), as well as values up to an order of magnitude greater or smaller have been tested.

**Concentration of oxygen at the follicular boundary** ($Y_o$). Since this parameter cannot be estimated, an arbitrary value of 134 μM (corresponding to ~3 ml$^{-1}$ at s.t.p.) was taken as being the highest value expected on the basis of arterial plasma concentrations.

**Rate of consumption of oxygen** ($C$) by isolated granulosa cells. These cells were obtained from Scottish Blackface sheep within 1 h of slaughter. Follicles measuring <2 mm diameter were punctured with a micropipette and transferred to Medium 199 (Flow Laboratories, Irvine) containing 5.56 mM glucose and 0.25 mM sodium pyruvate. The cells were partially disaggregated by gentle pipetting and oocytes and debris were discarded. They were centrifuged and washed in fresh medium and transferred to a 2-cm$^3$ chamber containing a pre-calibrated oxygen electrode (Clark Electromedical, Bottisham, Cambridge) which was connected to a potentiometric recorder/plottter (Servoscribe IS, Smith Industries, London). Measurements were made at 37°C with constant stirring. Similar experiments were carried out in medium containing pregnant mares' serum gonadotrophin (PMSG) (1 IU ml$^{-1}$, Organon, Oss, The Netherlands).

At the end of the experiment (40–70 min) the cells were recovered and centrifuged and the protein content of the pellet was estimated colorimetrically (Bradford, 1976). The original volume of the cells was then determined from a calibration curve constructed by measuring the protein content of packed granulosa cells contained in capillary tubes of known volumes. Thus, both the rates of oxygen consumption per unit protein and per unit volume were obtained.

**Results**

The rates of oxygen consumption ($C$) by granulosa cells standardized per unit weight of protein or per unit volume are presented in Table I. A cell volume of 1 mm$^3$ corresponded to 175 μg protein. Incubation with PMSG raised the rate of respiration by 30%. The rate was constant above 30 μM l$^{-1}$ or 15% saturated, as is illustrated by a representative result (Figure 1).

At lower oxygen levels the curve extended exponentially, but it was not possible to estimate the new relationship because precise measurements were not possible at such levels.

The oxygen concentration gradient for large pre-antral follicles ($a = 0.15$ mm) is expressed as $Y(r)/Y(a)$ versus $r/a$ (Figure 2). The slopes, which are approximately linear, have not been extended to the abscissa because the rate of oxygen consumption at low levels was unknown. The three curves depicted in Figure 2 represent different values of $\lambda$. The middle one, $\lambda b = 145.34$, was calculated on the basis of the estimated values of the variables, i.e., $D_o = 7 \times 10^{-8}$ cm$^2$ s$^{-1}$, $C = 2.18$ nM mm$^{-3}$ min$^{-1}$, $Y_o = 134$ μM l$^{-1}$. The other two curves have been calculated for values either an order of magnitude greater or smaller than $\lambda b$ and represent the effects of raising or lowering $D_o$ or $C$ by the same degree. The results show that most of the oxygen has been consumed within the outer 5% of the radius even under the most favourable conditions expected for diffusion. Whilst estimates of $Y_o$ at the centre of the follicle (i.e., in the oocyte) are not justified, the clear implication of the results...
obtained with this model is that it approaches zero.

Discussion

The radial diffusion model predicts that most of the oxygen entering the follicle is consumed in the outer 5% of the radius, which corresponds to the outer layer of cells only (~10 μm thick). On the basis of an assumed oxygen concentration at the epithelial boundary and a rate of respiration estimated in vitro, it is predicted that the granulosa cells of a large pre-antral follicle consume 0.22 nmol min⁻¹. Somewhat unexpectedly, the model predicts that follicles consume oxygen as a proportion of their radius (a) rather than a². The boundary concentration of oxygen cannot be measured, and will vary according to the vascular perfusion and other factors, but the high value was not chosen arbitrarily; it corresponds to that expected in arterial blood, which may not be unrealistic considering the high rate of blood flow in the ovary. If, on the other hand, the external concentration of oxygen is low and is being consumed in the exponential portion of the curve, the model will not accurately predict the gradient, yet the conclusion that the follicular core is virtually anoxic will still hold. Considering the stated estimate of oxygen consumption per follicle and that each ovary contains ~200 pre-antral growing follicles (unpublished observations), the granulosa cells alone would account for ~0.7% of the total ovarian oxygen uptake as measured in auto-transplanted organs, 0.135 ml min⁻¹ (Baird et al., 1973). Before considering the implications of a steep oxygen gradient in the follicle, it is important to appraise critically the underlying theoretical assumptions of the model.

This model has been developed to draw attention to the physical problem of oxygenation of the follicular core. It is based on a small number of variables, which can be estimated, yet it can only give an indication of the complexity of the situation in vivo, and it ignores dynamic changes resulting from perfusion. The follicle is assumed to be radially symmetrical with a uniform capacitance coefficient for oxygen (Piper et al., 1971). Provided that the epithelium has the physical characteristics of a single homogeneous phase, the rates of diffusion can be predicted from the concentration gradient. This assumption is valid provided that diffusion is not facilitated by removal of oxygen from solution, other than by the uptake into a relatively even distribution of mitochondria (Kreutzer, 1970). The assumption that the diffusion pathway is wholly through a cellular continuum is an approximation because of the existence of extracellular space. The extracellular compartment has been ignored for present purposes since it is comparatively small and cannot provide an independent pathway because oxygen passes readily through cell membranes.

Follicular oxygen concentrations are attenuated as a result of a high rate of consumption by granulosa cells. The rates estimated in the present study are consistent with those obtained using similar methods for ovarian cells of other species (Fujita, 1928; Barker and Schwartz, 1953; Hamberger et al., 1971). Measurements of respiration rates in isolated cells have frequently been taken to indicate the physiological rates, although the assumption cannot be verified in the present case (Kleiber, 1975; Schmitz-Nielsen, 1984). It is reassuring, however, that if the rate of consumption has been overestimated even by as much as an order of magnitude, the boundary layer of cells would still cause a steep oxygen concentration gradient into the follicle. In view of this result, the 30% rise in the rate of respiration during PMSG stimulation will have a negligible effect on the gradient.

The prediction of a steep oxygen gradient in pre-antral follicles should not be extrapolated to the situation in Graafian follicles except with the utmost caution. A new compartment is present at Graafian stages (the antrum), in which the consumption of oxygen will be negligible, and the diffusion coefficient will be greater than in cells which will increase the penetration of the gas (Krog, 1919; Tai and Chang, 1974; Jones and Kennedy, 1982). There is also the geometrical asymmetry and thinner, more permeable epithelium in pre-ovulatory follicles (Payer, 1975) which would add considerably to the complications of developing a model. The oxygen tensions in follicle fluids measured following aspiration are highly variable, although higher than those estimated for smaller follicles. Mean values in these fluids were similar to those in venous blood (Shalgi et al., 1972; Fraser et al., 1973; Edwards, 1974; Knudsen et al., 1978) and a rise in oxygenation during antrum formation may contribute to the stimulation of oocyte maturation (Zeilmaker et al., 1972). The antrum characterizes the mature follicle of the majority of species, yet its functions are not well understood. One possibility is that it emerged during the co-evolution of body and follicular sizes as an adaptation to the problem of oxygenating an avascular structure. This conjecture is consistent with our observation that the antrum begins to form in most species when a follicular radius of ~0.15 mm is reached, i.e. independently of the sizes of either the primordial or mature follicles.

If the oxygen concentration in multi-laminar follicles is as rarefied as the model would suggest, there is the physiological problem of how growing oocytes generate energy. In this respect they are presented with a similar problem to that of parasitic helminths living in the relatively deoxygenated environment of the gut (Barrett, 1981), although oocytes do not have the option of anaerobiosis (Masui and Clarke, 1979). Possibly some of the energy requirements of growing oocytes are met through the metabolic continuum which exists between the granulosa cells and oocytes (Moor, 1983).

As a consequence of the progressive rarefication of dissolved oxygen, the outer layer of granulosa cells would be expected to be the most active zone for steroid biosynthesis; and a rising level of oxygen, which is anticipated to follow ovulation, could be a potent stimulus for secretion from cells undergoing luteinization. Both side-chain cleavage of cholesterol and aromatization of androgens to oestrogens require molecular oxygen (Ryan, 1959; Halkerston et al., 1961). A gradient of biosynthetic activity has been demonstrated by the preferential distribution of key enzymes in the peripheral granulosa layer of rat Graafian follicles (Zoller and Weisz, 1978, 1979; Zoller and Unelov, 1983), which is also optimally placed for the diffusion of hormonal products into neighbouring capillaries. It is, however, recognised that pre-antral follicles are much less competent to perform steroid biosynthesis than are Graafian types (Roy and Greenwald, 1985), and the functional significance of the oxygen gradient in these stages therefore requires further investigation.

Acknowledgements

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Alterations to the microtubular cytoskeleton and increased disorder of chromosome alignment in spontaneously ovulated mouse oocytes aged in vivo: an immunofluorescence study

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Abstract. Alterations in the organization of the microtubular cytoskeleton and chromosome alignment were examined by tubulin immunofluorescence and DAPI staining during in vivo ageing of naturally ovulated, metaphase-arrested oocytes of CBA/Ca mice in the fallopian tubes. In oocytes isolated from young mice on the day of oestrus, a few hours after ovulation, when they are still tightly surrounded by cumulus, the anti-tubulin fluorescence is almost exclusively restricted to the metaphase spindle. Only some faintly staining foci are observed in the cytoplasm, which presumably represent cytoplasmic MTOC not involved in spindle formation. The spindle is usually barrel-shaped or slightly pointed at its poles and does not possess astral fibres. In oocytes aged for more than 12 h in the fallopian tubes cytoplasmic asters develop, while microtubules seem to become gradually lost from the spindle, preferentially in its central area near the chromosomes. Astral fibres are observed radiating out from the polar centrosomes into the cytoplasm. In oocytes free of cumulus, and consequently more than 24 h post-ovulation, a pronounced shrinking of the spindle is observed. The mean pole-to-pole distance becomes significantly reduced in postovulatory aged cells. At the same time astral microtubules in the cytoplasm appear to become gradually depolymerized. Age-dependent alterations in the microtubular cytoskeleton do not seem to result from a changed pattern of the post-translational detyrosylation of \( \alpha \)-tubulin in certain sets of microtubules. In freshly ovulated oocytes chromosomes in most spindles are well ordered and precisely arranged at the equatorial plane. In 11% of the cells only, there was dislocation of one or several of the chromosomes from the spindle equator. By contrast, 61.4% of bipolar spindles of postovulatory aged oocytes have chromosomes displaced from the centre of the spindle towards one of the spindle poles. The implications of the observed alterations in the microtubular cytoskeleton, shrinking of the spindle and increased disorder of chromosome alignment are discussed with regard to predisposition to aneuploidy and reduction of developmental potential of postovulatory aged oocytes.

Introduction

The aetiology of the maternal-age-related increase in aneuploidy which in humans is responsible for Down’s syndrome is one of the major unsolved problems of human cytogenetics (for references see Hassold and Jacobs 1984). Several hypotheses have linked the high level of nondisjunction in older women to the spindle apparatus or its components in those oocytes remaining longest in the ovary and being exposed to numerous noxious agents before maturation and ovulation (e.g. Penrose 1965; Alberman et al. 1972; Kaufmann 1985). There are several reports demonstrating that exposure of organisms and germ cells to antimicrotubular agents or certain environmental hazards can induce malorientation of half-bivalents (La Fountain 1985 a, b) and aneuploidy (Sugawara and Mikamo 1980; Tease and Fisher 1986). However, no data exist for the spindle apparatus in mammalian oocytes with regard to the maternal-age effect, although there have been a number of structural studies of mammalian oocyte spindles (e.g. Szöllősi et al. 1972; Szöllősi 1975; Wasserman and Fujikura 1978; Schatten et al. 1985). In two studies alterations of the oocyte spindle due to pre- and postovulatory ageing were observed (Mikamo 1968; Szöllősi 1971, 1975). Small spindle fibres, individual microtubules and finer details of the spindle structure may have evaded observation in these previous investigations using light microscopy, and/or detection by electron microscopy of randomly oriented sections. We have therefore employed immunofluorescent techniques to visualize the entire microtubular cytoskeleton of individual oocytes, which also permits analysis of structure and alignment of the chromosomes in the same cell.

Before comparing the spindle structure associated with natural ageing of the female by examining oocytes of young and old females, it seemed important to look first at those events which are clearly associated with degeneration of the spindle during the postovulatory ageing of oocytes in the fallopian tubes. Although induction of aneuploidy at all ages in the human seems to result mainly from first-division meiotic, i.e. preovulatory, errors (for reviews see Bond and Chandley 1983; Hassold and Jacobs 1984), data indicate that second-division errors also contribute, although to a much lesser extent (Bond and Chandley 1983; Hassold and Jacobs 1984). Premature chromatid separation which could predispose to aneuploidy has been described in postovulatory aged oocytes of the mouse (Rodman 1971), and hypotheses linking postovulatory over-ripeness to aneuploidy in humans have been proposed (German 1968). The developmental capacity of in vivo- and in vitro-aged oocytes is much lower than that of freshly ovulated ova (Jutten and Bavister 1983; Gianfortoni and Gulyas

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and methods

Materials

Stocks of inbred CBA/Ca mice were obtained from Bantin and Kingman (Hull). Animals were bred and housed in our departmental colony under a 12 h photoperiod (lights on at 7 a.m.). The oestrous cycle of mature females from 6 weeks of age or older (up to 4 months) was recorded by vaginal smears. Samples were analysed by phase-contrast microscopy and classified by a method based on that of Allen (1922). Ovaries were usually obtained at autopsy between 8 and 11 a.m. when oocytes were expected to have been ovulated no more than 4–8 h before (Flurkey et al. 1982). These oocytes were always tightly surrounded by cumulus cells, and therefore termed ‘freshly ovulated’. Several mice were also killed late in the afternoon of the day of oestrus (6–7 p.m., approximately 15 h after ovulation). Oocytes freed from their ovaries were then often only loosely surrounded by or completely free of cumulus. Oocytes characterized as ‘postovulatory aged’ came from animals in metoestrus, 1 day after oestrus and therefore at least 28 h after ovulation. These oocytes were always free of cumulus.

Ovaries and oviducts of animals in oestrus or metoestrus were washed in prewarmed (37°C) Hank’s medium. Oocytes with or without cumulus were isolated from the ampullae and freed of cumulus by exposure to hyaluronidase (81 IU of type III hyaluronidase (Sigma), 2–5 min). Oocytes were then washed with a modified M2 medium containing 5 mg/ml BSA (Fulton and Whittingham 1978). The zona was removed with 0.5% pronase (Boehringer, Mannheim). After several washes with M2 medium oocytes were prefixed for 4 min in 1% paraformaldehyde in 50 mM Pipes, 5 mM EGTA and 2 mM MgCl, pH 6.9, except where stated otherwise. For immunofluorescence oocytes were then extracted for 50–60 min in a microtubule-stabilizing buffer containing 25% glycerol, 10 mM mercaptoethanol, 25 mM HEPES, 0.5 mM MgCl, 10 mM EGTA, 25 mM phenylmethylsulfonylfluoride and 2% Triton X-100 (modified after Bershadsky et al. 1978 and Balcacz and Schatten 1983). After attachment to poly-L-lysine-coated slides, cells were immersed for additional extraction and fixation in cold methanol (–10°C) for 8 min and rehydrated in PBS. Reaction with the first antibody was carried out either for 60 min at 37°C or at 4°C overnight with one of the following antibodies: (1) a polyclonal, high-affinity antibody to porcine brain β-tubulin (Füchtbauer et al. 1985; a kind gift from Dr B. Jockusch); (2) a monoclonal antibasin antibody directed against the tetrasylated form of α-tubulin (YL1/2; Kilmartin et al. 1982; Wehland et al. 1983; a kind gift from Dr J. Kilmartin); or (3) a polyclonal, affinity-purified peptide antibody reacting specifically with the tetrasylated site of α-tubulin (GLU-anti-tubulin; Gunderson et al. 1984; a kind gift from Dr J.C. Bulinski). After three washes with PBS, labelling with the second antibody was performed [fluorescein (FITC)- or rhodamine (TRITC)-labelled goat anti-rabbit Ig (a kind gift from Dr B. Jockusch), FITC- or TRITC-labelled rabbit anti-rat Ig or sheep anti-rabbit Ig (Sigma), respectively]. The second antibody alone did not label oocytes, and the enzymatic removal of cumulus and zona had no influence on microtubule configurations. Double staining of the different forms of α-tubulin was performed in the following sequence to prevent nonspecific binding: permeabilized, coverslip-attached cells were first reacted with the monoclonal anti-α-tubulin (YL1/2), washed and exposed to the polyclonal anti-α-tubulin (glu-anti-tubulin). After thorough washing, oocytes were stained by the TRITC-labelled anti-rabbit antibody, washed carefully, and finally exposed to FITC-labelled anti-rat antibody. After the antibody reactions had been completed and the last washes in PBS carried out, chromosomes were stained for 5–10 min in 1 μg/ml DAPI in PBS. After an additional wash, cells were immersed in 20% glycerol/PBS containing 1 mg/ml diazobicyclooctane (DABCO); coverslips were sealed with nail varnish.

Microscopic observation was performed on a Leitz Ortholux II equipped with the NPL Fluorot 63 (1.32) and 100 (1.32) and the Pleomopak filter system (system A for DAPI, system I2 for FITC-, and system N2 for TRITC-fluorescence). Photographs were taken on Kodak TriX Pan (400 ASA).

Prints with a final magnification of 2000–4000× were used to determine the pole-to-pole distance of spindles. Only those spindles which were straight and flatly spread out in one plane of view were evaluated.

Results

Spindles of spontaneously ovulated oocytes surrounded by cumulus

The typical features of the spindles of freshly ovulated CBA/Ca mouse oocytes at metaphase II are consistent with descriptions of oocyte spindle structure observed in other strains of mice by light and electron microscopy (Sató and Blanched 1979; Szöllösi et al. 1972) and by immunofluorescence (Schatten et al. 1985). As is characteristic for this mammalian cell, oocytes do not possess centrioles (Szöllösi 1985), an effect which may also be based on structural alterations in the spindle, chromosomés, or cytoskeleton of the oocytes (e.g. Marco et al. 1986, 1984). To elucidate whether the spindle is, in fact, altered and how degeneration proceeds in oocytes aged in the oviduct, we have examined metaphase II oocytes using the CBA mouse as a model system. Although these mice ovulate only a small number of oocytes at each cycle compared with other strains, an age-related increase in aneuploidy has been reported in CBA mice (Gosden 1973; Fabricant and Schneider 1978; Brook et al. 1984), and they have a relatively short reproductive span of life. Data obtained in this investigation could therefore be of value with regard to maternal age-related increases in aneuploidy when future research on freshly ovulated oocytes of aged animals is carried out (U. Eichenlaub-Ritter et al. 1986, in preparation).

Since the hormonal environment does not only control and initiate oocyte maturation in the ovary (for references see Masui and Clarke 1979) but may also affect the quality of ovulated ova and their environment in the fallopian tubes (e.g. Sherman et al. 1984), we have examined only spontaneously ovulated oocytes. The day of ovulation has been determined by vaginal smear analysis. Cumulus tightly surrounding the oocyte is indicative of freshly ovulated oocytes, postovulatory aged oocytes losing their association with the cumulus mass.

Our study demonstrates that characteristic structural alterations of the spindle and cytoskeleton accompany postovulatory ageing of the mouse oocyte. There is tentative evidence suggesting that this predisposes to aneuploidy.
et al. 1972). Microtubule-organizing centres (MTOC) recognized by an antibody directed against pericentriolar material (Callarco-Gillam et al. 1983) form ring- or disk-like centrosomes at the poles of the metaphase spindle. The spindle of CBA mice is therefore barrel-shaped, as observed in other strains of mice (Schatten et al. 1985), and resembles the typically anastral spindle of plant cells (Figs. 1a, 2, 3). Microtubules seem to fill the region between the poles and the chromosomes in a uniform fashion in freshly ovulated oocytes. In the brightly fluorescent spindle only the space occupied by chromosomes in its central, equatorial part is devoid of staining (Fig. 1a, b). Sometimes, a few interpo-
lar microtubules are observed to extend across the equator of the spindle. Rather, the spindle seems to be composed of two half-spindles. In apparently freshly ovulated oocytes, it was rare for tubules to be observed which extended from the polar centrosomes into the cytoplasm in an astral fashion. Although the diameter at the poles of most spindles is only slightly smaller than that at the spindle equator, some of the naturally ovulated oocytes possess more pointed spindle poles (Fig. 1c). This appears to be due to tighter bundling of tubules near the poles in these cells rather than to experimentally induced depolymerization of peripheral microtubules, because the fluorescence is especially bright and pronounced near the spindle poles. The cytoplasm of freshly ovulated oocytes generally seems to be quite devoid of microtubules, and neither astral structures nor individual, single microtubular fibres were observed. In particular in preparations in which oocytes were directly extracted in the microtubule-stabilizing buffer with-
out prefixation in formaldehyde cells contained faintly staining dots of tubulin-reactive material in the cytoplasm (Fig. 2) and occasionally small asters. These could originate from an association of some short microtubules or tubulin with cytoplasmic MTOC which become lost during fixation. The spindles of such cells show the same characteristics as after fixation. However, cytoplasmic foci are never associated with many microtubular fibres even in the directly extracted oocytes if they have been ovulated recently.

Comparison of oocyte spindles from one individual mouse stained either by the polyclonal anti-β-tubulin or the monoclonal YL1/2 antibody shows that there is no difference in the structure and shape of the spindle stained by either antibody (Fig. 3). A brighter fluorescence is observed and, concomitantly, a more pronounced halo for spindles reacting with the polyclonal antibody (Fig. 3a, b). There is also no indication that either the monoclonal antibody directed against tyrosylated α-tubulin or the polyclonal antibody reacting with the detyrosylated form of α-tubulin depicts only certain classes of microtubules or does not bind to any type of tubules in the metaphase spindle of freshly ovulated oocytes (Fig. 2b, c). The same has been found in spindles of other organisms (Gunderson et al. 1984). Since the monoclonal YL1/2 antibody reacts only with the tyrosylated form of α-tubulin it gives much better resolution in the microtubule-dense oocyte spindle and thus allows more accurate measurements of spindle size compared with the polyclonal anti-β-tubulin. Therefore it was employed in most of the experiments in which spindle length was determined.
with cumulus decreases dominantly stages loses immunoreactivity of tubulin, are washed under They were cells. This 7 equatorial plane, has apparently moved from Fig. 6a-d. Figs. 4, 5, many microtubules are found in the cytoplasm, and contrast to the region of the spindle (Figs. 4, 5). Interestingly, the astral microtubules in the cytoplasm can be specifically stained by antibodies directed against the tyrosylated and detyrosylated form of α-tubulin although staining with the peptide antibody against detyrosylated α-tubulin is usually weaker (Figs. 4, 5). In astral fibres it depicts mainly the central, focal region of the aster.

Table 1. Pole-to-pole distance of oocyte spindles from mice aged 6 weeks to 4 months

<table>
<thead>
<tr>
<th></th>
<th>No. of cells</th>
<th>Mean spindle length</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly ovulated, + cumulus</td>
<td>48</td>
<td>26.4 µm</td>
<td>+0.3 µm</td>
</tr>
<tr>
<td>Postovulatory aged, − cumulus</td>
<td>38</td>
<td>21.7 µm</td>
<td>+0.7 µm</td>
</tr>
</tbody>
</table>

Microtubule cytoskeleton of spontaneously ovulated oocytes that age in vivo

Mouse oocytes aged in vivo in the fallopian tubes for 15–30 h or more are always either completely free of cumulus or only loosely surrounded by a corona of cumulus cells. This appearance is conspicuously different from the sticky cumulus mass of the earlier postovulatory period. They were treated sequentially with the two enzymes and washed under identical conditions to the freshly ovulated oocytes. In oocytes isolated from females late in the day, which are still very loosely attached to cumulus, the spindle loses immunoreactivity of tubulin, preferentially in its central area containing the chromosomes (Fig. 7a). In contrast, the region near the spindle poles of such presumably intermediate stages of oocyte ageing is still brightly fluorescent. In contrast to freshly ovulated oocytes, many microtubules radiate out from the centrosomes into the cytoplasm (Fig. 7b, c). Other aster-like microtubular aggregates are widespread in the cytoplasm, and not confined to the region of the spindle (Figs. 4, 5). Interestingly, the astral microtubules in the cytoplasm can be specifically stained by antibodies directed against the tyrosylated and detyrosylated form of α-tubulin although staining with the peptide antibody against detyrosylated α-tubulin is usually weaker (Figs. 4, 5). In astral fibres it depicts mainly the central, focal region of the aster.

 Pronounced shrinking of the spindle is observed predominantly in oocytes completely devoid of cumulus (Fig. 7d, e). Measurements of the pole-to-pole distance of spindles from freshly ovulated and in vivo-aged oocytes show that the average spindle length of 26.4 µm in oocytes with cumulus decreases significantly (t-test; P < 0.001) to a mean length of 21.7 µm in the postovulatory aged cells (Table 1). Concomitantly, spindle poles become much more pointed, while microtubules are still associated with the centrosomal area (Fig. 7). The rest of the spindle contains more or less disordered microtubule bundles, which become thinner towards the central area of the spindle. Some of the oocytes still display large cytoplasmic asters (Fig. 6), although a proportion also appear to lose these structures. Even in oocytes which have passed through the ampullae and into the upper part of the oviduct, remnants of the spindle remain. Bipolarity may be lost but microtubules are still associated with centrosomal foci. Tubulin fluorescence at the chromosomes is nearly abolished (Fig. 7f).

Since the changes seen in the cytoskeleton of detergent-extracted aged oocytes could be due to differences in resistance to permeabilization and extraction of cells or sensitivity of microtubules to depolymerization, spindles of freshly ovulated oocytes were exposed to the extraction buffer for extended periods of time (between 40 and 80 min). However, they never showed the same changes in microtubule pattern as were observed in aged oocytes. Only the diffuse background staining was stronger in cells extracted for shorter times, and spindle structure was more often obscured by remaining, light-scattering organelles. The typical appearance of astral microtubule arrays pointing away from the centrosomes into the cytoplasm and the formation of large cytoplasmic asters or shrinkage of the spindle are never found in freshly ovulated oocytes even after thorough extraction. Thus, they seem to be characteristic of aged cells.

It appeared in most of the aged oocytes that the spindles had moved towards the centre of the cell from their original peripheral position. However, we cannot determine the topographic location of the spindle with confidence, because flattening of the extracted cells may give a false impression of the actual positioning of the mitotic apparatus.

Chromosome alignment in freshly ovulated and postovulatory aged mouse oocytes

Another typical feature of freshly ovulated oocytes is the highly ordered alignment of chromosomes at the equator of the metaphase II spindle. In most oocytes, chromosomes are located right at the spindle equator in the centre of the spindle, halfway between the flat spindle poles. As
Table 2. Percentage of oocytes from mice aged 6 weeks to 4 months, which show spindles with displaced chromosomes

<table>
<thead>
<tr>
<th></th>
<th>No. of animals</th>
<th>No. of oocytes</th>
<th>Spindles with Non-displaced</th>
<th>% Displaced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly ovulated, + cumulus</td>
<td>13</td>
<td>82</td>
<td>73</td>
<td>9</td>
</tr>
<tr>
<td>Postovulatory aged, — cumulus</td>
<td>10</td>
<td>44</td>
<td>17</td>
<td>27</td>
</tr>
</tbody>
</table>

Displacement of chromosomes from the spindle equator can also be seen in postovulatory aged oocytes of other strains of mice. Owing to the absence of pigment in eggs of Swiss Albino mice, phase-contrast microscopic observation of post-ovulatory aged oocytes reveals the same characteristic features of the spindles in these mice as are seen in CBA mice by immunofluorescence: shortening of the spindle and dislocation of chromosomes from the spindle equator (Fig. 8a, b).
Not a single cell was observed, however, in which one of the chromosomes was completely outside and independent of the spindle or was forming its own 'minispindle'. This has been suggested as one possible cause for the induction of aneuploidy during the second division in mouse oocytes (Szollosi 1975). Also, even among the aged cells that were completely free of cumulus there were very few oocytes in which the spindle had transformed from being bipolar to multipolar with random scattering of chromosomes (Fig. 7f). Rather than disintegration of the entire spindle apparatus and loss of bipolarity it looks as if a gradual weakening of spindle structures and loss of some microtubules from it occurs, preferentially in its centre near the kinetochores, together with displacement of chromosomes from the spindle equator in metaphase II during the ageing of the oocyte in the fallopian tube.

Discussion

This communication demonstrates structural alterations in the spindle during postovulatory ageing of the mammalian oocyte in the fallopian tube. Some of the observed changes, such as the general disorganization of the spindle and its displacement from the cell cortex into its central cytoplasm, have already been described in earlier reports (Szollosi 1971). Whilst the present study cannot confirm or exclude the existence of topographic changes in the location of the spindle, immunofluorescence has enabled us to analyse the details of the microtubular cytoskeleton and to detect structural rearrangements which have previously remained unnoticed. First, it is worthwhile to point out that the spindle in the aged oocyte is no longer anastral and barrel-shaped, as it appears shortly after ovulation, but becomes more fusiform and attains a number of astral microtubules radiating out into the cytoplasm. At the same time a number of large asters are formed throughout the cell.

Could these alterations be explained by a gradual decrease in the critical concentration of tubulin in an ageing oocyte? Centrosomal MTOCs are apparently not only associated with the spindle poles in mammalian oocytes but are also present at the periphery of the cell (Schatten et al. 1985; Marco et al. 1985). Faint tubulin staining and association of these foci with short microtubules may already be observed in some of the freshly ovulated oocytes (see Fig. 2). Upon lowering of the critical concentration of tubulin during ageing they might attain the capacity to induce more microtubule polymerization away from the main spindle body. Concomitant redistribution of polar MTOC into the cytoplasm would explain the more fusiform shape of the spindle. However, if the critical tubulin concentration decreases more microtubules in the spindle near the kinetochores and a general enlargement of it would be expected. This has never been found in aged oocytes. In contrast, fluorescence is gradually lost from the spindle, preferentially in the centre of the metaphase II spindle (compare Fig. 7a), and the pole-to-pole distance becomes significantly reduced (see Table 1).

Since very old oocytes not only have short spindles but have also lost some of their cytoplasmic astral microtubules, it can be assumed that if the critical concentration of tubulin is altered, it increases rather than declines in the ageing oocyte. However, the first alterations in the microtubular cytoskeleton seem to be caused mainly by a redistribution of microtubules, and not by a general change in the critical concentration of tubulin in the cytoplasm. The loss of nucleating/anchoring capacity for microtubules at the kinetochores of the chromosomes could explain the formation of astral fibres and gradual depolymerization of spindle microtubules concomitant with the assembly of microtubules at other available MTOC in the cytoplasm of ageing oocytes. Since these asters disappear and the spindle becomes decomposed only upon prolonged ageing for more than 24 h, the critical concentration of tubulin does not seem to become dramatically increased until a long time after ovulation.

As demonstrated again recently in an immunofluorescence study (Schatten et al. 1985), kinetochore microtubules of the second maturation spindle of inseminated mouse oocytes become disassembled during ana/telophase about 6-8 h after ovulation. At this time, and even more so later on during pronuclear formation and migration, asters are formed at the many cytoplasmic MTOC in the fertilized oocyte (Marco et al. 1985; Schatten et al. 1986). The present observations suggest that comparable events occur in unfertilized, ageing oocytes. Thus, moderately aged oocytes also posses many cytoplasmic asters (Figs. 4, 5), and fluorescence is often diminished in the central area of the spindle of aged cells, probably due to depolymerization of microtubules at the kinetochores (Fig. 7a). Whereas in the freshly ovulated, fertilized eggs aster formation occurs about 6-12 h after sperm penetration, similar changes are pronounced in aged oocytes 12 h after ovulation and later. In general, however, alterations in the organization of the cytoskeleton in the two situations are broadly similar.

It therefore appears possible that transformations of the cytoskeleton of oocytes during ageing are among the processes which are to some extent independent of, but accelerated by, fertilization, as has been shown for some translational events (Howlett and Bolton 1985). Transient changes in the organization of the cytoskeleton of the aged egg may enable the cell, once fertilized, to catch up with the normal developmental schedule, which may be critically important for coordination of the expression of the molecular programme of development with the necessary cytoskeletal alterations and optimizing exchange of signals with the maternal environment. Evidence of catch-up growth during cleavage stages has been observed in rats (Shalgi et al. 1985).

Both translation and post-translational modification events of existing proteins seem to occur in ageing oocytes.
irrespective of fertilization (Howlett and Bolton 1985). The post-translational modification of α-tubulin in which a glutamine residue becomes tyrosylated at its carboxy terminus first but can be detyrosylated again later, probably preferentially on polymerized microtubules (Acre and Barra 1985), could be one way of controlling the function of microtubules in a cell (Gunderson and Bulinski 1986). Therefore, shifts in the distribution of both forms of α-tubulin could go hand in hand with the structural alterations in aged oocytes. However, comparison of the staining pattern of both antibodies in the half-spindles of freshly ovulated and aged oocytes show only slight differences in the presence of either tyrosylated or detyrosylated α-tubulin. In mitotic cells detyrosylated tubulin seems to be sparse if not absent from astral microtubules (Gunderson et al. 1984; Gunderson and Bulinski 1986). Unlike this, staining of centrosomal foci (Fig. 2c), cytoplasmic asters (Figs. 4c, d, 5b) and astral fibres (Fig. 6b) of meiotic spindles by the anti-glut-tubulin antibody suggests that there are either general differences in detyrosylation pattern of α-tubulin in mitotic and meiotic cells or the observed staining of astral microtubules is due to the special constitution of mammalian oocytes which do not possess centrioles typical for mitotic animal cells. Furthermore, the appearance of detyrosylated α-tubulin in astere microtubules could be related to a gradual shift in the oocytes cytoplasm from a division/mitosis state to an interphase state since glut-tubulin, for instance, is found in some of the microtubules associated with the centriolar MTOC in animal cells during interphase (Gunderson et al. 1984).

An important consequence of the redistribution of microtubules from the spindle into the cytoplasm of the oocyte during ageing seems to be the shrinkage and 'weakening' of the spindle (cf. Table 1). Probably of even greater significance for induction of aneuploidy in aged cells, the alignment of chromosomes in postovulatory aged oocytes is much less ordered. In freshly ovulated oocytes about 11% of the cells show irregularities in chromosomal alignment. This compares very well with the cytogenetic chromosomal studies of preimplantation embryos in young CBA mice, in which 7.2% were aneuploid. Since most of the errors in chromosome distribution in man seem to occur at the first maturation division, displacement of chromosomes from the spindle equator at MI could reflect a preceding nondisjunction event. Similarly, it could indicate the predisposition of certain chromosomes to become maldistributed during anaphase II. The high incidence of chromosomes located outside the equatorial plane in aged cells suggests that disorder increases with the postovulatory age of the oocyte, and thus also raises the risk of malsegregation of chromosomes. It seems improbable that all the 61.4% of cells possessing displaced chromosomes are destined to produce aneuploid offspring. However, a correlation between the number, type and incidence of chromosome displacement and the occurrence of aneuploidy has also been suggested by studies of spread metaphase spindles of human lymphocytes (Ford and Lester 1982). The reduction in developmental potential noticed for in vivo-fertilized oocytes after experimentally induced over-ripeness (for reference see Bond and Chandy 1983) or for in vivo- and in vitro-aged oocytes before in vitro fertilization (Juettner and Bavister 1983; Gianfortoni and Gulyas 1985), is expected to be due in part to aneuploidy arising during the second maturation division (for references see Szölőzi 1975). Apart from alterations of other components of the cytoskeleton (e.g. the actin distribution; see Maro et al. 1986) the dramatic increase in numbers of oocytes with less well-ordered chromosomes after ageing of the egg adds weight to the suspicion that the risk of cyogenetic errors will greatly increase during ageing. The present study emphasizes that there are correlations between spindle structure, chromosome order, ageing and, possibly, induction of aneuploidy. Assessment of spindle structure and chromosome alignment in extracts, lightly spread out cells may therefore become a useful tool for estimating the predisposition to aneuploidy in other species also, and especially in humans. We are presently investigating whether similar alterations of the cytoskeleton and alignment of chromosomes to those observed in post-ovulatory aged oocytes can also be found in freshly ovulated oocytes of aged females.

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Similar changes in spindle structure and chromosome distribution as reported for spontaneously ovulated oocytes in the present study have recently also been described for aged murine oocytes after hormonally induced ovulation (Webb et al., J Embryol Exp Morphol 95).
Scaling of follicular sizes in mammalian ovaries

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(With 6 figures in the text)

The scaling of ovarian follicle and oocyte sizes according to body weight \((M, \text{ ranging from } 0.005-500 \text{ kg})\) has been analysed using data obtained from 22 mammalian species in nine orders. The diameters of non-growing (primordial) follicles were correlated significantly with body weight, the relationship being described by the allometric formula \(y = 0.028M^{0.10}\). The mean size at which growing follicles began to accumulate extracellular fluid was approximately the same in all species, 0.3 mm diameter. Graafian follicle sizes varied allometrically with body weight as a result of differences in the volumes of follicular fluid rather than those of oocytes, which were relatively similar in eutherian mammals. The statistical significance of the correlation between Graafian and body sizes was increased when the dimensions for an ovulatory quota of follicles were combined because follicles in polyovulating species were disproportionately small. The total Graafian surface areas and volumes were then predicted from body weight by \(58.4M^{0.65}\) and \(18.5M^{1.96}\), respectively. Follicular dimensions in the three species of primates were significantly greater than predicted by the allometric relationship. The exponents of these relationships show that the total volume of a set of preovulatory follicles varies approximately isometrically with body weight and, therefore, with the presumptive hormone distribution volume \((M^{1.9})\). The hypoallometric relationship of follicular surface area demonstrates that, during the course of the evolution of body size, the surface area for secretion has not increased to match the dilution of hormones in the body pool.

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</tbody>
</table>

Introduction

A number of variables that characterize patterns of reproductive physiology are scaled allometrically with physical size, evidently contributing to the optimization of reproductive success. Such relationships have been well documented in the cases of variation in litter size, age at puberty, and a number of other variables in mammalian reproduction (May & Rubenstein, 1984), but scaling of the sizes of the reproductive organs has received scant attention.
In primates, testicular weights, which are indicative of spermatogenic capacity, present allometric variation, but superimposed upon this are the effects of sperm competition in some species (Harcourt, Harvey, Larson & Short, 1981). A scaling relationship exists in the ovary between the size of the store of non-growing (primordial) follicles and body weight (Gosden & Telfer, 1987), but the sum of these units is only a small proportion of the organ size overall. Most of the volume of young adult ovaries derives from Graafian follicles or corpora lutea, depending on the phase of the ovarian cycle.

Parkes (1932) reported that Graafian follicle diameters vary linearly with body weight when the axes are transformed logarithmically, implying an allometric relationship, but this conclusion was based on only seven species. His finding is verified in the present paper in which the study has been extended to other follicle stages using a larger set of data. Allometric expressions have been obtained to describe relationships, and these may guide theory about the underlying physiological bases of follicle size and scale.

Materials and methods

Sources of ovarian data

Data were obtained from young, adult nulliparous individuals either at autopsy or at ovariectomy. The species are listed in Table I. Measurements of Graafian follicles were taken shortly before the expected time of ovulation, whereas those of smaller stages were not so constrained. The ovaries were fixed, embedded in paraffin wax, sectioned serially at 7 or 8 μm and stained with haematoxylin and eosin.

<table>
<thead>
<tr>
<th>Code no.</th>
<th>Common name</th>
<th>Specific name</th>
<th>Order</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bandicoot</td>
<td>Isoodon macrourus</td>
<td>Marsupialia</td>
</tr>
<tr>
<td>2</td>
<td>Common shrew</td>
<td>Sorex araneus</td>
<td>Insectivora</td>
</tr>
<tr>
<td>3</td>
<td>Hedgehog</td>
<td>Erinaceus europaeus</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Elephant shrew</td>
<td>Elephantulus myurus</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Pipistrelle bat</td>
<td>Pipistrellus pipistrellis</td>
<td>Chiroptera</td>
</tr>
<tr>
<td>6</td>
<td>House mouse</td>
<td>Mus musculus</td>
<td>Rodentia</td>
</tr>
<tr>
<td>7</td>
<td>Wood mouse</td>
<td>Apodemus sylvaticus</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Bank vole</td>
<td>Clethrionomys glareolus</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Field vole</td>
<td>Microtus agrestis</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Norway rat</td>
<td>Rattus norvegicus</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Guinea pig</td>
<td>Cavia porcellus</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Plains viscacha</td>
<td>Lagostomus maximus</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Laboratory rabbit</td>
<td>Oryctolagus cuniculus</td>
<td>Lagomorpha</td>
</tr>
<tr>
<td>14</td>
<td>Domestic cat</td>
<td>Felis catus</td>
<td>Carnivora</td>
</tr>
<tr>
<td>15</td>
<td>Domestic dog</td>
<td>Canis familiaris</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Sheep</td>
<td>Ovis aries</td>
<td>Artiodactyla</td>
</tr>
<tr>
<td>17</td>
<td>Swine</td>
<td>Sus scrofa</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Cattle</td>
<td>Bos taurus</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Horse</td>
<td>Equus caballus</td>
<td>Perissodactyla</td>
</tr>
<tr>
<td>20</td>
<td>Common marmoset</td>
<td>Callithrix jacchus</td>
<td>Primates</td>
</tr>
<tr>
<td>21</td>
<td>Rhesus monkey</td>
<td>Macaca mulatta</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Human</td>
<td>Homo sapiens</td>
<td></td>
</tr>
</tbody>
</table>
Mensuration of ovarian follicles and oocytes

The mean diameters of non-atretic follicles and of oocytes in Graafian follicles were obtained in the equatorial tissue sections from the average of the maximum diameter and the diameter at right angles. They were based on 8–30 measurements from 4–8 individuals in each species. Follicles were measured at 3 distinctive stages of development: (1) primordial follicles in which the epithelium was unilaminar and squamous and a small oocyte was present; (2) solid, multilaminar follicles in which formation of pools of follicular fluid was incipient; (3) mature Graafian follicles shortly before ovulation. The larger Graafian dimensions among species exceeding 1 kg body weight were obtained by measuring fresh materials with calipers. In the carnivores, primates and some artiodactyls, the data were collected from published studies. The surface areas and volumes of Graafian follicles have been estimated because these variables could be physiologically more significant than the follicular diameter (D). The estimates were based on the assumption that follicles are spherical with surface areas and volumes corresponding to \(4\pi(D/2)^2\) and \(1.33\pi(D/2)^3\), respectively. The morphological appearance of follicles upheld this assumption in all cases except the domestic bitch, in which the surface area presented for diffusion to and from the vascular theca will have been underestimated because of the natural folding of the follicular wall.

Body weight and numbers of primordial and ovulatory follicles

The body weights and the numbers of ovulations per cycle (ovulation rates) were obtained for young adult individuals during the course of the study, or were obtained from published sources (e.g. Altman & Dittmer, 1972). The ovulation rate has been taken to indicate the numbers of preovulatory follicles when estimating dimensions collectively. The mean numbers of primordial follicles in pairs of ovaries were obtained during a concurrent study which was based mainly on the same specimens (Gosden & Telfer, 1987).

Statistics

The mean values for each of the variables were used for analysing interspecific patterns. They were transformed to common logarithms for graphical representation and for testing whether relationships were consistent with the allometric formula, \(y = aM^b\). Linear regression analysis was carried out by the method of least squares and the 95% confidence limits were calculated.

Results

A correlation matrix for the major variables is given in Table II and selected allometric relationships are depicted with their formulae in Figs 2–6.

The diameter of oocytes in Graafian follicles was correlated with body weight at the 5% level of statistical significance (0.05 > P > 0.02); a regression line has not therefore been fitted (Fig. 1). The diameter of oocytes in Graafian follicles varied among eutherian mammals from 0.058 mm in small rodents to 0.113 mm in sheep; in the marsupial species it was 0.133 mm.

Diameters of primordial follicles ranged from 0.014–0.093 mm. When plotted against body weight on logarithmic scales, a linear correlation was obtained (P < 0.01) in which the coefficient of determination \(r^2\) was 0.45 (Fig. 2). The interspecific differences in sizes of these follicles were mainly due to the volumes of ooplasm, which were disproportionately large in relation to body size in rabbits and cats. These sizes showed statistically significant correlations with both the numbers present at young adult ages and with the sizes of Graafian follicles and of oocytes (P < 0.01) (Table II).
Table II

Correlation matrix for logarithmically transformed values for body weight and ovarian parameters

<table>
<thead>
<tr>
<th></th>
<th>Number of primordial follicles</th>
<th>Ovulation rate (n)</th>
<th>Diameter</th>
<th>Surface area</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primordial follicle</td>
<td>0.953</td>
<td>-0.270</td>
<td>-0.460</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovulation rate (n)</td>
<td></td>
<td>0.552</td>
<td>0.730</td>
<td>-0.422</td>
<td></td>
</tr>
<tr>
<td>Diameter</td>
<td></td>
<td>0.669</td>
<td>0.676</td>
<td>-0.368</td>
<td>0.769</td>
</tr>
<tr>
<td>Primordial follicle</td>
<td></td>
<td>0.880</td>
<td>0.902</td>
<td>-0.673</td>
<td>0.687</td>
</tr>
<tr>
<td>Graafian follicle (GF)</td>
<td></td>
<td>0.868</td>
<td>0.905</td>
<td>-0.684</td>
<td>0.691</td>
</tr>
<tr>
<td>Surface area GF x n</td>
<td></td>
<td>0.921</td>
<td>0.930</td>
<td>-0.475</td>
<td>0.686</td>
</tr>
<tr>
<td>Volume</td>
<td></td>
<td>0.869</td>
<td>0.905</td>
<td>-0.684</td>
<td>0.691</td>
</tr>
</tbody>
</table>

Fig. 1. Variation between mean diameters of oocytes in Graafian follicles and body weight (see Table I for species code list for Figs 1–6).
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**Fig. 2.** Variation between mean diameters of primordial follicles and body weight. Here, and in the following Figs, the line of best fit obtained by the least squares method is presented with the 95% confidence interval and allometric formula (see Table I for species code list for Figs 1–6).

**Fig. 3.** Allometric relationship between the surface area of individual Graafian follicles and body weight (see Table I for species code list for Figs 1–6).

Mature follicles were characterized by the presence of a follicular antrum in all species examined. The volume of follicular fluid contributed more than 50% to the total volume of mature follicles exceeding 0.4 mm diameter, the percentage being greater in the larger follicles. The epithelium of mural granulosa cells was 3–9 cell layers thick, without any systematic variation.
Fig. 4. Allometric relationship between the volume of individual Graafian follicles and body weight (see Table I for species code list for Figs 1-6).

Graafian dimensions ranged from 2–5 orders of magnitude above the minimum values observed in the shrew and Plains viscacha, depending on whether the diameter, surface area or volume was being compared (Figs 3–6). These dimensions were correlated significantly with the diameter of both oocytes and primordial follicles, although only about half or a third of the total variation was so accounted for ($r^2 = 0.47$ and 0.33, respectively). Differences between species in body
weight could account for most of the variation in Graafian sizes and was fitted by the allometric formula \( r^2 > 0.75 \) (Table II).

A greater proportion of the variation in follicular sizes was encompassed by the allometric relationship when the dimensions of a set of preovulatory follicles were combined rather than considered singly, this being indicated by a rise in \( r^2 \) from 0.75 to 0.85 (Table II). The impact of this manoeuvre was attributed to the exceptional data from the Plains viscacha more than any other single species, since this rodent, which weighs 2-4 kg, combines a high ovulation rate with diminutive follicular size. Apart from this interactive effect and the negative relationship to Graafian size, the ovulation rate was not correlated significantly with the other variables being studied. The allometric exponents for total Graafian surface areas and volumes were 0.651 ± 0.063 and 1.056 ± 0.103, respectively; the second of these being not significantly different from unity. Graafian dimensions in the three species of primates were, in most of the comparisons made, significantly greater than would be expected on the basis of body weight (Figs 5, 6).

**Discussion**

The results demonstrate, inter alia, that most variation between species in the sizes of Graafian follicles and, to a lesser extent, of primordial follicles can be accounted for by differences in adult body weight. The scaling relationships are consistent with the general allometric formula, \( y = aM^b \). In contrast to the follicle and to the spermatozoan (Cummins & Woodall, 1985),
which is scaled negatively with body weight, oocytes of eutherian mammals were relatively similar in size, thus confirming the results of earlier studies (Austin, 1976). Attempts have been made to find physiological explanations for the allometric expressions obtained, but, first, the principal assumptions underlying the study will be discussed.

The value of allometric formulae as devices for describing general relationships between two variables and for predicting one variable from the other rests on the assumptions that the data are taken from a representative sample of species and of individuals and that measurements are reliable. The present sample was restricted by the availability of specimens of sufficient quality for providing most, if not all, of the seven measurements sought; consequently, it includes a number of domesticated species and laboratory stocks. There were no indications, however, of systematic differences in the raw data obtained from these animals compared with other species.

The list of species represents nine taxonomic orders and the body weights range from 0.005–500 kg with order of magnitude differences in Graafian follicle sizes. Such large differences within
correlated variables, in combination with logarithmic transformation of data, reduces the impact of individual variation on the fitting of a straight line by regression analysis. Compared with that arising from measurement errors, a greater proportion of the total variation is probably due to that existing naturally within species and even within ovaries in the size and numbers of preovulatory follicles.

Allometric variation of Graafian follicle sizes is perhaps the most notable finding in this study. It would appear to be due to variation in the volume of follicular fluid, since the thickness of the membrana granulosa was relatively constant. Allometric scaling has presumably evolved to adapt follicular function to the changing physiological conditions of increased body size. Ovarian follicles have two major functions: they support and control the maturation of oocytes, and secrete hormones which integrate sexual physiology and behaviour. Since the range of oocyte size was small, Graafian dimensions might be expected to subserve the second function.

On a priori grounds, a more significant correlation would be anticipated between body weight and Graafian dimensions when considered collectively rather than individually, since follicles are integrated in development for ovulation and for secretion of hormones into the body pool. This expectation was confirmed, but additional data are required to determine whether an interactive effect of numbers and sizes of preovulatory follicles exists continuously across a wide range of species, or is confined to species with very high ovulation rates where the importance of scaling is obvious. In the Plains viscacha (Lagostomus maximus) and the Elephant shrew (Elephantulus myurus), ovulation rates vary from 200-800 and from 50-180, respectively (Weir, 1971; Tripp, 1971), which appears to have affected Graafian sizes in accordance with theory. The norms of body mass based on a regression line for the other species would predict that their Graafian volumes would be 22.0 and 0.23 mm³ instead of the observed values of 0.01 and 0.02 mm³. The ovarian mass and surface area required for accommodating and ovulating the predicted follicle sizes in such large numbers may not be physiologically realistic.

The hypothesis that the total surface area presented for secretion by a set of preovulatory follicles matches the distribution volume for oestrogen and other hormones is not supported by the present results, since a hypoallometric exponent of 0.65 was obtained. Plasma oestrogen levels are independent of body weight. The exponent suggests a closer relationship to the body surface area \( M^{0.67} \) than to body volume \( M^{1.0} \). The isometric variation between the volumes of Graafian follicles and body weight, of which the hormone distribution volume is a fixed proportion (Stahl, 1967), would be expected if follicles consisted entirely of a homogeneous population of secretory cells. The majority of space in most species is, however, extracellular, and there is only a shell of theca and granulosa cells, with most metabolic activity confined to the periphery (Gosden & Byatt-Smith, 1986). Isometric variation is therefore unexpected, unless the follicular fluid, which contains high concentrations of steroid hormones (Edwards, 1974), acts as a hormone store and is dynamically involved in the maintenance of circulating hormone levels. Variation in Graafian size might also be explained, in principle, on the basis that the maximum dimensions determine the size of the corpora lutea. Being vascular and relatively more solid than the follicles, corpora lutea might be expected to vary isometrically with the distribution volume. This hypothesis may, however, not fully account for their dimensions because of the existence of accessory corpora lutea and of heterogeneous luteal cell populations in some species (Schwabl & Niswender, 1985). Isometric relationships have not been found among other endocrine organs (Brody, 1945; Harcourt et al., 1981), and there is therefore no general rule of fixed apportionment of organ space within the abdomen. On the basis of the present information,
there would appear to be little support for the view that simple geometrical scaling factors dictate follicular sizes, possibly because of the complexity of physiological factors.

Besides providing a general description of scaling relationships, allometric expressions can also draw attention to exceptional data and, hence, indicate peculiar physiological or anatomical features. In primates, the disproportionately large size of Graafian follicles is of particular interest when considered in relation to the remarkably high levels of oestradiol-17β during the preovulatory phase. The levels are about 3-10 fold greater than those in non-primate species which are invariably < 0.5 pmol ml⁻¹ (e.g. for non-primates: Scaramuzzi, Caldwell & Moon, 1970; Noden, Oxender & Hafs, 1975; Smith, Freeman & Neill, 1975; Austad, Lunde & Sjaastad, 1976; Hodges, Henderson & McNeilly 1983; and for primates: Weick et al., 1973; Korenman et al., 1974; Reyes, Winter, Faiman & Hobson, 1975; Nadler, Graham, Collins & Gould, 1979).

Such notable differences are not conspicuous among the other gonadal steroids in either females or males. They cannot reflect simply a distinction between those species having menstrual cycles and those which do not, since especially high levels of oestradiol are present among the New World monkeys, which do not menstruate (Wolf, O'Connor & Robinson, 1977; Harlow, Heam & Hodges, 1984). The elevated oestrogen levels in primates could be due to larger follicles. It is equally possible, however, that they reflect a slower rate of clearance from the circulation, resulting from greater plasma sex steroid binding activity and consequent protection from metabolism (Corvol & Bardin, 1973; Sitteri et al., 1982).

The norms of Graafian follicle scaling may not be obeyed by some bats. This tentative conclusion is based on limited information gained from another species because the ovaries of pipistrelle bats were aneuploidy. The ovaries of the Lesser horseshoe bat (Rhinolophus hipposideros) contains one or two Graafian follicles which measure 0.3 mm in diameter (Harrison Matthews, 1937). The follicular volume in this bat, although similar to that of terrestrial mammals of similar size (e.g. common shrew), is considerably less than that predicted by allometry for the known ovulatory quota.

The present study has been mainly concerned with the effects of interspecific rather than intraspecific differences in body weight, because the latter were comparatively small in the samples obtained. It is known from studies of other variables that allometric relationships across a range of species may not apply within any given species (Calder, 1984), and there is tentative evidence of this qualification with respect to follicles. The Merino sheep has a mean ovulation rate of 1-2, with Graafian follicles measuring 6 mm, whereas in the highly fecund Booroola breed the values are 5.2 and 4 mm (Driancourt, Cahill & Bindon, 1985). The total Graafian surface areas and volumes are therefore 241 and 261 mm² and 321 and 174 mm³, respectively, in the two breeds. These findings contradict the expectation that the total volumes should be the same.

The allometric relationship observed for Graafian follicles cannot be simply accounted for by scaling up from that observed among primordial follicles, since different compartments are involved in the two types, namely the ooplasm and the antrum. A physiological basis for scaling of primordial follicle sizes and for the exceptional cases, rabbits and cats, is even more obscure than that for Graafian follicles. Primordial follicle size was significantly correlated with several of the other variables, but these relationships are unlikely to be causal.

In contrast to the scaling of follicle sizes at either end of the growing phase, the morphogenetic transition from the preantral to the antral stage occurs at a similar size in all species. In mice, this is initiated when 2000-3000 granulosa cells have accumulated and, as cell volume is approximately the same, it is probably similar in other animals (unpubl. obs.). In a few species, follicle mature without forming an antrum (Nicholl & Racey, 1985), but the dimensions hardly exceed...
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the upper size limit for solid follicles in others. The striking uniformity of size at this stage is yet another puzzling observation without a clear explanation at present. Experimental studies are needed to test whether it reflects the physical limitations of diffusion of respiratory gases or metabolites, or represents a developmental requirement for accumulating a critical number of granulosa cells for commitment to separate pathways in the Graafian follicle.

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Any suitable data or materials which could extend the present study to additional species would be received most gratefully by the authors.

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Numbers of follicles and oocytes in mammalian ovaries and their allometric relationships

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(With 2 figures in the text)

The numbers of ovarian follicles present in the reserve pool of non-growing ('primordial') stages at the commencement of adult life in 19 species varied allometrically with both body weight ($M$, in kg) and with maximum life expectation ($L$, in years), the mathematical expressions being $27700M^{0.47}$ and $820M^{1.58}$, respectively. These allometric relationships, which could not be accounted for by differences in reproductive behaviour patterns or ovulation rates, indicate that species differences in the size of the follicular store, perhaps accompanied by more parsimonious utilization, could be a life strategy which guarantees fecundity throughout most of the lifespan. The number of follicles in humans at menarcheal age is commensurate with body size, and follicular deficiency at menopause in mid-life may have therefore arisen adventitiously with lifespan extension.

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Introduction

The processes of oogenesis and follicle formation in mammalian ovaries have been completed by birth or shortly thereafter. The dynamics of these developmental processes and of subsequent follicle utilization are therefore expected to ensure that individuals can eke out their limited store of germ cells throughout life, whilst providing sufficient flexibility for fertile cycles to continue under favourable external conditions.

There is a large body of knowledge showing the effects of scaling on physiological parameters, many of which vary with body weight ($M$) according to the allometric formula $y = aM^b$; but there is little information about scaling of factors relating to reproductive system ageing (Calder, 1984). The present study was designed to test: (a) whether the provision of follicles for adult life varies allometrically with body weight and (b) the relationships between the numbers of follicles and body weight on the one hand and the maximum life expectation of the species on the other. To achieve a uniform basis for comparisons, the follicular populations of peripubertal ovaries have been studied, since developmental states at puberty are comparable in different species and only follicles which are potentially capable of contributing to fecundity will be present.
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* Mean number per pair of ovaries
** Ratio of the number of growing follicles to the size of the total follicular population
NUMBERS OF OVARIAN FOLLICLES

Materials and methods

Data were obtained from non-pregnant representatives of 19 species among 8 orders (see Table 1). Ovaries were obtained either at autopsy or at ovariectomy and prepared as serial histological sections 8 μm thick and stained with H. & E. They were collected without regard to the stages of the sexual cycle since the numbers of primordial follicles vary independently and there is the practical difficulty of finding a comparable stage among the variety of patterns, which included reflex ovulation in some species.

The average number of small, non-growing ('primordial') follicles in each species was obtained by counting in every 10th, 20th or 40th section (depending on organ size) for 4-8 individuals. Either the nucleus or, when single and distinctive, the nucleolus, was used as a marker and the total numbers of follicles were estimated by multiplying by both the sampling frequency and a correction factor (Abercrombie, 1946). Growing follicles were counted in the same sections, these being defined by those ranging from unilaminar follicles with a growing oocyte and cuboidal epithelium to Graafian sizes. Atretic follicles were included in the counts when the marker remained distinct. In the rare cases of binovular and polyovular follicles, it was the follicle rather than the enclosed oocytes that was counted. An attempt was made, however, to reduce this additional source of variation by choosing individuals and breeds in which the body size was close to that characteristic of the species. Average values for body weight (M, in kg) and ovulation rate at young adult ages were obtained from either unpublished or published sources. In most species, there was sufficient reliable data for estimating the maximum adult lifespan by subtracting the characteristic pubertal age from total longevity.

Data for the following 5 species were extracted from studies in which comparable cytological methods have been used. Those for domestic swine and cattle were obtained from Erickson (1966, 1967). In laboratory mice (strain A) and rats and humans, the numbers of follicles at pubertal ages were obtained from regression lines, which were based on large sets of data which extended across most of the lifespan (Block, 1952; Mandl & Shelton, 1959; Jones & Krohn, 1961). The regressions were obtained using an exponential model, N = Ae^{-bt} since this produced a good fit with each data set (coefficients of determination, r^2 = 0.94, 0.80, 0.49, respectively) and facilitated estimation of follicle numbers at puberty and the fractional disappearance rates for small follicles in adult life.

Results and discussion

The species characteristic number of follicles per individual at the onset of fertile life varied hypoallometrically, the body weight exponent being 0.475 ± 0.038 (Fig. 1). Since r^2 was 0.91, most interspecific variation was accounted for by differences in adult body weight rather than in numbers or frequency of ovulations or reproductive pattern. The range of species tested did not, however, include those having prodigious ovulation rates, and it will be interesting to compare the numbers of small follicles on a body weight basis in the Plains viscacha, Lagostomus maximus (Weir, 1971), and the Elephant shrew, Elephantulus myurus (Tripp, 1971), when suitable data become available. With the single exception of the domestic bitch, the great majority of follicles in all species were uniovular and, therefore, allometric relationships for follicles can be read for oocytes. The apparently straightforward scaling of the numbers of follicles or oocytes according to body weight contrasts with the pattern in primate testicles which vary in size and, presumably, therefore in gametogenic capacity, according to reproductive behaviour (Harcourt, Harvey, Larson & Short, 1981).

The allometric relationship between the maximum longevity of a species (L, in years) and body weight was found to be 11.8M^{0.20} (Sacher, 1959), which agrees with the present finding of 9.85M^{0.25} for the adult lifespan. A comparison of allometric exponents indicates that the numbers of follicles at puberty rises more steeply with a unit increase in body weight than with life
expectation. This implies that follicular redundancy increases in larger animals. The same conclusion is indicated by the hyperallometric relationship existing between follicle numbers and adult longevity, for which the exponent is $1.592 \pm 0.188$ ($r^2 = 0.84$) (Fig. 2). The slope of the graph does not strictly match the variation in redundancy within the follicular population since ovulation rates tend to vary inversely with body size. It should be pointed out, furthermore, that the distribution of redundant follicles may be asymmetrical. In the Greater horseshoe bat ovulations occur only in the right ovary (Harrison Matthews, 1937), whereas it appears that follicles are divided approximately equally within pairs of ovaries.

![Graph showing variation in follicle numbers with body weight.](image)

**Fig. 1.** Variation with body weight in the numbers of primordial follicles per individual at the beginning of adult life. The relationship is depicted by linear regression analysis with the 95% confidence intervals and the allometric formula being shown. See Table I for species code list.

The increased follicular store size in larger species could be an important adaptation for greater span of adult life. It is presumably due to a greater number of oogonial mitoses during the pre- or perinatal period, although species differences in the rates of germ cell wastage could also contribute to the pattern at puberty. The surplus provision of follicles would appear to provide a wide margin of physiological safety, which has been postulated for other phenotypic characters (Gans, 1979). An alternative theory suggests that a large store reflects the dynamics of postnatal follicle utilization, since follicle numbers are distributed exponentially or mult-exponentially by age, with the store size being substantially reduced or even exhausted at the end...
of life (Faddy, Gosden & Edwards, 1983). Accordingly, these dynamics present the ovary with the physiological problem of having to dispose of growing follicles by atresia at young ages when there is a surplus above the small and steady requirements of ovulation. The well-known capacity for superovulation following unilateral ovariectomy or priming with exogenous gonadotrophins may well be an incidental result of a surplus of follicles during the cycle.

A consideration of life history strategies for producing and maintaining germ cell numbers should take account of the rates at which primordial follicles disappear as a result of atresia and recruitment for ovulation. The numbers or fraction of growing follicles are far too variable to be useful indicators of the rate of depletion of the follicle store (Table I). Furthermore, these measurements do not take account of primordial follicles which disappear without having entered the growing follicle population. On the other hand, the slopes of the exponential age distributions of small follicles take account of this potential factor, although data is at present confined to only three species. The slopes obtained for mice, rats and humans are $-1.136 \pm 0.053$, $-0.402 \pm 0.025$ and $-0.043 \pm 0.008$, which correspond to follicle population half-lives of 0.27, 0.75 and 6.99 years, respectively. Thus, the dynamics of follicular utilization would appear to be scaled inversely with body size, indicating that longer-lived species utilize their follicles more conservatively. Data from many more species will be required before any attempts can be
made to test rigorously for allometric variation and, by extension, to determine whether a balance exists between the size of the store and the rate of utilization, so leading to similar residual numbers of follicles near the end of life. Such hypothetical inter-relationships may, however, be difficult to detect because of superpositional effects of the variability of life patterns. For instance, the numbers of follicles were commensurate with body weights in the two species of bats studied (Fig. 1), but they lie far to the right of the distribution with respect to adult longevity based on the limited information which suggests a lifespan of 10–20 years in the natural environment (Fig. 2, Stebbings, 1977). Compared with rodents and insectivores of similar size, these species may depend on parsimonious utilization of follicles in order to prevent premature loss of fecundity. At present, there are no observations to support this expectation, although the lowering of metabolic rate during hibernation could provide a mechanism.

Primary ovarian failure has rarely been reported to occur before the end of life in animals except among a few inbred and mutant strains of rodents (Finch & Gosden, 1986). There, however, the notable exception of menopause in our own species which is usually attained in mid-life and is considered to be primarily a result of follicular deficiency (Gosden, 1985). Since the size of the human follicular population at puberty is consistent with the norms predicted on body weight (Fig. 1), menopause cannot be attributed to an antecedent deficiency. Early ovarian failure in our species could potentially be caused by a comparatively high rate of follicular attrition, but evidence is lacking. There is, however, a prima facie case for arguing that menopause has arisen adventitiously during evolution. Menopause may be a by-product of the extension of lifespan beyond expectation based on body size since, in contrast with the maximum longevity of approximately 100 years, allometry predicts a figure of around 30 years for our species.

We thank Kay Grant for technical assistance, Dr J. Cohen for suggesting improvements in the manuscript and Drs J. Clarke, A. F. Dixon, Janet M. Nicholson, P. A. Racey, J. Searle and Suzanne Ullmann for providing specimens. The project was supported by a grant from the Medical Research Council.

REFERENCES

A method has been developed to permit the early stages of follicle development to be investigated in vitro. Ovaries from litters of CBA/C3H/Bi mice were dissected and cultured in collagenase for 30 minutes at 37°C. Ovaries were isolated by repeated pipetting using a Gilson pipette. Follicles were cultured in medium 199 supplemented with ITS (Insulin, TRANS-2,4-diaminobutanoic acid) at 37°C in 5% CO2. The culture medium contained 20% bovine serum albumin. When the follicles were cultured for 6 days in the presence of LH, they continued to develop and grow in size. In later stages of development, the follicles were identified using autoradiography. Metaphase spreads were maintained in these conditions for up to 1 week. In order to identify the follicles at different stages of development, the method of counting the number of oocytes in the follicles was employed. This approach allowed the identification of the follicles at different stages of development, providing valuable information about follicle growth and development.
A novel technique for isolating and culturing mouse ovarian follicles.

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A method has been developed to permit the early stages of follicular development to be investigated in vitro. Ovaries from 8 - 11 day CBA/C57BL6 F1 hybrids were bisected and incubated in collagenase for 30 minutes at 37°C. The follicles were isolated by repeated pipetting using Gilson pipettes. Follicles were cultured in Medium 199 supplemented with 10% donor calf serum at 37°C in 5% CO2. The isolated follicles consisted of an oocyte with 1 to 3 layers of epithelial cells, generally stripped of adhering stromal cells. When the follicles were cultured on a plastic substrate the granulosa cells soon became adherent and began to spread, disrupting the follicle. The oocyte was shed within 1 - 3 days. To maintain the structural integrity of the oocyte-granulosa unit the follicles were embedded in a matrix of collagen extracted from rat tail tendons. 20 µl samples were transferred to the wells of a Terasaki plate, excess fluid was removed and 10 µl of collagen gel solution added. The gel was set and transferred to a second well containing more collagen gel solution. The final gels were set and cultured as above. The collagen gels can be fixed and processed for assessment by histology and autoradiography. Preliminary results indicate that the follicles can be maintained in these conditions for several days and may continue to progress to later stages of development. Although some follicles are damaged by the procedure, follicles with 4 or more intact layers of granulosa cells have been identified after 8 days in culture.

A quantitative cytological study of polyovular follicles in mammalian ovaries with particular reference to the domestic bitch (Canis familiaris)

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Summary. The incidence of polyovular types in the growing follicle population was estimated using quantitative cytology. Of 15 species studied, polyovular follicles were recorded in the following species and in ascending order of abundance: rabbits, rhesus monkeys, humans, cats, dogs. The incidence in bitches was 14% in animals aged 1–2 years but only 5% at 7–11 years old. The frequency of the various types of polyovular preantral follicle varied inversely with the numbers of oocytes per follicle and the probability of finding a follicle with more than 5 oocytes was remote. In young ovaries the frequency was constant in the early stages of growth but decreased in the largest preantral stage. The pattern in ageing ovaries was, by contrast, one of declining frequency such that few if any polyovular types completed development. The ovary of the ageing bitch was also characterized by a higher incidence of degenerating follicles and a much smaller pool of primordial stages. Polyovular follicles were larger than uniovular types at comparable stages which were defined by the number of granulosa cell layers. Their oocytes were smaller but the overall ooplasmic mass was increased with a corresponding increase in the mass of granulosa cells.

Introduction

The majority of ovarian follicles contain only a single oocyte although many authors have drawn attention to instances in which two or more oocytes are present. Binovular and multiovular follicles (henceforward called polyovular follicles) have been reported sporadically in most mammalian species since Von Baer’s discovery of the oocyte (Von Baer, 1827). The early literature on polyovular follicles was primarily descriptive histology and was reviewed by Hartman in 1926, but subsequent work has mainly contributed to the range of species in which these are found rather than attempting the larger task of quantitation (see Mossman & Duke, 1973). Progress towards obtaining reliable data and, hence, making inferences about the origin and developmental potential of these structures has undoubtedly been hampered by the problem that polyovular follicles tend to occur infrequently in most species.

Interest in polyovular follicles has stemmed from two questions, namely, their ontogenesis and their contribution to fecundity. These follicles have frequently been regarded as pathological entities and this has led many investigators to pay attention to the incidence of oocyte death and the role of sex steroid and gonadotrophin concentrations (Lane, 1938; Bodemer & Warnick, 1961a; Kent & Mandel, 1970). The changing hormonal ‘balance’ after puberty provided an explanation for the apparent fall in the incidence of polyovular follicles as animals matured (Kent, 1960). The early inquiries were, however, influenced by a limited knowledge of reproductive endocrinology and the widespread and erroneous belief that oocytes are formed continually throughout life. Polyovular follicles are also of interest because they present a natural experiment in which the general rule of one oocyte:one follicle has been altered with possible developmental significance for the follicle and its various compartments.
The present study was devised to obtain quantitative data about polyovular follicles at different developmental stages in order to identify patterns, to draw inferences about ontogenesis and to make predictions about the contribution of these structures to ovulation at different ages. The data also provided an opportunity to make an initial judgment whether the development of polyovular follicles is substantially different from that of uniovular types.

These objectives depended on the availability of ovaries having a sufficiently high incidence of polyovular follicles. Since published reports rarely present numerical data and where these exist they are not based on a standard classification of follicles, a survey was carried out to identify a suitable species for detailed study. The domestic bitch was chosen on the basis of having a higher frequency of polyovular follicles than did any of the other species studied.

Materials and Methods

Incidence of polyovular follicles in different species. The proportions of growing follicles containing 2, 3, 4 or more oocytes were estimated using histological specimens which had been collected during an earlier study (Gosden & Telfer, 1987). Fifteen species were examined in toto (see Table 1) with at least four young adult individuals representing each species except for man for which only one ovary from each of two patients was available. A minimum of 1000 follicles was examined for each species. The nucleus of the oocyte was used as a marker for counting the follicles and, when appropriate, adjacent sections were searched to determine the complete set of oocytes in polyovular follicles.

On the basis of the findings, additional specimens were obtained from bitches for further studies.

Source and preparation of specimens. Ovaries were obtained during routine veterinary spaying of cross-bitch bitches weighing 3.8-21.5 kg. The animals comprised a group of 10 virgins aged 1-2 years old and another group of aged 7-11 years with reproductive histories. All animals were anoestrous and healthy at the time of surgery.

The reproductive tracts were immersed in buffered formalin for transit to the laboratory where the ovaries were dissected and fixed in Susa for 24 h. One ovary from each pair was prepared by serially sectioning at 10 pm and staining with haematoxylin and eosin.

Classification of follicles in bitch ovaries. Each follicle was classified according to the 'stage' in the development continuum ('stage') and the 'type' of follicle, as defined by the numbers of oocytes present. The follicle stages were based on the scheme used for rat ovaries by Mandl & Zuckerman (1951) in which the appearance of the oocyte and the number and morphology of the granulosa cell layers are the defining variables (Fig. 1). The follicle types were defined according to the numbers of oocytes present; i.e. type 1 contained a single oocyte, type 2 contained two oocytes, and so on to type 5+. Follicles were also distinguished according to their qualitative appearance: those having a wrinkled oocyte(s) and/or pyknotic granulosa cells were deemed to be degenerating ('atretic'). The data presented in the 'Results' comprise only follicles which appeared to be healthy because it was frequently not possible to assign degenerating follicles to a definite stage and type.

Protocol for counting bitch follicles. A sampling frequency of every 20th serial section was used because a single bitch ovary may yield more than a thousand 10 pm sections. Primordial (non-growing) follicles and uniovular growing stages were counted using the oocyte nucleus as a marker as this is small and discrete and normally single. The total number of follicles per animal was obtained by multiplying by (a) the sampling frequency (20), (b) a correction factor because of overcounting biases, and (c) 2 to obtain the value for a pair of ovaries. The correction factor was obtained by a standard method based on the mean diameter of the nucleus and the section thickness (Mandl & Zuckerman, 1951).

Primordial follicles could not be characterized as polyovular because the boundaries of these small follicles are not easily resolved using the light microscope. The procedure for counting the numbers of polyovular growing follicles differed from that used with the uniovular types. Having identified at least one marker in a polyovular follicle, adjacent sections were searched to determine the total complement of markers (oocytes) present. In general, the added step was important only in larger follicles (stage V) in which the oocytes were more widely dispersed. The product of the raw counts and the sampling frequency produces the estimated numbers of polyovular follicles in an ovary. This method may, however, overestimate the actual number present because follicles carrying a larger number of dispersed oocytes are more likely to be detected in the samples. This problem is not amenable to a simple theoretical approach for obtaining correction factors, as has been the case for uniovular follicles, because there are several markers present and any given section may not contain all of them. Since an examination of every section is impractical, a correction factor was obtained by the following empirical method. The number of polyovular follicles at an intermediate stage (III) was counted in 200 consecutive sections in each of 3 ovaries. The correction factor was obtained by dividing this number by the number obtained from sampling every 20th section. Pilot studies showed that the correction factor was accurate only marginally after applying the corrected values.

Measurements of follicle and oocyte dimensions. A random sample of 206 uni- and polyovular follicles was used to measure follicles and oocytes in their equatorial planes, as follows. (1) The mean follicular diameter was obtained by...
measuring the maximum and minimum diameters using a microscope fitted with an ocular micrometer. The boundary of the follicle was defined by the outer circumference of the granulosa layer because the theca layer was indistinct in small follicles. (2) The diameters of oocytes and their nuclei were measured by a similar method. (3) Precise measurements of nucleolar diameters were obtained using an image-shearing device with oil immersion optics ($\times 1000$) which provide a theoretical resolution of less than 0.2 μm (Swyte & Rosberry, 1977).

Results

Incidence of polyovular follicles in different species

Uniovular follicles were the predominant type in the animals investigated. In 10 of the 15 species studied polyovular follicles were not detected although further and more extensive searches sometimes revealed small numbers of binovular types. The incidence of polyovular follicles in the other 5 species varied from 1% in the rabbit to 14% in the bitch; in human ovaries 3% of the follicles were either bin- or trinovular (Table 1). In each ovary the numbers of follicles of the different types varied inversely with the numbers of oocytes that they contained.

The follicle profile of the anoestrous bitch ovary

The follicles were confined to a narrow cortical band and the large volume of medullary tissue was afoollicular and fibrous. Primordial follicles were frequently clustered but the boundaries of these small groups were not clearly circumscribed at the light microscope level (Fig. 2); consequently, groups of oocytes which may be predisposed to polyovular development could not be identified at this stage. Polynuclear oocytes were rarely encountered. Some primordial follicles appeared to be in the process of degeneration but atresia was more clearly characterized in growing follicles in which the oocyte appeared wrinkled and the granulosa cells pycnotic. Polyovular follicles (Fig. 3) could not be confused with atretic follicles containing a fragmented oocyte since a distinct nucleus was present in each of the oocytes. About 10% of the growing follicle population in young ovaries was atretic by these criteria, as opposed to 30% in the older bitches. The proportions of the different types of polyovular development were remarkably consistent from stage to stage. Anovular ‘follicles’ were observed occasionally in ageing ovaries; the space formerly occupied by the oocyte had been invaded by granulosa cells.

The total numbers of primordial follicles per animal were highly variable (Table 2). The mean in young ovaries was 85 800 (s.e. 18 600) compared with only 2750 (s.e. 900) in the older group ($P < 0.05$ using the Kruskal Wallis non-parametric test). The primordial follicle was the most abundant stage present and, like the other stages, suffered substantial losses during ageing. In contrast to these reductions, the percentage of follicles in the growing stages rose substantially since the relative age-dependent losses in the growing and non-growing sub-populations differed.
Table 1. Frequency of polyovular follicles in 15 mammalian species

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<td>Field vole</td>
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<td></td>
<td>&lt;0.10</td>
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<tr>
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<td>&lt;0.10</td>
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<td>&lt;0.10</td>
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<tr>
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<td>0.91</td>
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<td>3.61</td>
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<tr>
<td>Bitch</td>
<td>Canis familiaris</td>
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<td>8.89</td>
<td>2.97</td>
<td>2.08</td>
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<tr>
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<td>Ovis aries</td>
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<td>Marmoset monkey</td>
<td>Callithrix jacchus</td>
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<td>&lt;0.10</td>
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<td>Macaca mulatta</td>
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<td>0.30</td>
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<tr>
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<td>Homo sapiens</td>
<td></td>
<td>2.72</td>
<td>0.19</td>
<td>&lt;0.10</td>
</tr>
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</table>

Fig. 2. The ovarian cortex of a young adult bitch which contains numerous primordial follicles in clusters. H & E, ×160.

Whereas in young animals only 10% of follicles were classified as growing, over 50% were growing in the older group.

Polyovular follicles were present in all animals with the single exception of a young bitch, except when stated otherwise, has been included in the presentation of data. They were found in each of the four stages of preantral growth (II–V) in young ovaries and at stages II–IV in the older groups (Tables 3 & 5). Overall, their incidence was similar to that obtained during the pilot study using a small sample (Table 1), being 14% in the young group but only 5% in the old animal.
Polyovular follicles 141

Fig. 3. Detail of the cortex of the bitch ovary showing a growing follicle containing 5 oocytes (centre). H & E, × 320.

Table 2. A quantitative profile of the follicle population of anoestrous ovaries in young adult and ageing bitches

<table>
<thead>
<tr>
<th></th>
<th>Young animals</th>
<th>Ageing animals</th>
</tr>
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<tr>
<td>No. of animals</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>1–2</td>
<td>7–11</td>
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<tr>
<td>No. of primordial follicles (range)</td>
<td>5400–214 000</td>
<td>1200–6150</td>
</tr>
<tr>
<td>No. of growing follicles (range)</td>
<td>3470–13 600</td>
<td>1720–6770</td>
</tr>
<tr>
<td>% growing follicles</td>
<td>10</td>
<td>51</td>
</tr>
<tr>
<td>No. of polyovular growing follicles (range)</td>
<td>423–5930†</td>
<td>62–238</td>
</tr>
<tr>
<td>% polyovular growing follicles</td>
<td>14</td>
<td>5</td>
</tr>
</tbody>
</table>

†Excludes one animal in which polyovular follicles were absent.

Table 3. Numbers (and s.e.m.) of growing follicles in young bitch ovaries classified according to the stage of development and number of oocytes ('type')

<table>
<thead>
<tr>
<th>Follicle stage</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5+</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>4002 (585)</td>
<td>378 (104)</td>
<td>119 (47)</td>
<td>56 (34)</td>
<td>49 (34)</td>
</tr>
<tr>
<td>III</td>
<td>2089 (32)</td>
<td>210 (77)</td>
<td>90 (43)</td>
<td>40 (57)</td>
<td>27 (20)</td>
</tr>
<tr>
<td>IV</td>
<td>972 (132)</td>
<td>147 (60)</td>
<td>59 (30)</td>
<td>20 (13)</td>
<td>5 (5)</td>
</tr>
<tr>
<td>V</td>
<td>1251 (209)</td>
<td>128 (60)</td>
<td>13 (14)</td>
<td>4 (3)</td>
<td>2 (2)</td>
</tr>
</tbody>
</table>
Fig. 4. Percentage frequency distribution at successive stages of development of polyovular follicles in ovaries of (a) young bitches (N = 10) and (b) ageing bitches (N = 5). Values given are mean + s.e.m.

The consistent proportions of polyovular follicles from stage to stage were striking in view of the variable numbers of follicles present (Fig. 4a). In young animals, it was only at stage V that proportions of these follicles fell and this effect was restricted to types 3+ (P < 0.01). The number at any given stage appeared to decline approximately exponentially as the numbers of oocytes increased. Since estimating the incidence of follicles with more than 5 oocytes was hazardous because of sampling errors at low frequencies the tabulated results and histograms have not been extended beyond type 5+ in which the less common types were combined. The largest number of oocytes observed in a single follicle was 9 in this series although up to 14 were observed in other bitches. A simple probability table was constructed to indicate the chances of encountering growing follicles of a given type and stage (Table 4). This demonstrates that the probability declines steadily from left to right as the numbers of oocytes increase, in contrast there is less variation on the vertical scale which represents successive stages of follicle growth. Clearly, a large number of follicles must be sampled to obtain reliable estimates of follicles with 4 or more oocytes.

The incidence of different follicle types in older bitches demonstrated a pattern similar to that described in the younger group (Fig. 4b) although there were two major differences between
Firstly, there was the overall reduction in numbers and incidence of polyovuly as was mentioned above. Secondly, the consistent proportions of polyovular follicles from stage to stage was lost. Despite having sampled hundreds of sections of ageing ovaries polyovular follicles were never detected at stage V and only a few binovular types were represented at the preceding stage (Table 5). The probability table suggests that the chance of finding a binovular follicle at the earliest stage of growth in older animals is only slightly lower than that for young ovaries whereas that of finding other types rapidly approaches zero (Table 6).

Dimensions of follicles and oocytes

The data were aggregated from young and old animals since the size of follicles and oocytes were independent of age. The diameter of oocytes increased in direct proportion to that of the follicle until the latter had reached approximately 300 μm at stage V when the oocyte had attained
Fig. 5. Variation in the diameters of oocytes in uni- and polyovular follicles at different stages of development in bitch ovaries. Values are means ± s.e.m.

Fig. 6. The proportion of follicle volume occupied by the oocyte(s): variation with the stage of development and the numbers of oocytes per follicle. Values are mean and s.d. because the s² values were too small to be drawn.

its mature size of 90–110 µm. Whilst the size of oocytes was similar in polyovular follicles at stage II, at subsequent stages it was always greater in uniovular types (Fig. 5). Frequently, the oocytes were packed closely to one another near the geometric centre of the follicle (Fig. 3) but when they were more scattered it appeared that the peripheral oocytes were smaller than the central ones.

Polyovular follicles were substantially larger than uniovular types at the same stage of development. This difference, which was 2–3-fold in volume, could be accounted for by the greater volume of oocytes in toto and larger numbers of granulosa cells. The granulosa cell volume was deduced from the difference between the follicle volume and the total volume of oocytes, the assumption that the volume of individual granulosa cells is constant and that there is extracellular space at preantral stages. Despite the smaller sizes of individual oocytes these volume in polyovular follicles was increased. The relationship between the volume of the oocyte...
and that of the follicle was not constant and when expressed as proportions there was a tendency for the granulosa cell component to rise during development (Fig. 6). The proportion occupied by oocytes was greatest at stage II for uniovular follicles, this being significantly greater than in comparable polyovular types ($P < 0.05$). At later stages the proportions were relatively even between types.

### Discussion

The quantitative profile of follicles in the bitch ovary conveys the impression that polyovular development is neither a spurious nor a pathological phenomenon but is a natural polymorphism arising from a spectrum of possible numerical combinations of oocytes and pregranulosa cells. Uniovular development is the dominant mode in all species investigated but polyovuly is widely distributed albeit at varying frequencies (see also Bocharov, 1966; Mossman & Duke, 1973).

These new data are consistent with, but do not prove, the hypothesis that polyovular follicles are predetermined during folliculogenesis. Concrecence was not observed between growing follicles and this possibility is apparently denied by the stable or falling incidence of polyovuly at successive stages. A third explanation for the presence of polyovular follicles proposes that either polynucleate or mononucleate oocytes divide (Hartman, 1926; Iguchi et al., 1986) but this can be refuted in the present examples because of a deficiency of the former with a lack of excess numbers of follicles with an even number of oocytes. Whilst there is circumstantial evidence for the predetermination of polyovular follicles the latter cannot be claimed to pre-exist in the primordial follicle pool since there is neither cytological nor physiological evidence of intercellular metabolic coupling. Light microscopy gives only a suggestion of physical intimacy between oocytes but fine structural details must be clarified if the possibility of synchronous development is to be inferred. Cortical oocytes occur in dense plaques in young ovaries of species having abundant polyovular follicles, but density-dependence cannot be the only causal factor since intimately clustered oocytes occur in some species which rarely present polyovuly (e.g. sheep).

One of the striking features of the data is the inverse relationship between the numbers of oocytes per follicle and the numbers of follicles. The frequency distribution suggests that a stochastic process has been operating although this hypothesis cannot be verified in the adult ovary since utilization and death of follicles has modified the original population and, additionally, incipient polyovular follicles cannot be identified. The conclusion that polyovuly arises from random events during folliculogenesis conflicts with most of the suggestions in the older literature. On the basis of early claims that polyovular follicles were less common after puberty than in the immature ovary (Davis & Hall, 1950; Dawson, 1951; Bodemer & Warnick, 1961b) some workers obtained evidence that the incidence of these follicles depends upon the balance of sex steroids and pituitary gonadotrophins (Kent, 1959; Graham & Bradley, 1971). The present results do not deny a possible role for these hormones, but the predetermination hypothesis proposes an explanation for polyovuly on the basis of cellular interactions occurring during folliculogenesis which may or may not depend on sex steroids and the gonadotrophins.

Polyovular follicles are less common in older bitches than in animals beginning their reproductive life: they are fewer in number and comprise a lower percentage of the growing follicle population. As more specimens become available it will be interesting to determine whether polyovular follicles become progressively more sparse throughout life. Whilst the probability of finding a binovular follicle was only slightly reduced during ageing, those for types 3 and 4 were considerably lower and other types were entirely absent. Since the animals were anoestrous it is unclear whether there is a hormonal basis for these findings. The age effect could be due to a reduced frequency of predetermined polyovular follicles in the reserve pool as a result of increased utilization at young ages and/or decreased viability of follicles entering their growth phase. The nature of the stimulus that prompts primordial follicles to start growing is not known, although it is
widely assumed to be a spontaneous event. If it is conjectured that this event occurs independent in oocytes and that activation can be transmitted to physiologically coupled oocytes it is to be expected that the probability of utilization will vary with the number of oocytes within a follicle. This model is in accord with the observed changes in the follicle profile. On the other hand, a diminishing frequency of polyovular follicles at each successive stage of growth suggests that these are less viable in old ovaries. The environment of the ageing ovary may be less capable of providing physiological support for the growth of follicles, and this is consistent with the observed high incidence of follicle death.

Oocytes in polyovular follicles start their development at a size similar to that of oocytes in uniovular follicles. Size differences emerge, however, at stage III when two granulosa cell layers are present but the oocytes undergo morphologically normal development. This may suggest some developmental lagging but it could also be argued that the stages of uni- and polyovular follicles are not comparable. This hypothesis is difficult to evaluate from a morphological approach.

Polyovular follicles were larger than uniovular types at the same stage and it will be interesting to determine whether these differences continue into the antral stages. The number of granulosa cells inferred from measurements of follicle and oocyte volumes rose pari passu with the total number of oocyte(s), which may indicate that cellular interactions are co-ordinating the growth of the compartments. There would appear from the morphological evidence to be no reason to suspect that oocytes in young ovaries are much less viable in polyovular follicles compared with the non-polyovular follicles and it is likely that this can reach preovulatory maturity, each being enclosed in a separate cumuli oophori (Dederer, 1934; Davis & Hall, 1950; Andersen & Simpson, 1973).

In addition to the problem of onogenesis, polyovular follicles have attracted interest because of their potential contribution to the quota of oocytes shed at ovulation and, hence, to the species characteristic litter size. According to the present results this contribution is expected to be small even in species having relatively abundant polyovular follicles and it is likely to be only a minor factor in older animals. The quantitative significance of these follicles can be illustrated by a simple example. If it is assumed that all oocytes in stage V follicles are equally viable and that the selection of dominant follicles is random, the chance of ovulating a polyovular follicle of any type would be a product of their incidence at that stage and the number of follicles selected for ovulation. We can see from the data that the pool of follicles available for ovulation contains 10 times as many uniovular follicles as polyovular follicles. Assuming that the mean ovulation rate in the bitch is 0.06 and using the values obtained for follicle type at stage V, we can estimate that the probability of a binovular follicle being ovulated will be 0.06. Because estimates based on the mixed group of follicles at stage V do not represent the ovulatory pool of follicles, any probability values are likely to be overestimates. In view of these predictions and the observed age change it appears that the rising fecundity during the first half of the reproductive lifespan, as measured by the frequency of dizygotic twins in women (Bulmer, 1970) and the successively larger litters in young primates (Kennedy & Kennedy, 1972), is probably due to a higher ovulation rate rather than a contribution of polyovular follicles.

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Bodemer, C.W. & Warnick, S. (1961b) Polyovular follicules


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Follicular status at the menopause*

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The store of ovarian follicles which is formed before birth progressively diminishes as a result of follicle death (atresia) and recruitment towards ovulation and will become barren in time. Depletion has occurred in the human ovary at or shortly after the menopause bringing reproductive potential, which has been declining for several years, to a final halt. Several factors can affect the timing of menopause and it appears that changes in the rates of follicle death have a greater impact than differences in the initial numbers of follicles. It is argued that the precocious loss of fecundity in humans, as compared with virtually all animals, has arisen adventitiously as a consequence of the evolution of long life relative to body weight.

Key words: menopause/ovarian follicle/depletion/atresia/evolution

Introduction

Menopause is the final menses and signifies that the reproductive lifespan has ended. It occurs at a median age of 50–51 years in most populations but is earlier in undernourished and malnourished women (Gray, 1976). Reproductive potential begins to wane much earlier judging by the impaired ability to conceive (Fédération CECOS et al., 1982), the irregular menses that herald menopause (Trelor et al., 1967; Vollman, 1977; Metcalf et al., 1981a, b) and the rising incidence of chromosomally defective zygotes (Bond and Chandley, 1983). Whilst the ovary does not carry sole responsibility for lost potential there can be little doubt that its age changes affect fecundity during the reproductive years and set an upper limit on the fertile lifespan. The evidence for this conclusion and the biological significance of early follicle loss and menopause are discussed in the following sections.

Numbers of follicles remaining at menopause

The morphology of the ovaries of post-menopausal and young fertile women are strikingly different (Figures 1 and 2). The weight of the adult ovary decreases from 7 to 4 g after menopause owing to the absence of large follicles and fresh corpora lutea (Nicosia, 1986).

The cortex of the post-menopausal ovary contains superabundant stroma but lacks follicles (Boss et al., 1965) (Figure 2). It has been suggested that, as in most senile animals, small numbers of both non-growing (primordial) and growing follicles remain after menopause (Costoff and Mahesh, 1975; Baker and Franchi, 1986). This conclusion appears to be without any quantitative foundation; moreover, the postmenopausal status of the subjects was not demonstrated. In a recent study of Canadian women aged 45–55 for whom well-documented endocrine profiles and menstrual histories were available the ovary was found to be virtually or completely afolicular after menopause (Richardson et al., 1987). Less than one thousand follicles remained in the ovaries of the women with irregular cycles (i.e. perimenopausal) whereas 10 times as many persisted in those who were of similar age but still cycling regularly. Thus, it appears that ovulation can occur in ovaries with a very small follicle store and that depletion is responsible for human menopause. The cytological evidence of follicle depletion is consistent with endocrine signs; the blood production rate for oestrogen being > 0.074 mg/24 h before menopause but only 0.006 mg/24 h afterwards. The circulating levels of FSH and LH rise accordingly (Gosden, 1985a). The follicular status is much the same whether women undergo menopause at about 50 or prematurely (<40 years) although in some circumstances early menopause is not necessarily equated with follicle depletion (Board et al., 1979; Aiman and Smenteck, 1985).

Such early depletion of oocytes in the human ovary is biologically remarkable since, apart from in a few strains of laboratory mice, follicle attrition does not take place in most species until the end of life, if at all. Such early senility of the human ovary is also exceptional in comparison with other organs. The numbers of neurons in, for example, the cerebral cortex and hippocampus decline in older people but these changes occur later in life, more variably and less completely than those of oocytes (Daura et al., 1985).

Dynamics of follicle formation and utilization

Among vertebrates it is only in mammals, birds and possibly elasmobranch and cyclostome fishes that oogonial mitoses are confined to pre-adult ages (Tokarz, 1978). In the other vertebrate groups (reptiles, amphibians and bony fishes) oogenesis recurs at every breeding season and this enables the fecundity to rise markedly as the animal grows larger and older. In humans the numbers of follicles present at a given age will be predicted by the algebraic product of the initial size of the follicle store and the rate of subsequent attrition. That the store is formed before birth is of great biological significance for it implies that germ
cell capital must serve the needs of reproduction throughout life and can be neither increased nor replaced.

Approximately 7 million human germ cells are formed in the ovarian rudiment but only ~2 million remain at birth and only 300,000 by menarcheal age (Block, 1952; Baker, 1963). Evidently this reduction occurs without ovulation and must be attributed to follicle death (atresia) affecting both growing and non-growing follicles. This process appears to continue during adult life because the store size far exceeds the maximum expected ovolutions (400).

The relationship between age and the numbers of primordial follicles has been well-characterized in mice. It is exponential or multieponential, implying that a constant fraction (rather than a constant number) of follicles disappear in unit intervals of time (Faddy et al., 1983). The size of the follicle store at birth varies between 9400 and 13,100 in different strains, but in one strain (CBA) it is exhausted by mid-life (450 days) (Jones and Krohn, 1961a). Other strains possess a residue of follicles until at least 600 days old, yet the ovulation rate is similar to CBA. The precocious sterility of CBA mice is due to a relatively high rate of loss of non-growing follicles. Early loss of follicles is therefore attributable to a high rate of follicle death rather than to ovulation or a small initial store.

It is far more difficult to obtain precise estimates of the rate...
Negative influences

Antibodies to partial ovariectomy
Ionizing cytotoxic substances
Dietary restriction
Genetic predisposition
Hypophysectomy

Factors affecting the age at which the ovarian follicle store is exhausted in humans and laboratory rodents

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<th>Delayed follicle loss</th>
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<td>Hypophysectomy</td>
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<tr>
<td>Cytotoxic substances</td>
<td>Dietary restriction</td>
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of small follicle utilization in the human ovary because the data are limited and highly variable. There can be no doubt, however, that follicle numbers dwindle progressively. The rate of loss is consistent with an exponential model because the data of Block (1952) and Gougeon (1984) are distributed log-normally (Figure 3). On close inspection of the data there are, however, tentative signs that the numbers of follicles during the last decade of menstrual life and at the normal menopause may be overestimated when the exponential model is extrapolated. This must be so if the ovary is to be depleted of follicles by age 50-51. Block’s data (1951, 1952) can be fitted using maximum likelihood methods either to an exponential model, in which the number of follicles \( y \) at a given age \( x \) is predicted by \( \frac{44,600x}{e^{413,100 - 16,600x + 164x^2}} \) (see Figure 3). In either case the resulting curves approach the baseline earlier than the asymptotic curve produced by a simple exponential relationship. These revised curves imply that follicles are being used at a faster rate in older ovaries. If substantiated, human follicle population dynamics are different to mice, in which a constant fraction of the population is being lost, and to rats, in which the fraction declines with time (Edwards et al., 1977).

Factors affecting the size of the follicle store at mid-life

Several factors can affect the rate of follicle attrition. Most are negative influences although two factors are known to extend the survival of the follicle population in mouse and rat ovaries (Table I).

The numbers of follicles at the beginning of adult life vary in a manner predicted by the body size of the species (Figure 4). Short-lived species possess comparatively few follicles which disappear rapidly; this contrasts with the parsimonious utilization of a large store in longer-lived animals (Gosden and Telfer, 1987). Evidently, the number of follicles and their rate of loss are determined by genotype at both the species and the individual levels. For example, there are substantial differences in follicle dynamics between different strains of mice (Jones and Krohn, 1961a, Faddy et al., 1983), premature menopause can be inherited in humans (Mattison et al., 1984) and early ovarian failure tends to be the rule in chromosomally abnormal individuals (e.g. Down’s and Turner’s Syndromes) (Russell and Altschuler, 1975; Reyes et al., 1976).

Certain cytotoxic agents are known to cause follicle death and the list of these is lengthening as research progresses (Dobson and Felton, 1983). The vulnerability of oocytes varies widely between species and at different stages of development. The sterilizing effects of ionizing radiation are well-known; it is estimated that the effective dose in humans is about 400 r (4 Gy), much higher than that for highly sensitive mouse oocytes (Baker, 1971). Potentially toxic substances readily gain access to the oocyte from the bloodstream because the follicle wall is porous (Baukloh et al., 1985). A number have been implicated in premature menopause, namely, organophosphorus pesticides, alkylating agents and components of cigarette smoke (Mattison, 1985). Some diseases are associated with premature sterility but in many cases it is difficult to distinguish direct effects of disease from the consequences of treatment.

Whilst many factors are potentially able to shorten fertile life by reducing the numbers of follicular oocytes there is no known way of delaying human menopause. Long-term inhibition of ovulation resulting from serial pregnancies or steroidal contraception has not been demonstrated to reduce the rate of small follicle utilization; neither would this be expected in view of the
negligible proportion that are ovulated and the continuing recruitment at all ages and in different physiological states. The natural ageing process of follicle attrition is slowed, but not halted, in rats and mice by hypophysectomy or underfeeding (Jones and Krohn, 1961b; Lintern-Moore and Everitt, 1978; Nelson et al., 1985; Meredith et al., 1986) but this has not been demonstrated in humans.

The long-term impact on the follicle population will depend on whether it is mainly the initial size of the store or the rate of follicle disappearance (i.e. atresia) which is affected. It will also depend on the age of the individual because a greater proportion of menstrual years will be lost when a child is affected as opposed to an adult. Mattison (1985) has compared the effects of varying the store size on the one hand with the rate of follicle disappearance on the other. He fitted Block's data to a simple exponential model from which he deduced that a critical number of about 3500 small follicles are present at the normal age of menopause. When the numbers of follicles at birth were reduced to 75 or 50 or 25% the critical number was reached at ages 47, 44 and 37 years, respectively, rather than at 50. Alternatively, if the population half-life was reduced by the same percentage points the ages corresponding to the critical follicle number were 38, 25 and 12. These predictions are based simply on the properties of exponential and linear relationships and they rest on the dubious assumption that the two variables are independent. They are instructive, however, because they draw attention to the much greater impact of changing the rate of atresia than the size of the follicle store. They are consistent with the aforementioned results with CBA mice in which high follicle death rates and early sterility were linked, and with the general recognition that removal of one ovary has a hardly perceptible effect on menopausal age.

Pre-menopausal fecundity

The ovary has an ability to maintain a surprisingly constant frequency and number of ovulations whilst follicle reserves continuously dwindle. Ovulations can occur as late as 16 weeks before menopause (Metcalfe et al., 1981b) when follicle reserves are very low. This ability depends on controlled maturation of the follicular store, surplus follicles being discarded by atresia. Fecundity is not, however, precisely the same throughout the menstrual years judging by the maternal age distribution for dizygotic twinning, which peaks at 36 years (Bulmer, 1970). This rise has been attributed to the maturation of an additional dominant follicle as a consequence of increased gonadotrophic stimulation rather than to polyovular follicles, which, at least in the domestic bitch, are less frequent later in life (Telfer and Gosden, 1987).

As the follicle store becomes depleted during the closing years of menstrual life recruitment of follicles is expected to become intermittent and may interrupt the normal regular sequence of cyclical events. Because of paucity of growing follicles the capacity to superovulate in response to exogenous gonadotrophic hormones is also anticipated to diminish with age, as is the case in ageing animals (Gosden, 1985b). The increasing proportion of follicles that are growing may be an indication that a larger proportion (if a smaller number) are being recruited to compensate for a diminishing store (Block, 1952; Gougeon, 1984). This could also result from a slower rate of development once follicles begin growing, although unlikely in view of rising levels of FSH and LH. There is a further explanation: that the rate of atresia is lower at intermediate follicle stages, as in ageing (Gosden et al., 1983). Finally, it should be recognized that the respective of changes in growth/atresia rate, the proportion growing follicles will rise terminally because the number reflects the follicle store when they started to grow some time earlier. There is little evidence which suggests that residual follicles in the ageing ovary are intrinsically absent from the well-documented predisposition of the oocytes to undergo chromosomal non-disjunction (Bond and Clarke, 1983).

The menopause in evolutionary perspective

In addition to its medical and social significance, menopause is an important problem for human evolutionary biology. As pointed out in an earlier section, the loss of oocytes is uniquely in humans by comparison with other mammals (Finch, Gosden, 1986). According to a sociobiological argument menopause may have evolved under conditions which favor the investment of the limited energy of older females in the protection and nurture of the offspring of kin rather than in their own reproduction (Mayer, 1982). This hypothesis implies that the early onset of post-reproductive life is caused by rather than simply an effect of ovarian failure. It rests on the untested assumption that life expectation among earlier hominids was sufficiently long for three successive generations to coexist.

According to another view, menopause arose not from any selective advantage conferred by a post-menopause but in extenuation with the evolution of extended life-span in our species. The number of human follicles at menarche is commensurate with the scale of body size (Figure 4), also an allometric (scale) relationship exists between the maximum expected longevity in body size in mammals (Sacher, 1959). Therefore, the number of follicles are positively correlated with longevity (Gosden and Telfer, 1987). In animals, the size and dynamics of the follicle store ensure that an adequate provision lasts until old age. During human evolution, however, it would seem that longevity became extended beyond the norms predicted by body size (15 to ~100 years) without a corresponding rise in follicle numbers or sufficiently parsimonious utilization of follicles. Consequently, the ovaries become sterile before the end of life. The existence of a long post-reproductive phase of life is, according to this view, a problem of explaining the evolution of longevity rather than of premature ending of fecundity.

References


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FOLLICULAR STATUS AT THE MENOPAUSE

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The menopause is the final menses and is a sign that irreversible loss of natural fertility has occurred. In most European nations it occurs at a median age of 50-51 years. The main characteristics of the endocrinological profile of postmenopause are a low circulating level of oestradiol (<10 pg/ml) combined with elevated FSH (>40-50 mIU/ml). These indicate primary ovarian failure, a conclusion that has now been verified by finding that exhaustion of the small follicle store coincides closely with the final menstrual cycle. Thus, the menopause is predetermined by the algebraic product of the initial size of the follicular store and the subsequent rate of attrition of small follicles. Irregular cycles in late menstrual life herald the approaching menopause and may be signs of erratic recruitment of follicles from a nearly depleted store. Certain types of menstrual dysfunction during the perimenopause may be accounted for by an alternative explanation: that there is defective neuroendocrine control of ovulation, as has been well-established in those strains of rats and mice that become acyclic in mid-life. It has been postulated that the menopause is an evolutionary adaptation for supporting reproduction in younger, optimally fertile individuals, but this hypothesis has to be squared with the evidence of allometric studies from a wide range of species that the size of the follicular store at the onset of menstrual life is commensurate with body mass. Menopause could therefore have evolved adventitiously with the extension of longevity in humans beyond expectations based on body size.

Physiological factors underlying the formation of ovarian follicular fluid

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Introduction

In common with all other cells, the oocyte and granulosa cells are bathed in extracellular fluid. It has, however, become conventional to reserve the term 'follicular fluid' for that fraction of the extracellular fluid that accumulates in the antrum of larger follicles. This pool of fluid is of considerable biological significance since its composition indicates the environment in which the oocyte and granulosa cells are growing and maturing. Furthermore, it buffers the internal environment of the follicle against the influence of external conditions presented by the blood stream.

The chemical composition of follicular fluid has been studied extensively and found to consist of substances derived from blood as well as from local secretion and metabolism. Particular attention has been paid to the proteins and hormonal steroids. Rather than attempt a comprehensive review, this paper will focus on general physical characteristics of the fluid and the physiological factors that influence its formation. These properties determine the rate at which extracellular fluid is accumulating and, hence, the size and morphogenesis of the Graafian follicle. It is important to reveal the mechanism and dynamics of follicular fluid formation if the composition of the fluid is to be fully understood.

Ontogenesis of the follicular antrum

Follicles do not possess a major pool of extracellular fluid from the beginning of their development. The primordial follicle consists of a relatively small oocyte surrounded by a single layer of squamous cells which are destined to become the granulosa cell layer (membrana granulosa). Pools of follicular fluid appear when the granulosa cells have passed through about 11–12 mitotic cycles and a solid follicle containing 2000–3000 cells and with a diameter of 150–400 μm has been built. These pools coalesce to form a single spherical cavity, the antrum, which is central and bounded by a layer of granulosa cells of uniform thickness except at the pole where cumulus cells are attached. The antrum characterizes the mature follicles of most mammals but it is not universally found. Ovulation is said to occur from 'solid' follicles in a number of insectivores (Mossman & Duke, 1973). These ovulations occur precociously because the follicles scarcely exceed the dimensions at which the antrum would normally form.

Quantitative aspects of follicular fluid formation

There is evidence which suggests that the size of follicles at the time of antrum formation varies with body size (Parkes, 1932; McNatty, 1978) although the raw data show that variation within a species is of a magnitude similar to that between species. There can be no doubt however that the
size of follicles shortly before ovulation varies with body size. When the volume of a preovulatory set of follicles is plotted against body weight on logarithmic axes, an isometric relationship is obtained (Gosden & Telfer, 1987). Most of this volume is extracellular space except in a few species in which the small follicles contain a diminutive antrum (e.g. *Sorex araneus*). Body size is not the sole arbiter of Graafian follicle volume since relatively small follicles occur in species that release prodigious numbers of eggs, although the collective volume remains commensurate with body weight (Weir, 1971). The size of the mature Graafian follicle is genetically determined and is of physiological significance. The volume of the antrum will influence the concentration of hormones and metabolites in the extracellular fluid and, consequently, their biological actions. Formation of the antrum requires the combined actions of FSH and oestrogen (Goldenberg et al., 1972) and it is anticipated that the volume changes will also be regulated by polypeptide and/or steroid hormones. In this regard, it may be significant that the concentrations of oestrogen and androgen in the antrum are very different in follicles that are expanding compared with those that are shrinking (atretic) (McNatty et al., 1979).

After Robinson (1918), it has been customary to distinguish three phases of follicular fluid formation. Primary fluid ("liquor") is produced until shortly before ovulation when the rate of accumulation rises abruptly. Secondary fluid is produced at this time, and presumably under the influence of the surge of gonadotrophic hormones. Tertiary fluid is produced in the collapsed follicle after ovulation. Robinson regarded primary fluid as having an intracellular origin whereas secondary fluid has been attributed to transudation from the thecal capillaries, a conclusion which has been upheld by many later commentators. Much less attention has been given to tertiary fluid it will not be considered further here.

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**Fig. 1.** The upper panel depicts the expansion of the antrum in mouse follicles which are either proceeding to ovulation (A–B) or, in the absence of an ovulatory stimulus, to formation of a follicular cyst (A–C). The lower panel represents the volume of the antrum at different stages and the estimated rates of fluid accumulation expressed on logarithmic axes. Measurements are based on histological sections and assume linear rates of growth.
In view of the long-established distinction between primary and secondary fluids it is surprising that this has not been quantified. The volume of the antrum and rate at which it is expanding can be estimated simply by making measurements of tissue sections. It is assumed that artefactual shrinkage is a minimal and constant factor and that the rate of expansion is more-or-less constant at each phase. Results which were obtained from mouse ovaries have verified the distinction between the fluids (Fig. 1). When standardized for differences in surface area, the results indicate that the rate of extracellular fluid formation during preovulatory swelling rises about 50-fold above that of the previous phase. When the gonadotrophin surge is lacking, swelling continues (Laing et al., 1984), but it is at a slower rate than that of follicles undergoing preovulatory activation, strengthening suspicion that hormones stimulate this process (Fig. 1). Antral follicles take much longer to grow to full size in large species than in small ones. Calculations based on data obtained by Turnbull et al. (1977) and Driancourt et al. (1986) for the sheep indicate that a distinction can be made between the rates of primary and secondary fluid formation, these being of the same order of magnitude as in mice. By comparison with the secretion/absorption of fluids across many other membranes, the rate of follicle swelling is sluggish, adding to the practical difficulties of investigating its formation. In, for example, the anterior chamber of the eye, aqueous humour is produced at a rate of about 2 μl.min⁻¹ whereas the corresponding value for ovine follicular fluid during the preovulatory phase is 20 μl.day⁻¹.

The membrana granulosa: an epithelial layer

Epithelial cells either line cavities or cover surfaces of the body and, as a consequence of their location, can regulate the internal environment of body compartments. These cells are therefore
Fig. 3. Scanning electron micrograph of the inner surface of the mural epithelium of a sheep Graafian follicle. The granulosa cells present a regular mosaic with relatively smooth surfaces. No large channels are visible opening into the antrum. × 2025.

expected to be specialized for promoting/restricting the movements of particular ions and molecules between compartments. Since granulosa cells are an epithelial type, they may be expected to exhibit these functions. The granulosa cells of growing follicles are typically polygonal although the outer layer is columnar and rests on a delicate basement membrane (Fig. 2). Up to about 10 cell layers develop in preantral follicles but these attenuate when the antrum expands because the follicle wall is compliant and mitotic activity has virtually ceased. The epithelial surface facing the antrum is relatively featureless: it is uniform and lacking in conspicuous channels or processes (Fig. 3). The granulosa layer is the same thickness throughout except at the pole containing the cumulus oophorus in which cellular proliferation continues (Gosden et al., 1983).

The morphology of the follicular epithelium suggests a structure that is highly permeable to water and dissolved substances. In contrast to so-called ‘tight’ epithelia (e.g. frog skin, toad bladder trophoblastoderm), occlusive junctions are not found between cells, although other junctional complexes exist for maintaining structural integrity and intercellular communication (Albertini & Anderson, 1974; Fig. 4). Granulosa cells are separated by channels measuring 20 nm which permit molecules of up to $M_r$ 500,000 in size to penetrate and reach the antrum (Zachariae, 1958; Albertini & Anderson, 1974; Payer, 1975; Cran et al., 1976). The density of cell packing decreases centrifugally and is further reduced in the cumulus cells during mucification. Thus, the extracellular fluid of the follicle is a continuum and sub-compartments are not well-defined by structural barriers. Hence, many potentially toxic substances carried in blood can reach the oocyte (Baukloh et al., 1985).

Small spherical spaces containing extracellular fluid appear in the granulosa layer of some species when the antrum is incipient. The significance of these Call–Exner bodies is not known although it has been suggested that they are sites of active secretion of follicular fluid (Bramhall, 1956) and may be related to the enlargement of the Golgi apparatus (Hadek, 1963). A major
role is doubtful because they remain separate from pools of primary follicular fluid and exist independently of an antrum in human granulosa cell tumours.

**Chemical composition of follicular fluid**

Chemical studies of secondary fluid have been encouraged by accessibility in large follicles. There is, however, a paucity of information about primary fluid in small follicles (<1 mm) and the extent to which the composition of these fluids differs remains unclear. Particular attention is being paid here to electrolytes since the organic components of follicular fluid have been reviewed thoroughly elsewhere (Edwards, 1974; McNatty, 1978; Lenton, 1988).

Table 1 shows the concentrations of the principal electrolytes in large (mainly preovulatory) follicles of 5 species together with the values for plasma/serum. No major concentration gradient exists across the follicle wall. There is tentative evidence, particularly from smaller follicles, that the concentrations of K⁺ are greater in follicular fluid than in blood. This interesting finding parallels the situation in the oviduct (Borland et al., 1980) and could indicate active inward transport of the cation. It requires verification because the possibilities that either K⁺ had leaked from damaged cells or that atretic follicles had been sampled were not excluded. Furthermore, any results obtained with fluids obtained post mortem must be considered unreliable because of the rapid changes that take place (Edwards, 1974; Knudsen et al., 1978). In view of variable sample quality, earlier claims that electrolyte concentrations during the ovarian cycle and at different stages of follicle development require re-examination. Measurements of Na⁺ are probably more reliable than those of K⁺ because this cation is abundant in extracellular fluid but scarce in cells. The slight, but significant, elevation

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**Fig. 4.** Channels between adjacent granulosa cells are visible in this rabbit Graafian follicle. The cells are connected by a junctional apparatus resembling (a) gap junctions and (b) maculae adhaerens. \*90 000.
Fig. 5. A Call–Exner body situated in the wall of a rabbit Graafian follicle. These structures frequently contain long strands of moderately electron dense material suspended in the extracellular fluid and in apposition to the epithelial cells. The apical faces of these cells are smooth although there are long processes in the vicinity (arrow). × 10 000.

Table 1. Electrolyte concentrations (mmol. l¹⁻¹) in the fluids of large follicles (FF) as compared with plasma (P) or serum (S)

<table>
<thead>
<tr>
<th>Species</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Cl⁻</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FF</td>
<td>P/S</td>
<td>FF</td>
<td>P/S</td>
<td>FF</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>124</td>
<td>145</td>
<td>4.4</td>
<td>4.6</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>143</td>
<td>154</td>
<td>5.4</td>
<td>5.4</td>
<td>140</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>133</td>
<td>125</td>
<td>7.0</td>
<td>4.3</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>136</td>
<td>6.2</td>
<td>5.7</td>
<td>144</td>
</tr>
<tr>
<td>Sheep</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>149</td>
<td>149</td>
<td>4.7</td>
<td>4.9</td>
<td>107</td>
</tr>
<tr>
<td>Pig</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>128</td>
<td>143</td>
<td>15.9</td>
<td>5.2</td>
<td>10.3</td>
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<tr>
<td></td>
<td>142</td>
<td>147</td>
<td>7.6</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>145</td>
<td>140</td>
<td>4.9</td>
<td>4.8</td>
<td>9.7</td>
</tr>
<tr>
<td>Cow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>132</td>
<td>9.2</td>
<td>149.5</td>
<td>3.1</td>
<td></td>
</tr>
</tbody>
</table>

*Follicular fluids were obtained post mortem.

References:


Shalgi et al. (1972)

Chong et al. (1977)

David et al. (1973)

Burgoyne et al. (1979)


Schuetz & Anisowitz (1974)*

Chang et al. (1976)*

Knudsen et al. (1979)


Olds & Van Demark (1957)*
of Na⁺ in follicular fluid of some species (e.g. pig, rabbit), although not all (e.g. sheep), may result from active transport processes (see below). No concentration gradients have been identified for the other ions (Cl⁻, Ca²⁺, Mg²⁺).

These results together with those obtained with many small organic molecules strengthen the conclusion that most substances can diffuse freely into or out of the follicle. This does not need to imply, however, that equilibrium conditions exist; indeed, this is doubtful since the follicle is never static because of continuous changes in volume and metabolism.

The protein concentrations have attracted most attention because hormones have to gain entry to the follicle to influence the granulosa cells. In toto they range from 50 to 100% of normal plasma values but they are not present in equimolar proportions (McNatty, 1978). Larger proteins penetrate more slowly than small ones and the concentrations present at a particular stage can be expected to vary with the flux of water, the surface area to volume ratio of the follicle and the permeability of the thecal blood vessels. Follicular fluid contains most of the plasma proteins, albumin being the most abundant in both cases. Plasma proteins of Mₙ ≥ 850 000 are absent (Shalgi et al., 1973) and some large molecules produced by granulosa cells (e.g. proteoglycans) probably do not escape from the follicle (Ax & Ryan, 1979). The follicular wall therefore behaves like a coarse molecular sieve and a blood–follicle barrier exists. The gonadotrophins, FSH and LH, are not excluded by this barrier, although present at lower concentrations than in blood (McNatty et al., 1975).

Respiratory gases and acid–base balance

Oocytes are isolated from the capillary circulation because the follicular epithelium is avascular. As a consequence of diffusion through layers of respiring cells, the concentration of oxygen around the oocyte is expected to be attenuated to an extent which will vary with the size and form of the follicle. A steeply descending inward concentration gradient has been predicted for large preantral follicles by a simple mathematical model (Gosden & Byatt-Smith, 1986) but Graafian stages are not so amenable to modelling because of their multicompartamental structure and asymmetry. Until direct measurements of the Po₂ can be made, inferences will have to be drawn from the analysis of follicular fluid whilst recognizing that these values could be substantially different from those of the local environment of the oocyte.

<table>
<thead>
<tr>
<th>Species</th>
<th>Po₂ (mmHg)</th>
<th>Pco₂ (mmHg)</th>
<th>HCO₃⁻ (mmol. l⁻¹)</th>
<th>pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>54.3</td>
<td>35.1</td>
<td>—</td>
<td>7.27</td>
<td>Shalgi et al. (1972)</td>
</tr>
<tr>
<td>Human</td>
<td>103.5</td>
<td>43.2</td>
<td>—</td>
<td>7.34</td>
<td>Fraser et al. (1973)</td>
</tr>
<tr>
<td>Pig</td>
<td>51.0</td>
<td>45.0</td>
<td>28.0</td>
<td>7.41</td>
<td>Knudsen et al. (1978)</td>
</tr>
<tr>
<td>Pig</td>
<td>—</td>
<td>52.1</td>
<td>27.3</td>
<td>7.34</td>
<td>R.G. Gosden &amp; R.H.F. Hunter (unpublished)</td>
</tr>
</tbody>
</table>

Respiratory gas tensions in follicular fluid have seldom been measured and the available data are highly variable (Table 2). Much of this variability is probably artefactual because there are several sources of potential error. The data are, however, sufficiently consistent to conclude that the fluid is not anoxic and the Po₂ may even be close to that of normal ovarian venous blood. Clarification is required. Measurements of the Pco₂ and pH are within the venous range and more consistent. Since follicular fluid has a similar composition to plasma, the pH will be buffered by carbonic
acid and protein (Shalgi et al., 1972). The pH of the extracellular fluid, which is 7.3–7.4 in the antrum, may differ locally as a result of differences in metabolism and secretion, e.g. hyaluronic acid and chondroitin sulphuric acid.

**Physiology of follicular fluid formation**

The morphological and chemical evidence discussed so far leads to the expectation that most molecules will move freely across a porous follicular wall according to their concentration gradient. They strengthen the early view that transudation from capillaries is responsible for secondary (and perhaps other) follicular fluid (Robinson, 1918; Burr & Davies, 1951). The possibility remains, however, that more than one mechanism exists for the transport of fluid, as is the case in other 'leaky' epithelia. In the gall bladder active outward transport of salt is followed by the net movement of water down its osmotic gradient (Spring & Ericson, 1982). A similar mechanism, perhaps controlled by hormones, could explain the formation of follicular fluid provided that the polarity of salt transport was reversed. An alternative suggestion has been made that hydrolysis of polymers of glycosaminoglycans in the antrum could raise the osmotic potential and cause follicles to swell leading to ovulation (Zachariae, 1957; Zachariae & Jensen, 1958). Either hypothesis would explain why the shrinkage of the antrum and pycnosis of mural granulosa cells occur concurrently in atretic follicles. The latter one is no longer favoured, however, because the colloid osmotic pressure is not elevated during the preovulatory phase and ovulation occurs without a raised intrafollicular pressure (see below). The question of whether salt transport is involved has not been answered. It appears that follicles possess osmotic properties because they shrink when immersed in hypertonic saline, demonstrating that the epithelium is sufficiently semipermeable.

The rate and direction of net water movement between the two compartments, namely plasma (strictly interstitial fluid/lymph) and follicular fluid, depends on the magnitude and sign of the chemical potentials. In the absence of temperature or hydrostatic pressure gradients these potentials are predicted by the depression of the freezing point of the fluids. The extracellular fluids of the sheep ovary are isotonic, with an osmotic pressure equivalent of 300 mosmol.kg⁻¹ (Table 3). Whilst the result appears to deny that water enters the follicle as an osmotic consequence of secretion of solutes into the antrum, there is evidence that gradients <1 mosmol.kg⁻¹ can produce substantial water transport (Spring & Ericson, 1982). In view of the slow accumulation of follicular fluid, particularly at early stages, it is not justifiable to dismiss the active transport hypothesis.

<table>
<thead>
<tr>
<th>Ovarian venous plasma</th>
<th>Ovarian lymph</th>
<th>Follicle &gt; 6 mm diam.</th>
<th>Follicle 2–6 mm diam.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.557 ± 0.006</td>
<td>-0.587 ± 0.021</td>
<td>-0.548 ± 0.005</td>
<td>-0.551 ± 0.005</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. for 7–11 observations.

If an osmotic gradient is being considered the Na ion is the prime candidate for active transport because of its abundance and the established role of the Na,K-ATPase in water transport in some epithelia. This suggestion is supported by the slight excess of Na in the antral fluid of some species and the binding of ouabain to granulosa cell membranes (R. G. Gosden, unpublished). The fact that granulosa cell monolayers do not form domes of secreted fluid, as do other transporting
The relation between current and voltage in the pig follicle wall as obtained in a voltage clamp experiment (for method see Civan, 1983). The current required to maintain a predetermined voltage was measured and the transmural resistance has been calculated using Ohm’s Law (see Table 4). The open circuit voltage and short-circuit current are found at the intersections between the line and the $x$ and $y$ co-ordinates, respectively: these are close to zero in this structure.

The movement of ions across a membrane can establish a transmural potential difference which can be measured electrophysiologically to provide evidence of active transport. When this voltage is electronically clamped to zero the current passing (‘short-circuit current’) indicates the direction and net movement of charged particles which can then be identified by inhibiting transport mechanisms (Ussing & Zerahn, 1951; Civan, 1983). This method has become conventional for studying transport processes in epithelia and has now been applied to the isolated follicle wall of the pig. The open-circuit potential difference (i.e. unclamped) was found to range from +0.5 to −0.5 mV and the short-circuit current was close to zero (Fig. 6). These data provide no support for the active transport hypothesis, but nor do they deny it. McCaig (1980, 1985) has tackled the same question by the alternative approach of measuring the potential difference of superfused mouse follicles using microelectrodes. As the microelectrode advanced, voltage changes were encountered: firstly as surface epithelial and granulosa cells were traversed successively, in which the membrane potentials were $-21.9 \pm 0.4$ mV, and finally, as the potential difference fell to $+1.2 \pm 0.3$ mV, when the antrum was entered (Fig. 7). This small antral potential difference became more positive near the time of ovulation or after treatment with metabolic inhibitors. Since the resistance did not change concomitantly it was inferred that active transport was responsible for the difference.
Fig. 7. Electrical potentials recorded using a glass microelectrode traversing the wall of a mouse follicle and entering the antrum. A number of intracellular potentials (negative-going, upward deflections) are recorded before a steady, slightly positive potential is reached in the antrum (McCaig, 1980).

Since there are transmural differences in the concentrations of relatively impermeable charged molecules (proteins and glycosaminoglycans) conditions favour the existence of a Gibbs-Donnan equilibrium. The small (if significant) transmural potential differences could therefore be developed merely from diffusion of the major ions. The equilibrium potentials for these ions can be calculated from their concentrations on either side of the follicle wall using the Nernst equation assuming that the activity coefficients are the same in the two fluids (Borland et al., 1977). The potentials corresponding to our data from the pig are: Na\(^+\), \(-0.69 \pm 0.05\) mV, K\(^+\), \(+0.41\) mV, Cl\(^-\), \(-0.40 \pm 0.47\) mV. These are consistent with the measured electrical potentials. However, this does not, however, rule out active transport because leaky membranes are less able to hold charge. Definitive testing of active transport will require direct measurement of Na\(^+\) and Cl\(^-\) fluxes.

The specific electrical resistance of the follicle wall can be calculated from the relation between the transmural potential difference and the current being passed (Fig. 6). This relationship is linear which indicates that the follicle wall behaves as a simple ohmic conductor without significant rectification. It is concluded that the current is probably conducted by a paracellular rather than a transcellular pathway.

<table>
<thead>
<tr>
<th>Table 4. Comparison of electrical properties of the ovarian follicular wall with those other mammalian epithelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epithelium</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Ovarian follicle (unstripped)</td>
</tr>
<tr>
<td>Trophoderm</td>
</tr>
<tr>
<td>Proximal convoluted tubule</td>
</tr>
<tr>
<td>Ileum (unstripped)</td>
</tr>
<tr>
<td>Gall bladder</td>
</tr>
</tbody>
</table>
transcellular route, which is consistent with the evidence that hydrated channels exist between the cells. The follicle wall resistance is sufficiently low that the epithelium can be classified with those that are electrically ‘leaky’ (Table 4). This resistance is in fact the sum of the resistance of the ovarian surface epithelium and the follicle itself, of which the latter may be the minor component.

Up to this point it has been assumed for the sake of argument that the total number of particles present (i.e. osmotic potential) determines the rate and direction of net water transport. Chemical potential is, however, influenced by differences in temperature and hydrostatic pressure (Patton, 1965). Whilst it might not be expected a priori that the temperature inside follicles would be different from that of the body core, the surprising claim has been made that rabbit follicles have a temperature 2–3°C lower than the ovarian stroma (Grinsted et al., 1980). If substantiated, this finding adds another, albeit minor, factor to the list of those responsible for the formation of follicular fluid.

Finally, it is necessary to discuss critically the evidence for transudation. The follicle wall is very permeable to water (Peckham & Kiechhofer, 1959) and although the hydraulic conductivity of granulosa cells has never been measured, it is assumed that most water enters paracellularly. Transudation requires a hydrostatic pressure gradient from capillary to antrum although the pressures have been found to be similar (~17 mmHg) (Blandau & Rumery, 1963; Espey & Lipner, 1963; Rondell, 1964). Accurate measurement under physiological conditions is difficult and small, undetectable gradients could still account for the slow accumulation of follicular water. According to this hypothesis, a change in interstitial pressure, perhaps under the influence of hormones, would lead to swelling of the follicle which would bulge into the lower pressure region of the ovarian bursa or peritoneal cavity, as occurs when the ovarian vein is experimentally clamped to raise intra-vascular pressure. There is evidence of stromal oedema and increasing leakiness of the thecal capillaries during the periovulatory period (Morris & Sass, 1966; Byskov, 1969; Bjersing & Cajander, 1974), but the existence of pressure gradients remains to be demonstrated.

Transudation provides the most satisfactory explanation for the formation of secondary follicular fluid which is occurring relatively rapidly. It might be argued, however, that it is an unwieldy force for the morphogenesis of the antral follicle and for the controlled production of primary fluid. That other mechanisms are involved at early stages of development may be inferred from studies of mouse ovaries in organ culture in which follicles were found to undergo formation and limited expansion of the antrum in the presence of FSH (Ryle, 1969). Since a vascular supply was absent it seems likely that antrum formation requires active secretion. It is not clear whether any secondary fluid can be produced under similar conditions although this appears to be doubtful because rat ovaries ovulating in vitro did not undergo a normal increase in weight (Osman & Lieuwma-Noordanus, 1980).

Throughout much of this review, there has been an assumption that follicular fluid is homogeneous whereas, in reality, microenvironments undoubtedly exist. Not only would these be anticipated in the vicinity of the oocyte and its investment of cumulus cells but also within the mural granulosa cell layers as a result of local metabolism/secretion and fluxes across the follicle wall. And even within the antrum unstirred layers may be encouraged by mucification. A major focus of future research should therefore be the charting of these differences.

**Conclusions**

The composition of follicular fluid in Graafian follicles is similar but not identical to ovarian venous plasma. Differences between the two fluids are attributed to a blood–follicle barrier, which restricts the passage of large molecules, and to the existence of a hypothetical active transport mechanism and/or a Donnan equilibrium, reflected by the distribution of small permeant ions. The follicle epithelium has been characterized as ‘leaky’ on the basis of both chemical and electrical criteria.
The rate of follicular fluid accumulation is much greater during preovulatory activation of the follicle (secondary fluid) than at preceding stages (primary fluid), suggesting that gonadotrophin hormones have a major influence on the rate of swelling. The evidence for water transport following an osmotic gradient set up by active transport of Na⁺ has been inconclusive. The conventional view that fluid forms from transudation of plasma rests on circumstantial evidence and is less likely to account for primary than for secondary fluid. The discussion has focussed on the fluid in the antrum principally because so little is known about the fluids which lie in the narrow extracellular spaces and bathe the granulosa cells and oocyte. A major challenge for future research will be elucidation of the compositions of these microenvironments which, in contrast to the bulky antral fluid which will buffer change, should be sensitive indicators of fluctuating biosynthetic activity and of optimal conditions for cell culture.

We thank Kay Grant and Robert Nichol for technical assistance and Dr Colin McCaig for helpful discussion and permission to reproduce some of his results. Financial support has been generously provided by the Medical Research Council, Agriculture and Food Research Council and the Wellcome Trust.

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Electrophysiological properties of the follicle wall in the pig ovary

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Summary. The transmural potential difference and short-circuit current of the porcine Graafian follicle have been measured in an attempt to test whether antral fluid accumulates as a result of active transport of salt. The values obtained by mounting explants of follicle wall in Ussing chambers were close to zero and the specific electrical resistance was only 99 \( \Omega \cdot \text{cm}^2 \). The elemental composition of the follicular fluid was similar to that of ovarian venous plasma with the exception of follicular Na\(^+\) which was slightly more abundant. Bicarbonate concentrations were slightly lower in follicular fluids. These findings were interpreted as evidence that the follicular wall is a leaky epithelium and, therefore, any charge resulting from net ion transport will be shunted along low resistance paracellular pathways.

Key words: Pig ovary; follicular fluid; active transport; trans-epithelial potential difference; short-circuit current; specific resistance.

Graafian follicles are characterized by an epithelium of granulosa cells which encloses a fluid-filled space, the antrum. The volume of the extracellular space in mature follicles is genetically determined and varies allometrically with body weight\(^1\). The rate of antrum expansion is slow at first but rises during the phase of preovulatory maturation\(^2\). The timing of this rise indicates that either the pituitary gonadotrophins or other hormones involved in preovulatory activation of the follicle are also responsible for stimulating the movement of fluid from blood to the antrum. However, the physiological mechanisms underlying water transport in the follicle are poorly understood.

The most widely held hypothesis for the accumulation of follicular fluid is based upon a presumptive hydrostatic pressure gradient across the follicle wall\(^3\). Experimental evidence is lacking, and the formation of small antra in cultured follicles would seem to indicate that other mechanisms are operating\(^4\). Furthermore, in many epithelia osmotic gradients created by active ion transport are responsible for net water movement\(^5\). The chemical composition and osmolality of follicular fluid and blood plasma are similar\(^6,7\), yet, this does not necessarily deny a role for active transport of salt because shallow osmotic gradients can cause net movement of water where the hydraulic conductivity of an epithelium is high\(^8\). Whilst the latter parameter has not been measured in follicles it is presumed to be high because tritiated water and small molecular weight markers rapidly enter the antrum\(^9,10\).

Progress towards understanding ion transport in epithelia has depended heavily upon electrophysiological measurements, most notably of transmural potential difference (PD) and short-circuit current (SCC) under voltage clamp conditions\(^11\). Such methods have been particularly fruitful when studying electrically tight epithelia, such as frog skin and toad urinary bladder\(^12,13\). The electrical properties of the ovarian follicle wall have been seldom studied and measurements obtained using Ussing chambers have not previously been published. This paper reports the results of experiments on the preovulatory pig follicle, a structure which is large enough to be studied in this apparatus.

Materials and methods. Preovulatory follicles measuring 7–9 mm diameter were obtained from 5 Large White \( \times \) Landrace pigs weighing approximately 95 kg. Oestrus was detected by teasing with a boar and ovaries were obtained surgically between 6–36 h of the normal 40-h preovulatory phase. Anaesthesia was induced with pentobarbitone sodium and maintained with a mixture of oxygen-halothane-nitrous oxide. The ovaries were exteriorized through a midventral laparotomy.

Fluids were drawn into syringes from a number of follicles and blood was taken from the ovarian vein. They were centrifuged immediately and stored frozen at \(-20^\circ\text{C}\). The ovaries were ligatured and severed at the hilum. The bulging walls of several large follicles were then dissected and immersed in Hepes-buffered Medium 199 (Flow Laboratories, Inc.).

Figure 1. Ussing chamber and apparatus for voltage clamp experiments on the porcine follicle wall. The specimen (S) was spread flat and held in position with stainless steel pins in the perspex chamber. The chamber halves, which were thus separated by the follicle wall, contained identical mammalian Ringer's solution (R) at \(38^\circ\text{C}\) which was circulated by rising gas bubbles (95% \( \text{O}_2, 5\% \text{CO}_2\)). The lower half of the figure shows the electromotive force (battery) with the arrangements for measuring current and voltage. Calomel electrodes immersed in a saturated solution of KCl and silver/silver chloride electrodes (A) in Ringer's solution (R) were connected to the Ussing chamber by salt bridges (SB).
Irvine), containing glucose (5.56 mM) which was chilled on ice and gassed with oxygen during transport to the laboratory (30 min after excision).

The elemental composition of the thawed fluids was determined using ion-sensitive microelectrodes (Na⁺, K⁺), colorimetry (Ca²⁺, Mg²⁺) and titration methods (Cl⁻). The HCO₃⁻ composition of freshly obtained samples was measured using an automated blood gas analyser (Radiometer Ltd.). Undamaged patches of follicle wall were mounted in a specially constructed Ussing chamber (Jim's Instruments Manufacturing Inc., Iowa City, USA). The internal cross-sectional area of the chamber was 0.16 cm². The apparatus was based on a conventional design 5–12 (fig. 1) and loaded with mammalian Ringer solution (pH 7.4) containing glucose (5.56 mM) and sodium pyruvate (0.25 mM). Recordings were made at 38°C after allowing the specimens to adjust to the experimental conditions for 30 min. Measurements of cellular respiration using an oxygen electrode demonstrated that the tissues were still alive at the end of experiments.

Results: The spontaneous PD of the follicle under open circuit conditions was < 1 mV and the short-circuit current when the voltage was clamped to zero was < 2 μA (table 1). In view of these low values, it was necessary to verify that the apparatus was functioning correctly. Frog skin was therefore mounted in the chambers and recordings were made at room temperature with the appropriate Ringer solution. High transepithelial PDs (> 70 mV) and electrical resistances (> 1000 Ω cm²) were obtained as expected.

The resistance of the follicle wall was calculated from the slope of the current-voltage relationship obtained by passing current from an external source at predetermined voltages (fig. 2). In every follicle tested the relationship crossed the axes close to the origin and was linear for at least the range from −35 to +35 mV. Specific resistances, which averaged 59 Ω cm², were calculated on the basis of the area of exposed follicle surface.

The elemental composition of follicular fluid and plasma was identical with two exceptions (table 2). The concentrations of Na⁺ were higher but those of HCO₃⁻ were lower in follicular fluid (p < 0.01).

Discussion: Numerous studies have shown that, apart from the high concentrations of locally produced hormones and the absence of suspended cells in healthy follicles, the composition of follicular fluid is similar to that of blood. This conclusion appears to hold for most if not all species, although some minor variations have been found. For example, some, but not all, studies of porcine follicular fluid have found greater concentrations of K⁺ in the follicle than in blood 17–20. Such differences may, however, be attributed to using post-mortem material or sampling damaged or atretic follicles. In the present study of samples obtained under anaesthesia, K⁺ concentrations were the same in the two fluids. On the other hand, a shallow but significant Na⁺ gradient was found. This result agrees with findings in rabbits 21 and may indicate the existence of active ion transport by the Na pump. The significance of this gradient for the formation of follicular fluid is obscure at present because most studies have been unable to demonstrate any osmotic gradient across the follicle wall. Whilst potential effects of anaesthetics on ion transport across membranes should normally be taken into account it is doubtful whether they apply in the present situation. Large changes in the flux of Na⁺ would be required to explain the observed increase in ionic concentrations in follicular fluid, which amounts to 0.2–0.4 ml, and in the case of the isolated follicular tissue it is expected that anaesthetics would have been washed out or vaporized at an early stage.

Experimental evidence that active ion transport is occurring across an epithelium can be obtained electrophysiologically. In the only substantive study reported, McGaughey 22 described a small, variable transepithelial PD in mouse follicles. Further, he demonstrated that this PD was metabolically coupled and changed with physiological state during the oestrous cycle. However, Mathews and Lipner 23 were unable to detect a PD in either rabbit or rat follicles. The present findings that the PD and short-circuit current across the pig follicle wall are

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Table 1. Electrophysiological characteristics of the porcine follicular wall

<table>
<thead>
<tr>
<th>Transmural potential difference (mV)</th>
<th>Short-circuit current (μA)</th>
<th>Specific resistance (Ω cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+0.2 ± 0.1</td>
<td>&lt;2</td>
<td>58.9 ± 2.1</td>
</tr>
</tbody>
</table>

4 animals with 2 or 3 follicles from each means ± SEM given.

Table 2. Concentrations of major electrolytes and bicarbonate ions in porcine follicular fluid and ovarian venous plasma (mmol·l⁻¹)

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Cl⁻</th>
<th>HCO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular fluid</td>
<td>141.1 ± 0.3*</td>
<td>3.80 ± 0.25</td>
<td>2.30 ± 0.03</td>
<td>0.75 ± 0.02</td>
<td>95.3 ± 1.7</td>
<td>26.0 ± 1.0*</td>
</tr>
<tr>
<td>Plasma</td>
<td>137.5 ± 1.1</td>
<td>3.77 ± 0.41</td>
<td>2.27 ± 0.05</td>
<td>0.77 ± 0.04</td>
<td>95.7 ± 3.4</td>
<td>29.9 ± 1.1</td>
</tr>
</tbody>
</table>

5 animals with 4 or more follicles from each. * significantly different compared with plasma (p < 0.01 by Student's t-test).
small are consistent with both of the earlier studies. It is not yet clear whether these are biologically negligible. Such findings could be interpreted as denials of the active transport hypothesis for follicular fluid formation, at least during the more rapid phase of preovulatory expansion. They also show that the specific electrical resistance of the follicle wall (including the granulosa and ovarian surface epithelium) is low and, therefore, electro-chemical gradients are unlikely to be stable. It can be concluded then that the results are not in conflict with the hypothesis which will require other methods for further testing. The near or complete absence of a measurable PD and short-circuit current across the follicle wall is not particularly surprising since the same properties are encountered in other 'leaky' epithelia, e.g. gallbladder, choroid plexus, renal proximal tubule, small intestine. In these cases, the cell membrane resistance typically exceeds the transepithelial resistance by a large margin and the linear current-voltage relationship indicates that current is being conducted paracellularly, a conclusion which agrees with the absence of morphologically recognisable 'tight' junctions between granulosa cells. Consequently, it is probable that the major pathway for entry of water into the antrum is paracellular, although the forces involved, whether active transport and/or transudation, require further clarification.

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The CBA mouse as a model for age-related aneuploidy in man: studies of oocyte maturation, spindle formation and chromosome alignment during meiosis

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Abstract. To elucidate the possible mechanism of disturbances in chromosome segregation leading to the increase in aneuploidy in oocytes of aged females we examined the meiotic spindles of CBA/Ca mice. Employing immunofluorescence with an anti-tubulin antibody, and human scleroderma serum, as well as 4'-6-diamidino-2-phenylindole (DAPI) staining of chromosomes the microtubular cytoskeleton could be visualized, and the behaviour of chromosomes and centromeres of oocytes spontaneously maturing in vitro could be studied. The morphology of spindles during the first meiotic division was not conspicuously different in oocytes from young and aged mice as far as the cytoskeletal elements were concerned. Neither multipolar spindles nor pronounced cytoplasmic asters appeared in oocytes of mice approaching the end of their reproductive life (9 months and older). Oocytes of aged females also did not exhibit any sign of premature separation of parental chromosomes at prophase, obvious malorientations of bivalents, or significant lagging of chromosomes during anaphase and telophase. Metaphase I with all bivalents aligned at the spindle equator appeared to be a relatively brief stage in oocyte development compared with pro- and prometaphase. Therefore, already slight disturbances occurring in the timing of the developmental programme which leads to a premature anaphase transition may be responsible for the high incidence of chromosomally unbalanced gametes in aged females, rather than non-separation and lagging of chromosomes during late anaphase and telophase. In a second set of experiments we compared in vivo the morphogenesis II spindles of spontaneously ovulated oocytes obtained from animals at different ages. Previous studies have shown that spindle length and chromosome alignment may be altered in cells predisposed to aneuploidy. To distinguish between the significance of the chronological age of the female and the physiological age of the ovaries (as indicated by the total number of oocytes remaining) we examined the spindle apparatus in young (3-4 months old) and aged (9 months and older) mice as well as CBA females which had been unilaterally ovariectomized (uni-ovx) early in adult life and were approaching the end of their reproductive life at 6-7 months of age. Measurements of the pole-to-pole distance implied that spindle length may be related to maternal age. In oocytes of aged (9 month), uni-ovx (6 month) as well as 6-month-old sham-operated controls the metaphase II spindle was significantly shorter than in oocytes of young mice. By contrast, chromosome disorder and displacement was most pronounced in the aged and uni-ovx mice whilst most oocytes from young mice and moderately aged sham-operated controls exhibited a more regular alignment of chromosomes. These results, which are consistent with recent findings in CBA mice of an increased rate of aneuploidy in females approaching the end of their reproductive life, are discussed with respect to the hypothesis that the aetiology of aneuploidy rests on the critical timing of different events in oocyte development.

Introduction

Maternal age remains one of the main risk factors in the aetiology of aneuploidy in man (Hassold and Chiu 1985). The risks for women at the end of their reproductive span are so high that, according to Hassold and Jacobs (1984), the majority of oocytes ovulated at this time can be predicted to be aneuploid. Despite the significance of these differences the origin of the relationship between maternal age and the increase in chromosome aberrations remains obscure. Theories which have been proposed relate predisposition to non-disjunction to various disturbances and different critical events in oocyte development (see Bond and Chandle 1983, for review).

In order to detect possible mechanisms underlying the high incidence of non-disjunction in the oocytes of aged individuals we have employed immunofluorescence techniques to visualise the microtubular cytoskeleton, and, in particular, the spindle. We hoped to detect alterations in the structure of this organelle which is of vital importance for the proper separation of chromosomes. Since human oocytes are not readily available for such studies we have chosen the CBA/Ca mouse as a model. Unlike several other mice without a maternal age effect (e.g., Beerman et al. 1987) this strain exhibits an age-related increase in aneuploidy (Gosden 1973; Brook et al. 1984), although much less dramatically than in man.

Alterations in spindle structure resulting from the post-ovulatory ageing of oocytes in the fallopian tubes of young females have already been reported (Eichenlaub-Ritter et al. 1986). Based on the comparison of the cytoskeleton in freshly ovulated and postovulatory aged cells of young female mice we proposed that spindle shape, length and chromosome alignment in spontaneously ovulated oocytes are indi-
cators of a predisposition to non-disjunction and aneu-
ploidy (Eichenlaub-Ritter et al. 1986). In the present work we
extended our observations to the cytосkeleton and chro-
mosome alignment in metaphase II oocytes of aged females.
Since hormonal stimulation of ovulation may in itself affect
aneuploidy only cells which had been ovulated spontane-
ously in the natural oestrous cycle were examined. In addition,
oocytes spontaneously maturing in vitro after isolation
from follicles were observed during their first meiotic di-
vision, since it is at this stage that the majority of human
non-disjunction occurs at all maternal ages (Hassold and
Jacobs 1984).

Moderately aged unilaterally ovarioctomized (uni-ovx)
mice were included in the study as an additional experi-
mental group. Removal of one ovary early in life leads to a
premature ending of the reproductive lifespan in these mice,
the remaining ovary compensating for loss of oocytes from
the missing partner by ovulating about twice as many eggs
in each oestrous cycle (Thueng 1961). Whilst fertility is ini-
tially unchanged the reproductive potential of operated an-
imals is reduced in the long term as frequencies of foetal
deaths rise (e.g., Gosden 1979). Evidently some of these
deaths can be accounted for by aneuploidy which rises ear-
lier in uni-ovx animals than in controls (Brook et al. 1984).
Examining spindles of freshly ovulated oocytes of uni-ovx
CBA mice and control animals throughout their reproduc-
itive span should, therefore, allow us to distinguish differ-
ences in spindle structure and chromosome alignment that
arise from ageing of the female (chronological age), as as-
sembled to ageing of the ovary (physiological age). Thus, the
results may indicate whether environmental or hormonal
factors are mainly influencing the predisposition to aneu-
ploidy.

Materials and methods

Stocks of CBA/Ca mice were obtained from Bantin and
Kingman (Hull, UK). Animals were bred and housed in
our departmental colony under a 12 h photoperiod (lights
on at 7 a.m.). Unilateral ovariectomy was performed on
6- to 8-week-old CBA mice which were allocated at random
into two groups and either operated on the right or left
side to remove one ovary or sham-operated under tribro-
moethanol anaesthesia. The oestrous cycle of mature fe-
males was recorded as described previously (Eichenlaub-
Ritter et al. 1986). Freshly ovulated metaphase II oocytes
were obtained from intact young (2-4 month) and aged
(9 month and older) females, and from 6- to 8-month-old
uni-ovx mice, early on the day of oestrus. Comparable
sham-operated controls for the uni-ovx mice were also ex-
amined in each age group.

Isolation of oocytes from cumulus cells, removal of the
zona pellucida and preparation and extraction of cells for
indirect immunofluorescence were essentially the same as
described previously (Eichenlaub-Ritter et al. 1986). For in-
direct immunofluorescence the YL 1/2 monoclonal anti-tu-
bulin antibody (Kilmartin et al. 1982) which is specific for
the tyrosinated form of a-tubulin and binds to all microtu-
bular fibres in oocytes (Eichenlaub-Ritter et al. 1986) was
used. The second antibody was a fluorescein isothiocya-
nate (FITC)-labelled rabbit anti-rat IgG (Sigma). Although 4'-6-
diamidino-2-phenylindole (DAPI) staining of chromatin re-
veals centromeric regions of bivalents in preparations of
the first meiotic prophase, the centromeres of chromosomes
in the second meiotic division cannot be readily visualized
after staining with the dye. Some oocytes were therefore
labelled first with the YL 1/2 anti-tubulin followed by expo-
sure to an anti-centromere serum obtained from a sclero-
derma/Crest patient (the SC serum, Jeppson and Nicol
1986; kindly provided by Dr. P. Jeppson). In the double
immunofluorescence protocol to determine the position of
centromeres in metaphase II oocytes a tetramethylrhoda-
mine isothiocyanate (TRITC)-labelled anti-human anti-
body (DACOPATS) and the FITC anti-rat antibody (Sigma)
were used as second antibodies.

For examination of spindles in the first meiotic division
we tried originally to isolate metaphase I oocytes directly
from mouse ovaries. However, limited numbers of maturing
oocytes in the hormonally unstimulated mice, difficulties
in the prediction of the exact timing of meiotic events under
conditions of natural maturation, inconvenience of the per-
iod of time under which maturation normally takes place
(the later part of the dark cycle), and the apparent fragility
of maturing oocytes directly recovered from antral follicles
led us to use an in vitro maturation system. To recover
oocytes, large follicles of dioestrous mice were punctured
with fine steel needles. Liberated oocytes were freed of ad-
hering follicle cells by repeated gentle pipetting with a
mouth-operated micropipette (Ø 100 μm) in prewarmed,
modified M2 medium (Fulton and Whittingham 1978) con-
taining 15 mg/ml BSA, 2 mM calcium lactate, 22 mM sodi-
um lactate, 4.15 mM sodium bicarbonate, and buffered
with Hepes to pH 7.4. Oocytes of individual animals were
then collected in the same medium with 5 mg/ml BSA and
cultured for 4 to 16 h at 37°C during which time they
underwent germinal vesicle breakdown (GVBD) and polar
body formation. Since we did not want to interfere with
the intrinsic developmental programme of oocytes by ar-
esting and collecting them in medium containing matura-
tion inhibitors we had to be taken that the time taken
to liberate oocytes from any given individual never exeeded
15-20 min. Consequently, oocytes underwent GVBD and
polar body formation more or less synchronously.

Microscopic observation was performed using a Leitz
Ortholux II equipped with the NPL Fluotar 63 (1.32) or
100 (1.32) and the Ploemopak filter system for FITC,
TRITC and DAPI fluorescence, or on a Zeiss Axiosplan
microscope equipped with the NPL 63 (1.4) and 100 (1.3)
objectives and the appropriate filter combinations. Photo-
graphs were taken on Kodak Tri-X Pan (400 ASA).

Prints with a final magnification of 2000-4000 x were
used to determine the pole-to-pole distance of spindles.
Only those spindles which were straight and spread out
flatly in one plane of view were evaluated.

Results

Spindle and chromosome positioning in oocytes during the
first meiotic division

As in other reports on the development of oocytes in vitro
(e.g., Speed 1977; Wasserman and Fujiiwara 1978; Polanski
1986), large oocytes isolated from young, dioestrous CBA
mice undergo GVBD between 2-4 h after the start of cul-
ture. Data on the proportion of oocytes which spontane-
ously mature, and the timing of different steps in maturation
will be published elsewhere (Eichenlaub-Ritter, in prepara-
tion). Light microscopic observation of developing oocytes
revealed that ana-/telophase and polar body formation begin after 9–10 h of culture. Thus, to obtain spindle preparations of late prometa-/metaphase stages of the first meiotic division oocytes were examined after 8 h. It was hoped to detect disturbances in chromosome alignment of such cells which could indicate a predisposition to non-disjunction. However, although most oocytes isolated after 8–9 h of culture already possess a bipolar spindle in a metaphase configuration with two flat poles as noticed previously (Wasserman and Fujiwara 1978) the bivalent chromosomes were still predominantly in a prometaphase configuration, i.e., not all bivalents were positioned in the equatorial plane. Usually one or more bivalents were still located in one or the other half of the spindle, apparently in the process of approaching one spindle pole or reorienting towards the other (Fig. 1a, b). Much more rarely spindles were found in which all bivalents were nearly aligned at the equator.

The same was observed in oocytes cultured for longer periods of time, the largest proportion of spindles which had not already proceeded further in division being in a prometaphase configuration. Therefore, the transition between prometa-/meta-/anaphase seems to be only brief. Only among oocytes isolated after extended times of culture (more than 16 h) did the proportion of “metaphase” stages increase (Fig. 1c-e). In such cells the centromeres of some bivalents often appeared to have become prematurely split into their daughter halves (Fig. 1e). However, cells which had not started to form a polar body by this time seemed to have become arrested in first division and could be taken as a reference when comparisons of chromosome alignment in cells of young and old mice were made. Since it is not possible to tell prometaphase stages of chromosome alignment in oocytes from a metaphase with a “displaced bivalent the positioning of bivalents in the dynamic process of maturation does not seem to be a suitable parameter to reveal predisposition to non-disjunction. This situation is unlike that in arrested metaphase II oocytes in which all chromosomes become precisely aligned at the equator.

Fig. 1ab. Spindles of in vitro maturing oocytes of young and old CBA mice during the first maturation division. a, b Young mouse 11 h of culture: most prophase cells which have not yet proceeded further into anaphase possess a bipolar spindle with a few astral microtubule-organizing centres attached laterally to the spindle body (arrows in a); bivalents are still in a prometaphase configuration some aligned at the equator, others still positioned closely to a pole or reorienting (arrow in b); brightly staining regions depict the centromeric heterochromatin of parental chromosomes mostly pointing towards opposite spindle poles (arrowhead in b). c-e Young CBA mice; after 16 h of culture cells with chromosomes in a “metaphase” configuration accumulate in which all bivalents are located at the equatorial plane; however, most seem to be blocked in metaphase I; double immunofluorescence with the scleroderma antiserum indicates that some of the centromeres of parental chromosomes may have prematurely separated (arrow in e). f, g Young CBA 10 h of culture; during anaphase parental chromosomes separate and migrate sequentially and not simultaneously towards opposite spindle poles. h, i Most oocytes of 9-month-old CBA possess normal ana/telophase spindles in which all chromosomes have migrated to the spindle poles. k, l The only example found of a spindle with possibly lagging chromosomes, in an oocyte from an aged mouse, however, the cell could also still be in a late anaphase stage in which the polar caps of the spindle have already become depolymerized but chromosomes are still migrating as in f, g; in telophases with contracted interpolar parts of the spindle “lagging” chromosomes were not observed. a, c, f, h, k Direct anti-tubulin immunofluorescence; b, d, g, i, l corresponding DAPI image; e indirect anti-centromere immunofluorescence. Bar represents 10 \( \mu m \)
and do not migrate any further (Eichenlaub-Ritter et al. 1986).

If oocytes of aged females are, in fact, more sensitive to environmental hazards and drugs as has been proposed several times (e.g., Tease and Fisher 1986) one might expect to find a more fragile or a smaller spindle apparatus. We therefore, measured the pole-to-pole distance of the spindle in the first meiotic division. However, initial screening showed that there is already much variation in spindle length in any population of oocytes from young individuals which have been fixed at the same time after induction of maturation. Apparently, spindles become more compact and shorter during prometaphase, and cells from one individual will always contain a slightly heterogenous population of earlier and later prophase stages. Taken together with the fact that oocytes from young and old mice show intrinsic differences in the timing of their developmental programme (Eichenlaub-Ritter, in preparation), spindle length cannot therefore be taken as a quantitatively measurable indicator of alterations in the cytoskeleton which might contribute to predisposition to non-disjunction.

Thirdly, we tried to determine the proportion of anaphase cells in which chromosomes were lagging during their separation. However, while it is possible to detect non-disjunction in other cell types, especially if they possess only a few chromosomes because some chromosomes clearly move behind the migrating front of the others, parental chromosomes of mouse oocytes do not migrate simultaneously to opposite spindle poles in meiotic anaphase, even in young animals (Fig. 1f, g). Some bivalents which probably possess fewer chiasma connections are separated and reach the poles early after the anaphase transition while others are still migrating. Thus, apart from the few cells which are in anaphase at any given time of culture and fixation, even in a relatively synchronous culture, the existence of sequential separation of bivalents prevents us from drawing conclusions on the relative risk of non-disjunction. In the much more common stage of early telophase, which is characterized by the presence of an unconstricted interpolar spindle part (Fig. 1h, i), we detected only one oocyte from an aged female in which some chromosomes might have been lagging and left in the central part of the spindle (Fig. 1k, l). In late telophases, in which midbodies are constricted, there was never any evidence of lagging chromosomes.

**Fig. 2a-h.** Metaphase II spindles and chromosomes of spontaneously ovulated, fresh oocytes from CBA mice of different ages. a, b 3-month-old CBA; c, d 9-month-old CBA; e, f 6-month-old sham-operated control; g, h 6-month-old unilaterally ovariectoimized (uni-ovx) CBA. Spindles in oocytes of aged mice appear to have a reduced pole-to-pole distance. In most oocytes of young CBA and 6-month-old sham-operated controls chromosomes are nicely aligned at the spindle equator while they are much more spread and irregularly aligned in a large number of oocytes from 9-month-old CBA and 6-month-old uni-ovx animals. a, c, e, g Indirect anti-tubulin immunofluorescence; b, d, f, h DAPI stained. Bar represents 10 μm

**Fig. 3.** Immunofluorescence with the scleroderma antiserum confirms that chromosomes in most metaphase II oocytes of young animals are aligned at the equator and only slight scatter is found (a, b) while centromeres are considerably spread around the equator in the oocytes of aged mice (c, d); arrows depict the amphitelic oriented centromeres of daughter chromosomes. a, c DAPI stained; b, c indirect anti-centromere immunofluorescence. Bar represents 10 μm
In general, characteristic features of the first meiotic spindles were virtually identical in young and old mice. Among the many cells obtained from aged mice none were found to have a prematurely disjoined bivalent with one half-bivalent located on its own in one spindle half. Even in prophase cells in which the spindle had been damaged during the attachment of the cell to the slide homologous chromosomes seemed to remain attached at their telomeres. We also found no case in which one or few bivalents formed their own "mini-spindle" outside the main spindle body. In oocytes of young as well as old mice, small astral arrays of microtubules can be found on the lateral margin of the spindle, characteristically connected with most of their tubules to chromosomes/centromeres located close by. However, no tripolar spindles were detected. In both types of cells, microtubules and astral arrays were only rarely found in the cytoplasm, outside of the spindle, once the cells had reached late prometaphase.

**Spindles and chromosomes in spontaneously ovulated, metaphase II-arrested oocytes from young, old and uni-ovx mice**

As in the first meiotic division, the morphology of metaphase II-arrested spindles is similar in spontaneously ovulated oocytes of young and old mice. Spindles of both types of cells have slightly pointed or flat spindle poles as is characteristic of recently ovulated oocytes, and do not possess many astral microtubules extending from the poles into the cytoplasm (Fig. 2). No small astral foci of microtubules were ever present in the cytoplasm of the freshly ovulated cells of old CBA mice. Only upon postovulatory ageing does a distinct network of cytoplasmic asters form as has been observed in young animals (Eichenlaub-Ritter et al. 1986). However, critical examination of spindles in the oocytes of aged mice reveals that the pole-to-pole distance is shorter in spindles of the 9-month-old animals than in 2- to 4-month-old mice, in spite of the fact that they have not aged visibly following ovulation (Fig. 2). This impression can be verified by quantitative measurement (Fig. 4). While the mean spindle length amounts to 26.4±/−0.3 μm in the freshly ovulated oocytes of young animals (n=48), that of old CBA mice is 22.7±/−0.7 μm (n=43; P<0.001 by Student’s t-test). The same holds true for oocytes of uni-ovx mice which are approaching the end of their reproductive life (6 to 8 months old). Although spindle structure resembles that of young animals, the pole-to-pole distance is significantly shorter with 23.3+/−0.5 μm (n=27; t-test: P<0.001). Spindle length in sham-operated controls of the same age is also shorter than in young mice (23+/−0.3 μm, n=36; t-test: P<0.001) showing no significant difference compared with the uni-ovx mice (Fig. 4). Thus, the spindle length of freshly ovulated oocytes seems to be mainly related to the chronological age of the mother.

By contrast, a feature which is correlated with ending of reproduction rather than age per se and, possibly, with aneuploidy induction, is the degree of order of chromosome alignment on the spindle in the oocyte. In young animals (up to 4 months old) only 11% of cells showed irregularities in alignment; most oocytes showed all chromosomes precisely located at the spindle equator (Fig. 2b), although on staining with the anti-centromere antibody the kinetochores were seen to be already slightly separated in the equatorial plane (Fig. 3a, b). A sharp rise in percentage of spindles with displaced chromosomes (64.6%) occurred, however, in the 9 month and older CBA mice in which one or many chromosomes were located closer to one spindle pole in one of the half spindles (Fig. 2d). Centromere staining confirmed the zig-zag pattern of alignment of chromosomes in many oocyte spindles of aged animals (Fig. 3c, d). The percentage of oocytes with displaced chromosomes is also very high in oocytes of 6 to 8 month-old uni-ovx mice (58.3%), while in sham-operated controls of the same age only 22.7% exhibited marked irregularities in chromosome alignment (Fig. 2e–h; Fig. 4).

**Discussion**

Our intention in the present study was to assess and compare the properties of spindle structure and function in oocytes of old and young females of the CBA mouse, and to seek clues to possible factors which may be involved in predisposition to non-disjunction, particularly in relation to maternal ageing. However, differences between spindle structure in the first meiotic division of oocytes from young and aged CBA/Ca mice are either too subtle to be detected by light microscopic immunofluorescence techniques or do not exist. Our in vitro maturation studies confirm earlier reports that meiotic bivalents and a number of microtubule-organizing centres (MTOC) are involved and capable of organizing a bipolar spindle in the absence of centriolar MTOCs (Szöllösi et al. 1972; Wassarman and Fujitaya 1978), and this is achieved irrespectively of the age of the female from which oocytes were obtained. Bipolarity could be determined by a centrosome which has so far not been defined in molecular terms (Miazza 1984). Moreover, our observations imply that chromosomes are also intimately involved in stabilization and capture of microtubules from cytoplasmic focal MTOCs which are thereby recruited for spindle formation. This can be deduced from the presence...
of many asters in the cytoplasm of cells in early prophase which disappear gradually once GVBD has occurred. Even the small lateral foci of microtubules on the prometa-/meta-
phase spindles are usually near to some chromosomes to which they are connected whereas the cytoplasm of the developing prometaphase cell becomes gradually depleted of astral fibres. Recruitment of cytoplasmic MTOCs for formation of a spindle has recently also been reported for mouse oocyte chromosomes, arrested at metaphase II and recovering from nocodazole (Maro et al. 1985).

Our first suspicion that attachment of a half bivalent to one of such non-polar organizing centres might cause non-disjunction or maldistribution of this chromosome, thereby possibly resulting in a maternal age related increase in aneuploidy could not be confirmed in the present study. Focal microtubule arrays are associated not only with spindles of old but also of young mice, and apparently disappear in most cells towards anaphase.

Maldistribution of chromosomes seems not to be caused either by the preferential failure of chromosomes in oocytes from old animals to form functional kinetochores which attach to the spindle. If this happens one would expect occasionally to find single bivalents in the cytoplasm of oocytes from aged females, but this was never observed (although it does occur in oocytes recovering from nocodazole (Eichenlaub-Ritter, in preparation)). In addition, cells with more than two poles or with an extra spindle were never found. Individual bivalents of untreated cells seem to be unable to form a separate minispindle as a result of which both parental chromosomes could remain unseparated in the cytoplasm of the female gamete during polar body formation. Hypotheses which attempt to explain the maternal age effect for human aneuploidy on the basis of fewer chiasmata or premature separation of univalents in oocytes of aged females (e.g., Henderson and Edwards 1968; Crowley et al. 1979), receive little or no support from the present study nor from some other recent reports (Beermann et al. 1987). No oocyte was ever observed to contain a univalent. Even in those cells in which the spindle had been destroyed during the extraction or settling of cells on the poly-lysine covered slides, chromosomes appeared to be still physically firmly attached at their telomeres with centromeres pointing away from each other as normal. The DAPI-stained preparations should also have readily shown whether a bivalent had disjoined precociously during prometaphase, but there was no evidence for this.

A more direct indicator of predisposition to aneuploidy in oocytes seemed to be the degree of order with which chromosomes are aligned in the metaphase II spindles. In accordance with recent findings about the occurrence of aneuploidy in offspring of mice approaching the end of their reproductive life we could see that a high percentage of oocytes obtained from CBA mice at ages when fertility is nearly lost (the 9-month-old intact and 6-month-old uni-

ovx animals) had spindles with displaced chromosomes whilst control females of the same age as the uni-ovx as well as younger ones still had neatly aligned chromosomes. In contrast to the greater disorder of chromosome alignment in postovulatory aged cells, displacement of chromo-

somes in fresh oocytes of mice approaching the end of their reproductive life is probably related to a preceding non-disjunction event at anaphase I, and not one which might be anticipated in the second maturation division. Our studies confirm the notion that chromosome alignment in metaphase arrested, spontaneously ovulated oocytes is, in-
have other causes. Oocytes isolated from female mice always appeared to be recently ovulated as judged from the presence of a compact cumulus mass. Secondly, we did not detect any changes in the cytoskeleton of such oocytes characterizing postovulatory ageing, e.g. formation of cytoplasmic asters, or astral spindle tubules.

The suggestion that age-related aneuploidy is due to hormonal imbalance associated with the loss of follicles is not new but has lacked supportive evidence at the cellular level. Whilst the present results do not demonstrate a direct causal relationship between hormonal status and cell morphology or aneuploidy they give some tentative evidence for the postulated interrelation between non-disjunction and hormonal control. Dependence of the period of time needed to undergo GVBD and polar body extrusion in vivo and in vitro maturing oocytes on the hormonal status of the female have already been demonstrated in CBA mice (Polanski 1986). Furthermore, correlations between the incidence of non-disjunction and altered timing in stages of maturation have been noticed in hormonally stimulated prepubertal mice (Hansmann and Theuring 1986). Hormonal imbalance towards the end of the reproductive span as a possible factor in the aetiology of aneuploidy have also been postulated for both ageing mice and women (Lyon and Hawker 1973; Crowley et al. 1979). Our evidence indicates that only subtle disturbances in timing of maturation in primary oocytes of CBA mice may be sufficient to lead a prometaphase oocyte with still unordered bivalents into a premature anaphase stage. Could this be sufficient to predispose the cell to non-disjunction? We have no immediate proof of such a notion but the misalignment of chromosomes at metaphase II which has been demonstrated in a large proportion of those oocytes coming from females approaching the end of their reproductive period are most likely the result of a non-disjunction event in the first meiotic division. Translated into human terms, the irregular cyclicity and prolonged menstrual cycles occurring during the pre-menopausal period (Treloar et al. 1967) may well also disturb oocyte maturation and chromosome distribution leading to the high levels of aneuploidy observed over this period in women.

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The number of clonal precursors of the follicular epithelium in the mouse ovary

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Summary. The number of clonal precursors of granulosa cells in mouse ovarian follicles has been estimated using a technique based on the phenomenon of random X-chromosome inactivation of somatic cells and the use of an X-linked alloenzyme variant of the glycolytic enzyme PGK-1. The granulosa cells of follicles were oligo-clonal in origin and founded by a small number of cells (about 5) which was consistent with histological observations. When the analysis was extended to two sub-compartments of the follicle, the mural and cumulus granulosa cells, the results indicated that the cumulus and mural granulosa cells had a common origin.

Keywords: granulosa cells; primordial follicles; phosphoglycerate kinase; ovary

Introduction

The oocyte develops within an envelope of granulosa cells which multiply to form a solid follicle with several cell layers. The entire granulosa cell population, which numbers about 50,000 in mature follicles of mice, is presumed to be derived from a single (and sometimes incomplete) layer of squamous cells associated with the small oocyte in primordial follicles. It is not known whether the pre-granulosa cells of individual follicles are derived from one or several ancestral cells, nor is it certain whether they all contribute to the mature epithelium. Distinct populations of granulosa cells have been identified by their antigenic differences (Erickson et al., 1985) and a gradient of biosynthetic activity exists across the epithelium according to the distribution of several enzymes (Zoller & Weisz, 1978, 1979; Zoller & Enelow, 1983). Whether these sub-populations of granulosa cells have distinct developmental lineages is unknown.

Once a follicle has accumulated 2000–3000 cells to form a solid multilaminar structure it subsequently undergoes a morphogenetic transition in which the extracellular space expands to form the antrum of the Graafian follicle (Gosden & Telfer, 1987). The epithelium of the latter is uniform in thickness except at the pole where granulosa cells support the oocyte. Thus, two morphologically distinct sub-populations exist: those forming an epithelial lining of the follicle (mural granulosa cells) and those in which the oocyte is embedded (cumulus granulosa cells). Differences in secretory products have been found in cumulus and mural granulosa cells (Gilula et al., 1978) but it is not known whether these differences are related to differences in developmental lineage.

The present study was designed to estimate the numbers of clones and their relationships in differentiating granulosa cell populations of mouse follicles using two alloenzymal variants of an X-linked enzyme, phosphoglycerate kinase-1 (PGK-1), to detect tissue mosaicism. The numbers of clonal precursors have been estimated from the proportions of the two phenotypes present which are distinguishable by their differences in electrophoretic mobility. These results have been compared with the numbers of somatic cells which, on morphological grounds, are intimately associated with oocytes in primordial follicles.
Materials and Methods

Analysis of granulosa cell heterogeneity using PGK alloenzymes. Granulosa cells were obtained from a colony of CBA/Ca x CBA/Ca-PgK-1a mice maintained at the Department of Zoology, University of Edinburgh. These animals were heterozygous for the two electrophoretic variants (A and B) of the X-linked glycolytic enzyme PGK-1 and, as a result of X chromosome inactivation (Lyon, 1974), each cell and its descendant form one or other expression of the enzyme.

Mice were sacrificed 21–28 days old (i.e. pre-pubertal) with 10 i.u. PMSG (Organon Laboratories, U.K.) i.p. to stimulate antral follicle development. Animals were killed by cervical dislocation and their ovaries were excised and placed in a dish containing Hepes-buffered Medium 199 (Flow Laboratories, Irvine, U.K.). The large follicles were dissected using watchmakers' forceps and transferred to 50 μl droplets of Medium 199 under paraffin oil. The granulosa cells were expressed by gentle teasing and the follicle wall (mainly theca-stroma cells) and the oocyte were discarded.

In the first series of experiments granulosa cells, which included both mural and cumulus cells, from 75 follicles were transferred by pipette to separate plastic tubes. In the second series mural and granulosa cells from 34 follicles were isolated separately by rupturing the follicle wall and transferring the oocyte in its associated cumulus mass to another droplet of medium where the oocyte was removed by pipetting. The mural and cumulus granulosa cell components from individual follicles were transferred to separate tubes for processing. In both series the cells were centrifuged at 15 000 g for 40 sec at room temperature in an Eppendorf centrifuge. Excess medium was removed and the cells were resuspended in 7 μl of a lytic buffer containing 50 mm-triethanolamine hydrochloride (Sigma), 0·3 mg dithioerythritol (Sigma)/ml, 0·5 mg bovine serum albumin/ml, and 2 mg digitonin (Sigma)/ml (Ansell & Micklem, 1986). Each sample was snap-frozen on solid CO2 and stored at −70°C until enzyme activities were measured. PGK-1 enzyme activity is stable at −70°C for up to 3 months (J. D. Ansell, unpublished).

Relative proportions of PGK-1A and PGK-1B activity were measured using an assay based on that described by Bucher et al. (1980) and modified by Ansell & Micklem (1986). PGK-1A and 1B alloenzymes were separated on Titan III cellulose acetate membranes (Helena Labs, Texas, U.S.A.) by electrophoresis for 40 min at 15 mA (constant current) at 4°C. The electrophoresis buffer was a barbitol citrate buffer pH 8·8 containing 2 mM-EDTA, 10 mM-sodium citrate, 5 mM-magnesium sulphate, 20 mM-sodium barbitol and 0·25 mg adenosine monophosphate (AMP; Sigma)/ml. Ten samples were applied per gel, approximately 0·5 μl per application. The substrate for PGK-1 (1,3-diphosphoglycerate) is unstable and is prepared in situ after electrophoresis by a series of linked enzyme reactions (aldolase, glycero-3-phosphate dehydrogenase, and glyceraldehyde phosphate dehydrogenase) catalysing the conversion of fructose 1,6-diphosphate to the substrate for PGK-1 in the presence of nicotinamide adenine dinucleotide (NAD) and inorganic phosphate. ATP produced by the action of PGK-1 on its substrate generates 6-phosphogluconolactone from glucose through a second series of linked reactions catalysed by hexokinase and glucose 6-phosphate dehydrogenase. The principle of the autoradiographic assay is that a proportion of the glucose substrate is universally labelled with 14C and that the phosphate-containing products of 14C-glucose metabolism will covalently bind to a polyethylene imine thin-layer chromatographic (t.l.c.) membrane. Unconverted 14C-glucose can be washed off. A solution of the enzymes and substrates described above was spread evenly over the t.l.c. membrane. The Titan III membrane was then applied making a sandwich of t.l.c. membrane, substrate solution and Titan III membrane. This was incubated for 45 min at 37°C. When a PGK band was present on the Titan III membrane, 14C-glucuronolactone was formed and bound to the t.l.c. membrane, forming an image of the PGK band. After washing and drying the t.l.c. membrane, this band was visualized by the application of Kodak X-ray film to the membrane. The autoradiographs were scanned with an automated microdensitometer (Chromoscan 3, Joyce Loebl, Gateshead, U.K.) and the areas under the peaks were integrated to estimate the proportions of the products.

Cytological studies of primordial follicles. The numbers of somatic cells in primordial follicles were counted in serial histological sections from a similar group of animals. The ovaries were fixed in aqueous Bouin's fluid for 24 h, embedded in epoxy resin and sectioned at 3 μm with a glass knife. The sections were stained with haematoxylin and cosin and examined at ×1000 using oil immersion optics. Through a camera lucida attachment the outlines of oocytes and their surrounding pre-granulosa cells in primordial follicles were drawn. The numbers of pre-granulosa cells were then determined by tracing cells through successive drawings.

Results

Numbers of granulosa cell clones

The results of measuring PGK activity in the granulosa cells (cumulus and mural cells combined) of mouse follicles are shown in Fig. 1: of 75 follicles (12%) contained one of the two phenotypes, A or B, and the other follicles contained various proportions of the two, demonstrating that each population had been founded by more than one cell. These results are shown with
those obtained the frequency of PGK-1A activity in peripheral blood (107 samples) during the course of another study. The distribution is comparable in both tissue types.

The large variance in the follicle data implies that the actual number of progenitors is small, and this has been confirmed with the use of the binomial equation. The binomial statistic relates variance to mean as follows:

\[ n = p(1 - p)/\sigma^2 \]

where \( p \) = the mean percentage value obtained for PGK-1A activity, \( 1 - p \) = the mean value of the B allele, \( \sigma^2 \) = the sample variance, and \( n \) = the mean number of clonal precursors. The estimate of the variance between samples indicates the number of clones of cells contributing to the tissue (Nesbitt, 1971; Stone, 1984). If there is only one founder cell the enzymic phenotype of the resultant tissue will be either A or B. If many cells are found the tissue ratio of A:B is expected to be similar and close to 1:1 in all samples. Intermediate situations can be analysed by application of binomial statistics to the variance of phenotypes between follicles. From the application of the binomial equation to data obtained from granulosa cells it is predicted that on average 5 precursor cells give rise to this population in a mouse follicle. Examination of alloenzyme phenotypes may be used to determine whether or not the clonal descendants of these precursors are distributed randomly between mural and cumulus sub-populations of the granulosa membrane. The binomial statistic for 34 follicles in which mural and cumulus granulosa cells were examined separately predicted 5-2 precursor cells in toto with 10% monoclonal follicles, confirming the previous findings. The proportions of total enzyme activity which was due to the single phenotype PGK-A were compared for the mural and cumulus cell sub-groups by linear regression analysis (Fig. 2). The numbers and phenotypes of the two sub-groups were linearly related, proportions being highly correlated (\( r = 0.89, P < 0.01 \)) and indicating that both cell types were derived from the same precursor pool.

Numbers of morphologically identified pregranulosa cells

A frequency distribution for the numbers of (presumptive) pregranulosa cells in 40 primordial follicles is shown in Fig. 3. Most follicles were associated with 3–6 squamous cells, the average being 4-8 (s.d. 2.05). Follicles with 1 and >11 somatic cells were not observed. Occasionally, naked oocytes were encountered.

Discussion

The value of the analysis of mosaicism of X-chromosome expression for the investigation of cell lineages has been demonstrated previously. In particular PGK-1 has been useful for the analysis of the clonal origin of tumour and haematopoietic tissues (Woodruff et al., 1986; Micklem et al., 1987). This is the first report for ovarian cells. The results have verified the assumption which was made a priori that granulosa cells express only one X chromosome for otherwise monoclonal types could not have occurred and, moreover, the proportion of total PGK activity which is due to the A phenotype was 0-69 which compares with the number found in other polyclonal tissues. A mean value greater than 0-50 is expected because of the presence of alleles of an inactivating locus (Xce) which affects the probability that a particular X chromosome will be inactivated (Cattanach & Papworth, 1981; Forrester & Ansell, 1985).

The technique for measuring enzyme activity has been demonstrated to be sufficiently sensitive for use even with the small numbers of cells obtained from a single cumulus oophorus (< 2000). Reliable estimates of the number of clones in follicles rest on the assumptions that differential cell multiplication and death are insignificant as appears to be the case for the following reasons. Firstly,
Fig. 1. Frequency distribution of the percentage of phosphoglycerate kinase activity (PGK) which is of the A phenotype in granulosa cell (cumulus and mural cells combined) from 75 individual mouse follicles (unhatched) and 107 samples of peripheral blood (hatched area). The frequency is expressed as a proportion of the total in each sample.

Fig. 2. The relationship between the proportions of PGK-1A activity in the mural and cumulus granulosa cells obtained from 34 individual mouse follicles. A straight line has been fitted by the method of least squares. The product moment correlation coefficient (r) = 0.899.
Granulosa cell clones in mouse ovary

very few apoptotic bodies are present in normal healthy follicles of the type obtained during PMSG treatment (Braw & Tsafriri, 1980). And, secondly, on the basis of tritiated thymidine autoradiography, it appears that the granulosa cell population in solid follicles of adult mice is multiplying uniformly but asynchronously, although at late stages in Graafian follicle development cell activity is restricted to the cumulus region (Gosden et al., 1983). The pattern of cellular activity in immature mice is not known but in rats there is no differential growth at these ages (Hirshfield, 1986). Polyploidy is another potential source of error when estimating the relative amounts of PGK activity and, hence, the estimated number of clones. It has been claimed that some pig and cow granulosa cells are polyploid (Coulson, 1979) but microdensitometric measurements of Feulgen-stained nuclei plainly show that murine cells are diploid (E. Telfer & R. G. Gosden, unpublished results).

The presence of the two alloenzymic phenotypes in widely varying proportions provides unequivocal evidence that the populations are oligoclonal. The binomial statistic suggests that the average number of clones is 5, but this figure should be used as a guide rather than carrying the weight of numerical precision. Follicles with a single alloenzyme phenotype are uncommon. These may indicate monoclonality or the chance coincidence of cells with the same phenotype in the primordial follicle. The average number of clones corresponds closely to the numbers of observed pre-granulosa cells, supporting the hypothesis that they are the progenitors of the mature follicle. The founding of the granulosa cell population of most follicles by more than one cell is reminiscent of other organ structures and its significance may be that plurality confers developmental flexibility.

Whilst detailed analysis of the spatial distribution of clones will require different methods, measurements of PGK activity in the two major cellular subpopulations gives some indication of the clonal relationships between them. The results show that the cumulus cells, which represent approximately 20% of the total population, were founded by a number of precursors similar to that for the granulosa cells. The high degree of correlation between the proportions of alloenzymes in cumulus and granulosa cells indicates that these precursors were probably the same. If the separate layers forming the follicle were founded by separate clones these proportions would not be expected to be highly correlated. Further, the correlations indicate that before the differentiation of cumulus cells there was extensive proliferation and cell mixing of granulosa cells by the time the antrum had formed and that a large number of these (probably > 200) generated the cumulus mass.
These results do not suggest what mechanisms are driving the differentiation but might indicate that a critical number of granulosa cells must be present before the cumulus can differentiate.

There is abundant evidence that in the mature follicle the granulosa cells differentiate to serve different functions. For example, the cumulus cells produce glycosaminoglycans in response to FSH (Eppig, 1981). Evidently, these differences have not emerged purely from the cell lineages but will have to be understood on the basis of other factors, such as cell position and interactions between granulosa cells with the stroma–theca and oocyte.

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The kinetics of pre-antral follicle development in ovaries of CBA/Ca mice during the first 14 weeks of life

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Abstract. The kinetics of ovarian follicle growth and death have been estimated in virgin inbred mice using a compartmental model and data obtained from differential follicle counts in histologically sectioned ovaries. The results showed that both growth and death rates are dependent on stage of development, defined by the compartments, and age, indicated in the model by step functions with transitions at 20 and 60 days of age. During the initial phase of postnatal ovarian development, large numbers of follicles disappeared from the non-growing reserve as a result of the combined effects of follicle death and recruitment into the growing population. The reduced death rate after 20 days led to a secondary peak in the numbers of follicles at intermediate stages. In contrast to these fluctuations, the number of large follicles, including pre-ovulatory types, were remarkably constant after this age and the rate of outflow stabilized at two to three follicles per day after an initially high value. This rate is sufficient for the normal ovulation rate in a 4-day oestrous cycle with a small surplus of follicles undergoing atresia. The rates of migration through successive stages of development decreased during ageing as large follicles began to emerge at the approach of puberty: this result may indicate that the recruitment of small growing follicles is influenced by a feedback effect.

The ovarian follicle is a composite structure consisting of (usually) a single oocyte and a granulosa cell epithelium, with the differentiation of a theca-stroma layer at later stages of development. It develops as a functional unit, but the population of ovarian follicles must be coordinated and controlled in order to produce sets of ovulated oocytes at regular intervals. Folliculogenesis in the mammalian ovary is normally completed before birth or during the early postnatal period, and the finite store of oocytes formed have to serve the requirements of reproduction for the rest of life. The factors responsible for controlling the utilization of follicles and oocytes are therefore matters of considerable theoretical and practical interest. Clearly, the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are involved in this process since hypophysectomy prevents follicle maturation (Greenwald, 1974). It is, however, far from clear how the succession of small and medium-sized follicles is maintained, since these are not acutely dependent on the pituitary gland. Knowledge of kinetics...
is an important foundation for progress in this field, since changes in the rates of follicle growth and/or death at different ages and in different physiological states can indicate the stages at which regulation of follicle recruitment towards ovulation is occurring. The analysis of this problem requires different approaches to those which have been successful for describing the developmental continuum of other epithelial tissues, since each proliferative unit is non-renewable and follicle death ('atresia') evidently plays a crucial role in controlling the numbers of mature follicles.

A number of methods have been used to study the kinetics of ovarian follicles, but these have generally given incomplete accounts because death rates have not been analysed (Bullough, 1942; Peters & Levy, 1966; Pedersen, 1970: Read et al., 1981). A compartmental model was introduced to overcome this particular problem and to give an overall impression of follicle development during the entire span of life (Faddy et al., 1976). In the present study this model has been applied to the analysis of temporal changes in follicle numbers and, for the first time, has employed non-parametric statistics to identify features of the data before fitting the model and obtaining parameter estimates. This has required a large amount of data in order that patterns in the profile of the follicle population can be reliably detected. The subjects of study, CBA/Ca mice, were investigated during the first 14 weeks of life, with the aim of providing a detailed description from which it may be possible to deduce the stages and ages at which major regulatory influences are being exerted. Earlier work has shown that changes in follicle utilization occurring early in life have a lasting influence on the functional potential of the ovary (Jones & Krohn, 1961a; Faddy et al., 1983).

MATERIALS AND METHODS

The data were obtained by differentially counting follicles in serially sectioned ovaries obtained from 95 inbred CBA/Ca mice aged from 1 to 98 days post-partum. Over 40% of the observations were made during the first 25 days of life. Since earlier studies showed that the size of the follicle population is surprisingly variable in syngeneic mice at the same age (Faddy et al., 1983), attention has been given to controlling a number of variables which are known to be or are suspected of being responsible for individual differences.

The animals were housed in thermostatically-controlled rooms at 21°C with a photoperiod of 14 hr beginning at 07:00 hours G.M.T. They were provided with a pelleted diet and water ad libitum. Mice were selected at random from litter sizes ranging from 5 to 10 pups. Adult animals were killed by cervical dislocation between 14-30 and 15:30 hours G.M.T., since a circadian rhythm may exist in the follicle population profile (Sahu, 1985). The age at which vaginal opening occurred, being indicative of the first ovulation, was identified by daily examinations. Vaginal smears were taken daily by lavage from animals aged more than 40 days when cyclic activity had been established. The smears were examined microscopically to determine the day of pro-oestrous which was chosen as the day of sacrifice in all adult animals. At autopsy, pairs of ovaries were dissected and fixed in aqueous Bouin's fluid; serial paraffin sections were cut at 7 μm and stained with H & E.

Follicles were counted in every tenth section using the oocyte nucleolus as a marker. Exceptionally, the oocyte nucleus was used as the marker of primordial follicles because a single distinct nucleolus was absent. The total numbers of follicles at a given stage were obtained by multiplying by both the counting frequency and a correction factor (Abercrombie, 1946). Every section was examined for the presence of the nucleolar marker in oocytes of the less abundant follicles at the largest stage which were therefore counted individually. To determine the
reproducibility of these counts, follicles in one immature and one adult pair of ovaries were counted on six separate occasions.

The stages of follicular development were classified according to the criteria of Mandl & Zuckerman (1951) in which the number and morphological appearance of granulosa cell layers are the decisive features. Follicles were classified as follows: 'primordial' follicles having a single layer of squamous cells (stage I); those with a single layer of cuboidal cells and a growing oocyte (stage II); those with two cell layers (stage III); three cell layers (stage IV) and four or more cell layers, irrespective of whether an antrum was present (stage V+). These stages are illustrated diagrammatically in Fig. 1.

The qualitative appearance of follicles, whether healthy or atretic, was not taken into account during the counting because stage I follicles were too small for changes to be recognized, and degenerating follicles at stages II–IV were very rare. Atretic follicles could, however, be recognized at stage V+, but provided they were clearly identifiable (i.e. atresia had not advanced to the degree at which the nucleolar marker had disappeared or the number of cell layers had become indeterminate), they were included in the counts. It was felt that these atretic follicles should be so included, as they must have been recruited from previous stages and thus not to have included them would have resulted in some bias (although probably slight) in the estimates of the parameters of the model.

The data were analysed using a compartmental model for ovarian follicle kinetics which describes the growth of follicles through these stages (Faddy et al., 1976, 1983; Halpin et al., 1986). The basic assumptions of the model are that follicles can migrate from the reserve population of stage I follicles into stage II and so on by growth until the final stage (V+), and they can leave any of these stages by death. There is experimental evidence demonstrating that follicles are not stationary after the primordial stage (Pedersen, 1970). A further assumption is that there is no increase in the size of the reserve population after birth. The numbers of follicles in each of the four growing stages are therefore interdependent, since they are influenced by preceding stages and, in turn, influence the stages that follow. The rates of migration and death are age-dependent and are represented as follows: four migration rates—\( v_1(t) \) (from I to II), \( v_2(t) \) (from II to III), \( v_3(t) \) (from III to IV) and \( v_4(t) \) (from IV to V+), and five death rates—\( \mu_1(t) \) (from I), \( \mu_2(t) \) (from II), \( \mu_3(t) \) (from III), \( \mu_4(t) \) (from IV) and \( \mu_5(t) \) (which represents the egress from V+ as a result of both death and ovulation). The mathematical properties of such a model may be summarized by mean numbers of follicles at each stage (\( \lambda_i(t) \), \( i=1, 2, \ldots 5 \)) given by the solution of the differential equations:

\[
\frac{d\lambda(t)}{dt} = v_{i-1}(t) \lambda_{i-1}(t) - [v_i(t) + \mu_i(t)] \lambda_i(t)
\]  

(1)

Fig. 1. The five discrete and successive stages of ovarian follicle growth in mice upon which the compartmental model was based. Stage I represents the non-growing reserve of primordial follicles and V+ is the terminal stage which includes antrum-containing follicles in addition to the larger pre-antral types depicted here. Each horizontal bar indicates 20 \( \mu \text{m} \).
with \( v_0(t) \) and \( v_0(t) \) both zero since growth into stage I and out of stage V+ is not permitted (Fabby et al., 1976). This model describes the process of follicular maturation from birth to adulthood in terms of the numbers of follicles in each of the five stages of development, or compartments. On the basis of the estimated growth and death rates, curves have been obtained which depict the changes in mean numbers of follicles present.

**RESULTS**

The data are shown in Fig. 2 together with age-dependent curves (broken lines) determined from the data by non-parametric regression techniques (Silverman, 1985). These techniques smooth the highly dispersed data points into curves, without any parametric assumptions influencing the shape of the curves, to provide estimates of the average numbers of follicles in the five stages of growth over the period 0–98 days of age. Certain features of these follicle profiles are apparent: the steady decline of small follicles in stage I, the triphasic profiles of stage II, III and IV follicles with troughs occurring at around 20 and 60 days, and the steady number of stage V+ follicles after about 20 days.

Our fitting of a parametric compartmental model to the data represents an attempt to interpret this behaviour by making inferences about the controlling components of growth and death of follicles throughout the 98 day period. Given the form of equation (1), governing the compartmental model (see Faddy et al., 1976), and a desire to mimic the non-parametric curves shown in Fig. 2, it is clear that some form of age dependence in the growth and death rates of follicles must be incorporated into the model. Age dependence can be represented by the pragmatic choice of step-functions with change points corresponding to the troughs in the triphasic follicular profiles of stages II, III and IV (cf. Faddy et al. 1976) where biphasic step-functions were used on a much less extensive data set). The resulting parametric compartmental curves giving the mean numbers of follicles in the five stages are also shown in Fig. 2 (continuous lines) and they correspond fairly well to the non-parametric regression curves (broken lines). Three estimates for each growth and death rate, corresponding to the triphasic step functions, are shown in Fig. 3. Significance should not necessarily be attached to the change points of these triphasic step functions at 20 and 60 days or, for that matter, to the three phases. These phases are in a sense arbitrary devices which represent our attempt to mathematically model a complex process in a tractable way with the step-functions describing the dominant changes that occur in the growth and death rates. Faddy & Jones (1987) describe in more detail statistical aspects of this approach, involving an interplay between non-parametric and parametric analyses of the data. Table 1 indicates the average numbers of follicles which are expected to leave the compartments per day, estimated from the mean numbers shown in Fig. 2 and the rates shown in Fig. 3.

The model assumes that the stock of follicles is fixed at birth, and on this basis it predicts that the follicular pool has a mean size of 8177 ± 642 at this time. During the initial phase the numbers of primordial follicles decline steeply as a result of death and recruitment to stage II. The death rate is about twice as high as the growth rate. The rates of growth through successive stages are high and those attaining stage V+ evidently terminate development by death at this stage, since ovulation does not occur at these ages. Stage V+ follicles were present in virtually all animals after day 9.

---

**Fig. 2.** A graphical representation of the numbers of follicles (stages I–V+) in 95 virgin CBA/Ca mice ranging from 1 to 98 days post-partum. The data were obtained by differential counting of the follicle stages. The continuous line shows the fit of the parametric curves obtained from the model and the interrupted line shows the corresponding non-parametric regressions.
Fig. 3. This figure shows the estimates of the migration and death rates (see equation (1)) with their standard errors (in parentheses) for each of the five stages of follicular development and three temporal phases.

Table 1. Average numbers of follicles leaving the compartments (per day) either by growth to the next stage or death

<table>
<thead>
<tr>
<th>Age</th>
<th>I+</th>
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The intermediate phase encompassed the age at which sexual maturity was attained. Vaginal opening occurred between days 23 and 29 when corpora lutea appear for the first time. The number of stage I follicles in 20-day-old ovaries is approximately 5000, which is less than 60% of the original store size at birth. The continuing attrition of the stock occurs more slowly than in the initial phase because of a reduced death rate, recruitment into stage II being similar.
Ovarian follicle kinetics

This results in the second peak at stages II, III and IV. There was no evidence that follicles die at the intermediate stages II and III, confirming the impression obtained histologically that atresia in growing follicles is mainly confined to the larger stages. The rate of movement to stage V+ was slower than in the previous phase, although this estimate is somewhat imprecise.

At the beginning of the final phase the primordial pool size was about 2300, which is less than 30% of the original population. This phase is characterized by a further significant decrease in the death rate of follicles from the primordial pool. The death rate is less than half that occurring during the preceding phase and, for the first time, the rate at which follicles initiate growth exceeds the death rate.

As a result of the almost constant mean numbers of follicles in stage V+ after about 20 days of age there is little statistical evidence of movement of follicles from this stage. Since there must be some removal by ovulation, the rate of removal was constrained to the value 0.06 in the final phase, which is equivalent to the egress of about ten follicles in a 4-day oestrous cycle (see Table I), corresponding closely to the observed ovulation rate in this strain (unpublished observations).

DISCUSSION

This is the first study of ovarian follicle kinetics which has been designed to follow the detailed temporal changes in follicle utilization from birth to maturity under strictly controlled conditions. A clearer picture has emerged of the fluctuating pattern of the follicular population profile using non-parametric regression analysis, which in turn was a useful aid to guide the fitting of the model to the highly dispersed data points. The resulting compartmental model provides an opportunity to infer whether kinetics of particular follicle stages coincide with the rising and falling levels of gonadotrophic hormones and with the emergence of large follicles before and corpora lutea at puberty. The model also provides a basis for comparing follicle kinetics in animals in different physiological states, such as pregnancy, and following experimental manipulation, such as hemi-ovariectomy and pharmacological inhibition of ovulation.

In view of the genetic uniformity of the animals used and the controls employed for physiological and environmental variables, it is surprising that the variation in the data was not substantially less than in earlier studies (Jones & Krohn, 1961a; Faddy et al., 1983). The repeatability of the counts made by a single observer is indicated by the coefficients of variation; these ranged from 3 to 7% for six replicates in young and old animals alike. This result suggests that most of the variation can be attributed to biological factors rather than technical imprecision, and we therefore conclude that wide variation is probably a normal characteristic of the follicle population. Recent theoretical work has shown that substantial random variation can be present in stochastic compartmental models (Matis & Wehrly, 1981; Faddy, 1985) which leads us to the conclusion that some of the variation observed may be 'structural'. The extent to which variation during the life history of any given pair of ovaries contributed to the observations cannot be traced, since longitudinal studies cannot be carried out on individual animals. There is, nevertheless, a clear impression that follicle development is a highly ordered process; this is to be expected since it is leading to the regular maturation of sets of pre-ovulatory follicles throughout adult life.

The major findings of this study are based on changes in the estimated follicle growth and death rates with age and development. It would be expected that temporal changes should be continuous rather than discrete; step-functions provide only an approximation to reality here, but one that facilitates estimation of the many parameters necessary to specify the model, and they
are consistent with describing the developmental continuum in terms of five discrete stages. Any physiological significance of the three age phases used should therefore be drawn with caution. These phases were identified non-parametrically by the troughs which occurred in the numbers of follicles at intermediate stages II–IV. It is reassuring that the first transition at about 20 days of age corresponds to that observed in an earlier study of CBA mice (Faddy et al., 1983). This immature phase of life encompasses the period from follicles commencing growth until the peak numbers of large (V+) stages are attained, when the ovary has acquired the capacity for ovulation in response to exogenous gonadotrophins (Gosden, 1985). Thus, day 20 marks the beginning of a phase when influences from follicles approaching maturation or undergoing atresia might be expected.

One characteristic of the initial phase of postnatal ovarian development is the very high rate of death in the non-growing pool of primordial follicles. More than twice as many small follicles die compared with the numbers of those undergoing development; if this high death rate were not attenuated during adult life it would lead to precocious sterility. The present studies cannot shed any light on the cause(s) of death though it is interesting to speculate whether primordial follicle death represents a continuum of germ cell losses which began during oogenesis (Beaumont & Mandl, 1962; Baker, 1963). Small follicles in neonatal ovaries may require a ‘priming’ effect by gonadotrophins to initiate growth (Eshkol et al., 1971; Baker & Scrimgeour, 1980), but it appears to be unlikely that a gonadotrophin deficiency is responsible for the substantial death rate since small follicles are not regarded as being acutely dependent on pituitary gonadotrophins and, besides, these hormones circulate in high concentrations during juvenile life (see below).

In contrast to the declining death rates, the daily rates at which follicles begin to grow (I–II) do not show any significant change during the ages studied. This finding is consistent with the view that the initiation of growth is independent of changes in gonadotrophins and other follicle stages and corpora lutea. The control of recruitment into the pre-ovulatory subpopulation does not therefore appear to involve feedback to source. The sum of the rates of primordial follicle growth and death determines the steady loss from the follicle store, which is described by a multi-exponential curve. The rate of follicle attrition should not, however, be regarded as inexorable, since the store is depleted more slowly in hypophysectomized animals and in intact animals on restricted diets (Jones & Krohn, 1961b; Nelson et al., 1985).

The predictions of the model and the morphological evidence agree that follicle deaths are comparatively rare events at intermediate stages of development (II–IV) but occur frequently at stages I and V+. Stages II–IV appear to be coasting towards a critical stage when selection for ovulation occurs and their numbers closely reflect those entering the growing population a few days earlier. The parameters for these intermediate stages are similar during the first two phases, which cover the period 0–60 days of age. One of the more remarkable findings is that subsequently there is a major decline in the rates of follicle growth at stages II–IV. The onset of the third phase does not appear to be related to any obvious physiological event involving major changes in hormone secretion, such as puberty, although it is interesting that it is close to the age at which animals in this strain become fully fertile. Studies using the labelled mitoses technique for estimating follicle growth have also reached the conclusion that the stage-specific rates of progression are highest for follicles developing during infancy (Pedersen, 1970). Interestingly, the first spermatozoa to be produced by the pubertal testis also undergo rapid development, although different factors in the two gonads are expected to underlie this apparent similarity (Janca et al., 1986).

Differences in the rates of growth of follicles at given stages may depend on a balance between stimulatory and inhibitory influences present at different ages. The circulating levels of...
the gonadotrophins, FSH and LH, reach peak levels during days 9–15 of postnatal life (Dullaart et al., 1975) but these high levels are not sustained in later life, apart from the cyclical peaks at the peri-ovulatory time. In view of this evidence the numbers of hormone-sensitive follicles would be expected to rise with the corresponding hormonal changes, but in fact they actually reach a nadir at around 20 days and there are no signs of a changing growth rate during the following phase. The gonadotrophin levels do not, therefore, appear to have a dominating influence over follicle kinetics up to multilaminar stages at immature ages.

The alternative hypothesis that inhibitory influences control follicle recruitment should be considered, despite a present lack of firm evidence. In view of the spontaneous flow of large numbers of follicles into the growing population recruitment into mature stages must be controlled in order to avoid an explosive expansion in ovarian volume and possible superovulation. At prepubertal ages a reduction in numbers is achieved by the large scale atresia of gonadotrophin-dependent follicles up to the end of the third week (Peters, 1969). Atresia of antral follicles continues at postpubertal ages when it is required for finely adjusting the numbers which are proceeding towards ovulation (Hirshfield & Midgley, 1978). The slowing down of follicle growth at intermediate follicle stages in peri- and postpubertal animals contributes to the control of these numbers. The temporal association of this phenomenon with the appearance of large follicles leads us to speculate whether these follicles release inhibitory substances, but there is, as yet, no experimental evidence for this hypothesis. Of possible relevance to this question is the fact that bovine follicular fluid has been found to inhibit the initiation of follicle growth in immature mice (Peters et al., 1973) and, whilst current knowledge denies importance to primordial follicles as a controlled stage, there is a possibility of analogous effects on intermediate follicles.

The function of the ovary is the production of fertile oocytes at regular intervals and the provision of hormones for initiating and supporting pregnancy. One of the key observations in ovarian physiology is that the production of ovulatory follicles is finely controlled to maintain approximately similar numbers of ripening follicles during life, despite a dwindling store or in the face of experimental reduction in ovarian mass (Lipschutz, 1928). The present results show that controlling processes are at work during pre-antral stages, since the spontaneous recruitment of follicles from the store leads to a stabilized subpopulation of follicles at stage V+ from about 20 days of age. Fine tuning of follicle numbers at this stage, which requires the gonadotrophins, leads to the production of a normal set of ovulations. The compartmental model has provided a quantitative description of the emergence of the adult pattern of follicle development, but other methods will be required for investigating the biological variables responsible for the underlying parameters of growth and death.

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REFERENCES


Ovarian cyclicity and follicular recruitment in unilaterally ovariectomized mice.

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Running headline: Unilateral ovariectomy in mice

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Summary. Halving the numbers of follicles in young adult mice by unilateral ovariectomy results in compensatory ovulation and a reduction of the total number of short oestrous cycles of about 25%. During the first 2 months after ovariectomy, which were studied in detail, the combined numbers of multilaminar preantral follicles and antral follicles in the single ovary rose to approach those in a pair of age-matched control ovaries; there was a corresponding fall in the incidence of morphologically recognised atretic follicles. The impact of ovariectomy on the numbers and dynamics of small follicles was studied using a compartmental mathematical model, the findings indicating that complex, time-dependent changes were occurring in this group. The rate of egress from the primordial pool and the movement from stage-to-stage increased, although moderating later, to drive recruitment into the antral stage. Paradoxically, the death rate of small follicles increased. Accelerated ovarian ageing after unilateral ovariectomy is consistent with the earlier loss of fecundity.
Introduction

The ability of organs to undergo compensatory functional hypertrophy following injury or partial extirpation is widespread among vertebrates, but the extent to which normal function is restored varies with the species, age and organ in question. In the case of mammalian ovaries, the capacity to restore a full quota of ovulations following hemi-ovariectomy appears to be universal at young adult ages in polytocous species; in mono-ovulating species, the corresponding response is the ripening of a single follicle at normal cycle frequency. Such observations helped to shape hypotheses about the extrinsic neurohormonal control of ovarian function and led to formulation of the "law of follicular constancy" (Lipschutz, 1928).

According to this theory, hemi-ovariectomy alleviates negative feedback by sex steroids and inhibin on the pulsatile release of pituitary gonadotrophins, which thus produce a superovulatory response in the remaining ovary. Many studies have shown that circulating levels of FSH rise, although in most cases returning to baseline within a few days (Ramirez & Sawyer, 1974; Butcher, 1977; Otani & Sasamoto, 1982). The rapid adjustment of the ovulation rate, occurring within one or two cycles of surgery, implies that ovulations result, at least initially, from recruitment of additional growing follicles rather than of primordial ("resting") stages, as originally suggested by Lipschutz. Compensatory ovulation has therefore come to be regarded as depending on hormonal rescue of growing follicles from the surplus in the cohort. This conclusion is supported by the observed reduction of atretic types after unilateral ovariectomy (uni-ovx) (Hirshfield, 1983) and the ability of gonadotrophins to produce superovulatory responses by rescuing follicles otherwise undergoing atresia (Braw & Tsafriri, 1980). The role of the gonadotrophins is still not clear, however, because compensatory follicle maturation can, in some circumstances, occur without a concomitant
hormonal change (Gibson et al., 1979; Fry et al., 1987) and some evidence suggests that ovarian adrenergic nerves are involved in responses to uni-ovx (Gerendai et al., 1978; Curry et al., 1984). Nevertheless, the histometric studies lead to the prediction that at later ages, when the numbers available for recruitment are diminished, the proportion of follicles promoted each cycle would rise and the diminishing store of small follicles would eventually limit the ability to sustain a compensatory response (Peppler, 1971).

In the short term, this theory need pay little regard to any changes in the dynamics of small follicles. In the longer term, however, they are crucial because small follicles determine the timespan over which fecundity can be maintained. A number of important questions need to be answered. Is the store of small follicles exhausted earlier than expected purely on the basis of halving at young adult ages? If so, do oestrous cycles cease at an earlier age? Are the growth and/or death rates of preantral growing follicles altered? The present study was designed to provide a detailed description of preantral follicle dynamics after uni-ovx in an inbred strain of mouse and to determine when acyclicity begins. Data were obtained from differential follicle counting and analysed using an established mathematical model and with the results of a detailed concurrent study of normal animals less than 100 days old (Faddy et al., 1988).
Materials and Methods

Animals. Inbred CBA/Ca mice were obtained from the laboratory colony which was maintained at 21 °C with a photoperiod of 14 h beginning at 0700 h GMT. A pelleted diet and water were provided. Virgin mice were housed in pairs and vaginal smears were obtained 6 days per week by lavage after 38 days of age. Thirty-three mice were unilaterally ovariectomized and five were sham-operated on either the right or left side on a pro-oestrous day between 41-45 days old.

Follicle numbers and dynamics. Autopsy occurred between 4 and 50 days after surgery when the animals were 48-95 days old and presenting a pro-oestrous smear. The dissected ovaries were fixed in Bouin's fluid and serially sectioned in paraffin wax at 7 µm and stained with haematoxylin and eosin. The numbers of follicles were counted using the classification of Mandl & Zuckerman (1951) in which the numbers of cell layers and appearance of the granulosa cells define the follicle stages. The largest stage (VI) was subclassified according to whether the antrum was incipient (VIa) or fully formed (VIb). Differential follicle counts for each animal were carried out blindly at every tenth section except in the two large stages, V and VI, for which every section was searched. The nucleolus was used as a marker for counting and the raw counts were adjusted using correction factors to obtain estimates of total numbers.

Additional information about the morphological appearance of the larger stages, V and VI, was recorded. When 3 or more pyknotic granulosa cells were present in the equatorial cross-section, the follicle was considered "atretic". The numbers and proportions of atretic follicles at these stages were compared in operated and control animals using the Kruskal-Wallis analysis of variance of ranks.
The raw follicle counts were used to construct a mathematical model which describes the numbers of follicles and their rates of progress from stage-to-stage. Since follicle counts in the sham-operated animals were indistinguishable from those of age-matched intact animals in a concurrent study (Faddy et al., 1988) the compartmental model obtained for the latter was used for comparing changes in uni-ovx mice. For guiding the fitting of the data to the non-parametric regression methods were used (Faddy & Jones, 1987).

**Ovarian cycle history.** The frequency of ovarian cycles in 20 uni-ovx and 21 sham-operated mice were interpreted from vaginal smears using a conventional classification (Nelson et al., 1982). The animals were killed when they had presented at least 30 consecutive days of anoestrus. At autopsy, uni-ovx was verified, the remaining ovary was processed by histology (see above) and any animals showing signs of disease were excluded from the results.

**Results**

**Follicle numbers and dynamics.** The numbers of follicles in uni-ovx animals are presented in Fig. 1, classified by age of animal and stage of follicular development. The parametric curves, which were derived from the compartmental model (see Fig. 2), are shown together with the non-parametric curves and with the model for intact mice. It was necessary to fit a new model for the data from the uni-ovx group in order to faithfully describe the distribution of the counts. The model was biphasic with a phase transition was set arbitrarily at 67 days, approximately bisecting the data. On the basis of the known ovulation rate of 7-9 in this strain (Gosden, 1979), the daily rate of outflow from $V^+$ by ovulation and atresia from the largest stages ($V^+$) was constrained to 0.08 to provide a realistic number of follicles maturing
during each cycle given that approximately 30 follicles are present at V+.

Since different ages were chosen for the phase transitions in the two models it is difficult to compare the parameters. Nevertheless, it is clear that the rate at which follicles disappeared from the store of non-growing types (stage I) was greater than in controls during the first two months post-ovariectomy. The numbers of follicles moving from stage to stage in the operated group shows that approximately 18 follicles were being lost daily between 49 and 98 days (Table 1). This compares with about 13 per day in each ovary of intact animals at the same ages (see Faddy et al., 1988). This difference appeared to be due to higher growth rates although, in view of the negative correlation between growth and death rates \(r = -0.96\), the precise apportionment between these parameters is uncertain. The numbers of follicles at stage II were substantially lower in the uni-ovx group and, in view of the greater outflow from stage I and relatively normal numbers in the subsequent stage (III), it is inferred that many follicles died at stage II. There is, however, a correlation between the death rates at stages I and II \(r = -0.98\); consequently uncertainty also exists about the balance of losses between these two stages. The death rates at stages III and IV were negligible and the rates of growth were not altered at intermediate stages. Most follicles leaving stage II would therefore reach V+. The higher rate of outflow by ovulation was sustained by the marked rise in growth rate IV-V+, which was responsible for declining numbers at stages III and IV (Fig. 1).

The numbers of follicles appearing healthy at stages V and substages VIa and VIb were remarkably similar in the two groups of animals (Fig. 3). The preovulatory types (VIb) numbered 9-10, of which 1-2 were atretic. Most of the atretic follicles occurred at stage VIa, this preponderance over other stages being particularly marked in the intact mice, although statistically significant only at the second phase \((P < 0.01)\). Comparable patterns were
obtained when stricter criteria for atresia were tested (>20 pyknotic granulosa cells per section). When the numbers of atretic follicles were expressed as percentages of the numbers at the same stage it was evident that the incidence of atretic V and VIa follicles had been halved by uni-ovx.

**Ovarian cycle history.** The results of the longitudinal study of oestrous cycles are presented by 20 day intervals from 90 days old and excluding 3 animals on the basis of autopsy findings (Fig. 4). More than 90% of the animals of both groups presented regular short cycles cycles (<6 days) from 90 to 300 days of age. Approximately half of the cycles were 4 or 5 days long. An incomplete set of records showed that a similar pattern was occurring in the preceding 40 days. After 250 days of age, cycle frequency in the uni-ovx group diminished, this being a result either of additional days of cornified (oestrus) or predominantly leucocytic smears (di-oestrus). Prolonged periods of leucocytosis (indicative of pseudopregnancy) were rare. The frequency of cycles, whether 4/5 days in length or longer, declined prematurely in uni-ovx animals of which 50% had become acyclic at 340 days old, 40 days earlier than the controls. The uni-ovx group had presented a total of 15.3 4/5-day cycles after 90 days of age as opposed to 20.4 in controls over the same period. Evidently, cycle loss was permanent since few, if any, histologically identifiable follicles remained after a year of age.

**Discussion**

Compensatory follicle growth and ovulation following unilateral ovariectomy has been extensively investigated in recent decades although the first tentative signs of this phenomenon were noted two centuries ago (John Hunter, 1787). Most attention has been paid to the underlying physiological mechanisms and comparatively little is yet known about the long-term impact on fecundity and the dynamics of small follicles. The present results suggest
that uni-ovx slightly accelerates the ageing of the remaining ovary and probably has an influence on the dynamics of all follicle stages.

The rate at which small follicles are being lost rises during the first few weeks after uni-ovx. This confirms earlier studies which showed transient differences in follicle numbers in both rats and mice (Mandl & Zuckerman, 1951; Baker et al., 1980) and long-term changes in the preantral follicle population of other species (Dufour et al., 1979; de Reviers, 1987). Effects are less likely to be detected in short-term experiments because small follicles grow sluggishly.

The higher rate of follicle attrition indicated by the mathematical model is not sustained and there was evidence that parameters were age-dependent even during the relatively short period of detailed study. Age changes are to be expected because follicle exhaustion would otherwise have occurred before the observed onset of acyclicity. When the parameters for phase 2 are used to extrapolate the numbers of stage I follicles to 300 days of age only 7 follicles were predicted in the uni-ovx group compared with the more realistic figure of 300 in the controls. Furthermore, the numbers of follicles actually counted and the serum oestradiol levels in the two groups were not-significantly different at 170 and 250 days old (R.G. Gosden & C. Mobbs, unpublished).

Ablation of the majority of the total ovarian mass leads to more abnormal cycles and an earlier onset of anovulation (Meredith & Butcher, 1985; Nelson & Felicio, 1986). The present results show that no simple proportionality exists between ovarian mass removed and the reduction in cyclicity; removal of half the follicle population had a minor impact on the number and character of the cycles. While this finding further substantiates the conclusion that moderation of the initially high rate of follicle loss has
occurred, it is also predicted by the exponential character of the age distribution of stage I follicles. The CBA/Ca strain is characterized by early follicle loss (Jones & Krohn, 1961) and the present results appear to support the hypothesis that ovarian failure is the primary cause of lost fecundity (Krohn, 1962). The capacity to support pregnancy to term is lost at about 200 days of age in this strain irrespective of whether one or two ovaries are present; this is long before cyclical ovulation fails and is due to impaired uterine function and abnormal embryos (Biggers et al., 1962; Gosden, 1979; Brook et al., 1984; Eichenlaub-Ritter et al., 1988). The effects observed on the oestrous cycles are not confined to the strain in question because Thung (1961) has noted in a F1 hybrid mice, which have a much longer reproductive lifespan than CBA strain, that cycle frequency falls abruptly at 24 months in animals which were ovariectomized unilaterally at 13 months, and about 3 months later in controls.

Since the biology of preantral follicles is poorly known, explanations for changes after uni-ovx are bound to be conjectural. The high death rate amongst the smallest stages, normally found only in infantile mice (Faddy et al., 1987), appears to be paradoxical in view of the increased demands for recruitment. However, it should be pointed out that there are sufficient preantral growing follicles present to enable compensatory ovulation to be maintained for the normal course without additions from the primordial pool, neither does Hirshfield’s hypothesis (1982, 1983) require supernormal recruitment of these stages. Influences on small follicles could potentially be due either to changes in extrinsic neurohomonal feedback or to subtle intraovarian effects resulting from the changes in the numbers of large healthy and atretic follicles and corpora lutea.

While evidence for the influence of FSH on unilaminar follicles is scanty, there can be little doubt that the larger stages are responsive (Ryle, 1971).
Stages II and IV appear to be least affected by uni-ovx, but the increased rate of inflow into stage V+, which closely matches the requirement to double the ovulation rate, could be a result of hormonal stimulation of IV-V+. Alternatively, this parameter may reflect a larger number of follicles resulting from reduced atresia at stages V and VIa, comparable to Hirshfield's findings in the rat (1983). Thus, we reach the conclusion that while uni-ovx affects both small follicle dynamics and long-term fecundity, compensatory ovulation in the mouse can be explained by adjustments in the cohort of large preantral and antral follicles.
We thank the Medical Research Council for the award of a grant to R.G.G. and a scholarship D.J.B.

References


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Table 1. Average numbers of follicles entering and leaving successive stages of development in unilaterally ovariectomized animals at different ages.

These rates have been estimated from a compartmental model (Fig. 3). The columns have been broken horizontally at the transition between phases 1 and 2.

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Legends

Fig. 1. Variation in the numbers of follicles at different stages of growth (I-V+) in mice which were unilaterally ovariectomized at 6 weeks of age. Non-parametric regression lines (---) and the compartmental model (•••) have been fitted to the data (see Fig. 2). The third curve models the age changes in follicle numbers of normal animals.

Fig. 2. These parameters, derived from a compartmental model, estimate the growth (migration) and death rates (with standard errors) of follicles in unilaterally ovariectomized mice (see data and curves in Fig. 1).

Fig. 3. The numbers of morphologically healthy and atretic large follicles at three stages of development in intact and unilaterally ovariectomized mice. Upper panel: animals aged 42-66 days; lower panel: 67-100 days. Mean values +/- SEMs are given.

Fig. 4. The frequency of oestrous cycles in mice which were either sham-operated (open bars, n = 20) or unilaterally ovariectomized (solid bars, n = 18) at 6 weeks of age. In the upper panel all cycle lengths have been included, whereas in the lower one only cycles 4- or 5-days long are shown. Mean values +/- SEMs are given.
Phase 0-037
0-035
0-14
0
0
0-11
0-012
0-08
(constrained)

42-67 days

67-100 days
Fig. 3

Number of follicles

Follicle Stages

- Normal control
- Uni-ovx animals
- Atretic follicles
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Impact of exogenous progesterone on ovarian follicular dynamics and function in mice

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Department of Physiology, University Medical School, Edinburgh EH8 9AG;
*Department of Statistics, University of Birmingham B15 2TT.

Running headline: Progesterone and follicle dynamics

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The suppression of ovulation when conditions for conception are physiologically unfavourable, as occurs, for example, during pregnancy, established lactation and seasonal food/water shortages, is presumably advantageous since it is almost universally found in mammals. However, the causes of ovulation inhibition and its effects on follicular growth and death are not completely understood, despite extensive study.

Anovulation during pregnancy is generally thought to depend on diminished pulsatile secretion of gonadotrophic hormones, FSH & LH, and this has been demonstrated in humans and in some animals (Jeffcoate, 1986; Baird et al., 1984; Linkie & Niswender, 1972; Murr et al., 1974). Consequently, follicles are not expected to complete their maturation to Graafian stages (Richards, 1980). In some species, Graafian follicles are absent during most of gestation whereas in others they may form, but undergo degenerative changes rather than ovulate. There is little information about the impact, if any, of anovulation on the dynamics of preantral follicles. Since these follicles constitute a store which limits the maximum number of ovulations and the functional ovarian lifespan, the dynamics of the preantral stages should be clarified during pregnancy and in other states in which ovulation is inhibited.

The present study was designed to estimate the rates of follicle growth and death in mice given progesterone implants to induce a state of anovulation. This model was chosen because it was assumed to produce a relatively steady state condition which facilitates analysis, as opposed to the complex and changing physiological conditions of pregnancy and pseudopregnancy. The dynamics of preantral follicle growth and death have been investigated using differential follicle counts applied to an
established mathematical model (Faddy et al., 1976).

Materials and Methods

General procedures. Virgin female CBA/Ca mice were obtained from the laboratory colony. Their oestrous cycles were monitored by examining vaginal smears 6 days per week from 40-45 days of age until they were killed. Only those presenting 3 or more consecutive 4- or 5-day cycles at the beginning of the study were used.

Hormonal implants were made from 1 cm lengths of sterile Silastic tubing (Dow Corning, U.S.A.) with an internal diameter of 1.9 mm. They were packed with crystalline progesterone (Sigma, London) and sealed with silicone adhesive (Dow Corning, U.S.A.). The implants were pre-incubated overnight in sterile saline at 37 C before being inserted subcutaneously in the nape region during tribromoethanol anaesthesia (5mg/10g body weight). Since the effective life of the implants was about 35-40 days they were replaced every 30 days under brief anaesthesia.

The efficacy of treatment was tested in 24 animals which were divided randomly into three groups receiving at pro-oestrus either an empty implant or one or two hormonal implants. A blood specimen was obtained under anaesthesia by cardiac puncture after 16 days of treatment and the animals were killed by cervical dislocation before regaining consciousness. The ovaries and oviducts were dissected and examined microscopically (x25) for corpora lutea, follicle stigmata and ovulated oocytes. The progesterone concentrations in the sera were analysed by radioimmunoassay (Fraser, 1983). On the basis of preliminary studies one hormonal implant was shown to be sufficient to block ovulation and was used in subsequent experiments.
Follicle numbers and dynamics. Thirty-five animals were given either a control implant (n = 6) or a hormonal implant (n=29) at 55 days of age. Vaginal smears were obtained daily until autopsy which ranged from 5-91 days later and occurred on the morning of pro-oestrus in control animals. Another group of animals (n = 11), including 7 controls, were treated from the same ages with implants for up to 120 days.

Implants were examined at autopsy to confirm that crystalline hormone remained. The ovaries were removed, fixed in aqueous Bouin's fluid and prepared as 7 um serial paraffin sections stained with haematoxylin and eosin. Follicles with up to 4 granulosa cell layers were counted in every 10th section using the nucleolus of the oocyte as a marker; larger follicles were traced through every section. The total numbers of follicles per animal were estimated and assigned to stages of development (I-VI) using the classification of Mandl & Zuckerman (1951). Large follicles with an enlarged extracellular space were sub-classified according to whether the antrum was incipient (VIa) or had formed (VIb). Follicles at stages V and VI were considered "atretic" if they contained 3 or more pyknotic granulosa cells in the equatorial section.

The numbers of healthy and atretic follicles were compared in control and experimental animals at different ages. Since the data from animals with control implants and those from a detailed concurrent study of intact animals were similar (Faddy et al., 1988), the latter set of data and the model derived from it were used as the primary bases for comparisons. The dynamics of follicle growth and death in preantral follicles were estimated for the age range 55-98 days using a compartmental model (Faddy et al., 1976) and non-parametric regression analysis guided the fitting of the model (Faddy & Jones, 1987). The Kruskal-Wallis analysis of variance of ranks was used to compare numbers of large follicles in treated and control animals.
Results

Efficacy of hormonal implants. Normal vaginal smear patterns were abruptly halted in the hormonal implant group whereas animals in the control group continued to present normal oestrous cycles. In the former group, smears became persistently di-oestrous within two days of treatment and oviductal oocytes and hyperaemic corpora lutea were absent at post-mortem. The animals appeared to remain healthy throughout the study. Plasma progesterone concentrations were found to be 22+/-4 ng/ml (mean +/- SEM), these being significantly greater than controls, 10+/-3 ng/ml (P <0.001).

Follicle numbers and dynamics. In Figure 1 the raw data for 5 stages of follicular development, I-V+, are presented for animals from 55-91 days old. Stages V and VI were combined for modelling because fewer follicles were present. The numbers of follicles at stages II, III and IV were substantially (27-61 %) and significantly fewer (P <0.05) in the treated animals than in controls whereas the numbers at stages I and V+ were similar.

These data did not fit the model based on the follicle growth rates in normal mice by manipulating the death rates since constraining the model by this manoeuvre the sum of squares was significantly increased by 17.9 with 4 degrees of freedom and a high death rate was predicted at stages I-III, which was denied by morphological observations. Estimated growth and death rates from a new compartmental model devised for these data are shown in Fig. 2. The fit of this model to the raw data and a comparison with the non-parametric curve are demonstrated in Fig. 1 which also shows the curves derived from modelling data obtained from control CBA/Ca animals (Faddy et al., 1988). There was no evidence of age-dependency in the growth and death rates over this period. The curves obtained from the progesterone-treated group deviated significantly from the normal animals.
Similarities as well as differences were observed in the models for control and treatment groups. In both cases the death rates among small follicles were negligible, but the rate of growth from stage I was significantly greater in treated animals (0.016 cf. 0.0051, P <0.001). Additionally, the rates of movement between stages II-IV were appreciably greater in these animals (P <0.001), as indicated by the abrupt fall in numbers. The larger numbers of follicles predicted to be leaving and entering successive stages are shown in Table 1. No indication of the numbers moving into more advanced stages are given because the standard errors of the estimates are high, indicating that the parameter estimates are not precise.

Since follicle development in the anovulatory ovary is not suspended, growing follicles die at some stage. Most deaths occurred at larger stages, particularly stage IV, which checks an otherwise large inflow into the antral population and a consequent expansion of ovarian mass. The rate of recruitment into stage V+ is reduced by a factor of ten from an average of about 2 per day in normal animals.

The generally comparable numbers of stage V+ follicles as a whole obscures complex differences within this heterogeneous group. The model cannot provide precise estimates of the dynamics at this stage and so inferences have had to be drawn directly from the morphological evidence. Figure 3 shows the mean number of healthy and atretic follicles at stages V, VIa and VIb in three groups of animals aged 60-100 days. The normal controls refer to those animals from an earlier study (Faddy et al., 1988) and are shown here to demonstrate that the results for sham operated controls and the other control group are virtually identical. The mean number of large follicles found in each of the three stages were not significantly different in control and treated animals; however, the number of atretic follicles at each stage was significantly greater in the progesterone treated group (p<0.025).
The differences can be illustrated by the mean percentages of atretic follicles which clearly show an excess of atresia, which in some cases differed two-fold (Fig. 4). Differences between hormone-treated and control animals tended to be greater during the first 20 days than in the subsequent 100 days of treatment. The incidence of atresia rose in the controls whereas it remained at a more constant level of above 50% in the treated group.

Discussion

This study was based on the assumption that implants of crystalline progesterone maintain a steady anovulatory state, as has been known for many years (Robson, 1938). In this study the implants of progesterone were effective at blocking ovulation and producing circulating levels of progesterone comparable with those of early pseudopregnancy and pregnancy in mice (McCormack & Greenwald, 1974; Murr et al., 1974). The follicular physiology in the hormone-treated animals will differ, however, because of the absence of both active corpora lutea and gonadotrophic hormones from the placenta/decidua.

Few studies have examined the behaviour of preantral follicles under anovulatory conditions. This is surprising in view of the attention paid to other aspects of pregnancy physiology and general interest in the impact of steroid contraception on pathogenesis. Only one investigation of follicle dynamics in pregnant mice has been carried out and this employed the labelled mitosis technique (Pedersen & Peters, 1971). The results showed that follicles grew at similar rates to those in non-pregnant animals, but the rate of entry of follicles from the primordial pool to growing stages was reduced. To what extent the different results obtained by Pedersen & Peters compared with the present study can be attributed to physiological
differences remains conjectural.

The model obtained from the present study gives a holistic impression of the dynamics of follicles since it encompasses both follicle growth and death. The compartmental model shows that the growth and death rates at all stages of follicular development have been affected by progesterone treatment. A greater than normal rate of loss of primordial follicles was inferred in the treated mice, but this effect appears to be modulated with time.

The most striking finding of this study was the increased movement of preantral follicles from stage-to-stage during progesterone-treatment. The larger numbers of growing follicles did not all emerge as antral types but terminated development as large solid follicles. This interpretation by the model is evident from the abrupt fall in follicle numbers at the beginning of treatment. An alternative hypothesis to explain this fall, namely, that follicles were dying in large numbers at stage II is not supported by observations. The rate of initiation of follicle growth I-II doubled, but this would appear to be of limited duration since there was no evidence to suggest that treated animals were deficient in primordial follicles at 160 days of age. There is evidence in the rabbit that an accumulation of small follicles occurs during pregnancy and progesterone implant treatment (Setty & Mills, 1987) which, although implying different dynamics in this species, confirms that progesterone affects preantral follicles.

Studies of follicle dynamics in anovulatory mouse ovaries following hypophysectomy, in the hpg mutant or in immature animals have all found increased rates of preantral follicle growth (Faddy et al., 1976; 1983; 1988; Halpin et al., 1986). Whether a feedback relationship exists between large preovulatory follicles in cyclic mice or large moribund follicles in
anovulatory ovaries on the one hand and recruitment from the small follicle reserve on the other is a hypothesis worth testing. Since plasma FSH levels are lower in pregnant than non-pregnant animals it would appear unlikely that this is the effector for accelerated growth of small follicles. The existence of hypothetical feedback would ensure continuing replenishment of follicle recruits when physiological conditions favour the resumption of ovulation.

In contrast to the rapid movement of small follicles, the model predicts increased death of stage IV follicles. This paradoxical effect appears to counteract the increased growth of earlier stages. Although ovulation was blocked, follicles still proceeded to form an antrum, but the rate was considerably more sluggish. Clearly there is a problem when comparing numbers of large follicles in anovulatory animals and others having cyclic variation, but comparisons made for preantral follicles need have no regard to this factor (Numazawa & Kawashima, 1982).

All of these large follicles succumb to degenerative processes. The incidence of atresia as determined by morphological criteria is greater in the treatment group and is probably as a result of low concentrations of gonadotrophins. Persistence and accumulation of atretic follicles in pregnant and pseudopregnant rats (Osman, 1985; 1986) might explain the substantial increase in atresia noted at stage V+ in progesterone-treated mice.

In conclusion, this study has found effects of suppression of ovulation by progesterone on all stages of follicular development, including primordial. Further study is required to determine at which stages, if any, these effects are direct.
We thank Dr H.M. Fraser for kindly measuring serum progesterone and the Medical Research Council for a grant to R.G.G.

References


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Robson, J.M. (1938) Quantitative data on the inhibition of oestrus by testosterone, progesterone, and certain other compounds. J. Physiol. 92, 371-382.


Table 1. Average numbers of follicles leaving the compartments (stages) per day either by growth or by death in mice receiving progesterone implants, estimated from the compartmental model (Fig. 2).

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Fig. 2. This gives the estimated rates of follicle growth and death with standard errors in parentheses for each of the five successive stages in young adult mice treated with progesterone.

Fig. 3. Numbers of follicles at three stages of development in normal mice at pre-oestrus and in progesterone-treated, anovulatory animals. The follicles are classified on the basis of their histological appearance into healthy and atretic sub-groups. Vertical bars indicate the mean ± S.E.M.

Fig. 4. Variation in the percentages of follicles at three stages of development that were atretic in pre-oestrus cyclic mice and in anovulatory animals treated with progesterone implants. Each bar indicates the mean ± S.E.M.
Legends.

Fig. 1. A graphical illustration of the numbers of ovarian follicles in 27 mice which received an implant containing progesterone at 55 days old to inhibit ovulation (some data points are superimposed). The panels represent the numbers at successive stages of growth (I-V+), ranging from primordial to Graafian sizes. These data were modelled using non-parametric regression analysis (Silverman, 1985) and a compartmental model (see Fig. 2) (Faddy et al., 1988) and indicated by --- and ···, respectively. The continuous line is the predicted numbers in a large number of control animals for a wider range of ages (see Faddy et al., 1988).

Fig. 2. This gives the estimated rates of follicle growth and death with standard errors in parentheses for each of the five successive stages in young adult mice treated with progesterone.

Fig. 3. Numbers of follicles at three stages of development in normal mice at pro-oestrus and in progesterone-treated, anovulatory animals. The follicles are classified on the basis of their histological appearance into healthy and atretic sub-groups. Vertical bars indicate the mean +/- S.E.M.

Fig. 4. Variation in the percentages of follicles at three stages of development that were atretic in pro-oestrous cyclic mice and in anovulatory animals treated with progesterone implants. Each bar indicates the mean +/- S.E.M.
Fig. 1 cont.
Fig. 3

Key:
- □ = normal controls
- ◊ = sham controls
- ■ = progesterone implant group
- ☐ = atretic follicles

Mean percentage of atresia

Mean number of follicles

Follicle stage:
- V
- V1a
- V1b
Fig. 4

The character and significance of Call-Exner bodies in Graafian follicles of the rabbit.

STAGE V

Control

Progesterone treated

stage VIa

stage VIb

MEAN PERCENTAGE OF ATRESIA

DAYS IMPLANT

2 - 20  21 - 40  41 - 80  81 - 120
The character and significance of Call-Exner bodies in Graafian follicles of the rabbit

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Key words:
Call-Exner bodies, glycosaminoglycans, Graafian follicle granulosa cell, lectin, rabbit

Running headline: Call-Exner bodies
Summary. In a histological survey of 19 mammalian species, Call-Exner bodies of conventional size and appearance were found in only five, namely, human, rhesus monkey, rabbit, guinea pig and sheep. Rabbit ovaries were used for detailed characterization of these bodies using quantitative histochemistry, lectin binding and ultrastructural methods. Call-Exner bodies were topographically distinct lacunae of the extracellular space containing glycosaminoglycan/ proteoglycan complexes, staining being particularly intense with wheat germ agglutinin. Evidently, these macromolecular complexes become trapped in the Call-Exner bodies which possess a delimiting membrane which is similar to the suspended filaments yet distinct from the basement membrane of the follicle. While the staining characteristics of antrum and Call-Exner bodies were generally similar, the former did not possess either long filaments or membrane. The origin and significance of Call-Exner bodies requires further study, but it is clear that they are associated with secretion rather than necrosis.
In 1875 Call & Exner described new cellular structures ("Zellen") within the epithelial layer of the rabbit follicle. They tentatively equated them with newly-formed oocytes because of resemblance to primordial follicles. While the mistaken identity was soon apparent, the character of the so-called bodies of Call and Exner has been in dispute ever since. It has been suggested that the bodies are sites of liquefication following necrosis of granulosa cells (Thompson, 1919), a view which has been widely-held to the present day. On the basis of ultrastructural studies of mouse follicles, Hadek (1963) attributed them to secretory droplets forming in the Golgi apparatus of granulosa cells, although the small size of the droplets led Mossman & Duke (1973) to question whether they were the same structures. Honoré (1899-1900) suggested that the bodies are extracellular sites of accumulating secretory products. This view was endorsed by both Robinson (1918) and Motta (1965) who regarded them as dispersed parts of the follicular antrum.

In view of this confusion and the existence of only rudimentary histochemical information, the character of Call-Exner bodies has been investigated as part of more extensive studies of the extracellular environment of follicle cells. Since they are not universal in mammalian ovaries (Mossman & Duke, 1973; Motta, 1967) it was necessary to identify a suitable animal model before proceeding to histochemical and ultrastructural study.
Materials and Methods

The occurrence of Call-Exner bodies was investigated in 19 species using serial ovarian histological sections collected in an earlier study (Gosden & Telfer, 1987). The rabbit ovary was chosen for further study because Call-Exner bodies were always found in Graafian follicles, were readily identifiable and additional fresh tissues were available.

Ovaries from young oestrous does were fixed either by direct immersion in fixative following dissection and slicing into fragments or by perfusion during anaesthesia with ethyl carbamate (1.75 g/kg, i.v.). Tissues for light microscopy were either prepared in aqueous Bouin's fixative with subsequent sectioning at 5 μm in paraffin wax or they were fixed in formol-calcium fixative and sectioned at 12 μm in a cryostat. Specimens for electron microscopy were fixed in 1% glutaraldehyde for one hour followed by a further hour in 1% osmium tetroxide in 0.13 M Pipes buffer (pH 7.0). In some cases, the fixative contained 15% picric acid (aqueous, saturated solution). Dehydrated tissue was embedded in LR White resin (London Resin Company) and thin sections were cut with a Porter-Blum MT2 microtome using glass knives. Sections showing a silver-white interference colour were mounted on single hole type 1000 grids which were covered with Pioloform support film. Aqueous uranyl acetate followed by lead citrate were used for post-staining (Reynolds, 1963).

Wax sections were stained for the periodic acid-Schiff (PAS) reaction or, in order to demonstrate carboxylated and sulphated glycosaminoglycans (GAGs), with the cationic dye Alcian blue (1% w/v at pH 2.5 in acetic acid or pH 1.0 in hydrochloric acid). Dewaxed
sections were set aside for quantitative histochemistry. They were preincubated with either ovine hyaluronidase (1 mg/ml, Sigma, London) or chondroitinase ABC (1.7 U/ml, Sigma) in a humidified chamber for 3 h at 37°C (Delgado & Zoller, 1987) and mounted for microscopy. Optical densities of the Call-Exner bodies stained with Alcian blue at pH 2.5 were measured at 610 nm using a Vickers M86 scanning and integrating microdensitometer. Oil immersion optics were used with a total magnification of x500 and a mask size (A6) slightly larger than the maximum diameter of the Call-Exner bodies (approximately 30 μm).

Carbohydrates in the Call-Exner bodies were characterized further using lectins labelled with tetramethyl-rhodamine-isothiocyanate (TRITC) which were either conjugated in the laboratory or prepared commercially (Sigma, London). Dewaxed paraffin sections and cryostat sections were used to investigate the binding of seven lectins (see Table 1), with working concentrations ranging from 0.1-1.5 mg/ml of PBS. Sections were incubated in the lectins for 1 h at 37°C, either with or without an inhibitory sugar present (0.2 M) (Lis & Sharon, 1986). They were washed in PBS and mounted in PBS-glycerol (1:1, v:v) containing 0.1 μg/ml of a DNA binding fluorochrome (Hoechst 33342, Aldrich Chemical Co., Gillingham, Dorset). Specimens were examined by episcopic fluorescence using a Nikon Labophot microscope with differential excitation and barrier filters for the two stains (filter types G and UV, respectively).

Results

Call-Exner bodies of conventional size and appearance were clearly recognisable in Graafian follicles of human, rhesus monkey, rabbit,
guinea pig and sheep ovaries. They were not visible by light microscopy in the other species examined.

The Call-Exner bodies of rabbits varied in size from the limits of resolution of the light microscope (0.2μm) to about 30 μm diameter. Several bodies were usually recognisable in a single follicle section at low magnification (x100) but they could not be counted reliably because of difficulty identifying the smallest examples. They were represented in all Graafian follicles and in the largest multilaminar preantral types. The bodies persisted during early stages of atresia, but were absent in corpora lutea of pregnant animals.

Each Call-Exner body was approximately spherical and enclosed by a rosette of slightly columnar granulosa cells. They were located in both mural and stalk regions of the granulosa cell population and miniature bodies were found in the cumulus oophorus using electron microscopy. They were eosinophilic and presented a reticulated appearance with a peripheral membrane when stained with PAS or Alcian blue (Fig. 1). The ultrastructural appearance of the membrane was reminiscent of the follicular basement membrane, although the former was considerably thicker (30-50 nm with normal fixation, 45-75 nm when picric acid was added) (Figs. 7-10). Filamentous material was frequently present; it varied in quantity from zero (Fig. 9) to an extensively folded reticulum (Fig. 7). The membrane was always present and was the most reliable feature for identifying Call-Exner bodies. Globular electron-dense matter was associated with the filaments and membrane when treated with picric acid (Fig. 9, 10). These filaments and membrane, which were similar in appearance, were absent from the antrum and other interstitial spaces of the follicle (Fig. 7). Apart from the membrane, there was no physical barrier
between the various extracellular spaces since, except at occasional points of contact, granulosa cells were separated from one another by continuous intercellular clefts. Call-Exner bodies did not appear to be sites of granulosa cell necrosis: neither cellular organelles nor DNA fluorescence were seen (Fig. 4). Apart from being slightly columnar in shape, the cells organized radially around Call-Exner bodies were indistinguishable from other granulosa cells, and there was no obvious polarity of cytoplasmic organelles or lectin binding. Microvilli were absent, although larger processes were frequently present beneath the membrane (Fig. 9).

**Discussion**

Despite widespread affinity of plant lectins for ovarian cells some differential staining was observed. While small preantral follicles had little affinity for any of the seven lectins, the surface epithelium and cortex of the ovary stained with them all, sometimes intensely (Table 1). Call-Exner bodies were stained weakly or moderately (e.g. concavalin A) by a number of lectins, but avidly bound wheat germ agglutinin (WGA). This lectin also stained the antrum, granulosa cells of large follicles, basement membrane and the zona pellucida above all (Figs. 2, 3, 5). Its fluorescence was inhibited specifically by N-acetyl glucosamine (Table 1). The fluorescence in frozen tissue sections was less than in paraffin sections suggesting that formalin had precipitated and stabilized less of the GAGs; on the other hand, nuclear fluorescence from H33342 staining was partially quenched in wax sections fixed with Bouin's fluid.

The results obtained with WGA were comparable to those with Alcian blue. Hyaluronidase digestion substantially and significantly reduced the staining by Alcian blue; chondroitinase had a significant, if
less marked, effect (Table 2). While the cumulative reduction in optical density was not tested, the sum of the independent effects of the two enzymes amounted to about 50%. Satisfactory measurements of the antrum could not be made because the space was not spherical and stained material was unevenly distributed. Nevertheless, it was apparent that staining intensity in the antrum diminished in the largest follicles whereas that in the Call-Exner bodies did not vary with follicle size.

Discussion

There can be little doubt, if any, that the Call-Exner bodies of rabbit follicles are part of the extracellular space. Present findings concur with Honore (1899-1900), Robinson (1918) and Motta (1965) who concluded that the bodies are extensions of, and probably contiguous with, fluid in the antrum. The bodies appear to be sites at which locally secreted macromolecules are accumulating; they are not zones of granulosa cell necrosis.

Since the bodies are PAS positive it has been assumed that they contain "glycoprotein", although this is the extent of earlier histochemical studies (McKay et al., 1961). Preliminary characterization of the carbohydrate moieties of the proteoglycan/GAG complexes in Call-Exner bodies and antrum has been achieved using enzyme digestion combined with quantitative histochemistry and lectin binding. The results are consistent with other studies of rabbits and mice in showing that hyaluronic acid and chondroitin sulphate are major constituents (Odeblad, 1954; Zachariae, 1957; Tadano & Yamada, 1978; Fowler & Guttridge, 1987). Quantitative biochemical studies of
several species have indicated that chondroitin sulphate is the major GAG secreted by granulosa cells into the antrum, whereas hyaluronic acid predominated in the cumulus oophorus (Ax et al., 1985). While present results appear to imply abundance of hyaluronic acid in rabbit follicular fluid it would be hazardous to infer predominance because labelling by different lectins and the binding of Alcian blue to different GAGs is not equimolar. Moreover, the substantial residue of Alcian blue staining after treatment with hyaluronidase and chondroitinase points to significant quantities of other GAGs existing.

There are several possible reasons why the biology of Call-Exner bodies has been overlooked in recent years. The first is connected with the absence of the bodies in myomorph rodents: rabbits replaced rats and mice as the major laboratory models for experimental reproductive physiology many years ago. A second reason for neglect is the practical difficulty of isolating the small, scattered droplets for biochemical analysis. Histochemistry overcomes this problem to some extent, but experimental models are needed to investigate the formation of Call-Exner bodies from the interstitial space. In vitro methods are attractive and there are some signs of progress. Harrison (1961) reported that cultured rabbit granulosa cells form hollow vesicles which vary in size with gonadotrophic hormone stimulation. Furthermore, Torrance & Gosden (unpublished) have observed GAGs within unilaminar vesicles formed by ovine granulosa cells in collagen gel cultures. Further attention on Call-Exner bodies may reveal important new facts about the secretory activities of granulosa cells and the control of cellular adhesiveness. For the present, however, Call-Exner bodies continue to be of interest to histopathologists as aids for diagnosing human
granulosa cell tumours (Novak & Woodruff, 1967).

While the character of the Call-Exner bodies is now clearer, many questions remain. There is even a question whether it is justifiable any longer to use the term bodies because these structures are part of the continuous extracellular space and a discrete function has yet to be demonstrated. On semantic grounds, it is perhaps more accurate to call them Call-Exner spaces.

The differences between the macromolecular assemblages in the Call-Exner spaces compared with other parts of the extracellular space are surprising. Perhaps they arise from differences in microenvironment associated with the laying down of a membrane. Motta (1965) suggested that the membrane had formed by condensation but it has been difficult to find early stages in the formation of the lacunae in order to see whether suspended filaments appear before the membrane. Even without the membrane it is likely that such large molecules would be trapped by the intercellular clefts. The limited expansion of the Call-Exner spaces as opposed to the continued growth of the protoantral cavities is also a matter requiring further research. The mechanisms underlying production of follicular fluid are poorly understood and most attention has been given to putative physico-chemical gradients affecting the rate of transport and the location of accumulated follicular water (Gosden et al., 1988). It is questionable whether such gradients are able to provide a satisfactory explanation for the distinctive morphogeneses of the sub-compartments of the extracellular space. For this reason, attention should perhaps turn to other potential factors, such as the adhesiveness of cells and the possible modulating role of the proteoglycans.
We thank Adrian Sumner for providing access to a microdensitometer and Bill Rowand for help with the preparation of labelled lectins. This work was partly financed by grants from the Wellcome Trust.

References


Legends to Figures

Plate 1

Fig. 1. Rabbit Graafian follicle stained with Alcian blue to demonstrate glycosaminoglycans in the antrum and Call-Exner spaces (arrows). Nuclei have been counterstained with neutral red. (x 320).

Fig. 2. Small antral and multilaminar preantral follicles stained with TRITC-labelled wheat germ agglutinin. While background fluorescence is moderately high, the staining of the antrum, Call-Exner bodies (arrowed), basement membrane and zona pellucida of larger follicles is intense. (x168).

Fig. 3. Two Call-Exner spaces are visible in this formalin-fixed frozen section which was stained with TRITC-labelled wheat germ agglutinin. The extracellular material and granulosa cells throughout the follicle show affinity for the lectin. (x 670)

Fig. 4. The same specimen as in Fig. 3 is visualized by staining with a DNA fluorochrome (Hoechst 33342). The Call-Exner spaces are unstained, implying that they are not sites of cellular necrosis. (x 670)

Fig. 5. Call-Exner bodies prepared with Bouin's fixative and paraffin wax stain more extensively with wheat germ agglutinin than formalin-fixed frozen sections (cf. Fig. 3). (x 670)
Fig. 6. The affinity of Call-Exner bodies (C) and antrum (A) for TRITC-labelled concanavalin A shown here is less than that for wheat germ agglutinin. (x670)

Plate 2.

Fig. 7. A Call-Exner body of a rabbit Graafian follicle. This approximately spherical body contains a reticulum of filamentous material bounded by a membrane. The filaments and membrane are absent from the antrum and other interstitial spaces. Glutaraldehyde & osmium (x7000)

Fig. 8. The specimen in Fig. 7 is viewed at higher magnification to show details of the filamentous material which appears to consist of densely branching macromolecules. The suspended filaments and membrane have a similar appearance. (x40000)

Fig. 9. Smaller Call-Exner bodies often possess fewer suspended filaments, but a membrane is always present and can be considered as diagnostic of all such bodies. Long processes are frequently present (arrow). This specimen, which was fixed in the presence of picric acid, shows electron dense granules (probably proteoglycans) associated with the membrane. Glutaraldehyde & osmium (x20000)

Fig. 10. This specimen, which was prepared by the same method as in Fig. 9, demonstrates granules (12-20 nm diameter) at higher magnification. They are evenly distributed on the filaments and on the inner border of the membrane (arrow). (x40000)
<table>
<thead>
<tr>
<th>Lectin</th>
<th>Inhibitory Monosaccharide(s)</th>
<th>Call-Exner Body Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine max (soybean)</td>
<td>D-galactose, N-acetyl-D-galactosamine</td>
<td>Call-Exner body antigen</td>
</tr>
<tr>
<td>Vicia ervilagine (gorse)</td>
<td>L-fucose, D-glucose, sucrose, D-mannose, N-acetyl-D-galactosamine</td>
<td>Call-Exner body antigen</td>
</tr>
<tr>
<td>Phaseolus vulgaris (Wheat germ)</td>
<td>N-acetyl-D-glucosamine</td>
<td>Call-Exner body antigen</td>
</tr>
<tr>
<td>Canavalia ensiformis (Concanavalin A)</td>
<td>N-acetyl-D-glucosamine</td>
<td>Call-Exner body antigen</td>
</tr>
<tr>
<td>Phaseolus vulgaris (red kidney bean)</td>
<td>N-acetyl-D-glucosamine</td>
<td>Call-Exner body antigen</td>
</tr>
</tbody>
</table>

Table 1: Relative binding of TRITC-labeled lectins to carbohydrates in the Call-Exner bodies and antral spaces of Graafian follicles in the rabbit ovary.
Table 2

Measurements of Alcian blue staining intensity in C71-Exner bodies of rabbit Graafian follicles treated with hyaluronidase or chondroitinase.

<table>
<thead>
<tr>
<th>Group</th>
<th>Optical density (arbitrary units)</th>
<th>% Reduction in optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.5</td>
<td></td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>95.8 ± 5.0 (31)</td>
<td>71.6 ± 4.2 (31)</td>
</tr>
<tr>
<td>Chondroitinase</td>
<td>37.6</td>
<td>114.7 ± 4.2 (40)</td>
</tr>
</tbody>
</table>

Both enzyme-treated groups compared with controls (p < 0.001, Student's t test).

The density was significantly reduced in enzyme-treated groups compared with controls (p < 0.001, Student's t test).
Biology of Menopause
The Causes and Consequences of Ovarian Ageing

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Foreword: D. T. Baird

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Foreword

The impressive expansion of life expectancy which has occurred this century in most developed countries has been accompanied by a large increase in the number of elderly women. Although the average woman will spend over one-third of her life after the menopause, we know very little about the factors which determine the onset of ovarian failure. Ignorance in this area reflects our lack of knowledge of the process of growth, development and degeneration of follicles. In this monograph, Roger Gosden has placed in biological context the events surrounding the continued ageing of the reproductive system, including the ovaries, of which the last menstrual period is an obvious landmark.

The oocytes are amongst the longest surviving cells in the body. A finite number of oocytes are formed in fetal life, and when they are exhausted ovarian failure occurs. With this prolonged period of storage it is hardly surprising, therefore, that the incidence of congenital abnormalities due to chromosome abnormalities rises with maternal age. Cumulative exposure to radiation and other potential mutagenic agents increases the risk of errors in meiosis occurring. The normal functioning of the ovary as an endocrine gland is totally dependent on an adequate supply of healthy gametes. Hence, as the ovary ages a variety of abnormalities in its function, as indicated by a disturbance in the pattern of menstruation, become common. The experimental models in laboratory rodents which are available for the study of these observations are discussed with authority by Roger Gosden, who has made a life study of ovarian failure.

The social significance of the menopause varies greatly from one culture to the next. In Hindu India and certain parts of northern Nigeria the appearance of the first grandchild marks the end of the second "ashrama", or period of procreation, after which sexual activity should cease. The menopausal grandmother acquires a different, more prestigious, place in society and may be permitted to take a more active part in decision making. In Western society the menopause usually coincides with marked changes in personal circumstances, e.g. children leaving home, and is regarded by many as a depressing reinforcement of the ageing process. The decline in production of ovarian steroids, including oestradiol, results in short-term symptoms and long-term changes in the re-
productive and skeletal system. It is against the biological consequences of ageing that the risks and benefits of oestrogen replacement therapy must be viewed. This book is a timely summary of our present knowledge in this important area.

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Preface

There should be good reasons for adding to the current flood of scientific literature, especially since the task of writing a monograph is a long and lonely one. This book was written for research workers, clinicians and students who have a special interest in the biology of the ageing reproductive system, of which the human menopause is a central issue. It deals with the causes of ovarian failure in mid-life, the associated physiological and behavioural changes and the preceding decline in fecundity and fertility. Despite widespread interest in this subject, no one until now has attempted to tackle the subject as a whole. A number of books have been written for the non-scientific reader who is concerned about "menopausal problems", but the professional scientist and clinician are faced with a widely dispersed literature. I felt that there was still time for an author with sufficient temerity to bring together the many aspects of reproductive system ageing and show the extent to which these are interrelated.

This book is primarily concerned with human biology, but in some sections where direct evidence is lacking there are detailed accounts of animal research, and often this is interesting in its own right. By way of introduction to some of the chapters, I have outlined historical concepts leading to our present knowledge of the physiology and biochemistry of reproduction. This is intended to help readers who are not well acquainted with the subject to appreciate the less tractable problems of ageing. However, to maintain balance and economy, it has been necessary to be highly selective. Originally, I planned to shun all practical issues in reproductive medicine, but, as the book evolved, the provinces of the biologist and clinician seemed less distinct, and some mention of contraception and hormone replacement therapy became desirable. Nevertheless, these topics have been tackled primarily from the scientific standpoint, and I leave questions of clinical management of subfertility and postmenopause to others having appropriate experience and expertise. Discussion of the psychological changes of middle age, apart from those of a sexual nature or connected with ovarian ageing, are beyond the scope of this book, and as the title plainly indicates my subject is strictly the female of the species. Males are not exempt from ageing of their sexual functions, but a term other than "menopause" is needed for their less discrete changes. A good deal more research needs to be done before as detailed a story can be written about them.
Had I not received so much encouragement and support I might never have begun this volume, never mind completed it. My thanks are due first to Bob Edwards for launching me into a research field which has held my interest over the years and now forms the basis of this work. Helpful advice and criticism during the evolution of the final manuscript were given by several colleagues, namely, David Baird, Terry Baker, Tony Bramley, Ann Chandley, Tuck Finch, Don McLaren, Evelyn Telfer; and I appreciate guidance given by the editorial staff at Academic Press Inc. (London) Ltd. at all stages of the project. Nevertheless, I accept full responsibility for the more speculative hypotheses which are proposed or endorsed here. It is a pleasure to record unstinting support and service from librarians in the Erskine Medical Library, the Centre for Reproductive Biology and elsewhere in the university. Authors and publishers have kindly granted permission to reprint data; their contributions are specifically acknowledged in the accompanying tables and legends. Much of the illustrated material was prepared with the help of the Medical Illustration Unit of Edinburgh University, with many figures being drawn by the medical artist, Ian Lennox. Finally, I thank my wife, Carole, for patience and encouragement throughout the project and for important contributions at every stage: searching the literature, providing literary criticism and proofreading; and if there is any humanity in the way I have presented the science, it is mainly due to her influence.

April 1984

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Distribution of Menopause

1.1. Introduction

The reproductive system changes in appearance and function more obviously than any other system during maturation and ageing. Indeed, the human female lifespan can be divided into three phases according to menstrual activity, the first and last menses (menarche and menopause) marking the transitions. These two milestones of life are merely expressions of underlying continuous changes; they signify the attainment of hormonal thresholds determining the initiation and maintenance of ovarian cycles. These changes affect aspects of reproduction besides menstruation and may be expressed as variations in fertility during menstrual life.

The pattern of rising and waning reproductive potential is not peculiar to our species, but can be seen in other animals in conditions of captivity or domestication. It is, however, a universal pattern in vertebrates, amongst which some remarkable life cycles have emerged. For example, the bluehead wrasse (Thalassoma bifasciatum) changes sex during ageing, and both sexes of marsupial mice (Antechinus sp.) have a single burst of intensive reproductive activity which is quickly followed by sterility, senescence and death (Diamond, 1982).

According to the evolutionary paradigm, every life cycle, no matter how bizarre, must confer special advantages for reproduction of the species. Can, then, the menopause and the long postcyclic phase of life in man be of adaptive value? This is doubtful. Of course, the crucial facts of hominid evolution are lacking, but we can be fairly sure that early ovarian deterioration was not selected since evolutionary fitness can be maximized only among young, reproductively active individuals. Although postreproductive individuals no longer contribute directly to the gene pool of the next generation, it has been suggested that some benefits may accrue indirectly; they can expend energy in caring for offspring of their kin, thus increasing the survivorship of their line (Mayer, 1982). Quite apart from the difficulties of obtaining credible evidence for this hypothesis, it would appear that any benefits are far outweighed by the costs of menopause, namely, the increased morbidity and mortality of the offspring and mother during the declining years of fertile life. There are several indications that wom-
en are not physiologically well adapted to the postmenopause, further countering the argument that it has an evolutionary advantage.

Human menopause is probably a novelty, although its potential has existed for a long time because germ cells are continuously depleted from a non-renewable store. The potential for ovarian failure also exists in animals today. According to this viewpoint, menopause is an artefact of human civilization which emerged when our growing mastery of the environment increased our survivorship.

Our understanding of the origins of life patterns is bound to colour our attitudes to the menopause. It is, however, much more hazardous to draw firm conclusions in this area than on the surer ground of the physiology and biochemistry of menopause which form the basis of this book.

1.2. Terminology

The term “menopause”, which was introduced in the nineteenth century, means the time when menstrual cyclicity ceases irreversibly. It is popularly used to describe a vague period near the close of menstrual life, but in scientific and medical parlance it simply designates the last menses. This is usually, though not universally, identified retrospectively after 12 months of amenorrhoea. Often it is tacitly assumed that the underlying cause of menopause is primary and permanent ovarian failure. This distinguishes it from the loss of menses after hysterectomy, in which occult ovarian cycles may continue, and from seasonal ovarian involution in some animals, which is reversible. Postmenopausal vaginal bleeding is, of course, a continuing possibility, but extraovarian factors are usually responsible.

Wilbush (1979) states that it was de Gardanne (1816) who coined the term “la ménopause” to replace the more cumbersome description “cessation des menstrues”. This was shortened in the second edition of his book to “ménopause”. This term was soon absorbed into the English language because of its precision in expressing the essentially feminine nature of the condition. The tendency to use this word to describe gonadal atrophy in ageing men ought to be resisted.

Various prefixes have been added to describe the phases surrounding or involving menopause, but there are no generally agreed-on definitions of them. In this book the definitions recommended by the World Health Organization (1981) have been adopted. They are illustrated in Fig. 1.1 and defined as follows.

**Menopause**: Permanent cessation of menstruation resulting from loss of ovarian follicular activity.

**Perimenopause**: This term is used synonymously with the word “climacteric” (L. klimakter: rung of a ladder). It indicates a variable period spanning a
few years on either side of the menopause when characteristic symptoms and signs of ovarian involution are present.

*Postmenopause:* The entire span of life following the last menses.

*Premenopause:* This is an unsatisfactory term because it can imply any period preceding the menopause, although many authors restrict its meaning to a short but poorly defined period before the climacteric.

### 1.3. Historical Recognition

The statistical probability of surviving to the age of menopause was relatively low until spectacular developments occurred in public health and medicine earlier this century. However, sufficient numbers of women have survived to this age for writers even in the distant past to remark about the phenomenon of menopause. When the author of Genesis stated that "it ceased to be with Sarah after the manner of women", he indicated the familiarity of this natural event. Many other documents from ancient Greece and Rome and from medieval Europe confirm this knowledge, although we cannot rely on them for accurate information on the exact age of menopause in those times (Amundsen and Diers, 1970, 1973; Post, 1971).

In the past, the loss of menstrual activity was widely believed to be directly responsible for the physical and psychological distress of the climacteric. The catamenial flow was thought to be a route for eliminating "peccant matter and morbid humour, sometimes acrimonious and malignant, whose retention never fails to be extremely injurious . . . to the constitution" (Fothergill, 1776). This
concept of the role of menstruation was responsible for the stigma of impurity and the rituals attached to this stage of the cycle in some societies. Treatment of symptoms was carried out by "sage femmes" and "granny women" during recent centuries in Western Europe and was designed to counteract the retention of menses (menochezis) and thereby eliminate the accumulated "poisons". Such treatments involved traditional enemmagoues, but if these were unsuccessful, the excretion of retained "poisons" was promoted at alternative sites: through the skin and/or gut by purgatives, enemata, sinapisms, moxas, cupping or leeches or, finally, by the methods of the barber surgeons—phlebotomies or various "issues" (Wilbush, 1979).

These procedures were practised regularly as a means of increasing the attractiveness of women of high status. However, according to exhaustive studies of the historical literature by Wilbush (1979), menopausal symptoms or complaints were not recorded until the time of social upheavals in post-Revolutionary France. This period was remarkable for the growth of medical and paramedical French literature dealing with women, and especially with climacteric symptoms. Wilbush suggested that these symptoms are modified by social and historical conditions, a conclusion that is endorsed by recent cross-cultural surveys described in the next section.

During the nineteenth century, the time-honoured notions of the role of menstruation and the significance of menopause underwent revolutionary changes as a result of increasing scientific attention. The discovery of the effects of electricity on the body by Galvani (1737–1798) and others led to the conclusion that a wide variety of disorders might have an electrical basis. Georg Prochaska (1749–1820), a Viennese physiologist, proposed a neural mechanism for controlling the gonads, and thus, the effects of castration and the symptoms of the menopause were thought to be nervous reflexes or "ganglionic" disorders (e.g. Tilt, 1857).

Although the significance of autonomic factors in climacteric symptoms is accepted, the early views have been modified by the major endocrinological paradigm of our time. The evidence proving that the ovary is an organ of internal secretion was collated in an important work published by F. H. A. Marshall (1910). In subsequent decades the major task of isolating and chemically identifying the ovarian steroids was carried out, being substantially completed by the mid-1930s. This knowledge provided the basis for a new evaluation of the significance of menopause and its biological consequences.

Public interest in the menopause has increased in recent times because it has become popular for women to consult medical practitioners about climacteric symptoms. The profession can now offer an effective hormonal remedy, albeit a controversial one (Chapter 7). Interest has been heightened by a remarkable change in the age structure of developed countries during the past century. This change is illustrated by census data and projections for Scotland (Fig. 1.2), and similar trends are found elsewhere. The number of women of postmenopausal
age (i.e. age >49 years) has increased threefold since 1871, and as a fraction of the total population they have doubled to reach one-third. The reasons for this demographic shift are complex, involving interactions between fertility, age-specific mortality and migration. The present pattern is mainly attributed to high fertility in the past and a significant improvement of survival rates (Gray, 1976). In Scotland, life expectancy at birth increased from 43.5 to 74.4 years between 1871 and 1977 (i.e. 71%), whereas that at age 50 rose from 21 to 27.5 years (31%). Therefore, increased survival of children to middle age has had a greater impact on the size of the postmenopausal population than lower mortality rates in old age. Demographic forecasts are usually hazardous, but it is widely assumed that the size of the postmenopausal population will be stable during the next 2 decades unless there are radical and unexpected changes in mortality rates amongst the elderly. Consequently, the special problems of biological ageing, including menopause, will continue to be major forces in defining health care priorities into the next century.

1.4. Symptomatology

It is necessary to make some comment about the symptoms (i.e. subjective aspects) of the climacteric even though their biological significance is clouded by
associated social and psychological factors of middle age. In some societies, particularly in the West, where youth and beauty are emphasized, the menopause can be a social stigma, and it often coincides with domestic or professional crises in the family. In other societies (e.g. in Rajasthan, amongst some Indian women of high caste) the menopause is associated with an increase in social standing which approaches, but does not quite reach, that of men (van Keep and Humphrey, 1976; Ware, 1979). It is not, therefore, surprising that attitudes towards menopause and the frequency with which symptoms are reported vary in different cultural settings. Some general patterns have emerged from the many surveys of climacteric symptoms, and they can be illustrated by comparative studies of ethnic groups within the same country.

In an Israeli study of five distinct ethnic groups (Jews of central European, Persian, Turkish and North African origin, and one Arab group), substantially different attitudes to menopause were found even though the range of somatic complaints was similar (Maoz et al., 1977). Hot flushes (flashes) and sweating were the predominant symptoms. Persian women experienced the greatest degree of discomfort, and Europeans suffered the least. Arab women were the most positive in their attitudes to menopause and Persians the least positive. Although higher income and education are associated with fewer or less intense symptoms during climacteric years (Jaszmann et al., 1969a; van Keep and Humphrey, 1976), the explanation of the Israeli data is undoubtedly more complex than this. The authors pointed out that there is no simple dichotomy of symptoms according to social background (traditional versus modern) and emphasize the possible effects of the relationship with the spouse and his attitudes. In another cross-cultural study, significant differences were found between Cuban and Jewish women in the United States (Flint and Garcia, 1979). Cubans expressed more negative attitudes to menopause and, perhaps as a consequence, experienced more symptoms than Jews. The following factors may be important in determining individual and ethnic differences in climacteric symptoms: (1) the social significance of menstruation and the escape from the stigma of menstruation that follows menopause in some cultures, (2) the social significance of childlessness, (3) the social status of postmenopausal women, (4) attitudes of husbands towards their postmenopausal wives, (5) the level of socio-economic deprivation experienced at the time, (6) the degree of change in the role of women at this time and the availability of new or alternative roles and (7) the availability of medical help for perimenopausal problems (World Health Organization, 1981).

A large number of symptoms are associated with the climacteric, and they are similar in character whether menopause is spontaneous or induced. Several observers have reported that they are of greater intensity when menopause is precocious or follows ovariectomy (oophorectomy) (e.g. Chakravarti et al., 1977). It is difficult to distinguish symptoms specific to menopause from those that are due to other ageing processes or social factors. This distinction is, of course,
imperative for those involved in management of climacteric problems. Symptoms can be divided into two groups according to whether they involve genital tissue. Some of them have an obvious, direct causal relationship to the altered endocrine environment (e.g. irregular menstruation, dyspareunia), whereas others are related indirectly and have an autonomic basis (e.g. vasomotor disorders, perspiration, palpitations). Some psychogenic disturbances may be secondary to these symptoms (e.g. insomnia, loss of sex drive), but the aetiology of others is less obvious and probably more complex (headaches, irritability, depression, dizziness). There are, in addition, other consequences of menopause which are not immediately apparent but develop insidiously during the postmenopausal years (osteoporosis, atheromatosis).

Since most though not all women experience climacteric symptoms (80–85%) (Medical Women's Federation, 1933) and since similar symptoms are reported in cross-cultural studies, some of them evidently have a biological basis. The most common symptom is the hot flush, which is associated with another autonomic disorder, namely, inappropriate sweating (Thompson et al., 1973). In a study of women attending a general practice in Scotland, 48% reported flushing episodes close to the time of menopause; the peak incidence occurred during the first 2 postmenopausal years (Table 1.1). Some 11% of women began flushing shortly before menopause. A similar pattern of results was obtained from a Dutch population, though the incidence of symptoms was somewhat higher (Jaszmann et al., 1969a). Flushing has been claimed to be a specific symptom of the

<table>
<thead>
<tr>
<th>TABLE 1.1</th>
<th>Vasomotor Instability (Flushing Experience) of Women in Aberdeen*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flushing</td>
</tr>
<tr>
<td></td>
<td>Current</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>11</td>
</tr>
<tr>
<td>Menopausal</td>
<td>6</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>37</td>
</tr>
<tr>
<td>Years since menopause</td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>11</td>
</tr>
<tr>
<td>2–3</td>
<td>7</td>
</tr>
<tr>
<td>3–4</td>
<td>4</td>
</tr>
<tr>
<td>4–6</td>
<td>8</td>
</tr>
<tr>
<td>7–9</td>
<td>5</td>
</tr>
<tr>
<td>≥10</td>
<td>1</td>
</tr>
<tr>
<td>Not stated</td>
<td>1</td>
</tr>
<tr>
<td>Total number of women</td>
<td>54</td>
</tr>
</tbody>
</table>

* From Thompson et al. (1973).
menopause because it is observed so consistently. Certainly it is highly responsive to exogenous oestrogens. On the other hand, several of the 11 climacteric symptoms listed in Kupperman’s Index (Kupperman et al., 1959) have been found to respond in some studies (Hagen et al., 1982a), but some benefits are probably indirect. The relationship between endogenous oestrogens and the occurrence of these symptoms is not as clear-cut as hormone replacement studies would suggest because levels of oestrone and oestradiol are not necessarily lower in symptomatic women compared with asymptomatic women (p. 127). The biological bases of the major symptoms of the climacteric and postmenopause are described in Chapter 6.

1.5. Age at Natural Menopause

Until relatively recently few surveys of the normal age at menopause had been rigorously designed and analysed. Much of the early information was anecdotal or obtained by recall in small samples. The first large-scale study was carried out in the United Kingdom by the Medical Women’s Federation in 1933.

There are many pitfalls when designing surveys for this purpose, and they are reviewed admirably by Gray (1976). Most data show that the age distribution for natural menopause is broad and negatively skewed (i.e. a wider scatter of women at lower menopausal ages) (Fig. 1.3). The asymmetrical distribution results in mean values which are sometimes as much as 2 or 3 years less than the medians (Table 1.2). Most authors now choose medians to express their results since this parameter (the 50th centile) is a more meaningful indication of the central tendency of a population. The median is generally calculated by estimating the percentage of postmenopausal women at each age and transforming the percentages into probits or logits to make the distribution more symmetrical. The median is then calculated from the probit or logit mean (Finney, 1971).

No refinements of analytic technique can compensate for poor data. Surveys have often been based on selected populations (e.g. through a hospital or general

Fig. 1.3. Frequency distribution of age at menopause among American white women (n = 393). (From Treloar, 1981; reprinted with permission.)
<table>
<thead>
<tr>
<th>Country and year of study</th>
<th>Race</th>
<th>Age at menopause (years)</th>
<th>Study design</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Median</td>
<td></td>
</tr>
<tr>
<td>Australia, 1978</td>
<td>Caucasian</td>
<td>50.4</td>
<td></td>
<td>Walsh (1978)</td>
</tr>
<tr>
<td>Czechoslovakia, 1967</td>
<td>Caucasian</td>
<td>49.03</td>
<td></td>
<td>Magursky et al. (1975)</td>
</tr>
<tr>
<td>England, 1951–1961</td>
<td>Caucasian</td>
<td>47.49</td>
<td></td>
<td>Frommer (1964)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>49.82</td>
<td></td>
<td>McKinlay et al. (1972)</td>
</tr>
<tr>
<td>Finland, 1961</td>
<td>Caucasian</td>
<td>49.8</td>
<td></td>
<td>Hauser et al. (1961)</td>
</tr>
<tr>
<td>Germany (FDR), 1972</td>
<td>Caucasian</td>
<td>49.06</td>
<td></td>
<td>Hofmann and Soergel (1972)</td>
</tr>
<tr>
<td>India (Punjab), 1966</td>
<td>Asian</td>
<td>44.0</td>
<td></td>
<td>Wyon et al. (1966)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>44.68</td>
<td></td>
<td>Singh and Ahuja (1980)</td>
</tr>
<tr>
<td>Israel, 1963</td>
<td>Caucasian</td>
<td>49.5</td>
<td></td>
<td>Hauser et al. (1963)</td>
</tr>
<tr>
<td>Netherlands, 1969</td>
<td>Caucasian</td>
<td>51.4</td>
<td></td>
<td>Jaszmann et al. (1969b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51.7</td>
<td></td>
<td>van Keep et al. (1979)</td>
</tr>
<tr>
<td>New Zealand, 1967</td>
<td>Caucasian</td>
<td>50.7</td>
<td></td>
<td>Burch and Gunz (1967)</td>
</tr>
<tr>
<td>Papua New Guinea, 1973</td>
<td>Melanesian</td>
<td>47.3 (Not malnourished)</td>
<td></td>
<td>Scragg (1973)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43.6 (Malnourished)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scotland, 1970</td>
<td>Caucasian</td>
<td>50.1</td>
<td></td>
<td>Thompson et al. (1973)</td>
</tr>
<tr>
<td>South Africa, 1960</td>
<td>Negro</td>
<td>47.7</td>
<td></td>
<td>Abramson et al. (1960)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48.7</td>
<td></td>
<td>Benjamin (1960)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Frere (1971)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Frere (1971)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Switzerland, 1961</td>
<td>Caucasian</td>
<td>49.8</td>
<td></td>
<td>Hauser et al. (1961)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>49.8</td>
<td></td>
<td>MacMahon and Worcester (1966)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>49.31</td>
<td></td>
<td>MacMahon and Worcester (1966)</td>
</tr>
</tbody>
</table>
practice) rather than on random samples. Furthermore, many early studies were retrospective and therefore subject to inaccurate recall and unconscious bias. There was a tendency to round up estimates to the nearest 0 or 5, which led to an artificially high incidence of menopause at ages 40, 45 and 50 years (MacMahon and Worcester, 1966; McKinlay et al., 1972). Furthermore, most women recall only the year of their menopause and not the date, leading to further loss of precision. The net effect of these inaccuracies is a variable, but significant, underestimation of the age of menopause (Gray, 1976). The precision of recall in questionnaires has been studied by verifying the results with an independent source (Bean et al., 1979). Here 75% of women were accurate to ±1 year, but the results might have been less reassuring if the sample had been obtained from a less well-educated and less motivated population.

Most modern surveys take account of these design problems. Two methods are commonly used: (1) cross-sectional study design, in which age and menopausal status are determined at the time of interview and (2) longitudinal cohort design, in which groups of women are studied until they reach their natural menopause. However, even these designs are not free from bias since, for example, early menopause is more likely to be represented than very late menopause if there is an upper age limit for the study. Nevertheless, a great deal of useful data has been obtained through surveys. Most studies have found median ages of menopause between 49 and 51 in Western nations (Table 1.2).

Once the normal age of menopause has been established, attempts can be made to identify biological and sociological variables. Some variables that may affect menopausal age are discussed below.

Genetic Factors. It is undoubtedly true that the size of the ovarian follicle endowment laid down in the fetus and the rate of subsequent follicle utilization and death are under genetic control. This is particularly evident in some mutant mice and in chromosomally abnormal women in whom few follicles are found (p. 56). However, there are no studies of twins or families to assess critically the contribution of genetics to the normal variation of menopausal age. Despite assertions that menopause occurs later in northern and Anglo-Saxon peoples than in other populations (Flint, 1976), there is no proof that heritable factors are responsible. In the United States and South Africa, median menopausal ages of Negroid and Caucasian subpopulations differ by less than a year (Table 1.2). In some developing countries the onset of menopause is much earlier. Menopause occurs at age 44 in Punjabis of India (Wyon et al., 1966; Singh and Ahuja, 1980), at 43 or 47 in the different groups of the Bundi sub-population of Papua, New Guinea (Scragg, 1973), and at 40 in Mexican Indians (Chavez and Martinez, 1982). Such data might be accounted for by genetic isolation, but they can be explained more convincingly on other grounds, as indicated in the following sections.
**Nutrition, Body Size and Composition.** The lower menopausal ages of women in developing nations have often been attributed to a poor diet. In support of this hypothesis, the age of menopause was lower amongst malnourished women (43.6) than amongst those who were better nourished in Papua, New Guinea (47.3) (Scragg, 1973). Moreover, in well-nourished women there is an association between the percentage of body fat early in adulthood and the age of menopause (Sherman et al., 1981). Early nutritional adequacy seems to maximize the length of menstrual life. Other studies in the United States and The Netherlands showed that women who were lean and had small statures tended to have an earlier menopause (MacMahon and Worcester, 1966; van Keep et al., 1979). However, the effects of undernutrition on menopause are not as firmly established as are those on menarcheal age and length of lactational amenorrhoea (Bongaarts, 1980). Nor is it clear how severe and chronic undernutrition/malnutrition might cause early menopause. It is widely assumed that a poor diet will always be detrimental to reproduction, yet feeding mice only on alternate days has been found to delay the loss of oocytes and increase the age at which ovarian cycles cease after restoration to a normal diet (Nelson et al., 1985). These effects are associated with the generally improved physiological robustness and longevity of calorie-restricted rodents, but it is not clear whether they apply to our species. Rather than looking for factors that accelerate follicular atresia in the postnatal human ovary, we should pay more attention to the developing organ. Poorly nourished women are likely to have been delivered by mothers who were similarly deprived for at least at some seasons of the year when food shortages and a high agriculture workload leads to a negative energy balance. This situation may have adversely affected fetal oogonia during their limited period of proliferation, leading to a small follicular store at the beginning of life.

**Socio-Economic Factors and the Secular Trend.** The historical change in socio-economic conditions and the associated improvement in nutrition and somatic growth are responsible for the advancing age of menarche in Western nations (Fig. 1.4). This downward trend, which formerly proceeded at the rate of about 0.3 years per decade, has now halted in several of these countries where the mean menarcheal age is about 13 years (Tanner, 1978). For similar reasons, it has been claimed that the age of menopause has become delayed in recent history (Backman, 1948; Frommer, 1964), although the data are questionable and subsequent studies have not confirmed a secular trend (Burch and Gunz, 1967; McKinlay et al., 1972; Bengtsson, 1979; van Keep et al., 1979). The ages of menarche and menopause have not been found to be correlated in most population surveys, notwithstanding the extension at both ends of menstrual life in short, fatter women (see the section on “Nutrition, Body Size and Composition” above). There is some evidence that menopause occurs earlier in women of low social class and lower income (MacMahon and Worcester, 1966; Soberon et
Fig. 1.4. Secular decline in age of menarche in developed countries, 1860–1970. (From Tanner, 1978; reprinted with permission.)

al., 1966), but the differences are small and have not been observed consistently (Jaszmann et al., 1969b; Thompson et al., 1973).

Marital Status. Single women may reach menopause slightly earlier than married women. This effect is not accounted for by associated variables such as parity (Jaszmann et al., 1969b; McKinlay et al., 1972; Magursky et al., 1975; Brand and Lehert, 1978).

Parity. The relationship between the number of pregnancies and menopausal age is controversial, and there have been several negative findings (Medical Women’s Federation, 1933; Frommer, 1964; MacMahon and Worcester, 1966;
Jaszmann et al., 1969b; Thompson et al., 1973; Walsh, 1978; Ernster and Petrakis, 1981). Some of the controversy can be traced to the complex relationship between this variable and socio-economic factors. In two studies, one in London and the other in Mexico City, there was evidence that high parity was associated with later menopause in the high-income group but not in the low-income group (Soberon et al., 1966; McKinlay et al., 1972). At one time, high parity was thought to exhaust reproductive potential and possibly to advance menopause, but this hypothesis finds no convincing support (Flint, 1976). In addition, the contrary hypothesis that oocytes are used more parsimoniously and therefore conserved during pregnancy has no biological foundation (p. 50). Any effects of parity on menopausal age are more likely to be due to socio-economic factors.

Contraceptive Practice. Since the endometrium is sensitive to ovarian steroids even in extreme old age, oral contraceptives can conceal the transition to the postmenopause. Long-term contraceptive practice during the fertile years of life would not be expected to conserve oocytes for the same reason parity does not. Epidemiological studies have, however, found a slightly later median age of menopause according to consumption of contraceptive pills (van Keep et al., 1979). This surprising finding requires confirmation. There are no obvious reasons why any other contraceptive method should affect the timing of ovarian failure. In an Israeli study, menopause occurred at younger ages among women who had had many abortions (Pumpianski, 1967). This remarkable claim also requires close study.

Geography and Climate. As already stated, the menopausal age of women in tropical countries has been reported to be lower than that in boreal regions of the world. The effects of climate, if any, are likely to be indirect. Similarly, it is unlikely that living at altitudes above 2000–3000 m compared with lower altitudes in Chile and India has either a direct or specific effect on the time of menopause (Flint, 1976).

Smoking. Cigarette smoking has been associated with earlier menopause in a number of developed countries (Jick et al., 1977; van Keep et al., 1979; Lindquist and Bengtsson, 1979; Kaufman et al., 1980). The differences in menopausal age between women who had never smoked and those who had smoked >14 cigarettes per day since age 35 years or less was almost 2 years in one multicentre study (Table 1.3). These results are important because additional years of postmenopause aggravate the risk of fractured bones and other problems later in life (see Section 6.3). Tobacco smoke and tar constituents may exert many subtle effects on the function of ovaries. They have adverse effects on blood vessel walls and reduce the oxygen-carrying capacity of haemoglobin. It has even been suggested that they might affect neuroendocrine function and steroid metabo-
TABLE 1.3
Mean Age at Menopause among 656 Naturally Postmenopausal Women Aged 60–69 According to the Number of Cigarettes Smoked (Data from the United States, Canada and Israel)

<table>
<thead>
<tr>
<th>Number of cigarettes</th>
<th>Number of women</th>
<th>Mean age at menopause (years)</th>
<th>Mean difference from never-smokers (years)</th>
<th>SE of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Never-smoker</td>
<td>434</td>
<td>49.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>10</td>
<td>49.2</td>
<td>0.2</td>
<td>1.5</td>
</tr>
<tr>
<td>1–14/day</td>
<td>66</td>
<td>48.0</td>
<td>1.4</td>
<td>0.6</td>
</tr>
<tr>
<td>15–24/day</td>
<td>99</td>
<td>47.6</td>
<td>1.8</td>
<td>0.5</td>
</tr>
<tr>
<td>≥25/day</td>
<td>47</td>
<td>47.6</td>
<td>1.8</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*From Kaufman et al. (1980).

b Smoking status at the time of the interview. All smokers began smoking before age 35; all ex-smokers stopped smoking before age 35.

Distribution of Menopause

lism, further compromising the function of the ageing ovary. One product of smoking materials which is also present in urban air pollution, benzo(a)pyrene, might actually reduce the size of the follicular reserve because small amounts can kill mouse oocytes (Mattison and Thorgeirsson, 1978). Other smoking materials may also affect menopause, although surveys have not been conducted because these materials are used clandestinely in many places. Of particular interest is marihuana because of its widespread use and the reported effects of the chief psychoactive constituent, tetrahydrocannabinol, on reproductive functions, including pulsatile release of pituitary luteinizing hormone (LH) (Tyrey, 1980).

Disease and Drugs. A number of diseases are associated with altered (usually earlier) menopause (Flint, 1976), but it is difficult to distinguish the effects of advanced or chronic illness from iatrogenic effects. This subject is discussed further in a later section (Section 3.7).

In conclusion, the menopause is a universal characteristic of women, occurring at a median age of about 49–51 years in well-nourished societies. The estimated ages are remarkably consistent in view of the diversity of diets, life styles and environments of the peoples that have been studied. The explanation of menopause is therefore firmly in the province of the biologist.

1.6. Menopause in Animals

There is a bewildering array of physiological strategies for reproduction in mammals, and even closely related species have sometimes evolved quite differ-
ently in this respect. Only in monkeys of the Old World and in the great apes do we find menstrual cycles fairly similar in length and endocrinology to those of women. Strictly speaking, only these species can serve as potential models for studying human menstrual cycles and menopause.

Unequivocal evidence of spontaneous menopause has been obtained only in captive colonies of rhesus monkeys (Macaca mulatta). The menopause occurs between 20 and 30 years of age (i.e. close to the maximum longevity under these conditions) and is followed by hormonal changes that are consistent with primary ovarian failure (van Wagenen, 1972; Hodgen et al., 1977; Diershke et al., 1983). Pigtail macaques (M. nemestrina) may reach menopause at similar ages (Graham et al., 1979). The natural fertility pattern of great apes might be expected to resemble the human pattern even more closely. However, despite the greater longevity (c. 50 years), chimpanzees continue to show cycles until death, although they fail to conceive at advanced ages (Graham, 1979). Tentative signs of menopause have recently been recorded in two extremely old chimpanzees (Pan troglodytes) and in one pigmy chimpanzee (P. paniscus) at the Yerkes Primate Center (Gould et al., 1981). Despite limited data, it seems safe to state that a postmenopausal phase of life is relatively short and confined to a few animals of exceptional longevity under present husbandry conditions. The rarity of all apes and, to a lesser extent, monkeys, combined with the costs and patience required for keeping them until advanced age, are major limitations for menopause research.

Rodents are a poor compromise when animal models are needed for analysing certain aspects of ageing in the human reproductive system. Since rodents do not have menstrual cycles, they cannot ipso facto become menopausal, although many exhibit a prolonged postreproductive phase beginning at mid-life. No specific term has yet been coined for this phase; the neologism “oestropause” is superficially attractive but fails to convey the variety of endocrine and behaviour patterns of postcyclic life, which rarely find parallels in human biology. Despite some contrasting patterns of ageing in rodents and primates, which will be described further, the former will continue to be important models for tackling the most fundamental biological questions.
2

Oestrogen—Queen of the Realm of Reproduction

2.1. Discovery and Characterization

Present knowledge of the chemistry and biology of oestrogens is based upon discoveries made at the turn of this century, although Chinese chemists had obtained urinary preparations of oestrogens much earlier. In 1896, Knauer established the endocrine activity of ovaries by showing that the effects of castration were reversed by ovarian grafts. Marshall and Jolly (1906) proposed that follicular or interstitial ovarian cells are responsible for oestrous phenomena in animals, but they had no means of purifying the hormones. The first clues to the chemical identity and source of oestrogens were obtained by Allen and Doisy (1923), who found that extracts of porcine follicular fluid had trophic effects on the genital tracts of ovariec tomized rats. During the following decade, the most important discoveries were made by chemists who eventually characterized and synthesized the major ovarian steroids (see Doisy, 1972, and Butenandt and Westphal, 1974, for historical accounts).

Early attempts to isolate oestrogens were hampered by the limited amounts of hormones in porcine ovaries, but progress quickened when much larger quantities were found in the urine of pregnant women. In 1929, Doisy and Butenandt, working independently in the United States and Germany, respectively, obtained pure crystalline steroids and concluded that oestrone (called "theelin" in those times) was a hydroxyketone with the formula $\text{C}_{18}\text{H}_{22}\text{O}_{2}$. The following year in London, Marrian identified another oestrogen called "theelol", which later became known as "oestriol". Identification of a third oestrogen (oestradiol-17$\beta$ or "dihydrotheelin") was delayed until 1936 because of low tissue concentrations. Doisy's group eventually recovered 6 mg of the pure substance from a ton of porcine ovaries (MacCorquodale et al., 1936). Nearly a quarter of a century elapsed before this was shown to be the principal oestrogen of human ovaries (Zander et al., 1959). Meanwhile, vast amounts of animal tissue were being extracted to obtain crystalline progesterone and male sex hormones, which were identified in the early 1930s.
The natural oestrogens are a small family of molecules within the nation of steroids. The structures of the three classical oestrogens named above are shown in Fig. 2.1. They have in common the biological property of stimulating uterine growth and vaginal cornification in rodents (and other species). This characteristic defines all oestrogens, natural and synthetic. Their biological potency differs. Oestrone is only 15–25% as potent as oestradiol in bioassays, although comparisons are difficult because of interconversion in tissue. Single injections of oestriol are not uterotrophic because the nuclear receptor–oestriol complex is retained for only a short period, but continuous administration can result in normal oestrogenic responses (Anderson et al., 1975).

Urine of pregnant mares is another rich source of sex steroids. Besides the classical oestrogens, it contains specific oestrogens, equilin and equilenin, in which the B ring of the steroid nucleus is unsaturated (Fig. 2.1). Conjugated equine oestrogens are of therapeutic importance for climacteric and postmenopausal symptoms because they are readily absorbed from the gut and join the systemic circulation. A currently popular formulation used in the United States and, to less extent, in the United Kingdom consists of oestrone sulphate (48%), equilin sulphate (26%) and 17α-dihydroequilin sulphate (15%) with other conjugated oestrogens in small amounts ("Premarin") (Hammond and Maxson, 1982).

Since natural unconjugated oestrogens are relatively inactive after ingestion, new compounds have been sought. The first orally active oestrogen that became widely available is not a steroid but a stilbene derivative, diethylstilboestrol (Dodds et al., 1938). During the same period, the Schering Corporation found that oestradiol became orally active when an ethinyl group was attached to the

![Fig. 2.1. Structure of natural oestrogens, showing standard nomenclature of carbon rings in steroid nucleus (A, B, C, D).](image-url)
Fig. 2.2. Major pathways for biosynthesis of oestrogens in the ovary.
Oestrogen—Queen of the Realm of Reproduction

C-17 position on the molecule. These were the first of many discoveries of practical importance.

When the structures of steroid hormones were elucidated, it was apparent that these substances might be derived from cholesterol. This hypothesis was confirmed when deuterium was shown to be transferred from cholesterol to pregnanediol in pregnant women (Bloch, 1945). Subsequently, the availability of steroids labelled with $^{14}$C and $^3$H at high specific activity led to rapid progress in unravelling the biosynthetic pathway for oestrogen formation (Fig. 2.2). Side chain cleavage of cholesterol, involving at least three enzymes, leads to a C-21 steroid, pregnenolone, which is the precursor of the three main classes of ovarian steroids, namely, progestogens, androgens and oestrogens. That androgens are normal intermediaries in the biosynthesis of oestrogens was first indicated by the formation of oestrogen after administration of testosterone propionate to men (Steinach and Kun, 1937). Biochemical studies demonstrated that, although the ovary and placenta are the major sites of oestrogen production in women, a number of tissues have the ability to produce oestrogens. Aromatization of C-19 androgens to form phenolic C-18 oestrogens occurs in a specific cellular fraction containing the microsomes and is catalysed by a mixed-function oxidase in the presence of nicotinamide adenosine dinucleotide phosphate (NADPH) and molecular oxygen (Ryan, 1959).

The title of this chapter is adapted from words of F. L. Hisaw and signifies the central role of the oestrogens in the realm of sexual physiology and behaviour. Oestrogens are pre-eminently responsible for the sex-specific form and physiology of female mammals. They are essential for fertility, and widespread metabolic consequences follow their withdrawal. Few, if any, other groups of hormones match the oestrogens for biological potency on a molar basis or in the range of cell types affected. For these reasons, and because they are deficient in the postmenopause, they have been singled out for special consideration.

2.2. Production of Ovarian Oestrogen

The ovary is the main site of oestrogen production during menstrual and oestrous cycles. Lower blood hormone levels after castration and menopause are clear evidence of this. It is much less certain which ovarian cells are responsible for synthesizing oestrogens. At one time the theca interna of follicles was held responsible, but subsequent research tended to uphold the suggestion that "all ovarian tissues may secrete oestrogen... but that the follicular epithelium is the primary source" (Allen, 1941).

Leaving aside for a moment the vexed question of the site of production, let us examine some general requirements for biosynthesis of oestradiol-17β (henceforward called "oestriadiol"), the principal oestrogen of ovarian cycles in wom-
Production of Ovarian Oestrogen

en and most animals. Maturation of Graafian follicles and maximal production of oestradiol occur at the same phase of the cycle and depend on gonadotrophic hormones from the pituitary gland. Optimal trophic responses in ovaries of hypophysectomized rats require follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in combination (Lostroh and Johnson, 1966; Armstrong and Papkoff, 1976). The levels of gonadotrophins are controlled, in turn, by inhibitory and stimulatory feedback by steroids and, perhaps, by non-steroidal products of the ovary to bring about the normal sequences of growth, ovulation and luteinization (Fig. 2.3).

Growing follicles possess specific receptors of high affinity for FSH and LH in their cell membranes. The receptors are distributed unevenly. FSH receptors are found exclusively in granulosa cells, whereas LH receptors are present in theca cells, though appearing in granulosa cells during final stages of preovulatory maturation (Richards, 1979). Binding of gonadotrophins to their receptors initi-

Fig. 2.3. Neuroendocrine regulation of ovarian function: summary of major pathways and feedback. LHRH, luteinizing hormone releasing hormone; PIF, prolactin inhibiting factor; PRL, prolactin; FSH, follicle-stimulating hormone.
Oestrogen—Queen of the Realm of Reproduction

ates a chain of biochemical events. There is a conformational change in the hormone–receptor complex which allows binding to a regulatory protein and, hence, activation of adenylate cyclase. This enzyme catalyses conversion of adenosine triphosphate (ATP) to cyclic adenosine 3', 5'-monophosphate (cyclic AMP), which can phosphorylate protein kinases and bring about observable biological effects: growth, differentiation and secretion. Thus, interactions between gonadotrophins and their ovarian target cells lead indirectly to the many physiological changes of ovarian cycles.

There is substantial evidence that the rising levels of oestradiol produced by the dominant follicle(s) require cooperation of theca and granulosa cells. Falck (1959) isolated these cells from rat follicles by microdissection and transplanted them to the anterior chamber of the eye, together with vaginal epithelium to act as an oestrogen indicator. They produced oestrogen only when they were present together.

Short (1962) proposed a different mechanism of oestradiol production based on measurements of hormone levels in equine follicular fluid and luteal tissue. He suggested that the theca interna produces oestrogens and androgens, whereas luteinized granulosa cells produce progesterone. The ability of corpora lutea of some species (e.g. humans) to secrete oestradiol could then be explained by the presence of theca lutein cells. Short coined the term “two-cell type” theory, but this term is now also used to describe different mechanisms of oestrogen biosynthesis in other species.

Confirmation of Falck’s results has now been obtained from studies of rodents by combining the practical advantages of tissue culture with the precision of hormone radioimmunoassay. Theca cells released mainly androgens, and their secretory activity was increased by human chorionic gonadotrophin (hCG)/LH (but not FSH). Isolated granulosa cells tended to luteinize spontaneously and produced large quantities of progesterone but very little androgen or oestrogen. When the two cell types were combined in short-term experiments, larger amounts of oestradiol were formed (Makris and Ryan, 1975; Fortune and Armstrong, 1977). Other studies show granulosa cells possess aromatase (oestrogen synthetase), which is stimulated in a dose-dependent manner by FSH in the presence of testosterone (Dorrington et al., 1975; Erickson and Hsueh, 1978). Since this stimulation is blocked by actinomycin D and by cycloheximide, it is presumed that enzyme induction/activation involves de novo synthesis of RNA and protein (Wang et al., 1982). These results have been interpreted to mean that androgens (androstenedione and testosterone) synthesized in the theca interna under the control of LH diffuse into the follicle, where they are converted to oestradiol by granulosa cells primed by FSH.

This model of oestradiol production in rodent follicles has been called the “two-cell, two-gonadotrophin theory” (Armstrong et al., 1979) (Fig. 2.4). It is presumably based on a differential distribution of enzymes. Non-luteinized granulosa cells contain aromatase but are deficient in 17α-hydroxylase and C₁₇-20-
Fig. 2.4. Schematic diagram of ovarian follicular biosynthesis of oestrogen by co-operation of theca interna and granulosa cells. Aromatizable androgens (A) are synthesized in the theca layer in the presence of LH stimulation. They diffuse to the granulosa layer, where they are substrates for aromatization to oestrogen (E) in the presence of FSH. The gonadotrophins exert their actions by a chain of events: binding to receptors (R) in cell membranes, which leads to increasing intracellular levels of cyclic AMP (cAMP) and enzyme induction/activation. FSH and oestrogen have synergistic effects of inducing LH receptors in preovulatory follicles which increasingly produce progesterone. FSH receptors increase during follicle growth pari passu with granulosa cell number, although they may decrease later.
desmolase, which are obligatory for converting C-21 steroids to androgens in the Δ4 pathway. This deficiency is compensated for by the acquisition of enzymes by theca cells during Graafian follicle development (Bogovich and Richards, 1982).

This model is consistent with data obtained for some other species. Moor (1977) showed that theca cells were the source of androgens in cultured sheep follicles and that co-culture with granulosa cells was required for the formation of significant amounts of oestradiol. Direct evidence of the two-cell theory was obtained in the same species by Baird (1977a). He found oestrogen secretion from the ovary carrying the dominant (ovulable) follicle was inhibited >50% after infusing antiserum to testosterone into the ovarian artery. Since antibodies do not normally enter cells, the results imply that androgens leave cells before they are aromatized.

There are data in other species which are inconsistent with this model. As implied above, there are strong indications that theca cells are responsible for oestradiol formation in equine follicles (YoungLai and Short, 1970). In addition, when radioactively labelled acetate was presented to human granulosa and theca cells in vitro, all steroid intermediaries between cholesterol and oestrogens were recovered (Ryan et al., 1968). Oestrogens were produced via the Δ4 pathway in the granulosa cells, whereas Δ5 was the preferred pathway of theca cells (Ryan and Petro, 1966) (Fig. 2.2). Similar differentiation of the two cell types is seen in rabbit follicles (Patwardhan and Lanthier, 1977). Although these experiments show the presence of enzymes, it does not follow that these pathways are necessarily operating in vivo. To help answer this question, granulosa cells and follicular fluid were aspirated from dominant follicles in rhesus monkeys (Channing and Coudert, 1976). The levels of oestrogens in blood draining from the ovaries were hardly affected by this treatment, suggesting that the theca was responsible for secretion. A problem with this type of experiment is that peripheral granulosa cells probably make the major contribution to oestrogen production (Zoller and Weisz, 1978) and are the most difficult to remove. Nevertheless, it seems probable that there are genuine species differences in the biosynthetic role of various ovarian cell types.

Although far from decisive, there is some evidence of two-cell co-operation for the production of oestrogen by human ovaries. Isolated human theca cells have limited aromatase activity: they produce <1% of the amount of oestrogen produced by granulosa cells from the same follicles (Moon et al., 1978; McNatty et al., 1979a; Hillier et al., 1981). When combined in vitro, the two cell types produce more oestrogen than the sum of their independent production (Batta et al., 1980). Theca cells resemble their stroma cell precursors both in this respect and in their ability to secrete quantities of androstenedione and testosterone. Thecal androgen secretion rises during the follicular phase and is greater than that of an equivalent mass of stroma. However, the large bulk of stroma implies a substantial contribution to total ovarian production (McNatty et al., 1979a).
The ability of human ovarian follicles to produce oestrogen rises during the follicular phase because of the increased size and heightened aromatase activity in individual granulosa cells. Indeed, follicles that are presumed to be healthy and progressing towards ovulation are identified by large amounts of oestradiol in follicular fluid, rising to 2.5 \( \mu \text{g/ml} \). Androgen levels in preovulatory follicles become less predominant, but the rate of aromatization is not limited by lack of substrate (McNatty and Baird, 1978).

The largest healthy follicle measures 5–8 mm in diameter at the beginning of the menstrual cycle (Gougeon and Lefèvre, 1983) and contains FSH and oestradiol in its fluid (McNatty and Baird, 1978). The relatively high levels of plasma FSH at this stage promote aromatase activity (Erickson et al., 1979). Oestradiol production is self-reinforcing. It causes proliferation of granulosa cells (Rao et al., 1978); consequently, healthy follicles have more granulosa cells than non-ovulatory follicles of similar diameter (McNatty et al., 1979b), and the numbers of FSH receptors rise correspondingly. Oestradiol also promotes aromatase activity within cells (Fig. 2.4). These actions help protect the dominant follicle(s) from understimulation during the second half of the follicular phase, when levels of plasma FSH fall (Fig. 3.5), by increasing both the efficiency of trapping FSH and the sensitivity of granulosa cells. By this time, the follicle exceeds 12 mm in diameter, and increasing production of oestrogen may depend on pronounced LH pulses to stimulate greater production of androgen (Bäckström et al., 1982). Furthermore, as rat follicles approach ovulation, they become much more sensitive to the stimulatory effects of LH on the production of cyclic AMP and oestradiol (Uilenbroek and Richards, 1979). Such responsiveness is likely to follow the acquisition of LH receptors on granulosa cells, which depends on the joint action of FSH and oestradiol (Richards, 1979). Subsequently, these cells become increasingly active in producing progesterone, anticipating their morphological transformation to luteal cells.

Non-ovulatory follicles do not develop FSH-sensitive aromatase activity and do not therefore switch from androgen to oestrogen production (McNatty et al., 1979a; Mori et al., 1982). Androgen production continues during early stages of atresia (McNatty et al., 1979a). Androgens serve a complex role in follicular maturation; besides being obligatory precursors of oestrogens, they are also evidently involved in priming granulosa cells for induction/activation of aromatase by FSH (Hillier and de Zwart, 1981). Other experiments show that androgens can inhibit aromatase, and they have even been held responsible for atresia (Louvet et al., 1975). More research is required to clarify their role.

Increasing output of ovarian steroids during the first half of the cycle is a sign of follicular growth, whereas the changing profile of hormones represented (androgens to oestrogens to progestogens) is indicative of cellular differentiation and maturation in the dominant follicle. Since some circulating oestrogen is derived from extra-ovarian sources, it is useful to measure its blood production rate (BPR), the total production from all sources, glandular and extra-glandular
TABLE 2.1
Ovarian Steroids during the Menstrual Cycle and following Menopause: Representative Blood Production Rates, Plasma Concentrations and Metabolic Clearance Rates

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Blood production rate (mg/24 hr)</th>
<th>Peripheral plasma concentration (pg/ml)</th>
<th>Metabolic clearance rate (litres of plasma/24 hr)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ML&lt;sup&gt;c&lt;/sup&gt;</td>
<td>PMP&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.074</td>
<td>0.429</td>
<td>0.258</td>
<td>0.006</td>
</tr>
<tr>
<td>Oestrone</td>
<td>0.133</td>
<td>0.265</td>
<td>0.236</td>
<td>0.040</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>2.91</td>
<td>4.60</td>
<td>3.75</td>
<td>1.70</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.152</td>
<td>0.345</td>
<td>0.207</td>
<td>0.150</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.682</td>
<td>4.44</td>
<td>31.13</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> EF, early follicular phase.
<sup>b</sup> MC, mid-cycle.
<sup>c</sup> ML, mid-luteal phase.
<sup>d</sup> PMP, postmenopause.
<sup>e</sup> M, menstrual cycle.
Production of Oestrogen after Menopause

The BPR can be obtained indirectly by measuring the metabolic clearance rate (MCR) of the hormone. The MCR is the volume of plasma completely and irreversibly cleared of hormone in unit time (litres per 24 hr). This is estimated by infusing radioactively labelled hormone into a peripheral vein until blood concentrations are stable. At this point, the rate of infusion balances the rate of removal or metabolism, and the MCR can be calculated. The BPR is then obtained as the product of the MCR and the plasma hormone concentration.

The BPR of oestradiol rises from 0.03 mg/24 hr at menstruation to about 0.40 mg/24 hr shortly before ovulation (Table 2.1). Comparisons between peripheral and ovarian vein blood indicate about 95% of peak levels are formed by the "active" ovary containing the dominant follicle in monovular cycles (Baird, 1977b). Very little oestradiol is secreted by the human adrenal gland or formed extra-glandularly. Consequently, it has been concluded that "in reproductively active women oestradiol is secreted almost exclusively from the preovulatory follicle or the corpus luteum, and the blood production rate equals the secretion rate" (Baird, 1977b). Plasma levels of oestrone are similar to those of oestradiol, but cyclical variations are less marked because of a greater extra-ovarian contribution (Baird and Guevara, 1969). The ovary may also secrete oestrone sulphate, but secretion of oestriol has not been established (Baird, 1978).

Larger quantities of androgens than oestrogens are produced at all stages of the ovarian cycle (Table 2.1). Besides circulating as prohormones, they probably have important (but presently undefined) physiological actions and, in excess, can produce masculinization. Androstenedione is by far the most abundant ovarian androgen in ovarian vein blood, where its level exceeds that of peripheral blood levels by 20:1. Testosterone and dehydroepiandrosterone (DHEA) are also products of the ovary, judging by their fluctuating levels during the menstrual cycle and their small excess in ovarian vein blood (2:1). Plasma DHEA and androstenedione have marked diurnal rhythms, which indicates that large quantities are secreted by adrenal glands.

Progestogens are secreted in abundance by the ovary during the luteal phase. Over 90% of the BPR of progesterone (30 mg/24 hr) can be accounted for by the corpus luteum. Other C-21 steroids are secreted by follicles and corpora lutea (notably 17α-hydroxyprogesterone), but these show much less cyclical variation.

2.3. Production of Oestrogen after Menopause

In the preceding section, oestradiol was shown to be produced mainly by ovarian follicles, but few follicles remain at menopausal age (see Chapter 3). The end of menstrual life therefore marks the beginning of a new phase in which
circulating oestrogen is maintained on a lower plane. During the first 6 months after menopause, significant quantities of oestrogen may be secreted intermittently (Sherman et al., 1979; Metcalf et al., 1982). Presumably, they are derived mainly from residual follicles that grow sporadically but fail to reach maturity. Subsequently, plasma oestradiol falls to low, relatively stable values of less than 20 pg/ml for the remainder of life.

The changes in rates of production of oestradiol and oestrone after menopause are not the same. The mean BPR of oestradiol in postmenopausal women is only 0.006 mg/24 hr, which is about 20% of the nadir and only 1% of mid-cycle levels of the menstrual cycle (Table 2.1). The BPR and plasma levels of oestrone fall by only about 50% to 0.04 mg/24 hr and 25 pg/ml. Oestrone is, therefore, quantitatively the most abundant oestrogen of the postmenopause, though even greater amounts of the sulphate ester are present (Roberts et al., 1980). The oestradiol:oestrone ratio in peripheral blood falls to a value that is closer to that of castrated women (0.42 ± 0.07) and men (0.35 ± 0.04) than that of menstruating women (>1.0) (Baird, 1977). The ovarian contribution to circulating oestrogen after menopause has been difficult to measure because of technical limitations of assaying small amounts. Even in the absence of follicles, a small contribution is expected because other ovarian tissues evidently produce small quantities in vitro (McNatty et al., 1979). Barlow et al. (1969), using isotope dilution methods, were unable to detect any significant ovarian production. On the other hand, the concentrations of oestradiol and oestrone in ovarian vein blood were double those in peripheral blood, which suggests a residual capability for secretion (Judd et al., 1974a; Greenblatt et al., 1976). However, in another study the ovarian contribution to the circulating pool of hormones was insignificant in the majority of women (Longcope et al., 1980) and so other sources of postmenopausal oestrogen have been sought.

At first, the adrenal glands were suspected to be important sources because of long-standing evidence of sex steroid excretion in castrates (Parkes, 1937). This hypothesis seemed to be strengthened by discoveries that adrenocorticotropic hormone (ACTH) increased urinary oestrogens, whereas adrenalectomy had the opposite effect (Dao, 1953). But measurements of adrenal vein blood indicate that, although large amounts of androgen are secreted, only small quantities of oestrone are formed and production of oestradiol is negligible (Baird et al., 1969; Greenblatt et al., 1976). Thus we are led to the conclusion that oestrogens are formed extra-glandularly.

The first clear evidence of extra-glandular production of oestrogens was obtained from two ovarietomized, adrenalectomized women in whom urinary oestrogen levels rose after injections of testosterone propionate (West et al., 1956). Later, double isotope methods provided opportunities for using elegant quantitative methods. It became possible to measure the proportion of the total
Production of Oestrogen after Menopause

amount of androgen substrate that can be converted to oestrogen, this parameter being called the conversion ratio (CR) (Siiteri and MacDonald, 1973). There is now little doubt that the bulk of oestrone in normal postmenopausal women is formed by extra-glandular conversion of androstenedione of adrenal origin (Fig. 2.5). This situation is reminiscent of the formation of oestrogens during pregnancy, in which DHEA sulphate from the fetal adrenal gland is aromatized by the placenta. However, this substrate and its unconjugated form are not converted to precursors of oestrogens in non-pregnant women (CR <0.001) (Horton and Tait, 1967) but give rise to most of the urinary 17-ketosteroids.

The CR of androstenedione to oestrone is low, but this is compensated for by an abundance of precursor. After the menopause, the BPR of androstenedione is about 1.7 mg/24 hr, which is 50% of that during the early follicular phase of premenopausal women (Table 2.1). But extra-glandular formation of oestrogens

![Fig. 2.5. Schematic diagram showing the major source of oestrogen in postmenopausal women. Arrows indicate direction and line thickness indicates relative activity of the pathway. Feedback by cortisol on ACTH production is indicated by ⊗; feedback by physiological levels of oestrogen on gonadotrophin release has not established and could be insignificant. E₁, oestrone; E₁S, oestrone sulphate; E₂, oestradiol; Δ4-A, androstenedione; DHEAS, dehydroepiandrosterone sulphate; T, testosterone; LH, luteinizing hormone; ACTH, corticotrophin.](image-url)
does not fall correspondingly because of a rising CR. The CR is 0.013 before the menopause and 0.027 afterwards, although individual values are highly variable and probably change continuously rather than abruptly at menopause (MacDonald et al., 1967; Siiteri and MacDonald, 1973; Grodin et al., 1973). On the basis of these figures, the production rate of oestrone from androstenedione in postmenopausal women is therefore 1.7 × 0.027 = 0.046 mg/24 hr. Within the bounds of biological and experimental variation, this figure is not significantly different from the total BPR of oestrone obtained by other methods (0.04 mg/24 hr).

Oestrone can also be formed by peripheral conversion of androstenedione via oestradiol and testosterone (Fig. 2.5). This pathway is much less active than the alternative one because levels of testosterone are lower than those of androstenedione and because the CR for androgen to oestradiol is very low (0.001) (Longcope et al., 1969; Judd et al., 1982). Therefore, it has been concluded that most circulating oestradiol is formed by conversion of oestrone, for which the CR is more favourable (0.065) (Judd et al., 1982).

The adrenal gland is responsible for most of the circulating androstenedione and, hence, oestrone after the menopause. There are several reasons for this view. Androstenedione is not produced to any extent from extra-glandular conversion of DHEA or testosterone and must therefore be of glandular origin (Horton and Tait, 1966, 1967). Plasma levels of this androgen and those of oestrone (but not oestradiol) show a circadian rhythm that characterizes adrenal secretion; peak levels are seen at 0800 hr and a nadir occurs between 2000 hr and midnight (Vermeulen, 1976; Crilly et al., 1979). Suppression of corticotrophin (ACTH) release by corticosteroids causes plasma androstenedione levels to fall by about 50%, and there is an associated decline of oestrone (Vermeulen, 1976; Marshall et al., 1978). Similarly, plasma androgen and oestrogen levels are more than 50% lower after adrenalectomy, although testosterone is somewhat less affected if the ovaries remain (Vermeulen, 1980).

The most remarkable feature of postmenopausal oestrogen production, apart from its paucity, is its overwhelming dependence on adrenal function. The ovary has become almost redundant, and circulating oestrogen is no longer controlled by the gonadotrophins. The pituitary–gonadal feedback loop is virtually inactive. Although unproven, it is likely that exogenous oestrogen has little effect on endogenous production, in contrast to the situation in premenopausal women. Drugs or diseases that affect adrenocortical function are of considerable importance because they influence production of oestrogen substrate.

There is widespread evidence that plasma levels of five androgens fall during ageing (Zumoff et al., 1980; Meldrum et al., 1981a; Vermeulen et al., 1982) (Fig. 2.6). This cannot be explained by altered metabolic clearance rates (cf. Section 2.4) but may be due to diminished sensitivity to ACTH with less C₁₇-20-desmolase activity in the adrenal cortex. Although contrary data exist, some
Production of Oestrogen after Menopause

Fig. 2.6. Twenty-four-hour mean plasma dehydroepiandrosterone concentrations in normal ageing women. (From Zumoff et al., 1980; reprinted with permission.)

studies show a comparable fall of the Δ4 steroid androstenedione (Crilly et al., 1979; Roger et al., 1980). In some studies the changes were progressive from early adulthood, whereas in others they began shortly after menopause. Some authors have chosen the appellation “adrenopause” to describe these changes, which contrast with the rising androgen levels of prepuberty (“adrenarche”). Declining androgen levels are not associated with changes in cortisol production nor are they secondary to ovarian failure, since they occur in men as well as in ovariectomized women (Crilly et al., 1979). The reported effects of exogenous oestrogen on adrenal androgen production are therefore probably pharmacological actions (Abraham and Maroulis, 1975). Despite these changes in androgen levels, oestrogens do not normally become more scarce during the postmenopause, and they are affected by changes in body composition rather than age per se (Meldrum et al., 1981a).

Bilateral ovariectomy of postmenopausal women leads to lower peripheral blood levels of androgens, whereas oestrogen and progestogen levels are unaltered (Judd et al., 1974b; Vermeulen, 1976; Badawy et al., 1979). Androgens are the only group of steroids which rise after a large dose of hCG (Greenblatt et al., 1976). The major products of the postmenopausal ovary are androgens: androstenedione, testosterone, 5α-dihydrotestosterone and, in smaller quantities, DHEA and androstenediol. The principal steroid is testosterone, judging by the 15-fold excess in blood of ovarian veins compared with that of the periphery (Judd et al., 1974a). This would seem to imply that the ovary makes a significant contribution to peripheral blood levels and that testosterone is not fully accounted
for by peripheral conversion of androstenedione, despite a favourable CR (~0.1) (Calanog et al., 1977; Judd et al., 1982). The excess of androstenedione in ovarian venous blood is only fourfold; therefore, indirect production of oestrogen by the ovary is small. This conclusion is supported by observations that castration does not affect oestrogen levels after menopause.

Two types of cell are probably responsible for producing most of the androgens from postmenopausal ovaries. Stroma cells contain enzymes for converting Δ5-hydroxysteroids to Δ4-ketosteroids (Fienberg, 1969) and produce androgens when isolated in vitro (Mattingly and Huang, 1969; McNatty et al., 1979a). This activity on a unit mass basis is small but nonetheless significant because these cells are abundant and stimulated by postmenopausal gonadotrophins. Stroma cell hyperplasia and thecosis are common features of these ovaries, but they do not necessarily lead to increased androgen production (Mattingly and Huang, 1969). Hilus cells are the other important source of androgens and are found in small clusters close to the ovarian hilum (Fig. 2.7). They present the typical appearance of steroid-secreting cells, although containing cytoplasmic crystalloids of Reinke, which are found elsewhere only in human Leydig cells. Hilus cell hyperplasia and tumours lead to clinical virilization (Sternberg, 1949). Clinical evidence suggests they normally secrete testosterone and are sensitive to hCG/LH, but experimental evidence is hard to obtain because hilus cells cannot be isolated completely from stroma cells in vitro. Some results even suggest they produce oestrogens, but they seem anomalous and require confirmation (Dennefors et al., 1982).

Differences in body size and composition of non-glandular tissues are probably responsible for much of the variation in postmenopausal oestrogen production. Knowledge of this subject is limited and animal models, which could provide impetus to basic research, have not yet been identified because most animal ovaries produce oestrogen until advanced age. The rate of production of androgens and of their conversion to oestrogens rises with excess body weight, leading to higher peripheral blood levels with an elevated oestrogen : androgen ratio (Grodin et al., 1973; Edman et al., 1978; Vermeulen and Verdonck, 1978; Meldrum et al., 1981a), though there are contrary data (O’Dea et al., 1979). Positive findings are partly explained by aromatase activity in white adipose tissue and skeletal muscle (Nimrod and Ryan, 1975; Longcope et al., 1978), and wasting of these tissues in old age could account for exceptional reports that oestrogen levels decrease with increasing age postmenopause (Badawy et al., 1979). Other tissues are capable of producing oestrogen in small amounts, including liver (Smuk and Schwers, 1977), bone marrow (Frisch et al., 1980) and brain (Naftolin et al., 1975), but the balance of production in vivo has not yet been measured. Excess oestrogen production in the postmenopause is associated with obesity, as well as with hepatic disease and hyperthyroidism, and may become clinically manifested with vaginal bleeding (Siiteri and MacDonald,
Metabolism and Clearance

Fig. 2.7. Nest of hilus cells in a postmenopausal human ovary. Haematoxylin and eosin (X500).

1973; Southren et al., 1974). It might also lead insidiously to carcinoma of the endometrium and, perhaps, even of the breast, as obesity is known to be a risk factor for both of these diseases. There is, however, no simple connection between obesity and elevated plasma oestrogen, although associations are sometimes reported (e.g. Vermeulen, 1980), nor is there any close correlation between hormone levels and the presence of disease or the absence of climacteric symptoms. Siiteri (1981) has drawn attention to the likelihood that the free steroid fraction (which is increased in obese women) is of greater significance than total levels of oestrogen. Clearly, the factors determining oestrogenic activity in postmenopausal women are highly complex and probably change during ageing.

2.4. Metabolism and Clearance

The metabolism and clearance of the three classical oestrogens have been shown to be inter-related by in vivo experiments using radioactively labelled test substances. The fate of injected oestradiol is determined mainly in the liver, where it is converted to oestrone and oestriol. These products are excreted in urine after further metabolism or after conjugation with D-glucuronic acid or
sulphuric acid or both. Steroid esters are more polar than free steroids and are therefore readily excreted, but they can also be hydrolysed to liberate the biologically active oestrogen moiety.

At least 50% of injected oestradiol and oestrone find their way into bile. Only 20–25% of oestriol follows that route, which is why conjugated oestriol is excreted almost totally unchanged (Slaunwhite et al., 1973). Soon after injection of oestradiol a host of metabolites, mostly conjugated and many of them still unidentified, appear in bile fluid. After entering the intestine they are hydrolysed, mainly by the bacterial flora (Aldercreutz et al., 1977; Back et al., 1981). Most of the oestrogen which is reabsorbed is the unconjugated form (Back et al., 1981). It is either reconjugated locally in the gut mucosa and returned by portal vein blood to the liver to repeat the cycle or reconjugated in the liver itself; only a small amount escapes conjugation and joins the systemic circulation. Therefore, as in the case of bile salts, the entero-hepatic circulation minimizes the loss of oestrogen in faeces. For this reason, and because of its greater affinity for sex steroid–binding globulin (Burton and Westphal, 1972), oestradiol is cleared much more slowly than oestriol. It is not known whether ageing affects the entero-hepatic circulation of steroids, although extra-hepatic biliary obstruction in older women potentially increases the rate of urinary excretion of oestradiol. The most active biotransformations of oestradiol are shown in Fig. 2.8. They are essentially oxidative, with oestron being a key intermediary. There is competitive hydroxylation of the 5-carbon D ring and the aromatic A ring of the steroid nucleus (Fig. 2.8). Hydroxylation at C-16 leads to oestriol formation via 16α-hydroxyoestrone. Hydroxylation at C-2 results in 2-hydroxyoestrone, a major urinary metabolite, which is partially and specifically o-methylated to 2-methoxyoestrone or, to a smaller extent, reduced to 2-hydroxyoestradiol (Fishman, 1981). These catechol oestrogens are formed in the liver and at other sites, including the hypothalamus (Fishman and Norton, 1975). Their rate of formation is expected to fall at menopause as a consequence of less ovarian oestrogen, but

![Diagram of oestrogen metabolism](image)

**Fig. 2.8.** Major pathways of metabolism of oestradiol in women. Thick arrows indicate predominant pathways.
concomitant changes in body composition could also affect the relative activity of alternative metabolic transformations (Fishman et al., 1975). The oestrogenic activity of these substances is weak or non-existent, and they are remarkably evanescent. It has, therefore, been difficult to test whether they have a physiological role in gonadotrophin secretion or any involvement in the vasomotor disturbances of the climacteric (see Section 6.2).

After the menopause, representative values of metabolic clearance rates of oestradiol and oestrone fall by about 30% (Table 2.1). This order of change is similar to that of other steroids, which implies that common end organs, namely, the kidneys and liver, are at fault. Metabolic clearance rates may not change as abruptly at menopause as is indicated by Table 2.1; they may follow the steady downward trend of glomerular filtration rates during ageing (Goldman, 1977). Clearly, the reduced MCRs in older women tend to conserve any sex steroids present in the body.
The Follicular Store

3.1. Introduction

During the reproductive years, oestrogens are mainly produced by ovarian follicles and, in women and some animals, by their successors, the corpora lutea. Waves of follicular maturation are responsible for the hormonal manifestations of menstrual and oestrous cycles. Follicles are the developmental units of the ovary, each consisting of an oocyte enveloped by somatic cells. These cells are required for maintaining and controlling the development of the oocyte and, as described earlier, are the source of follicular oestradiol. This intimate relationship between germ cells and sex steroid-secreting cells contrasts with the situation in the testes, where their homologues are isolated from each other. Thus, ovarian oestrogen secretion falls when oocytes are depleted, whereas testosterone secretion continues from sterile testes. The biology of ovarian follicles must, therefore, be central to any discussion of the causes and timing of menopause. In the following sections the dynamic features of the follicular store are described and the age-related changes are discussed.

3.2. Formation of Oocytes and Follicles

In the late nineteenth century there was a lively debate on whether primordial germ cells are genuine progenitors of the definitive pool of the germ cells of later life. The school of Waldeyer (1870) claimed that they arise from proliferation of the epithelium covering the presumptive gonad, whereas Nussbaum (1880) stressed that a germ cell lineage is segregated from somatic cells at an early stage of embryogenesis. The second view was upheld by Weismann's (1885) theory of the continuity of germ plasm and is now well established.

Primordial germ cells of mammals are recognizable by their large size, low nuclear-cytoplasmic ratio and, in laboratory rodents and man, alkaline phosphatase activity at presomite and early somite stages. This enzyme is located in the Golgi apparatus and cell membrane, where it may be involved in the transport of metabolites. It is a useful marker for counting and tracing cells, and it enabled
early workers to identify primordial germ cells in the yolk sac endoderm of 30-day-old human embryos (Witschi, 1948). It was assumed that germ cells originated from primary endoderm, but doubts arose when they were found at the base of the allantoic rudiment in mouse embryos. This finding implied a mesodermal origin in the primitive streak (Ożdzeński, 1967). More recently, elegant microsurgical techniques have provided new opportunities of studying the embryonic source of germ cells. Chimaerism among germ cells of mice could sometimes be identified following injection of epiblast cells into host blastocysts (Gardner and Rossant, 1976). Since epiblast and primary endoderm cells are already committed cell lines, it is concluded that germ cells are derived from epiblast which may subsequently migrate to the yolk sac.

Human primordial germ cells, estimated to number 1000–2000 by alkaline phosphatase activity, migrate to the gonadal ridge by active and passive movements, possibly guided by chemotaxis. Many cells are lost during transit, and only those reaching the favourable mesenchymal environment of the ridge will normally survive. Colonization of the gonadal anlagen by primordial germ cells is completed by 44–48 days after conception in man (12–14 days in mice) (Peters, 1970). By this time they have greatly multiplied in number. During the subsequent 5 months of gestation in man the germ cells can be seen in the

Fig. 3.1. Variation in the total number of germ cells in human ovaries at prenatal and postnatal ages. [Reprinted with permission from T. G. Baker (1971), Radiosensitivity of mammalian oocytes with particular reference to the human female, Am. J. Obstet. Gynecol. 110, 746–761.]
ingrowing "sex cords" of coelomic epithelium, where they reach a peak number of about 7 million (Baker, 1963) (Fig. 3.1). The germ cells may be unevenly distributed between the two ovaries, the imbalance being most marked in birds, where in many species the left ovary is the only functional gland. In some strains of mice (e.g. C57BL/6J) the number of follicles and ovulations is significantly greater in the right ovary, whereas, in women, there is tentative evidence of a left-sided bias in the number of primordial follicles (data of Block, 1952, re-analysed; $P < .05$). It is probable that unequal numbers of germ cells in adult ovaries reflect the number formed during oogenesis and that the more deficient organ will be the first one to fail during ageing.

Oogonia undergo a limited number of cell divisions and then enter meiosis, from which time they are known as "oocytes". Leptotene oocytes appear at about 4 months of gestation, and most oocytes become arrested at the diplotene stage 3 months later (Fig. 3.2). Some will not emerge from this "resting" phase until they are recruited for ovulation several decades later. In most species, including our own, oogonia do not persist after birth, and since oocytes are non-proliferating, a newborn infant already possesses its lifetime store.

In order to develop, oocytes require the physical and nutritional support of an envelope of somatic (follicle) cells, which also serve to regulate their develop-

**Fig. 3.2.** Ovary of a human fetus at mid-gestation, showing an abundance of small oocytes, many of which have already reached diplotene of meiosis. Haematoxylin and eosin (X320).
Follicle formation is a protracted process commencing at 5–6 months of gestation, with a few naked oocytes remaining for several months postpartum. Progenitors of granulosa follicle cells are derived, at least partly, from the embryonic mesonephros (Byskov, 1975). This system of tubules, known as the "rete ovarii", communicates with the ingrowing sex cords of ovarian epithelium. The prototype follicle consists of a single layer of pregranulosa cells. These "primordial" follicles are ovarian storehouses for oocytes. When oocytes are absent from the fetal gonad, the organ remains a small and undifferentiated "streak" which is incapable of producing steroid hormones in quantity. Sex steroids and gonadotrophins are probably not required for these early developments in the ovary (Baker and Neal, 1974), although they have dominating effects on follicles at later stages.

3.3. Initiation and Maintenance of Follicular Growth

Initiation of follicle growth is first recognised when the somatic cells increase in number and change from squamous to cuboidal forms. At the same time, oocytes enlarge and increase their production of RNA (Lintern-Moore and Moore, 1979), and mesenchymal cells in the stroma form a wreath around the small follicle. The latter cells will differentiate later into the inner secretory and external fibrous layers of the theca. Follicles leave the store of primordial stages at all ages and independently of the physiological state of the organism (e.g. pregnancy and lactation). Virtually nothing is known about the mechanism that activates them. Since they are recruited at a constant rate, it has been suggested that their activity is triggered by some stochastic event within the oocyte. However, few biological processes are truly random, and growth initiation is likely to depend on interactions between germ and somatic cells. There is some doubt whether gonadotrophins are required at this early stage of development. In adult ovaries of hypophysectomized mice, follicles grow to large multilaminar stages in the absence of these hormones (Jones and Krohn, 1961b). On the other hand, follicle growth in neonatal mice is inhibited by administering antiserum to gonadotrophins (Eshkol et al., 1971), and follicles fail to grow in anencephalic human fetuses in which pituitary function is also impeded (Baker and Scrimgeour, 1980). Whilst gonadotrophins may be required for the commencement of follicle growth in fetal or neonatal ovaries, most evidence suggests they are required mainly for larger follicles in postnatal life.

After its commencement, follicle growth continues without interruption until preovulatory maturation, unless atresia intervenes. The human primordial follicle measures 0.1 mm in diameter and contains approximately 10 pregranulosa cells, but when mature it measures >20 mm and contains 60 million granulosa cells (Gougeon, 1982). By this stage it has accumulated pools of extra-cellular
fluid which coalesce to form an antrum. The follicle is called "Graafian" at this stage. In mice, primordial follicles are only slightly smaller than in women, but they form Graafian structures less than 1 mm in diameter, comprising only 50,000 cells. Stages of follicle growth are defined either by the number of granulosa cells or, more readily, by the number of cell layers (Figs. 3.3, 3.4).

Follicle growth is a lengthy process extending throughout several ovarian cycles. Unilaminar follicles in mice take at least 19 days to reach preovulatory size (Pedersen, 1970). In women, the corresponding period is probably greater than 3 months (Gougeon, 1982). Throughout adult life, murine follicles maintain a steady rate of growth which does not fall in middle age when ovulatory cycles cease (Faddy et al., 1983; Gosden et al., 1983b). Although comparable data

Fig. 3.3. Ovarian cortex of a young adult rhesus monkey, showing many primordial follicles, with follicular growth initiated at the inner boundary. Haematoxylin and eosin (X160).
The Follicular Store

Stage III (4)

Stage II (3b)

Stage I (1–3a)

Stage V (5b)

Stage VIa (6)

Stage VIb (7–8)

Stage VIc

OVULATION

Fig. 3.4. Classification of follicle stages for the mouse ovary according to Mandl and Zucker-
man (1951) (in roman numerals) and Pedersen and Peters (1968) (in arabic numerals). Primordial follicles (stage I/1–3a) grow through successive stages to antral (Graafian) size (stage VI/6–8) to ovulate and reorganize into corpora lutea or, in the absence of an ovulatory stimulus, enlarge to form follicular cysts (stage Vic). Follicles also terminate development in atresia at earlier stages, especially stages I, V and VIa.

cannot be obtained for human ovaries, gradual shortening of the follicular phase by 2–3 days over the menstrual lifespan (Vollman, 1977) would suggest that the velocity of growth of dominant follicles is increased unless ovulation occurs prematurely. A subtle, continuous change in the rate of growth can be attributed to corresponding changes in the pulsatile release of gonadotrophins, though this is a purely hypothetical suggestion and will be difficult to test (p. 89).

During the growth of follicle cells, their secretory activity and trophic requirements become differentiated. Granulosa cells possess receptors for many peptide and polypeptide hormones (FSH, LH, prolactin, growth hormone, LH releasing hormone and epidermal growth factor in rats), steroids (oestrogens, progesterone, glucocorticoids and testosterone), prostaglandins and even adrenergic agents. For normal maturation, they require a combination of extra- and intravascular factors at specific stages in development. A fine balance between oppos-
ing stimulatory and inhibitory factors determines the fate of the follicle, whether it goes on to preovulatory maturation or atresia. One aspect of this balance is illustrated in patients with polycystic ovarian disease (Stein-Leventhal syndrome), in whom low levels of FSH combined with large pulses of plasma LH prevent follicles from developing beyond small antral stages which secrete androgens (Giorgi, 1963; Yen, 1980). Production of oestrogen can be restored by giving supplementary FSH (Erickson et al., 1979). The failure of growing follicles to thrive in postmenopausal ovaries could be due to an inappropriate level or balance of gonadotrophic stimulation (Costoff and Mahesh, 1975). After the menopause, circulating levels of gonadotrophins rise steeply, with FSH becoming the more abundant of the two (see Section 4.4). Experimental studies have shown that excessive amounts of these hormones can suppress follicle cell growth (Rao et al., 1978), oestrogen secretion (Moor, 1974) and even ovulation (Friedrich et al., 1975). In contrast to the situation in women, rodents entering the anovulatory phase of life do not show the same elevation of gonadotrophin levels (Lu et al., 1979) and have a normal succession of follicle stages which terminate in atresia after Graafian follicle maturity has been attained (Gosden et al., 1983b).

3.4. Follicular Involution (Atresia)

Fewer than 0.01% of oocytes in women and 10% of oocytes in mice are ovulated. The remainder degenerate at various stages of follicular development at pre- and postnatal ages. Follicles undergoing degeneration are generally called "atretic", implying closure of the antrum (Gk. a, not; tretos, perforated). Small and medium-sized follicles are described in the same terms when moribund, even though neither of them possesses an antrum and different factors may be responsible for death. The greatest attrition of germ cells occurs prenatally during oogenesis. There are three waves of degeneration in human fetal ovaries involving (1) oogonia during mitosis ("atretic divisions"), (2) pachytene oocytes ("Z" cells), and (3) diplotene oocytes. These reduce the number of germ cells from about 7 million to about 2 million at birth in both ovaries combined, and half of these are morphologically degenerate (Baker, 1963). Germ cells are also lost in fetal and neonatal rodent ovaries (Beaumont and Mandl, 1962). Small, non-growing follicles (stage I) die in large numbers between birth and puberty, with losses continuing throughout adult life in CBA mice (Faddy et al., 1983). The death rate for human primordial follicles has not yet been estimated postnatally, though the data of Block (1952) would suggest that it is substantial.

Whereas preantral follicles at stages II–IV rarely appear degenerate, subsequent stages are highly vulnerable to atresia. Signs of atresia are normally seen first in the follicular epithelium and then in the theca and oocyte (Fig. 3.5). Apoptotic bodies, indicative of cell death, form in the mural population of
The Follicular Store

Fig. 3.5. Ovary of a rat aged 25 days, showing two stages of atresia of large follicles. In (1) the oocyte is still intact, although there are many pyknotic cells in the granulosa layer. At a later stage (2) the antrum is obliterated and the oocyte is fragmented. Haematoxylin and eosin (X125).

granulosa cells; the follicular antrum shrinks and may contain exfoliated cells. These changes seem to alleviate the normal inhibitory effects of granulosa cells on the oocyte, which then resumes meiosis spontaneously and may undergo "pseudocleavage" divisions (Fig. 3.5). Theca cells may hypertrophy and even luteinize in some species. Advanced stages of atresia are reached in 3 days in mice (Byskov, 1974), but the process probably takes much longer in human ovaries, depending on follicle size. There may be vigorous infiltration of medium-sized human follicles by connective tissue to form avascular scars (corpora albicantia) which persist for long periods. When larger follicles become atretic, they may form cysts lined by fibrous, non-glandular cells. In ageing rodents, follicular cysts containing a clear or haemorrhagic fluid appear in small numbers in advance of the anovulatory phase of life, when they are more abundant (Peluso et al., 1979). These cysts are continuously being formed from smaller non-ovulatory follicles, but rapidly undergo atresia (Gosden et al., 1983b).

Cell death occurs in many developing organs besides the ovary (e.g. muscle and spinal cord), but the mechanisms are likely to be organ specific. It has been suggested that cell death in the germ line is a consequence of a lethal load of mutations or errors of chromosome synapsis in some cells. There is, however, no experimental evidence that the bulk of germ cells are lost because of genetic faults. Moreover, oocyte death is not an inexorable process because the rate at
which the follicular store is lost can be retarded in adult mice by hypophysectomy (Jones and Krohn, 1961b; Faddy et al., 1983) and by long-term undernutrition (Nelson et al., 1985). On the basis of hypophysectomy studies, Jones and Krohn (1959) postulated that the pituitary gland produces an atresia-promoting substance, but the effects of these treatments on oocytes are more likely to be nonspecific involving lowered cellular metabolism.

Graafian follicles evidently can be rescued from atresia. They can regenerate in vitro with healthy oocytes (Hay et al., 1979) and are rescued in vivo by supplementary gonadotrophins (Peters et al., 1975; Braw and Tsafriri, 1980b). There are two distinct types of atresia according to whether follicles fail to be selected for ovulation (small antral stages) or fail to be ovulated after having reached preovulatory maturity (larger stages). In the former case, FSH-sensitive aromatase is not activated/induced (McNatty et al., 1979a), whereas, in the latter, follicles lose the aromatase activity they had acquired (Braw and Tsafriri, 1980a; Terranova, 1981). Large follicles are acutely dependent on gonadotrophic stimulation, and atresia follows abruptly after hypophysectomy (Braw et al., 1981). The normal process of atresia could arise from understimulation because of lower plasma hormone levels or redistribution of ovarian blood. Whatever the mechanism, atresia leads to production of oestrogen, declining as production of progesterone rises (Braw and Tsafriri, 1980a; Terranova, 1981), whilst gonadotrophin receptors disappear (Carson et al., 1979; Uilenbroek et al., 1980). Follicle differentiation follows an alternative biosynthetic pathway if the normal steps towards ovulation are not taken, and this leads to reduced responsiveness to trophic stimuli and final limitation of the ability to be rescued.

3.5. Recruitment of Ovulatory Follicles

Many follicles begin to grow during each ovarian cycle, but few emerge as large, ovulable Graafian types. The number of these so-called dominant follicles is a species characteristic and is remarkably constant within individuals, despite variations in cycle length or size of the follicular store. The ovulation rate in animals (i.e. number of ova shed per cycle) is controlled by the global actions of gonadotrophins on the two ovaries. Local interactions between follicles seem unimportant because the distribution of ova shed by a pair of ovaries in polymorphic species is binomial. Experimental support for a global mechanism has been provided by unilateral ovariectomy experiments, beginning with the historically important work of John Hunter (p. 115) and followed by many confirmatory studies of laboratory rodents. After surgery, the remaining ovary compensates for the loss of its partner by ovulating twice its normal quota of ova. This quota is maintained whilst sufficient stocks of follicles are available for recruitment, which is the greater proportion of normal cyclic life (Chatterjee and Greenwald, 1972; Brook et al., 1984). Lipschutz (1927) used such findings in support of his "law of follicular constancy", but he drew the erroneous conclusion that the
number of ovulatory follicles is regulated at the stage when they emerge from the non-growing store. Currently, it is thought that in rats, recruitment of the next set of preovulatory follicles is controlled by the surge of gonadotrophins that are associated with the dismissal of the present set by ovulation (Hirshfield and Midgley, 1978; Hoak and Schwartz, 1980). Thus, selection will take place among the population of small antral follicles, with the excess undergoing atresia.

In human ovaries, the ovulatory follicle is recruited from the population of antral stages at the end of the luteal phase preceding its ovulation (Baird, 1983). At this time there are about 6–30 antral follicles 1–15 mm in diameter in each ovary, but most of them, including all the large ones, are atretic (McNatty, 1982). Healthy antral follicles grow sluggishly, producing mainly androgens because plasma FSH levels are low during this phase (Fig. 3.6). Those that...
develop FSH-sensitive aromatase activity in their granulosa cells will increase production of oestrogen and proceed towards ovulation. Only one or two such follicles exist in toto at the beginning of the follicular phase, others having become atretic. At present, it is not possible to predict which ones are selected. Further growth of healthy follicles depends on the significant elevation of plasma FSH levels at this time. These levels fall during the mid-follicular phase because of feedback effects of follicular oestrogen or the putative non-steroidal inhibitor, inhibin (de Jong and Sharpe, 1976). Since exogenous gonadotrophins given at this time have led to multiple ovulations (Edwards et al., 1972), it is likely that lower endogenous levels of FSH prevent recruitment of supernumerary follicles. Once selected, the dominant follicle seems to go through a "coasting" phase in which its growth is not impeded by lower FSH (Brown, 1978), perhaps because an extensive vascular net in its theca promotes the trapping of gonadotrophins (Zelesnik et al., 1981).

There is clinical evidence to support the view that the number of follicles selected and the time of selection depend on a "window" of rising FSH secretion early in the cycle. Although ovarian responses to gonadotrophin therapy are highly variable in infertile women, a deviation in FSH levels of only 10% in a single individual may alter the number of ovulations (Brown, 1978). On the other hand, attempts to use plasma hormone profiles to predict ovulation rates in sheep have been disappointing, leading to the suspicion that FSH action is modulated by other factors (Scaramuzzi and Radford, 1983). In cynomolgus monkeys, removal of the corpus luteum resulted in early emergence of a new follicle in the opposite ovary in 90% of cases; hence, it has been suggested that gradients of progesterone determine which follicles are recruited (Goodman et al., 1977; Goodman and Hodgen, 1979). At present, there is no such evidence of local effects in women (Nilsson et al., 1982), although the search for modulating factors will continue. Recently, it was reported that human follicular fluid contains proteins that interfere with responses to gonadotrophins (DiZerega et al., 1983), but it is not known whether these proteins are involved physiologically in the selection of dominant follicles.

The number of ovulatory follicles selected during a cycle is affected by ageing. The incidence of dizygotic (but not monozygotic) twins increases with maternal age until about age 37 (Fig. 3.7). This increase is presumed to parallel the incidence of binovular cycles rather than reflect greater fetal survival in utero. A rising incidence of binovular cycles seems to proceed pari passu with shorter follicular phase length, and both might be explained by a rising output of pituitary FSH. This explanation is supported by the established dose relationship between FSH and ovulation rate, though there are few reports that FSH rises in normal women before the fifth decade (Reyes et al., 1977). The alternative explanations that binovular follicles are more common or that follicles are more responsive to FSH during ageing seem improbable.
Parity has less effect than age on the incidence of dizygotic twins (Fig. 3.7), though this too could be explained by FSH action. An effect of parity on ovulation rate is found in animals. The steadily rising number of pups born to mice during the first few litters is attributed to more ovulations rather than less prenatal mortality (MacDowell and Lord, 1925; Kennedy and Kennedy, 1972). The rise in body weight or the change in body composition of the mother resulting from earlier pregnancies could be important factors. For instance, expansion of the distribution volume for hormones might decrease feedback actions of gonadal hormones, or subtle changes in metabolic activity might affect neurosecretion or hormone action. Whilst these mechanisms are entirely conjectural, the phenomenon of "flushing" in sheep is concrete evidence of a link between energy balance/stores and ovarian function (Rattray, 1977). Increased caloric intake during the preovulatory period leads to 10–20% more ovulations per cycle provided that animals are prevented from becoming fat during summer months.

The steeply declining rate of dizygotic twins from age 37 occurs at a time when circulating amounts of FSH are, if anything, rising. This may be explained partly by increased fetal mortality among twin pregnancies of older women or by the waning fertility of the father, but waning ovarian function is likely to be more important. In twin-prone women, declining numbers of follicles available for recruitment will finally limit double ovulations (Bulmer, 1970) and may even bring about earlier menopause (James, 1979). That exceptionally fecund women should cease reproduction earlier may seem paradoxical, but data in rats show...
the number of ovulations is inversely related to the size of the follicular store (Land et al., 1974).

3.6. Follicular Dynamics throughout Life

The major characteristics of ovarian follicular dynamics have been stated already, namely, establishment by the time of birth of a large store of nongrowing follicles from which recruits are drawn at a constant rate into the growing follicle population. This population, in turn, supplies recruits for ovulation, and surplus follicles are eliminated by atresia. The fixed rate at which follicles are initiated into growth helps to account for demographic data which show that the age of menopause is relatively independent of a wide range of physiological and environmental factors, apart from those that actually destroy follicles (see Section 1.5).

The rates of follicle utilization and atresia have been studied in greatest detail in rodents. Two methods have been used. In the first, proliferating granulosa cells are labelled during the S-phase of the cell cycle with tritiated thymidine and analysed by autoradiography (Fig. 3.8). The rates of transit between successive

Fig. 3.8. Autoradiograph of an adult mouse ovary 1 hr after injecting tritiated thymidine. All multilaminar follicles are labelled by silver grains which indicate that follicle growth, once initiated, is continuous without interruption until the follicles either ovulate or become atretic. Haemalum and eosin (X125).
Fig. 3.9. Variations in the number of follicles of differing sizes (stages I–V+) in non-pregnant CBA mice between birth and senility. Data for virgin (x) and ex-breeder mice (®) are presented. Means ± SE have been estimated using a mathematical model. (From Faddy et al., 1983; originally published in the J. Endocrinol.)

Follicle stages can then be estimated from measurements of granulosa cell numbers and cell cycle time because once growth has been initiated there is no further resting stage (Pedersen, 1970). In the second method, the number of follicles at each stage of development (I–VI) is counted in histological sections. Rates of follicle growth and atresia can then be estimated at all ages using a mathematical model (Faddy et al., 1976). Data obtained by Jones and Krohn (1961a) have been fitted to this model (see Fig. 3.9 and Table 3.1 with Fig. 3.4 for the classification of follicle stages). At birth, about 9400 stage I (primordial) follicles are present in a pair of ovaries, no other stages being present yet (Faddy et al., 1983). Soon afterwards, a proportion of these follicles begins to move
Fig. 3.9. (continued)
through successive stages of development. The number present in each category is determined by stage- and age-specific growth and death rates in the precursor pool and are described by Poisson statistics (Table 3.1). More than half of the original stock of follicles is lost during the first 20 days of life. Since fewer than 10% of those leaving the non-growing store (437 our of 5059) reach stage II, more than 90% must have died. Differences between the number of follicles leaving and entering successive stages (see columns) are small (Table 3.1). Most of those reaching stage II proceed to become large preantral and antral follicles (V+), although they fail to mature and ovulate at prepubertal ages. The high death rate among small follicles before puberty (<5 weeks) prevents an explosive increase in the number of follicles in the growing population. After-
TABLE 3.1

Mean Number of Follicles (from the Fitted Model) in CBA Mouse Ovaries Leaving and Entering Five Groups (Primordial to Graafian) at Specified Ages throughout Lifea

<table>
<thead>
<tr>
<th>Age span (days)</th>
<th>Movement of follicles from and to successive groups</th>
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...wards, the death rate falls to produce the characteristic biphasic distribution of follicles of different stages (Fig. 3.9). In the CBA strain, continuing death in the primordial stages is responsible for the loss of more follicles from the store at all ages than are accounted for by those initiating growth. This explains why the ovary is depleted of oocytes at little more than 1 year of age, with early disappearance of ovarian cycles at 10–11 months. Since small follicles are lost continuously throughout life, all animals will eventually become sterile if their survival is prolonged. Therefore it is not surprising that neither pregnancy (Fig. 3.9) nor unilateral ovariectomy (Baker et al., 1980) has much effect on the number of small follicles remaining at advanced ages.

Far more follicles enter the growing population at most ages than are required for ovulation. In CBA mice, the number of follicles at each stage of growth reaches peak values during adult life at about 100 days (Fig. 3.9). These enable the ovary to superovulate when stimulated by supplementary gonadotrophins following removal of the contralateral ovary or treatment with exogenous gonadotrophins. The subsequent decline in the number of growing follicles is a
reflection of fewer being recruited from a dwindling store. Only 21 follicles entered the preovulatory stages V and VI at 330–360 days of age, whereas there were 10 times as many at ages 60–90 days (Table 3.1). Thus, the ability of the ovary to superovulate is expected to fall steadily during ageing, and this has been confirmed experimentally (Beatty, 1958; R. G. Gosden, unpublished). Unfortunately, present mathematical models cannot provide precise estimates of the dynamics of antral follicles because these follicles are fewer in number and fluctuate during the cycle. The number attaining preovulatory maturity is maintained in CBA mice until 300 days of age, when the natural ovulation rate begins to fall. The ability to sustain or increase the ovulation rate appears to depend on a reduction in the proportionate incidence of atresia in medium-sized follicles rather than on the recruitment of more follicles from the store (Gosden et al., 1983a).

Few workers have attempted a differential count of the number of follicles in adult human ovaries, the only substantial data having been obtained from cadavers by Block (1952). The reasons for the limited information on this important subject are plain. Premenopausal ovaries (if obtainable) can yield 2000 paraffin sections of 10-μm thickness, requiring weeks of laborious microscopy to obtain reliable follicle counts in just one ovary. Despite limitations of the data (viz. sampling every 200th section and no estimates of atresia), Block’s work seems to establish that numbers of primordial follicles fall continuously during adult life (Fig. 3.1). Their distribution by age would be expected to be an exponential or double exponential, as in rodents, but closer inspection of the data shows that a quadratic function provides a marginally better fit for human primordial follicles ($P = .065$). In view of the limited data, this conclusion must be tentative but, if confirmed, would establish that these follicles are lost at an increasing rate during ageing.

Despite the sampling errors which occur in most quantitative studies of the follicular population, the remarkable variations in primordial follicle numbers in individuals of the same age probably reflect real differences. Since the numbers at a given stage and age can vary as much as twofold in syngeneic mice (Fig. 3.9), biological differences have environmental causes, and these are likely to operate prenatally during oogenesis. Such variation in data adds further uncertainty to the already hazardous procedure of extrapolation. Nevertheless, if Block’s data are extrapolated to the median age of menopause of 50 in contemporary Swedes (Table 1.2), only a few hundreds or thousands of primordial follicles evidently remain. This suggests that primary ovarian failure causes the final cessation of menses in mid-life and the inability of postmenopausal ovaries to respond to gonadotrophins. Irregular menstrual cycles preceding the menopause can be attributed to irregular recruitment of multilaminar follicles, which decline in number in the fifth decade of life (Block, 1952). However, only after the
majority of small follicles have been lost (at about 36 years of age) does the menstrual cycle become most stable and regular (Treloar et al., 1967; Vollman, 1977; Fig. 3.10). Thus, age-related variations in the timing of the cycle probably depend to some extent on factors which are independent of the diminishing follicular store.

3.7. Precocious Ovarian Failure (Precocious Menopause)

Primary, irreversible ovarian failure occurs before middle age in some individuals, but the biological effects are essentially independent of age, except in

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Fig. 3.10. Representative section of the ovarian cortex of a 34-year-old woman, showing that small follicles are sparse by this age. Graafian stages (F) are present. Haematoxylin and eosin (X50).
children, in whom there are additional effects on adolescent growth and matur-
tation. This condition is diagnosed when acyclic women have plasma levels of
FSH exceeding 40–50 mIU/ml, a sign of an inactive negative feedback pathway
from the ovary (Goldenberg et al., 1973). This shibboleth is not, however,
entirely reliable and the possibility of pregnancy should not be ruled out (Rebar
et al., 1982). The limitations of present definitions could be overcome if more
stringent criteria were adopted (e.g. by extending the duration of amenorrhea),
but this would be at the expense of lost time in providing hormone replacement
therapy. The best criterion of menopause would be complete absence of ovarian
follicles in serial histological sections, but quantitative studies of this kind are
impractical on a routine basis and biopsy material is not always available (see
Section 3.6).

The follicular store may become depleted at any age after it is formed. There
is, however, no agreed-on lower normal age limit for the menopause, and so
widely differing and arbitrary limits have been used, ranging from 30 to 45
years. This unsatisfactory state of affairs is likely to continue because of the
difficulties of estimating menopausal age in the normal population (see Section
1.5). Thus, there are no reliable estimates of the frequency of precocious meno-
pause in any population. Even if data became available, they could not be
applied universally; if they could, by Western standards, many poor women in
parts of the developing world might be described as having precocious meno-
pause.

Cases of precocious menopause can be divided into two categories according
to whether or not substantial numbers of follicles remain. In many cases, there is
evidence that ovaries are afollicular, and a number of factors can account for this
condition.

Genetic Factors. A substantial proportion of all women presenting with pre-
ocious ovarian failure have an abnormal karyotype, hence the term “chro-
mosomally incompetent ovarian failure” (Tho and McDonough, 1982). The
most familiar cases of ovarian dysgenesis occur among women with X-chromo-
some monosomy (Turner's syndrome) or XX/XO mosaicism. The histological
appearance of ovaries in these individuals suggests that a primordial germ cell
population is established but fails to thrive. The timing of the loss of germ cells is
highly variable. Anovular streak gonads are common at pre- and postnatal ages
among XO individuals (Carr et al., 1968), but some ovaries have a population of
oocytes of normal appearance at full term which, in rare cases, provide an
opportunity for pregnancy before an early menopause (Reyes et al., 1976; King
et al., 1978). Oogonia and oocytes might fail to thrive either because of the
generalized debility of some fetuses or because of gene dosage effects. Unlike
somatic cells, in which only one member of each pair of X chromosomes is
genetically active, both sex chromosomes of 46,XX fetal oocytes are normally
active (Gartler et al., 1973) following reactivation shortly before meiosis (Monk and McLaren, 1981). Since single chromosomes do not compensate for the loss of their opposite number, there is a deficiency of gene products in oocytes of XO fetuses. Further progress in this field might be gained by the study of XO mice for, although they are more fertile than XO women, they too have fewer oocytes than do normal siblings, and consequently their reproductive lifespan closes prematurely (Lyon and Hawker, 1973; Burgoyne and Baker, 1981a). Apart from runts, XO mouse fetuses establish normal numbers of germ cells and the deficiencies of postnatal life have been traced to an excess of "Z" cells at the end of gestation (Burgoyne and Baker, 1981b).

Trisomy affects the follicle reserve in different ways depending on whether sex chromosomes or autosomes are involved. Most women presenting the X chromosome in triplicate have normal phenotype and fertility, although ovarian failure occurs early in a few of them (Villanueva and Rebar, 1983). In contrast, trisomies of chromosomes 18 (Edward's syndrome) and 21 (Down's syndrome) are associated with reduced numbers of both non-growing and growing follicles (Russell and Altschuler, 1975; Højager et al., 1978). Other types of autosomal trisomy probably effect gonadal development, but such fetuses are not viable anyway.

Most cases of streak gonads occur sporadically in the population but familial transmission is seen in a number of rare conditions, especially 46,XY and 46,XX gonadal dysgenesis (German et al., 1978; Simpson, 1983). It is difficult to establish whether specific genes cause precocious ovarian failure because opportunities for studying them are rare and there are additional practical and ethical constraints. However, on the basis of a few favourable pedigrees it has been possible to obtain evidence that premature ovarian failure can be heritable, transmission occurring via either maternal or paternal relatives and probably involving an autosomal dominant factor (Mattison et al., 1984). In mice, heritable factors clearly influence the size and rate of utilization of the follicular store, though differences between inbred strains almost certainly have a polygenic basis (Jones and Krohn, 1961a). Some gene mutations in mice having pleiotrophic effects cause ovarian dysgenesis. In the Steel mutant (SL) a streak gonad forms when primordial germ cells fail to proliferate (McCoshen, 1982), and mutant alleles of the W series are generally associated with germ cell hypoplasia and sterility (Mintz and Russell, 1957; Murphy, 1972).

**Immunological Factors.** Autoimmune phenomena may lead to premature sterility in rodents as a result of total destruction of germ cells. Such damage occurs when neonatal mice are thymectomized (Michael et al., 1981; Nishizuka et al., 1981), which may explain why mutant mice (nu,nu), which are congenitally deficient in T cells, are relatively infertile. It is interesting that ovarian
hypoplasia also occurs in children with ataxia telangiectasia in whom thymus tissue is also deficient or absent (Miller and Chatten, 1967).

A substantial proportion of women having premature menopause possess circulating anti-ovarian antibodies though signs of infiltration of the ovary by mononuclear cells is required to establish the presence of autoimmune destructive activity (Coulam and Ryan, 1979). In some cases, evidence of such damage coexists with autoimmune disease involving other endocrine glands with clinical expressions of Addison’s disease, hypoparathyroidism and Hashimoto’s thyroiditis (de Moraes-Ruehsen et al., 1972; Vasquez and Kenny, 1973). The aetiology of this complex of diseases is poorly understood. Autosensitization to common antigens expressed on cell surfaces may explain why several glands are involved simultaneously, although T cell function might also be impaired (Mathur et al., 1980a). Sometimes chronic yeast infections of the lower genital tract (Candida sp.) lead to menstrual irregularity and even ovarian failure, apparently as a consequence of autoimmune damage (Mathur et al., 1980b). The combination of these problems can also be explained by common antigenic epitopes in ovarian cells and the microorganism.

**Cytotoxic Drugs.** Clinical use of drugs in the treatment of cancer, rheumatoid arthritis and other serious diseases frequently leads to temporary cessation of menses. If treatment is prolonged, destruction of follicles may result in earlier menopause (Rose and Davis, 1977). These effects are obviously more important for young women and children than for the majority of patients at risk, who are much older (Warne et al., 1973; Himelstein-Braw et al., 1977, 1978). Damage can be inflicted inadvertently on the fetal gonad since alkylating agents such as busulphan are transmitted across the placenta following maternal administration (Diamond et al., 1960). Damage to proliferating primordial germ cells may be significant later in life because they have only a limited ability to undergo compensatory growth after withdrawal of treatment (Tam and Snow, 1981), and destruction of germ cells after oogenesis is serious because they are irreplaceable.

**Ionizing Radiations.** Extensive studies have been made of the cellular and genetic effects on the ovary of X and γ rays because these can penetrate deeply into tissues. Cell-killing activity varies with age, species and the manner of administration (single dose or fractionated). In rodent fetuses, the oogonal stage is the most radio-sensitive of all stages in oogenesis, although sensitivity rises again in early dictyotene when follicles are forming. Primordial follicles in rats and mice are among the most sensitive groups of cells. Fortunately, those of monkeys and women are far less vulnerable to irradiation, probably because of differing chromosome configurations and metabolism (Baker, 1971; Baker and Neal, 1977). Multi-layered follicles of all species studied so far are vulnerable to
irradiation because of their mitotic activity. Therapeutic exposure of the pelvic region in women can lead to amenorrhoea, but this is usually temporary if the dose is <400 R (fractionated). The outcome will vary according to age, with the probability of resuming cycles after treatment falling in older women.

Low-energy radiation is of little biological significance unless the isotope is actually incorporated into ovarian cells. Tritated nucleosides are especially hazardous for proliferating germ cells since most of the energy is dissipated within the nucleus (Baker and McLaren, 1973).

Other Disease Effects. Irreversible damage of the ovary during the course of disease is usually iatrogenic, although there are minor exceptions. Some infiltrative diseases (e.g. tuberculosis) have serious effects on the genital tract with involvement of the ovary, but permanent loss of ovulatory function is rare. Viral diseases (e.g. mumps) can cause oophoritis, which may be a more extensive problem than rare case reports would suggest (Morrison et al., 1975).

Non-infectious diseases, in addition to those mentioned above in the context of genetics and immunology, have profound effects on fecundity. Where there is a deficiency of 17α-hydroxylase, androgen and oestrogen production falls and pregnenolone and progesterone are metabolized by an alternative pathway, leading to excess production of mineralocorticoids. This rare disease presents symptoms of sexual immaturity and primary amenorrhoea, with castrate levels of gonadotrophins and oestrogen (Goldsmith et al., 1967). Ovarian failure is therefore due to a lack of oestrogen and a consequent inability of follicles to grow rather than to early depletion of the follicular store. Hence, these ovaries resemble more closely those of individuals with hypopituitarism or with the so-called resistant ovary syndrome, in which follicles are unresponsive to FSH (Jones and de Moraes-Ruehsen, 1969). On the other hand, precocious menopause caused by the congenital disorder of metabolism, galactosaemia, (Kaufman et al., 1981) may be due to formation of a smaller follicular store because galactose or its metabolites have toxic effects on germ cells in the developing ovary of animal models (Chen et al., 1981). This would explain why early menstrual failure is not overcome by feeding patients a galactose-restricted diet from infancy. Possible effects of inadequate diets on the age of menopause in normal women have been discussed elsewhere (see Section 1.5).

Blindness is thought to advance the timing of menarche, and there is now tentative evidence that it also leads to later menopause (Lehrer, 1981). Such results have been attributed to the activity of the pineal gland, which is affected by photoperiod, but there is little physiological evidence at present to support the claim that it affects the length of menstrual life.

Alcoholism. Chronic alcoholism has complex effects on nutrition and metabolism. It causes hypogonadism and infertility in both sexes (van Thiel and
The Follicular Store

Lester, 1976), but it is not known whether gonadal injury is permanent or reversible, and practical difficulties of working with alcoholic patients hamper further study. In adult rats, ethanol inhibits follicular growth and luteinization (van Thiel et al., 1978), but it remains unknown whether the size of the non-growing store of follicles is reduced. Of greater concern is the unanswered question of whether alcohol consumed during pregnancy causes lasting damage to the fetal gonad.

Partial Ovariectomy (Oophorectomy). Since oocytes cannot be replaced after birth, removal of any ovarian tissue potentially reduces the remaining period of fecundity. It has been postulated that the dynamics of ovarian follicle growth initiation are stochastic. This would imply that substantial amounts of tissue would have to be lost in order to cause an obvious advance of menopausal age, and this can be illustrated by a simple example. If it is assumed that the normal age for complete depletion of ovarian follicles in a woman is 60 years, then hemi-ovariectomy at birth would advance the time by 30 years according to a linear model of follicle utilization, but by only 5 years according to an exponential model. This hypothesis has not been verified in any primate species, but supporting evidence is provided by longitudinal studies of unilaterally ovariecomtomized CBA mice (Brook et al., 1984). Removal of one ovary at 50 days of age resulted in complete cessation of cycles by 330 days in half the population, whereas this point was reached 40 days later in controls with both ovaries present.

The dynamics of follicle utilization would also serve to minimize differences in menopausal age, despite considerable natural variation in the size of the follicle population. Nevertheless, the present policy of conserving healthy ovaries during gynaecological surgery should not be relaxed since even a small advance in age of menopause is usually undesirable.

3.8. Anatomy of the Postmenopausal Ovary

Follicular deficiency is the most striking morphological feature of the human ovary after menopause and has profound importance for ovarian physiology and pathology. As mentioned earlier, the ovary is not completely afollicular at the time of menopause, although it may eventually reach this state. The postmenopausal ovary is composed mainly of "stroma", which is a collective term for supporting (connective tissue) cells, contractile cells and interstitial cells. The interstitium is widely thought to be derived from theca cells that return to the stromal population after follicular atresia. Besides stroma, the only other bulky materials in postmenopausal ovaries are large fibrous bodies which are relics of corpora lutea of the final menstrual years (Fig. 3.11). The walls of
ovarian arteries, as elsewhere in the genital tract, become thicker and calcified during ageing, particularly in multiparous women (Fig. 3.12). In middle age, there is surface folding of the organ because atrophic changes within are not matched by corresponding dynamic changes of the ovarian epithelium. Later, the ovary shrinks to a small, pigmented structure, but is not entirely without endocrine activity (see Section 2.3).

There is a striking contrast of appearance between ovaries of postmenopausal women and aged sub-human primates on the one hand and those of acyclic rodents of middle age on the other. Apart from some mutants and inbred strains, most rodents still possess some ovarian follicles in extreme old age. At this time the rodent ovary is dominated by interstitial tissue and Graafian follicles, which undergo cystic enlargement ("overripening") rather than ovulation (Fig. 3.13). In later life, long after fertility is lost, these follicles may ovulate and luteinize to form functional corpora lutea of pseudopregnancy (p. 71). Rodent ovaries do not accumulate large residual fibrous structures, nor do their blood vessels undergo the same degree of sclerosis as in primates. Their interstitium may contain cells in which nuclear chromatin is arranged radially ("wheel cells"). Although this condition needs re-evaluation in the light of recent hormone assays, these cells have been interpreted as evidence of LH deficiency, since similar cells are seen

Fig. 3.11. Perimenopausal human ovary (age 51 years), showing abundant stromal tissue and two corpora albicantia (C). One cystic (atretic?) follicle (F) is present. There are no small follicles in this section. Haematoxylin and eosin (X5.5).
Fig. 3.12. Postmenopausal human ovary at low magnification. Blood vessel walls are sclerotic; stromal cells are abundant and interspersed with fibrous remnants of atretic follicles and corpora lutea. Haematoxylin and eosin (X50).

Fig. 3.13. Anovulatory ovary of a 16-month-old C57BL/6J mouse. There are few small or medium-sized follicles and no corpora lutea. Two cystic (anovulatory) follicles are present. Haematoxylin and eosin (X70).
in hypophysectomized animals and a normal pattern of chromatin can be restored by gonadotrophins (Crumeyrolle-Arias et al., 1976). Other cells within the stroma contain abundant lipochrome pigments which are responsible for the yellow-brown hue of ageing rodent ovaries. These pigments, which are found in primate ovaries to a lesser extent, probably represent indigestible residues of autolysis and phagocytosis of follicular and luteal tissue.

Pathological changes in ageing ovaries depend less on chronological age than on the time when follicles are depleted. Such changes appear precociously when animal ovaries become sterile following neonatal thymectomy (Michael et al., 1981) and ionizing irradiation (Peters, 1969), as well as in mutant and inbred strains characterized by early loss of oocytes (Murphy, 1972; Thung, 1961). These changes involve cellular proliferation as well as atrophy. Thung (1961) suggested that the former is due to hypergonadotrophism, but there are likely to be local contributing factors (e.g. rising androgen:oestrogen ratio) because pathological changes do not emerge evenly in the gland but begin at sites that are first depleted of follicles (unpublished observations). Stroma cell hyperplasia and hilus cell prominence in human ovaries are usually attributed to stimulation by postmenopausal levels of gonadotrophins (Sternberg, 1949). In mice, the surface epithelium grows inwards to mingle with the ovarian rete and form a complex internal structure of “testis-like” tubules, epithelial cords and “anovular follicles”. A peculiar form of follicular atresia may give rise to similar structures in rats (Crumeyrolle-Arias and Aschheim, 1981). In primates, ovarian epithelia tend to proliferate for a relatively short period, and they tend to form papillary projections (Graham et al., 1979). These differing patterns of proliferative activity in rodent and primate ovaries might therefore explain the higher frequency of tubular adenomata and granulosa cell tumours in the former group.
The Ovarian Clock

4.1. Early Concepts of the Menstrual Cycle

Periodic menstrual activity has fascinated humans since antiquity. Frequently it has acquired special cultural significance in religious ritual and superstition (Frazer, 1935; Lévi-Strauss, 1966). Among other notions, it was thought to be responsible for eliminating toxins accumulating in blood, which seemed to explain the associated pain, odour and appearance of the menstrual flow. This ancient view can be traced back to Hippocrates and was upheld subsequently by Aristotle, Galen and Pliny, remaining popular in Europe even into the nineteenth century. Early scientific speculation in the first half of that century was equally faulty. Too much attention was paid to the similarity in the length of the lunar cycle and the "typical" menstrual cycle (28 days). Indeed, the word "menses" is derived from this connection (L. mensis: month).

The first experimental evidence for a role of ovaries in the menstrual cycle is attributed to Percival Pott, a London surgeon, who reported that removal of both ovaries in a young woman for correction of "ovarian herniae" resulted in atrophy of her breasts and cessation of menstruation (Pott, 1775). Pott did not comment on the physiological significance of his findings, which went unrecognized for nearly 40 years. John B. Davidge, an American student of medicine in Edinburgh, learned of this case. His observation that loss of much larger quantities of blood by venesection than by menstruation did not interfere with menstrual flow led him to conclude that the latter "is attributable to a peculiar condition of the ovaries serving as a source of excitement to the vessels of the womb, rather than to the doctrine of repletion of the body" (Davidge, 1814). Despite this astute conclusion, the traditional views persisted for many years, and the ovary was not recognized as an organ of internal secretion until the end of the nineteenth century.

The foundations of present knowledge concerning the timing of ovulation and the cyclical fluctuations of ovarian hormones were laid in the 1930s, a golden decade of discoveries in reproductive physiology and biochemistry. Later, the neuroendocrine mechanisms controlling the cycle were studied in great detail in rats. Whilst information about primates was scarce, there was little reason to
doubt the general validity of the rat model except, of course, for the peculiar phenomenon of menstruation itself. But recent research casts doubt on this assumption. Some background information on the differing physiological mechanisms is therefore essential before proceeding to describe and explain the effects of ageing on the ovarian cycles of women and animals.

4.2. Physiological Regulation of Ovarian Cycles

During the 1920s there was a growing appreciation of the importance of feedback mechanisms in physiology. Their greatest proponent was W. B. Cannon, who coined the term “homeostasis” and broadcast the new concepts in his influential book The Wisdom of the Body (1932). Reproductive biologists grasped the implications for their own field, and it was soon recognised that gonadal function might be controlled by feedback interactions with the pituitary gland (Moore and Price, 1932). At an early stage the hypothalamus was suspected to be a key structure in reproduction, containing a “sex centre” to mediate actions of sex steroids on the underlying pituitary gland (Hohlweg and Junkmann, 1932). During the subsequent 50 years, a major goal of reproduction research has been to elucidate the neuroendocrine control mechanisms and identify pathways of communication between the brain and gland. Luteinizing hormone-releasing hormone (LHRH, also known as GnRH) was isolated from hypothalamic extracts and characterized in 1971 (Matsuo et al., 1971), the same year as Harris reviewed the early history of the field in the prestigious Dale Lecture (Harris, 1972). The neuropeptide LHRH is a key substance for reproduction because, as Harris had postulated much earlier, it is the signal by which information processed by the brain is passed to the pituitary gland.

A number of studies showed that the ovulatory surge of LH (and FSH) in rats is elicited by neural activity associated with the internal circadian rhythm. That a cerebral clock was responsible for timing ovulation was shown clearly by the inhibitory action of barbiturate administration during a “critical period” on the afternoon before oestrus (Everett and Sawyer, 1950). Continuous treatment of ovariectomized rats with oestradiol elicited ovulatory-type spikes of plasma LH at the same time on successive afternoons (Legan and Karsch, 1975). Therefore, the neural signal is a daily event and requires oestrogen for expression at the pituitary level. The signal triggers the release of LHRH from nerve terminals in the median eminence of the hypothalamus to bring about the ovulatory surge of pituitary gonadotrophins (Sarkar et al., 1976; Levine and Ramirez, 1982). However, the magnitude of the increase of LHRH in pituitary portal blood during the critical period is insufficient to explain the entire output of pituitary hormones, which must also depend upon rising sensitivity of gonadotrophs to LHRH (Aiyer et al., 1974).
Physiological Regulation of Ovarian Cycles

The timing of the critical period, which precedes ovulation by about 12 hr, is obviously an adaptation in this nocturnal species. The pacemaker is located in the rostral hypothalamus close to the suprachiasmatic nucleus, from which many circadian rhythms emanate. This location was demonstrated by the ability of bilateral lesions at the caudal margin of the optic chiasm to inhibit ovulatory surges of gonadotrophins. Basal hormone levels and follicular maturation remained unaffected by the lesions (Szentágothai et al., 1962; Blake et al., 1972). These results imply that gonadotrophin release in rats is under dual hypothalamic control. A rostral area is responsible for “cyclic” release whereas a medial basal area (the “hypophysiotrophic area”) maintains “tonic” secretion in the absence of extrinsic neural inputs. The stimulatory or so-called positive feedback effects of oestradiol are exerted at the rostral site (Goodman, 1978) but require neuronal connections with the basal hypothalamus for releasing LH (Tejasen and Everett, 1967).

General concepts of hormonal feedback in the ovarian cycle apply equally in rodents and primates (Fig. 2.3), but the hierarchical organization of glandular control mechanisms are different. Despite continuing controversy, recent evidence provides strong indications that the rhesus monkey ovary provides its own timing mechanism for the events of the menstrual cycle, rather than depending on a circadian rhythm generator in the brain. Neither the blood levels of gonadotrophins nor the hourly frequency (“circhoral”) with which they rise were affected by surgical disconnection of nerves projecting to the medial basal hypothalamus (Krey et al., 1975). In contrast to the aforementioned effects of these lesions in rats, the normal response of the primate pituitary gland to positive feedback effects of oestradiol was unaffected. These findings explain why the timing of the mid-cycle surge of gonadotrophins in primates is less obviously dependent on a circadian rhythm and why it is not interrupted by barbiturate anaesthesia. The emergent concept from these discoveries is that the ovary is the “clock” for the menstrual cycle, each phase being determined by the time taken to build an ovulatory follicle or destroy a corpus luteum (Knobil, 1974). This mechanism is in striking contrast to the cerebral clock of rats, which is coupled to the photoperiodic cycle (Everett, 1974).

Despite this progress towards understanding the physiology of the menstrual cycle, some difficulties remain. If the hypothalamus simply “drips” LHRH onto the underlying pituitary gland, then a role for the brain in processing interoceptive and exteroceptive inputs is abrogated. This apparent theoretical obstacle is diminished if the magnitude or frequency of LHRH pulses can be modulated (see later). Of even greater concern is the conflicting experimental finding of another highly respected research group. Norman et al. (1976), using a different technique for isolating the basal hypothalamus in monkeys, found that disruption of pathways from the preoptic area–anterior hypothalamus abolished ovulatory function and feedback control. However, Ferin et al. (1977) obtained evidence
supporting the original findings, which have been extended by Knobil’s group using a different approach. To identify the site of oestradiol feedback, the latter group abolished pituitary stimulation by endogenous LHRH by producing radio-frequency lesions in the hypothalamic arcuate nucleus. After treatment, it was necessary to infuse LHRH in pulses at hourly intervals in order to maintain physiological levels of gonadotrophins and pituitary responsiveness to oestrogen (Fig. 4.1). These pulses are thought to mimic the natural rhythm of neurosecr-

![Graph showing LH and E2 levels](image)

**Fig. 4.1.** Experimental study of the role of LHRH and gonadal steroids in the control of the menstrual cycle in rhesus monkeys. See text for details. EB, oestradiol benzoate; Ovex, bilateral ovariectomy; Lesion, hypothalamic lesion to disrupt secretion of endogenous LHRH. [Reprinted with permission from E. Knobil, T. M. Plant, L. Wildt, P. E. Belchetz and G. Marshall (1980), Control of the rhesus monkey menstrual cycle: Permissive role of hypothalamic gonadotropin-releasing hormone, Science 207, 1371–1373; Copyright 1980 by the AAAS.]
tion which is responsible for oscillation of pituitary hormone release (Clarke and Cummins, 1982). This infusion maintained follicular maturation and ovulation, as witnessed by successive increases of endogenous oestradiol and progesterone. Two major discharges of gonadotrophins occurred at an interval of about 1 month. This decisively proves that normal cyclic activity and feedback relationships in monkeys do not require mediation by release of hypothalamic LHRH. It is therefore no longer surprising that oestradiol elicits a surge of gonadotrophins when endogenous LHRH is neutralized with antibodies (McCormack et al., 1977; Fraser et al., 1984). Oestradiol can exert its actions as much as 48 hr after eliminating LHRH; thus, it too must be a gonadotrophin-releasing hormone (Wildt et al., 1981a).

Details concerning the control of ovarian cycles are available for few species, yet it is clear already that talk of "animal models" is hazardous. Ovarian cycles in ageing rodents can (and do) cease for reasons which differ from those in primates because they are controlled by different physiological mechanisms. Knowledge of the human menstrual cycle is bound to depend heavily on animal research, and the rhesus monkey provides the best available model at present. Cycles in the two species are similar in terms of phase lengths and circulating hormone profiles, but some differences exist (e.g. absence of a luteal phase oestrogen peak in monkeys) (Knobil, 1974). Clinical studies have shown that infusion of LHRH or its analogues in pulses every 60–120 min is sufficient to restore ovulation in patients with hypogonadotrophic amenorrhoea (Yen, 1983). If hypothalamic function is "permissive" as far as the control of human ovulation is concerned, we gain a new perspective in our understanding of events leading towards the menopause.

4.3. Patterns of Cyclical Activity throughout Life

Laboratory Rodents

Rodents' short lifespan and low cost, as well as the ease of recording their cycles by the vaginal smear technique, make them ideal subjects for study of the effects of ageing. Several longitudinal studies of ovarian cycles in rodents have been published.

The most comprehensive study involved inbred mice of the C57BL/6J strain aged 3–18 months (Nelson et al., 1982). Three phases of the lifespan were delineated according to the regularity of oestrous activity: I: an initial phase of relatively infrequent and irregular cycles in which cycle frequency increased linearly with age; II: a phase of maximum cycle frequency and stability; III: a phase of steadily declining cycle frequency (Fig. 4.2). The transition time between these phases varied within the ranges 3.5–4.5 and 10.5–13.5 months in
different cohorts of animals. Cycle lengths were shortest during phase II, but they were not precise reciprocals of cycle frequency because the latter is a global measurement, including individuals that stop cycling as well as those with genuine changes of cycle length. The normal oestrous cycle of young rodents lasts only 4 or 5 days because corpora lutea are inactive unless mating has occurred. Surprisingly, there is less uniformity amongst mice than amongst rats and "strings" of short cycles of the same length are not particularly common, despite genetic uniformity in inbred mice. The frequency of consecutive pairs of 4- and 5-day cycles in C57BL/6J mice was maximal for a short time in phase II. The longer cycles of the other phases resulted from additional days of either vaginal cornification or leucocytosis, reflecting different underlying endocrine states.

Formerly, it was thought that oestrous cycles in rodents continue into extreme old age, but many strains have now been shown to become acyclical in mid-life, at 12–18 months. This difference is probably attributable to the combined effects of (1) the greater tendency today to use inbred strains in which reproductive life is shorter and (2) better husbandry conditions which prolong survival. The C57BL/6J strain has a long postreproductive phase of life. Animals become
Patterns of Cyclical Activity throughout Life

acyclical at 13–16 months of age, although ovulation is still a possibility and the oocytes continue to produce moderate amounts of oestrogen acyclically for several more months. In contrast, healthy males of the same strain maintain production of testosterone into extreme old age (Nelson et al., 1975).

Oestrous cycles in rats follow patterns similar to those in mice. Cycle length is extended during ageing and days of vaginal cornification become more frequent, eventually replacing the normal cyclic pattern (Huang and Meites, 1975). In virgin rats of the now extinct inbred DA strain, persistent vaginal cornification (“persistent oestrus”) began at only 4–5 months (Everett, 1939), but in most outbred animals it occurs at 6–12 months (Nelson et al., 1982) and is slightly later in multiparous compared to virgin animals (Nass et al., 1982). Persistent oestrus in rats frequently lasts for months and is followed by a phase of repetitive pseudopregnancy-like cycles and, finally, anoestrous (Bloch and Flury, 1959; Huang and Meites, 1975; Aschheim, 1976; Lu et al., 1979). Spontaneous restoration of ovarian cycles, albeit infertile ones, following a prolonged anovulatory phase is not paralleled in women nor is it found in some strains of mice which are anoestrous for the greater part of postreproductive life (e.g. CBA, C57BL/6J).

Human and Other Primates

Cycle Length

The first carefully documented record of the frequency of human menses was published by Clos (1858). Like other workers in the nineteenth century, he was preoccupied with the supposed association with phases of the moon. His data are of interest today only because they portend the results of modern large-scale studies of the effects of ageing.

In 1967, Treloar et al. published a major longitudinal study of menstrual histories between adolescence and menopause. This monumental work involved Caucasian women enrolled at the University of Minnesota, who recorded personal details of vaginal bleeding, contraception, reproductive events and medical history continuously over many years. The project began in 1934 and progressively involved about 5000 women. Concurrently, Vollman (1977) was collating similar records of 691 Swiss women attending a general practice together with additional information about the frequency of ovulatory cycles estimated from nonspecific criteria, viz. rectal temperature, cervical mucorrhoea and intermenstrual pain. The results of these two independent studies are remarkably consistent.

When the average length of menstrual cycles was plotted against either chronological or gynaecological age (i.e. from menarche), J- or U-shaped curves were obtained (Fig. 4.3). The distribution of cycle lengths was skewed, and median values, which were lower than means, were more meaningful descriptions of
The Ovarian Clock

Fig. 4.3. Variation in the length of menstrual cycle from menarche (age 0) to menopause. A total of 30,261 cycles were reported by 594 women. (From Vollman, 1977; reprinted with permission.)
central tendency. In the American study, the distributions were divided into three zones (postmenarcheal, middle and premenopausal) for the entire menstrual span of 36 years (Treloar et al., 1967). Throughout this span the celebrated 28-day cycle tends to be modal at most ages (Fig. 4.4). Variation of cycle length is more striking when plotted according to gynaecological rather than chronological age, perhaps because values begin at a uniform stage of biological maturation. Beginning at a median of 29 days at menarche, cycle length descends steadily to 26 days at age 40 in Swiss women (gynaecological ages quoted) (Fig. 4.3). Mean values fall steeply from 35 to 30 days between ages 0 and 4. Subsequent descent is more gradual until a nadir of 27 days is reached at 29 years, this decrease being due exclusively to shorter follicular phases. Finally, there is a steep ascent to 44 days at the close of menstrual life. The frequency distribution of cycles of specified length is both complex and continuous (Fig. 4.4). During the postmenarcheal zone, cycles >29 days long tend to decline in frequency, whilst shorter cycles are steady. The pattern changes at 7 years (about 21–22 years chronologically) when short cycles (<24 days) are replaced abruptly by 25- to 28-day cycles. Further changes are seen from 30 years (or about 45 chronologically), shorter cycles becoming more common and longer ones declining correspondingly. In the final years of menstrual life, cycles 21–35 days long become less frequent, whereas very short (<21 days) and very long cycles (>35 days) comprise nearly half of all cycles.

![Cumulative Percentages](image)

**Fig. 4.4.** Cumulative percentage distribution of the individual lengths of menstrual cycles according to gynaecological age in women. (From Vollman, 1977; reprinted with permission.)
Thus, the range of cycle lengths is greatest near the beginning and end of menstrual life (Fig. 4.3); minimal variation is seen at a chronological age of 36 years (Treloar et al., 1967). The zone of irregular cycles preceding the menopause has been called the “menopausal transition.” It lasts about 6–8 years and its length is mostly independent of age at onset, although it is slightly shorter in women attaining a late menopause (Fig. 4.5). It is these women who have the greatest variation and extension of cycle length in the final two years of menses, and these factors might be responsible for their higher risk of breast cancer (Wallace et al., 1978).

Similar changes in menstrual cyclicity might be expected in sub-human primates, but data are rare. In a longitudinal study, van Wagenen (1972) observed three rhesus monkeys with oligomenorrhoea in their third decade of life. At ages 27 and 28 years, two of them finally stopped cycling, having evidently reached menopause (Fig. 4.6).

In conclusion, longitudinal studies show that menstrual life is not simply switched on at menarche and off at menopause with an intervening phase of regular cycles. As in rodents, there are continuous changes in frequency of ovarian cycles, signifying underlying changes of maturation and senescence of the reproductive system. It is anticipated that parallel changes will be found for other parameters, as indicated in the next section.
Cycle Quality

The quality of menstrual cycles is generally studied by measuring basal body temperature and steroid excretion rates since more direct and precise methods involving plasma hormone assay are difficult to organize in large-scale surveys and repeated venepuncture is stressful.

The incidence of abnormal cycles is shown in Table 4.1. Cycles with a hyperthermic phase of <10 days were considered infertile, and those lacking an obvious mid-cycle rise in temperature were presumed to be anovulatory (Döring, 1963). Such cycles occur at all ages, but they are most frequent near the beginning and ending of the menstrual lifespan (Collett et al., 1954; Vollman, 1977; Metcalf and MacKenzie, 1980).

Longitudinal studies of urinary hormone profiles in perimenopausal women in New Zealand provide a rare glimpse at the underlying patterns of ovarian changes. Women aged 36–55 years were selected soon after a break in their natural menstrual rhythms, and they collected weekly specimens of urine for steroid and gonadotrophin analysis (Metcalf et al., 1981a). Two main types of cycle were identified: (1) “short” cycles <45 days in length, in which gonadotrophin excretion was normal with a pronounced rise of pregnanediol, indicating ovulation; these cycles occurred sporadically up to 16 weeks before the last menses (Metcalf et al., 1981b); (2) highly abnormal cycles in which gonadotrophin excretion rates rose to the postmenopausal range for long periods of up to 8 months, sometimes associated with hot flushes. Thus, the probability of ovulation in any cycle is related more closely to cycle length than to age (Metcalf, 1979, 1983).

Elongated cycles are experienced by most perimenopausal women. They tend to be anovular with highly variable patterns of hormone excretion, but FSH and oestrogen are frequently elevated simultaneously for several weeks (van Look et al., 1977; Metcalf and Donald, 1979). Thus, the menopause is heralded by a
TABLE 4.1
The Proportion of Abnormal Menstrual Cycles during the Late Phase of the Reproductive Lifespan in Women

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Anovulatory cycles (%)</th>
<th>Cycles with short luteal phase (%)(^a)</th>
<th>Total abnormal cycles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31-35</td>
<td>7</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>36-40</td>
<td>3</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>41-45</td>
<td>12</td>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td>46-50</td>
<td>15</td>
<td>36</td>
<td>51</td>
</tr>
<tr>
<td>Approx. age(^b) (years)</td>
<td>Anovulatory cycles (%)</td>
<td>Cycles with short luteal phase (%)(^a)</td>
<td>Total abnormal cycles (%)</td>
</tr>
<tr>
<td>30-34</td>
<td>3</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>35-39</td>
<td>2</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>40-44</td>
<td>4</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>≥45</td>
<td>18</td>
<td>12</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^a\) <10 days.

\(^b\) The original data set has been retabulated and is approximate because it was tabulated according to gynaecological age rather than chronological age (after Gray, 1979).
high output of gonadotrophins in advance of the profound and sustained fall in ovarian secretion (Metcalf et al., 1982). The causes of prolonged secretion of oestrogen are not understood, though it seems likely that altered feedback relationships are at fault, with implications for follicular dynamics. Either dominant follicle(s) fail to become atretic after a normal period of preovulatory secretion or an unbroken succession of these types form. As a consequence of persistent secretion of oestrogen without opposing effects of progesterone, there is an increased probability of dysfunctional uterine bleeding among women of perimenopausal age (de Jong et al., 1974).

When urinary assays are used, subtle changes of endocrine function are likely to go unnoticed. Even before cycles become irregular, blood hormone measurements can indicate that the climacteric is imminent in women of middle age (Fig. 4.7). The hormone profiles are generally similar to those of younger women, but peak levels of oestradiol are lower during the follicular and luteal phases, whilst FSH levels are increased at most times of the cycle (Sherman et al., 1976; van Look et al., 1977). In rhesus monkeys, similar changes occur during the climacteric (Hodgen et al., 1977; Dierschke et al., 1983). Reyes et al. (1977) found circulating levels of LH and progesterone during the menstrual cycle tended to fall during ageing, but these findings have not been confirmed in other studies. Since pituitary production of FSH and LH depends on stimulation by the same neuropeptide, LHRH, it is conjectured that differential elevation of FSH is caused by lower output of follicular inhibin by ageing ovaries. This counterpart of testicular inhibin is capable of suppressing secretion of FSH (de Jong and Sharpe, 1976), but its physiological significance requires clarification.

The peculiar features of perimenopausal cycles are too variable to provide a practical basis for predicting the final menses. Urinary gonadotrophic activity may be elevated for weeks and is indistinguishable from that of the postmenopause. Oestrogenic activity is not a better guide since it fluctuates during the first few months after the menopause as a few remaining follicles make fruitless attempts to mature. The endocrine data confirm that the changes of the menopausal transition are not abrupt but rather involve the progressive loss of ovarian function.

The University of Minnesota study of menstrual histories has provided a limited basis for predicting the timing of menopause for the North American white population (Fig. 4.8). Variation in the probability that menopause has occurred has been plotted against the first record of amenorrhoea of given duration. The relationship is approximately linear, but the probability of menopause is higher at each point on the abscissa for older women. For example, there is a 13% chance that menopause has occurred at the first interval of 90–119 days of amenorrhoea in 47-year-old women, but the corresponding value is 30% at age 51 and 47% at age 54. Even after a year without menses, there is a residual 5–10% chance that vaginal bleeding will occur, assuming this is not due to genital
tract disease or withdrawal of exogenous hormones. This illustrates the probabilistic nature of conventional criteria of menopause.

4.4. Aetiology of Acyclicity and Menopause

A great deal is known about the biology of ageing in rodents. Strictly speaking, these animals cannot have a menopause, but they do have patterns of
reproductive senescence that are common to many species, including our own. Therefore, it is appropriate to begin by discussing the causes of ovarian ageing in rodents and then to compare and contrast the human menopause.

**Balance of Factors in Rodents: Ovarian or Neuroendocrine?**

Waning reproductive function in animals was formerly attributed to "wearing out" of ovaries. This view seemed to be upheld by the discovery that the follicular store is non-renewable, but it was rejected when significant numbers of follicles were found in postreproductive mice (Jones, 1970). There is now abundant evidence that extra-ovarian factors are at least partly responsible for failure of ovulation in ageing rodents.

The relative responsibilities of central versus peripheral factors in ovarian senescence have been weighed by means of ovarian transplantation experiments. If ovarian ageing limits the length of cyclical life, then replacement by younger ovaries should restore function. However, the first reports in rats showed that senile animals did not return to normal oestrous cycles after organ replacement, whereas old ovaries were revitalized in young hosts, showing that responsive
The Ovarian Clock

follicles remained (Aschheim, 1964–1965; Peng and Huang, 1972). Consequently, it was concluded that the ageing hypothalamo-pituitary unit is incapable of releasing ovulatory amounts of gonadotrophins even though its secretory activity is adequate for follicular growth and maturation.

When similar transplantation experiments were carried out with C57BL/6J mice, the balance of responsibility was found to be more complex (Felicio et al., 1983). Ovaries from young donor mice aged 5 months were grafted under renal capsules of ovariectomized hosts aged 5–30 months. The profiles of ovarian cycles which followed in the young–young combination and intact controls were similar, the only notable difference being earlier termination of cycles in the grafted animals, and this could be accounted for by ischaemic necrosis of follicles whilst grafts became established (Fig. 4.9). When ovaries were grafted into hosts of postcyclic age (17 months), cycles were restored for a shorter period of 3 months at 25% of control levels. Grafts showed even less cyclical activity in older hosts. These results indicate that ovulatory function is limited by ageing of the neuroendocrine control mechanisms in the host. Concurrent studies tested whether the feeble activity of young grafts in ageing mice was affected by the earlier history of endocrine function in the hosts. Tentative evidence of improved graft performance in long-term ovariectomized rats had been presented already (Aschheim, 1964–1965). This was confirmed in mice by finding that the number of cycles produced by grafts in hosts deprived of their own ovaries between 5 and 17 months approached that of 5-month-old controls. There were, however, fewer cycles amongst older hosts (Fig. 4.9).

![Fig. 4.9](image-url). Experimental study of effects of the age of the host and the duration of ovariectomy on the ability of ovarian grafts to restore oestrous cycles in ageing C57BL/6J mice. Grafts were obtained from young donor animals aged 3–5 months and inserted under renal capsules of syngeneic hosts at one of the following ages: 5, 17, 25, 30 months. The hosts had been ovariectomized either shortly before receiving grafts (O—O) or after a prolonged period of ovariectomy beginning at 5 months of age (•••••). An additional group of mice with their ovaries intact served as control (••••••). Means ± SEM are shown. (From Felicio et al., 1983.)
In conclusion, at least three groups of factors limit cyclical activity in these ageing mice. (1) Primary ovarian ageing is indicated by the ability of grafts to restore oestrous cycles. Similar conclusions were reached by Krohn (1962) in pioneering experiments with CBA mice, in which ovarian failure occurs in mid-life. (2) Extra-ovarian factors must be operating because graft activity was improved in long-term ovariectomized hosts. They might involve actions of ovarian hormones on hypothalamo-pituitary function. (3) Other extra-ovarian factors that are independent of the history of ovarian activity finally limit the maximum cycle extension in the lifespan. Since this limit is reached late in life, general debilitation might be responsible.

We can see then that a number of factors are operating in mice to bring about acyclicity, whereas in rats the hypothalamus (and possibly the pituitary gland) has been held to be wholly responsible. Nevertheless, there is now evidence that in Holtzman rats ovarian ageing is a contributing factor (Sopelak and Butcher, 1982), which shows we cannot simply assign species-specific causes of acyclicity. Future research should aim to elucidate how genetic and environmental variables influence the balance of factors which bring about the onset of reproductive senescence.

**Hormone Production and Feedback in Ageing Rodents**

Age-related changes in reproductive hormone levels begin in mice at 10–13 months of age when cycles are lengthening (Fig. 4.10). Peak preovulatory levels of oestradiol are undiminished though they begin their ascent later, perhaps because fewer follicles are recruited. The characteristic rise of progesterone on the periovulatory days is smaller, as is the ovulatory discharge of LH in both mice and rats (Cooper et al., 1980; Flurkey et al., 1982; Wise, 1982a). Most studies indicate that FSH secretion is not affected by ageing until after the time when ovarian cycles are disrupted. Progressive impedance of the mechanism for releasing LH is not a significant factor at younger ages because blood hormone levels rise far higher than are required for ovulation and the number of ova shed per cycle is not diminished until acyclicity is imminent. Nevertheless, this change is a notable indication of neuroendocrine dysfunction leading to anovulation with persistent oestrus in mid-life.

The ovaries of anovulatory rats and mice presenting persistent vaginal cornification are dominated by large Graafian follicles (Fig. 3.13). Since corpora lutea are absent, blood levels of progesterone and 20α-dihydroprogesterone are low. Apart from minor diurnal fluctuations, there are no cyclical rhythms of reproductive hormone levels. Plasma oestradiol levels are intermediate between those of castrates and cyclic animals at pro-oestrus (Lu et al., 1979; Felicio et al., 1980). The gonadotrophins are not substantially elevated. Steady and prolonged output of oestrogen, unopposed by progesterone, has pathological significance (Fig.
The Ovarian Clock

Fig. 4.10. Changes in plasma hormone levels during oestrous cycles in young (Y) and middle-aged (M) C57BL/6J mice. Top panel: oestradiol (E$_2$) was similar in Y and M at pro-oestrus, but fell to lower values in M. Middle panel: LH rose more slowly to peak values and subsequently declined more rapidly in M; basal levels were similar in both groups. Bottom panel: peak levels of progesterone (P) were lower in M than Y. (From Finch et al., 1980; reprinted with permission.)

4.11). Frequently, it leads to cystic glandular hyperplasia with fibrosis in the uterus. Furthermore, it stimulates mammotrophs which hypertrophy and produce prolactin copiously. Hyperprolactinaemia leads to galactorrhoea and tumours in mammary glands of susceptible animal strains. Unless exhaustion of the follicular store intervenes, increasing output of prolactin will lead eventually to luteinization of mature follicles and, hence, pseudopregnancy cycles later in life. Gonadal secretion will then change to a pattern of lower levels of oestrogens combined with high, fluctuating amounts of progestogens (Lu et al., 1979). The final, irreversible state of vaginal anoestrum in some mouse strains resembles the postmenopausal phase of women in some respects. Vaginal anoestrum signifies a deficiency of ovarian follicles and oestrogen. In the absence of feedback restraint, the gonadotrophs hypertrophy and plasma levels of gonadotrophins increase (Parkening et al., 1980; Gee et al., 1983). The lack of comparable changes in rats might be attributable to elevated prolactin in this species.
Aetiology of Acyclicity and Menopause

Loss of ovarian cycles in mid-life is associated with reduced central responsiveness to gonadal steroids, as shown by the inability of native or grafted ovaries to elicit ovulatory amounts of gonadotrophins. Defective positive feedback has also been demonstrated using ovariectomized animals by mimicking the endogenous hormonal changes with exogenous steroids. The amount of LH released by exogenous oestrogen or progesterone in rats primed with oestrogen has been shown repeatedly to be diminished in middle-aged compared with younger rats (see Blake et al., 1983). Impaired positive feedback responses to gonadal steroids are not restricted to this species but have been recorded in other rodents and even in the domestic fowl (Williams and Sharp, 1978). Whereas failure of positive feedback might be a general feature of ageing, there is little convincing evidence of impaired negative feedback responses to these steroids. It is widely presumed, though not yet proven by direct measurement, that inability to release sufficient LHRH is responsible for attenuated feedback effects.

Since physical and chemical lesions of the rostral hypothalamus lead to positive feedback failure and anovulation (Blake et al., 1972; Benedetti et al., 1976), this site is held to be responsible for the spontaneous loss of oestrous cycles. In support of this hypothesis, electrical stimulation of the preoptic area (or arcuate nucleus) stimulates ovulation in acyclic rats, though fewer ova are shed than in normal cycles. The amounts of LH released in these experiments indicated that pools of readily releasable LHRH and LH are adequate and that gonadotrophins are sufficiently sensitive (Everett and Tyrey, 1983). However, the question of pituitary responsiveness to LHRH is still unsettled, and further study is required to clarify conflicting claims. Where reduced responsiveness has been observed, it is attributed to the effects of ovarian secretions rather than to chronological ageing per se (Cooper et al., 1984).

Our limited knowledge of the basic physiology of hypothalamic function holds back progress in understanding age changes. It is attractive to consider whether loss of hypothalamic sensitivity to feedback information is due to a reduced concentration of nuclear receptors for oestradiol, which has been observed in the preoptic area (Wise and Camp, 1984), other age changes in the neuroendocrine apparatus being secondary effects of this. Target neurons for feedback of oestradiol are probably not the same as those which synthesize and transport LHRH from the rostral hypothalamus to the site of release in the median eminence (Shivers et al., 1983). Monoaminergic synapses are involved at unidentified stages in the neural pathways for both circadian and pulsatile release of gonadotrophins. Because of their position in the hierarchical organization, small changes in the activity of hypothalamic monoamines could create a cascade of functional disturbances at lower levels and in peripheral organs (Finch, 1976). This potential is illustrated by the serious effects on motor control of altered dopaminergic functions in ageing basal ganglia (Finch et al., 1981) and by the restoration of ovarian cycles by centrally acting drugs (see below). Hence, it may be significant that both concentrations and metabolism of noradrenaline are
reduced in anterior hypothalami of ageing female rats (Meites et al., 1982; Wise, 1982b). Levels of other putative neurotransmitter and neuromodulator substances may vary in middle age, but this catecholamine is of particular interest because of its probable involvement in releasing LHRH (Simpkins et al., 1979; Gallo and Kalra, 1983).

The hypothesis that altered neuronal activity contributes to the acyclicity of ageing is strengthened by the discovery that exogenous agents restore function. Progesterone was the first substance shown to have this effect, which was obtained after administration in small, repeated doses (Everett, 1940). To conclude from this that anovulatory rats must therefore have a deficiency of this hormone is too superficial since it avoids the question of how luteinization failed in the first place. Progesterone probably restores cycles by alleviating the insensitivity of the brain to oestrogen. Alternative explanations may have to be found for the efficacy of other agents, such as noradrenaline, dopaminergic agonists, ether stress, ACTH (Quadri et al., 1973; Huang et al., 1976), dietary supplements of L-tyrosine (Linnoila and Cooper, 1976) and decreasing photoperiod (Everett, 1970). The ability of catecholamine precursors to restore cycles when administered directly to the preoptic area supports the general belief that neuronal function in this area becomes defective in old animals (Cooper and Linnoila, 1980).

Although these experiments provide prima facie evidence of altered neuroendocrine function, it is important to appreciate the possibilities of cycle restoration by non-specific means. Restoration by substances given on a daily basis might be explained by the reinforcement of a circadian rhythm which has become damped during ageing (e.g. the adrenal rhythm) (Mosko et al., 1980). Treatments which produce only a single cycle should not be assumed to have affected the causes of acyclicity, and there may be more than one possible interpretation of cycle restoration. When a set of corpora lutea are introduced by any stimulus, momentum may be created for a series of short cycles in which luteal progesterone facilitates cyclic release of LH. The response to treatment then depends on endogenous levels of prolactin (which is luteotrophic) rather than continuation of exogenous agents. If prolactin levels are low, the anovulatory state is quickly resumed; if they are higher, a longer series of cycles commences. In rats with pituitary tumours secreting prolactin abundantly, short cycles may switch to the longer pseudopregnancy type (Everett, 1980).

The timing of loss of sensitivity to positive feedback depends upon the earlier history of ovarian secretions. This is evident from the ovarian transplantation experiments cited earlier and has been confirmed by the improved pituitary response to gonadal steroids in animals deprived of ovaries over a long period (Lu et al., 1981; Blake et al., 1983). Since long-term ovariectomy protects hypothalamo-pituitary function from deleterious effects of ageing, can excessive exposure to oestradiol early in adulthood accelerate the loss of cycles? An affirmative answer was obtained when intact adult rats were treated with large doses of oestradiol valerate (Brawer et al., 1978). Their arcuate nuclei had histo-
pathological changes indicative of neuronal damage (viz. hyperactive glia). Similar changes occurred spontaneously during ageing (Brawer et al., 1980) and could be induced by physiological levels of oestrogen (Mobbs et al., 1984). In either case, loss of ovarian cycles could not be explained by pituitary tumours but may have been due to defective function of LHRH-containing neurons.

A heuristic model has been proposed to explain the loss of cycles during

![Diagram](image)

Fig. 4.11. Schematic representation of neuroendocrine interactions involved in the loss of short ovulatory cycles in ageing rodents. Some pathological consequences of anovulation are shown: +, stimulation or lack of inhibition; −, inhibition; ↑, increased production; ↓, decreased production; E₂, oestradiol; P₄, progesterone.
ageing and after oestradiol treatment (Finch et al., 1980). Exposure of the rodent brain to gonadal steroids (particularly oestradiol) throughout life may lead to accumulated damage, and at some critical threshold, the mechanism of cyclic release of LH is impaired because of insensitivity to oestradiol. The threshold would be attained later in animals lacking ovaries or exposed to progesterone over a long period. Although a neural "memory" of oestradiol stimulation is hypothetical for the present, the model which is emerging is consistent with the general picture of reproductive system ageing in which complex interactions exist at different levels, making hazardous any attempts to assign strict hierarchical responsibility for dysfunction (Fig. 4.11).

Human Menopause

Human menopause is usually presented as a comparatively more straightforward case of peripheral organ failure. There is little doubt that primary ovarian ageing brings about the final cessation of menses, although closer study of the physiology suggests that other complex changes occur during the perimenopause. Few follicles remain in the postmenopausal ovary, and they fail to mature despite abundant circulating gonadotrophins of proven biological activity (see Section 3.8). This circumstance prohibits the restoration of cycles. The ability of monoamine precursors to reinitiate ovarian cycles in rats finds no convincing parallel in human biology, and reports of postmenopausal vaginal bleeding in women receiving levodopa (L-Dopa) should be explained on other grounds (Kruse-Larsen and Garde, 1971; Wajsbort, 1972). Conclusive proof that menstrual life is terminated by ovarian ageing could be obtained if normal cycles were resumed in postmenopausal women after grafting ovaries from young donors. However, such experiments are highly questionable from an ethical standpoint.

There are other grounds for supporting the "exhausted ovary" theory of human menopause and in particular the elevation of plasma gonadotrophins (Goldenberg et al., 1973). Circulating levels of FSH and LH rise after menopause approximately 13- and 3-fold, respectively, compared with those of the early follicular phase in cyclic women (Figs. 4.12 and 4.13). Thus, levels of both gonadotrophins reach the castrate range, with FSH being the more abundant of the two. These high levels are maintained for at least 1 decade after menopause, though most studies have found that they fall in the last years of life (Wide et al., 1973; Chakravarti et al., 1976; Crilly et al., 1981). In contrast to gonadotrophins, prolactin levels tend to fall slightly after menopause (Vekemans and Robyn, 1975). This is expected since the stimulatory effects of gonadal steroids on prolactin secretion are withdrawn at this time.

Increased blood levels of gonadotrophins are usually interpreted as a sign that the pituitary gland is no longer able to elicit a feedback response from atrophic ovaries. These elevated gonadotrophin levels are presumed to be due to rising
Fig. 4.12. Serum levels of FSH during the human menstrual cycle compared with average trends during ageing. (From Wide et al., 1973; reprinted with permission.)
concentrations of LHRH in pituitary portal blood, although dilution and rapid metabolism of LHRH during circulation have hampered attempts to obtain direct proof of this hypothesis. Hypothalamic levels of LHRH fall after menopause, but it is not possible to conclude whether this is due to more secretion or less synthesis of the decapeptide (Parker and Porter, 1984). Recent discoveries demand revision of classical concepts of feedback control and could significantly alter our view of postmenopausal gonadotrophins. It has been assumed that, since metabolic clearance rates do not change after menopause, increased blood levels of gonadotrophins reflect increased production (Kohler et al., 1968; Coble et al., 1969). This assumption must now be questioned because human and animal gonadotrophins have been shown to be pleiomorphic. Earlier measurements of hormone clearance rates in pre- and postmenopausal women employed
the same standard hormone preparations, but such comparisons are misleading because different types of gonadotrophins circulate in these two groups. The types found in young women are less acidic than those in castrates, postmenopausal women and men (Wide, 1981, 1982; Reader et al., 1983). A smaller content of sialic acid is physiologically significant because it leads to more rapid clearance from blood, which would aid the fine control of follicular maturation by allowing prompt responses to feedback information (Wide and Hobson, 1983; Wide and Wide, 1984). The increasing acidity of FSH after the menopause seems to be due to withdrawal of sex steroids, but such changes would appear to develop sluggishly, and they do not account for variable hormone levels during the normal menstrual cycle (Wide, 1982). Such findings do not call into question the present practice of using blood or urinary FSH activity as an indication of primary ovarian failure (Goldenberg et al., 1973). But it remains unclear whether the bulk of this activity is accounted for by increased production or decreased clearance of gonadotrophins.

The new concepts of how normal menstrual cycles are regulated endorse the current theory of the aetiology of menopause. In contrast to the situation in rodents, the human hypothalamus is unlikely to be responsible for acyclicity since it does not provide a stimulus for timing events in normal cycles. After menopause, pituitary responsiveness to LHRH remains and positive feedback responses to exogenous gonadal steroids are still possible (Odell and Swerdloff, 1968). Prolonged cycles during perimenopause are usually attributed to irregular recruitment from a dwindling pool of follicles, but primary ovarian ageing is not likely to account for shortening of the follicular phase during the middle zone of menstrual life. If this shortening is due to a greater growth velocity of the dominant follicle, it could be explained by frequency and/or amplitude modulation of LHRH pulses, perhaps rising abruptly before menarche (Wildt et al., 1980) and then more gradually during adult life. This explanation is purely hypothetical at the present time and will be difficult to test because pulses of FSH and, to a lesser extent, LH are difficult to quantify in women (Bäckström et al., 1982). But to continue this speculation, changes in neuronal activity in the hypothalamus could depend, in turn, on long-term changes in sensitivity to ovarian feedback, which have been postulated on other grounds (Dilman, 1971). In perimenopausal women, failure of oestrogen to exert positive feedback on LH release leads to anovulation with dysfunctional uterine bleeding (van Look et al., 1977, 1978). Experiments with rhesus monkeys show how this situation could arise as a result of decreased hypothalamic signal frequency. Following destruction of the pathway for endogenous release of LHRH, infusion of this peptide led to a rising FSH:LH ratio with irregular follicular maturation and anovulatory cycles when pulses were delivered at greater than the optimal interval of 1 hr (Wildt et al., 1981b; Pohl et al., 1983). These observations lead us to conjecture that there might be, after all, a link between perimenopausal changes in women and the neurohormonal basis of anovulation in rodents.
5

Fertility in Middle Age

5.1. Introduction

Twenty years ago, Krohn (1964) concluded from a far-reaching review of the literature that "there is no reasonable doubt, therefore, that (a) the likelihood of conceiving and (b) the size of a litter declines with increasing age in all the species for which there is any information at all". Subsequent studies have upheld his conclusion, although the number of species studied remains small and mainly confined to laboratory and domesticated groups.

A number of difficulties are encountered when measuring the effects of ageing on fertility and when designing gerontological experiments. The fertility of individuals of the same age and stock has a much larger coefficient of variation than most other physiological parameters (e.g. blood pressure, nerve conduction velocity), perhaps because individual survival does not depend on integrity of the reproductive apparatus. Furthermore, it is not always obvious whether infertility is due to normal and universal age changes ("eugeric" phenomena) rather than "pathogeric" changes which are secondary. For practical purposes, reproductive life in women is considered to begin at menarche and end at menopause, a span of 36 years in the developed world (Treloar, 1974). In contrast to wild animals, in which reproductive potential (fecundity) and actual performance (fertility) are closely matched, human fertility is modified by a host of factors which obscure any underlying trends of ageing. Some of the most significant of these are contraception, frequency of coitus, sterility of the male partner, age at marriage, prevalence of genital and other diseases, breast-feeding patterns and iatrogenic factors. It is the purpose of this chapter to review evidence for the age factor in human and animal reproduction, to discuss possible mechanisms and, finally, to reach some general conclusions of practical relevance for family planning in middle age.

5.2. Age-Specific Fertility

Animal Fertility

Small Laboratory Mammals

There have been many longitudinal studies of the fertility of laboratory mice, rats, hamsters and rabbits. Since these animals are polytocous (i.e. litter bear-
Fertility in Middle Age

Fluctuations in litter size throughout life provide an index of fertility. Control data in Fig. 5.9 show the number of offspring produced per uterine horn in outbred mice which were breeding ad libitum (total litter sizes are exactly double the values given). The age of sires was controlled because, although this variable does not affect litter size in mice (Finn, 1964), it may reduce the disposition to mate. The first litter is generally smaller than the second and, in some strains and species, peak litter size is not reached until the third or fourth pregnancy. This rise is mainly accounted for by increasing numbers of ovulations (p. 48). The phase of rising reproductive performance is followed by one of declining litter sizes which, towards the close of fertile life, is also characterized by less frequent deliveries (Asdell et al., 1941; Ingram et al., 1958; Jones and Krohn, 1961a; Soderwall et al., 1960). Some researchers have shown an intermediate phase of relatively constant litter sizes in mice, but such findings depend on how the data are expressed. Fertility data often fit a three-phase model when a "standardized curve" is constructed (Biggers et al., 1962). This manoeuvre was introduced to overcome statistical aberrations created by variation in the time when animals become infertile, but it rests on the assumption that fertility is lost for similar reasons in all individuals.

The length of fertile life is highly variable even among animals of a common stock. Reproduction commences at about 2–4 months of age in all the species named above and ceases 12 months later in most small rodents. Fertility is generally reduced by inbreeding in mice, but it rises in F1 hybrids (Jones and Krohn, 1961a; Roberts, 1961). Since mice live for 2–3 years, they spend the second half of the lifespan in a postreproductive phase, which also exists in rats, hamsters, rabbits, gerbils and guinea pigs (Talbert, 1977).

**Domesticated Animals**

Despite their abundance, farm animals have provided scant data on fertility in old age because it is unprofitable to keep infertile stocks, and records which have been obtained are often biased by selection for exceptional breeding performance. The available literature has been reviewed in depth by Krohn (1964) and Talbert (1977), who cautiously concluded that fertility decreases in ageing horses, cows, sheep and swine, as well as in cats and dogs. In view of artefacts that have been introduced by selective breeding practice in the past and in view of modern husbandry methods, it is not justifiable to extrapolate such conclusions to ancestral stocks of the same species or to extant wild relatives of domesticated animals. Reproductive senescence is rarely observed among wild animals because few survive long enough to become senescent. Even in species noted for longevity, such as African elephants, postreproductive individuals do not exist in wild populations (Perry, 1953).

**Primates**

Long-term fertility data for monkeys are scarce, but there are good prospects of future progress because the first animals to be born in U.S. primate research
centres are now approaching the end of life. Information about great apes is presently limited to 10 chimpanzees aged 35–48 years, apart from isolated records from zoos and private collections. Although this group continued to have regular menstrual cycles until shortly before the end of life (about 50 years old), only three conceptions occurred and only one of these led to a live infant (Graham, 1979). The percentage of fertile menstrual cycles was only 4%, whereas it was 25% when the same animals were 15–25 years old. Longitudinal studies of breeding performance of lower primates are almost as rare as those of apes. In one colony, three rhesus monkeys reached menopause at ages 25–28, each having carried 12–14 pregnancies (van Wagenen, 1972). The oldest mother to deliver a live baby was 19 years old but required caesarian section, and there was a higher frequency of abortions and stillbirths among all older, multiparous mothers. However, these data may not be representative of the whole colony and could have been affected by earlier surgical procedures. This reservation is endorsed by a preliminary report from the Wisconsin Regional Primate Center that normal pregnancy in this species can occur as late as 26 years of age (Dierschke et al., 1983).

**Human Fertility**

Live birth rates in Scotland are used to illustrate present fertility patterns in Western nations (Table 5.1). Fertility is much higher among women aged 25–29 years than in younger and older age groups. In the past, the age distribution for live births was bell-shaped (as it is today in many parts of the developing world); now it is skewed towards younger age groups. Apart from the greater frequency

<table>
<thead>
<tr>
<th>Age of mother (years)</th>
<th>1950</th>
<th>1980</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-19</td>
<td>20.98</td>
<td>32.52</td>
</tr>
<tr>
<td>20-24</td>
<td>128.59</td>
<td>112.65</td>
</tr>
<tr>
<td>25-29</td>
<td>147.77</td>
<td>130.54</td>
</tr>
<tr>
<td>30-34</td>
<td>108.16</td>
<td>67.30</td>
</tr>
<tr>
<td>35-39</td>
<td>60.88</td>
<td>20.49</td>
</tr>
<tr>
<td>40-44</td>
<td>18.16</td>
<td>3.73</td>
</tr>
<tr>
<td>45-49</td>
<td>1.26</td>
<td>0.18</td>
</tr>
<tr>
<td>15-49</td>
<td>69.86</td>
<td>63.82</td>
</tr>
</tbody>
</table>

*From the General Register Office, Scotland.
of teenage maternity, the fertility of all other age groups has fallen during the past 3 decades. The decline has been most rapid amongst older women. In 1950, women aged 40–49 were responsible for 4% of recorded deliveries in Scotland, whereas in 1980 they contributed only 1% of births. In many other countries similar patterns have been found, and though social and economic forces may bring about minor fluctuations, demographic forecasters expect these patterns will be maintained for at least the next 2 decades. Since demographic statistics from modern industrial societies cannot provide reliable estimates of human fecundity, it is necessary to turn to records from historical and traditional societies in which "natural fertility" has been less suppressed. Unfortunately, it is often these societies in which documentation of reproductive events and parental age is least satisfactory. Nevertheless, from a careful study of historical sources, Henry (1961) was able to conclude that fertility of married couples declined steadily from the third decade of life, approaching zero at age 50. This decline has been attributed to biological factors since it is remarkably similar in most societies that have been studied. Surveys of contemporary societies have shown that the average age at which fertility is lost (age at last live delivery) is about 40 (Gray, 1979), though it is earlier in places where gonorrhoea and genital tuberculosis are widespread and in some undernourished societies (Scragg, 1973; Chavez and Martinez, 1982). Therefore, the average age of sterility precedes that of menopause by up to 10 years. The demographic data do not distinguish women who are sterile from those in whom residual fertile capacity exists; consequently, the exponential decrease in the proportion of sterile women by age (Pittenger, 1973) does not necessarily apply to individuals. The population asymptote implies that the age at which a cohort becomes sterile is indeterminate, but claims of maternity after the sixth decade are not well documented. The record for late maternity is presently held by Ruth Alice Kistler, an American woman, who gave birth to a daughter in 1956 at the age of 57 years and 129 days (Fergusson et al., 1982). During the span of fertile ages the secondary sex ratio is fairly stable, tending to fall slightly with increased parental age (Russell, 1936; Garfinkel and Selvin, 1976).

Some of the most reliable estimates of natural fertility in married couples have been obtained from Hutterite communities in North America (Eaton and Mayer, 1953). The Hutterites are members of an Anabaptist sect who emigrated in the nineteenth century to escape religious persecution in Europe. Their beliefs strictly prohibit contraception, abortion and extra-marital coitus. The lack of any artificial restraints on fertility within the marital bond combined with a short duration of lactation and high standards of diet, housing and medical care have produced some of the highest fertility rates on record. These rates are close to the theoretical maximum, though presently falling as elsewhere in North America (Laing, 1980). The distribution of live births among Hutterites is far less skewed than it is in the American population as a whole because of greater fertility
TABLE 5.2
Fertility among the Ethnic Hutterites: Age-Specific Fertility Ratesa and Percentage of Sterile Couples by Age Groupb

<table>
<thead>
<tr>
<th>Age of the mother (years)</th>
<th>Annual number of live births per 1000 women</th>
<th>Sterile couples at end of age interval (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15–19</td>
<td>12.0</td>
<td>3.5</td>
</tr>
<tr>
<td>20–24</td>
<td>231.0</td>
<td>7</td>
</tr>
<tr>
<td>25–29</td>
<td>382.7</td>
<td>11</td>
</tr>
<tr>
<td>30–34</td>
<td>391.1</td>
<td>33</td>
</tr>
<tr>
<td>35–39</td>
<td>344.6</td>
<td>87</td>
</tr>
<tr>
<td>40–44</td>
<td>208.3</td>
<td>100</td>
</tr>
<tr>
<td>45–49</td>
<td>42.1</td>
<td></td>
</tr>
<tr>
<td>15–49</td>
<td>226.6</td>
<td></td>
</tr>
</tbody>
</table>


among older cohorts and because early marriage is discouraged (Table 5.2). The birth rate of Hutterites fell after the third decade of maternal age, and between ages 35 and 49 the percentage of women who ceased to reproduce rose from 10 to 100%. The average age at last confinement was 40.9 years, in accord with findings from other populations. The interval between confinement was surprisingly constant throughout reproductive life, apart from substantially longer intervals between the final two pregnancies. It must be remembered that these data show the natural fertility of married couples, and the contributions of male infertility and reduced coital rates are not recognizable. Such limitations have been overcome using records from programmes of artificial insemination by donor (AID) in which the male factor is uniquely controlled.

A large-scale survey of experience with AID in France has shown that female fecundity begins to fail at about the mid-point of menstrual life (Fédération CECOS et al., 1982). The subjects were presumed to be fecund on the basis of azoospermia of their husbands and the absence of gynaecological disease. The time taken for 50% of them to achieve a clinically recognizable pregnancy after joining the programme was 6 months for those aged less than 30, 7 months for those aged 31–35 and >12 months for older women (Fig. 5.1). These interesting results imply that fecundity begins to fall a few years before the age when menstrual cycles are most regular (see Section 4.3). They also have clear implications for women who wish to defer reproduction, but it should be emphasized that the prognosis for fertility in the fourth decade is still relatively good.
5.3. Biological Basis of Infertility in Middle Age

Occurrence of Fetal Wastage

In middle-aged rodents, the litter size falls in advance of a significant reduction in the number of corpora lutea (Jones, 1970). Although a larger proportion of ovulatory follicles fail to liberate their ova, the number of these faults is relatively small, and so the loss of fertility is attributable mainly to postovulatory failure (R. G. Gosden, unpublished observations). Most of the infertility arises from intra-uterine mortality of implanted fetuses, and in C57BL/6J mice, the death rate peaks after mid-gestation (day 10) when the allantois assumes primary responsibility for placentation (Fig. 5.2). Since dead fetuses are soft and macerated and cannot be evacuated from uterine horns in polytocous species without disturbing viable siblings, they are eliminated by resorption in utero, being reduced to a dark spot or "mole" by full term. There are statistical grounds for suspecting that when an entire litter is aborted systemic factors are
responsible, whereas individual fetal death occurs sporadically in the uterus, which is perhaps a reflection of local deterioration of the environment (Gosden et al., 1981). Some conceptuses are lost at other stages of gestation, particularly the transitional stages of fertilization, implantation and parturition (Blaha, 1964; Fabricant et al., 1978; Holinka et al., 1978; Mizoguchi and Dukelow, 1981).

Approximately 10-15% of all clinically recognizable pregnancies in women who are apparently fertile terminate spontaneously by abortion. Such figures underestimate the global proportion lost, which has been estimated to be as high as 78% (Roberts and Lowe, 1975). The extent of embryonic and fetal losses can now be estimated prospectively from an early postimplantation age by measuring the specific β subunit of hCG in urine (Miller et al., 1980). In a study of healthy, sexually active women, 62% of conceptuses detected by this method were lost by 12 weeks of gestation (Edmonds et al., 1982). Many of those lost were recognized only by a transient elevation of hCG. That human embryos are frequently lost at early stages, including preimplantation, was indicated by morphological studies of Hertig et al. (1959), who observed that 10 out of 34 embryos were abnormal during the first 2 weeks after conception. Some of these embryos are likely to have been chromosomally imbalanced, judging by preliminary results with in vitro fertilization (Angell et al., 1983). However, despite this progress, it is not yet possible to confidently estimate the total extent of pregnancy failure in normal women, and it is even more difficult to gauge the effects of ageing.

The incidence of spontaneous abortion in women rises with age (Fig. 5.3). Parity (birth order) might be an independent risk factor (Naylor and Warburton,
However, the probability of abortion for any given individual does not necessarily increase year by year as the survey data suggest. The relationship between age and abortion rate could be a statistical artefact of reproductive compensation (James, 1974a). For example, Aberdeen women with successful pregnancy histories restricted their reproductive activity during the fourth and fifth decades to a greater extent than abortion-prone women, who were then over-represented in these age groups (Billewicz, 1973). The artefact hypothesis predicts that the relationship between abortion incidence and maternal age (or parity) would be less marked in societies that encourage large families. Confirmatory evidence has been obtained from the Amish community in the United States (Resseguie, 1974), details of pregnancy wastage in Hutterite women being lacking at the present time. Nevertheless, it is probable that the rising incidence of fetal death is partly owing to biological effects of maternal age. Records from an in vitro fertilization and embryo transfer programme indicate that a large proportion of conceptuses are lost near the time of implantation. Between 15 and 21% of embryos conceived extra-corporeally implanted successfully in native mothers aged 20–39, whereas only 0–7% did so in older women (Edwards and Steptoe, 1983). Conceptuses in older mothers might be at greater risk at all stages of gestation, although it is most likely that transitional stages in development are most hazardous, as in animals. Those that survive gestation in mothers aged >39 are also more likely to die during the perinatal period (Naeye, 1983), but social factors contribute to this increased risk, as in the case of higher
mortality rates among women of high parity (Department of Health and Social Security, 1982).

Errors of Oogenesis and Embryo Development

Chromosomal Anomalies

Chromosomally defective fetuses form a large portion of human reproductive wastage at all ages. Cytogenetic surveys in several parts of the world and among different racial groups show that about 50% of all spontaneous abortuses of the first trimester are chromosomally aberrant, a smaller percentage being found at later stages of gestation (reviewed by Bond and Chandley, 1983). Since 10–15% of all clinically recognizable pregnancies terminate in abortion, a minimum of 5–10% of conceptuses are chromosomally imperfect in man. It is not yet clear whether sub-human primates have such a remarkably high incidence of anomalies, but in rodents the figures are almost an order of magnitude lower. The frequencies of various abnormal karyotypes among early abortuses have a characteristic pattern, as is illustrated by data obtained in France (Boué et al., 1975). In that survey, autosomal trisomy accounted for 52% of the anomalies recorded. All chromosome groups (A–G) were involved, though anomalies of the larger chromosomal groups, A and B, were proportionately under-represented. Only 15% of the anomalies were monosomies, most of these being 45,XO (potentially Turner’s syndrome). If aneuploidy arises only from non-disjunction and each chromosome pair affected migrates randomly to either pole of the spindle, trisomic and monosomic fetuses would be conceived in equal numbers. In fact, monosomy can arise also by failure of a single chromosome to be transported from the equator of the spindle (“anaphase lagging”). The observed lower frequencies of monosomies are not inconsistent with theory if these die at very early stages of gestation, as they do in experimental animal models (Gropp, 1976). The other major group of anomalies in the French survey was triploidy, comprising 20% of the total; the remaining groups included small numbers of fetuses with tetraploidy or structural defects of the karyotype.

Fortunately, the majority of chromosomally anomalous fetuses conceived are not well adapted to life in utero, and the incidence of chromosomal aberration in full-term babies is less than 1% at most maternal ages (Hook, 1981). The survivors are mainly trisomies of small chromosomes, yet even these are only a small proportion of the number conceived. The causes of prenatal death are poorly understood. In the case of monosomy, the absence of essential “housekeeping” genes carried by X chromosomes or autosomes can explain early embryonic death. In trisomy the effects of chromosomal imbalance are generally less severe, especially if sex chromosomes are involved; nevertheless, trisomy is invariably associated with some form of phenotypic disturbance with retarded growth of both embryonic and extra-embryonic tissue (Mittwoch and Delhanty, 1983).
1972; Gropp, 1976; Honoré et al., 1976). Eventually, the teratogenic effects of chromosomal imbalance will be explained by gene mapping and developmental biology. There are some indications already that the additional gene dosage is fully expressed in chromosomally aberrant cells. In erythrocytes and fibroblasts of Down’s syndrome patients (trisomy 21) there is 50% excess activity of two enzymes specified by chromosome 21, namely, superoxide dismutase-1 and phosphoribosylglycinamidase synthetase (see Epstein et al., 1982). Failure to suppress supernumerary genes responsible for these products may not be serious, but if this applies to genes responsible for membrane proteins, there are likely to be implications for cellular interactions, growth and migration. Such effects could be responsible for abnormal phenotypes and death through failure of a critical organ or simply by growth retardation (Polani, 1974).

The incidence of several clinically significant chromosome disorders in newborn babies rises with maternal age. The distribution is J-shaped for some disorders because of a small excess in incidence amongst newborns of teenage mothers compared with newborns of slightly older mothers (Smith and Berg, 1976; Erickson, 1978; Hook, 1981). In an American survey, 0.2% of neonates born to mothers less than 30 years old were chromosomally abnormal, but the incidence rose to 1.6% for those aged 40 and to 5.4% for 45-year-old mothers (Fig. 5.4). The most common anomaly among older mothers, Down’s syndrome, has been known to be associated with maternal age for more than a century. The incidence of trisomy of the following chromosomes rises at similar rates from different baselines: chromosome numbers 13 and 18 and X (Court Brown et al., 1969; Hook, 1981). A maternal age relationship has been found amongst most trisomic abortuses (with the exception of trisomy 16) but not for monosomy X, triploidy or mosaic karyotypes. Penrose (1933, 1961) demonstrated that Down’s syndrome in babies was related to maternal age rather than to paternal age or parity. Furthermore, since the maternal age distributions were bimodal in some samples, he proposed that they represent the superposition of two distinct Gaussian distributions, one of which has characteristics similar to those of mothers of normal infants (class A), with the other representing the higher incidence among late maternities (class B). The mean age of mothers in class B is about 38, 10 years greater than A. Down’s babies in class A, which is independent of age, arise either de novo from environmental factors or from hereditary causes such as structural translocations and parental aneuploidy, whether total or mosaic. Those in class B are usually more abundant (c. 60%), as depicted in the distribution for abortuses (Fig. 5.5). The risk of delivering a Down’s baby rises three- to fourfold every 5 years after age 30, although some data show the rate of ascent is less steep during the final years of fertile life (Collmann and Stoller, 1962; Hook, 1981). Such irregularities of the age distribution have obstructed attempts to fit equations, which would have theoretical implications for understanding the aetiology of trisomy. A simple exponential
function would indicate that a series of independent or accidental events is responsible for trisomy, but such a curve is only a rough approximation to the data. The discontinuous form of the age distribution has been attributed to statistical artefacts (reproductive compensation or limitation among mothers of affected children), interactions of age with environmental risk factors and increased fetal mortality in older mothers, but these require further study.

The reduction divisions of meiosis responsible for trisomy 21 have now been identified using stains which produce specific and reproducible banding patterns on human chromosomes. The use of chromosomal heteromorphisms for tracing the origin of supernumerary chromosomes depends on Mendelian principles of segregation and can be illustrated by the following simple example. In an informative mating in which four chromosomal variants are present, designated ab in the father and cd in the mother, a trisomic child with acd or bcd will have arisen by chromosomal non-disjunction at metaphase I in the mother. If the child had any of the combinations aac/aad/bbc/bbd, a fault at metaphase II in the father must have been responsible. By using such methods it has been possible to show that the majority of human trisomies originate, irrespective of maternal age, at
the first meiotic division of oocytes (Jacobs and Hassold, 1980; Mikkelsen et al., 1980). They also demonstrate that most triploid fetuses arise by dispermic fertilization (Jacobs et al., 1978; Lauritsen et al., 1979). However, the male is not exonerated from responsibility for aneuploid offspring because these have sometimes been traced to errors in spermatocytes. Direct examination of human spermatozoal karyotypes is not possible on a large scale, except by allowing penetration of hamster ova following removal of the zona pellucida. This method has shown that 5% of human spermatozoa are aneusomic (Martin et al., 1983), and it is expected that it will soon help to extend knowledge of paternal ageing. This may help to clarify the results of epidemiological studies, which are almost equally divided between those showing a slight positive effect of paternal age and those in which aneuploidy was independent of this factor (Roecker and Huether, 1983).

The incidence of trisomy at full term is the product of two probabilities:
chromosomal non-disjunction and survival in utero. It has been suggested that the incidence rises because of relaxed selection against anomalous fetuses in older mothers (Aymé and Lippman-Hand, 1982). This conclusion is not accepted by a number of authorities (Carothers, 1983; Hook, 1983; Warburton et al., 1983) and is at variance with the observed higher frequency in spontaneous abortuses (see Fig. 5.5) (Alberman et al., 1976; Hassold et al., 1980) and higher incidence of aneuploidy among zygotes and preimplantation embryos in older mice (see Bond and Chandley, 1983). Despite this latter finding, fewer abnormal pups are delivered at full term (Parsons, 1964; Goodlin, 1965). This situation might arise from competition between abnormal fetuses and their more vigorous siblings for some limited resource, such as the blood supply, in the compromising environment of older uteri.

The increase of trisomy in long-lived oocytes has sometimes been attributed to accumulated damage inflicted by agencies in the external environment. The smaller (if present) effect of paternal age could be explained by natural selection of the normal, more vigorous cells in the continuously proliferating male germ line. Although the loss of microtubules could lead to chromosomal non-disjunction by failing to provide a balance between the traction forces in the meiotic spindle, ageing effects must be indirect because the spindle is not assembled until shortly before the reduction division. It has been suggested that older oocytes are more vulnerable to ionizing radiation and chemical mutagens, but studies could not confirm this hypothesis (Tease, 1982; Golbus, 1983). Other environmental factors have been imputed, namely, atmospheric pollution, thyroid autoimmunity, fluoride in drinking water supplies, viral hepatitis (see Smith and Berg, 1976; Bond and Chandley, 1983) and, most recently, smoking (Kline et al., 1983). But the worldwide distribution of Down’s syndrome would seem to deny that environmental variables have a primary role, although they might explain temporal and geographic fluctuations in frequency (Collmann and Stoller, 1962; Evans et al., 1978; Read, 1982). In recent years, workers have increasingly concentrated their search for an explanation of age-dependent trisomy on internal factors.

Postovulatory deterioration of oocytes is one of the factors which has been considered. Zygotes are more frequently triploid or carry other abnormal features if fertilized after the short span of optimal ripeness (Austin, 1970). Since there is no oestrous phase at mid-cycle to synchronize human ovulation and insemination, German (1968) postulated that the risk of conceiving a Down’s zygote would increase with age because the probability of delayed fertilization is greater in older women who engage in coitus less frequently. The mechanism of aneuploidy has then been explained either by premature disjunction of chromatids in over-ripened oocytes (Rodman, 1971) or by failure of a chromosome in a subnucleus of a zygote to unite with those on the metaphase plate of the first cleavage division (Austin, 1970). However, German’s hypothesis has not been
substantiated statistically (Cannings and Cannings, 1968), nor is it compatible with the evidence that most chromosomal non-disjunction occurs before ovulation during the first reduction division.

Alternatively, it has been proposed that oocytes are qualitatively inferior when shed from follicles after a prolonged follicular phase (Hertig, 1967; Jongbloet, 1975). This hypothesis is not easily reconciled with the observed reduction in the length of this phase in ageing women (p. 73). Furthermore, phase length was not a factor for the fertility of women joining an AID programme, although the number of women with follicular phases <12 and >17 days is admittedly small (Broom et al., 1981). This hypothesis is, however, corroborated by a rising incidence of developmental anomalies and chromosomal imbalance following delayed ovulation in the toad, *Xenopus laevis* (Mikamo, 1968). It is much more difficult to test whether comparable effects of intra-follicular ageing occur in laboratory mammals because ova are more scarce and aneuploidy is comparatively rare. In these animals, ovulation can be delayed by inhibiting the release of ovulatory amounts of gonadotrophins using either barbiturates (Butcher and Fugo, 1967; Mikamo and Hamaguchi, 1975) or antibodies raised against LHRH (Laing et al., 1984). The maximum extension of Graafian follicle life is limited by atresia, which afflicts most follicles on the third day postoestrus. No statistically significant increase in trisomy has been obtained using these methods. Since only trisomy is associated with maternal ageing, the greater abundance of triploid and mosaic embryos after delaying ovulation with barbiturates indicates that cycle length extension has different effects from those of ageing.

According to Henderson and Edwards (1968), trisomy may arise at the first reduction division from random segregation of univalents formed by inadequate bonding of chromosome pairs established prenatally during oogenesis. Their hypothesis is based on an inverse relationship between maternal age and chiasma frequency in oocytes, which has been confirmed in several laboratories (Polani and Jagiello, 1976; Luthardt et al., 1973; Speed, 1977; Sugawara and Mikamo, 1983). Since chiasmata are formed during oogenesis, Henderson and Edwards proposed that there is a programmed sequence of utilization of oocytes in which those formed first are ovulated first, and so on. Since oogenesis continues over many weeks in primates (Peters, 1970), it is possible that changing conditions of the internal environment affect oocytes; even in mice, in which this process is brief, germ cells proceed asynchronously through meiosis (Dietrich and Mulder, 1983). For technical reasons it has been difficult to verify this “production line” hypothesis either by labelling premeiotic germ cells with radiolabelled nucleosides or by estimating the recombination frequency for linked genes. To confirm the hypothesis, this frequency should fall pari passu with chiasma frequency. This hypothesis, once widely held, is now receiving more critical appraisal. In Chinese hamsters there was little correspondence between the chromosome groups involved in univalent formation at metaphase I and those responsible for aneuploidy at the ensuing maturation division (Sugawara and Mikamo,
and some Other Anomalies of human cytogenetics. This would then be a final verdict cannot be passed yet on whether chromosome anomalies are preformed in the fetal ovary. If they are not, some hope remains for eventually controlling their production later in life.

Some evidence suggests that trisomy should be seen within the setting of the wider physiological changes of the ageing female. This hypothesis is less discrete than those mentioned so far, but there is some experimental support for suspecting that ageing of oocytes is an epiphenomenon of the general deterioration of the reproductive system. Brook et al. (1984) reported that in unilaterally ovariectomized mice, the rising incidence of aneuploid embryos during ageing began earlier and cyclical ovarian function terminated earlier than in controls. These results suggest that the risk of non-disjunction in oocytes is increased during the final cycles of life, irrespective of the chronological age of the mother. According to some workers, it is the prolongation of these cycles that is significant because spontaneous or drug-induced extension of cycle length in rats leads to more morphologically abnormal embryos (Fugo and Butcher, 1971; Page et al., 1983). However, in view of what has been said already about delayed ovulation, it is unlikely that longer cycles are wholly to blame for trisomy. Since the incidence of human trisomies in the population rises long before the irregular cycles of the climacteric, the possibility of subtle hormonal changes affecting follicular oocytes is worth considering. Ovarian steroids can impair segregation of meiotic chromosomes during maturation in vitro (McGaughey, 1977), and a case has been made for hormone-dependent changes in the timing of maturation leading to trisomy (Crowley et al., 1979). These views can accommodate the J-shaped age distribution for trisomy because ovarian cycles are more irregular at both ends of the menstrual span (see p. 72). Changes in the frequency of Down’s syndrome among young women have also been attributed to altered hormonal balance following oral contraception (Read, 1982). To extend the argument to a logical conclusion, any factor that advances the normal course of ovarian ageing and its associated hormonal changes might potentiate the increase in trisomy. This would then explain the excess of trisomic offspring among smokers (Kline et al., 1983) and among Turner’s syndrome patients, in whom pregnancy is sometimes possible before early menopause (Reyes et al., 1976; King et al., 1978). However, we should not be sanguine about untested hypotheses, especially when an earlier study was unable to demonstrate an association between Down’s syndrome and less regular menses or early menopause in mothers (Sigler et al., 1967). The maternal age factor is still one of the major unsolved problems of human cytogenetics.

**Other Anomalies of Development**

Germ cells are segregated from somatic cells at early stages of embryogenesis, and some of their descendants survive for most, if not all, of the lifespan. For
much of this span they are not multiplying, and natural selection cannot operate to maintain vigour in the population. Yet, apart from those becoming chromosomally imbalanced, their progeny after fertilization have the same fertile potential and longevity as the parents. Furthermore, epidemiological studies have found little evidence that germ cells accumulate fresh gene mutations during ageing, and the effects that have been found are attributed mainly to paternal age (Polednak, 1976; Roberts, 1979). The ability of germ cells to avoid the costs of ageing, which are manifest among somatic cells, has fascinated biologists for generations. Germ cells which succeed in producing progeny are presumably either peculiarly resistant to damage of their informational macromolecules or have highly efficient repair mechanisms, which perhaps involve events during meiosis (Medvedev, 1981). This special quality of germ cells applies to most species, including mammals (Suntzeff et al., 1962), a notable exception being the “Lansing effect” of the freshwater rotifer Philodina citrina. Eggs from ageing clones of this species become progressively less viable and new individuals arising from them become precociously senile, but this phenomenon seems to be confined to the parthenogenetic mode of reproduction in these simple animals (Lansing, 1947).

Finally, mention should be made of human neural tube defects, for although they probably arise from a vitamin deficiency or an environmental agency, rather than being a general feature of ageing, their incidence varies during maternal ageing with a U-shaped distribution. Above age 35, the incidence of spina bifida and anencephalus is independent of parity, as is the age-related increase of hydrocephalus (Record, 1961; Carter et al., 1968; Carter and Evans, 1973). Further study is required to discover whether the age factor is due to variable penetrance by the causal agent or to differential viability in utero.

The Maternal Environment

Changes with Age

Increased pregnancy loss in ageing animals cannot be accounted for by defective embryos alone. The balance of responsibility between the maternal environment on the one hand and embryonic factors on the other has been shown by transferring zygotes or preimplantation embryos from young to old animals and vice versa. In small rodents it is necessary first to make the hosts pseudopregnant by mating with a vasectomized male because the corpora lutea are not active spontaneously during normal oestrous cycles. Results have shown unequivocally that animals of middle or old age perform poorly, for less than 15% of embryos from young donors were successful in older hosts, whereas approximately 50% survived to term in hosts of similar age (Table 5.3). In the experiments shown in Table 5.3, and in subsequent confirmatory ones, embryonic losses were found at both pre- and postimplantation stages.
Biological Basis of Infertility in Middle Age

TABLE 5.3
Relative Contributions of Embryonic and Maternal Factors in the Loss of Fertility during Ageing: Comparison of Three Species Using Reciprocal Transfer of Embryos between Young and Ageing Animals

<table>
<thead>
<tr>
<th>Age of donor</th>
<th>Age of host</th>
<th>Embryos surviving to term (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>Young</td>
<td>49</td>
</tr>
<tr>
<td>Young</td>
<td>Old</td>
<td>8</td>
</tr>
<tr>
<td>Old</td>
<td>Young</td>
<td>5</td>
</tr>
<tr>
<td>Young</td>
<td>Young</td>
<td>50</td>
</tr>
<tr>
<td>Young</td>
<td>Old</td>
<td>2</td>
</tr>
<tr>
<td>Old</td>
<td>Young</td>
<td>13</td>
</tr>
</tbody>
</table>

Hamster*   Rabbitb   Mousec

*Blaha (1964).
cTalbert and Krohn (1966).

It has been suggested that embryos may be dying at the time of implantation because the normal development schedule of the embryo and endometrium are desynchronized. This state of affairs could arise from a reduced rate of cleavage, which has been observed in old hamsters (Parkening and Soderwall; 1973). However, this would not appear to be the principal factor because transfer of preimplantation embryos which are one day in advance of the development of the ageing host uterus does not subsequently improve pregnancy success. The time taken for transporting embryos along the oviduct is about 3 days in both young and ageing mothers, but obstruction of the channel by stones could affect fertility in some species (Adams, 1970), although ectopic pregnancy in the oviduct is most exceptional in animals of any age (Gosden and Russell, 1981).

At the time of implantation, the uterine epithelium is thrown into folds which probably support blastocysts during the transport and attachment phases. This support is impaired in ageing rodents in which luminal epithelia fail to undergo corrugation changes and engage one another (Finn, 1970). Furthermore, attachment of trophectoderm cells of the blastocyst to the uterine epithelium may be hindered because microvilli on the surfaces of the latter are morphologically abnormal (Smith, 1975) or sparse (see Fig. 5.6). That this deficiency is not dependent on the systemic environment has been shown by the failure of uterine epithelia of 6- and 30-month-old CBA mice to adopt the appearance of the host following transplantation between different age groups (Fig. 5.6). In apparent contrast, it has been suggested that steroid treatment of the human postmenopausal endometrium restores the normal morphology of epithelial surfaces (Nathan et al., 1978).

Preparation for and maintenance of postimplantation development depend on stimulation by oestrogen and progesterone (Psychoyos, 1973). Blood levels of these hormones have been compared in many studies of ageing rodents, and most
Fig. 5.6. Scanning electron micrographs of the luminal surface of uteri of CBA mice, showing a greater density of microvilli in (a) a young uterine graft in an old host compared with (b) an old uterine graft in a young host (X10,000). (From the late E. C. Jones, unpublished.)

results show that neither hormone is deficient (Spilman et al., 1972; Gosden and Fowler, 1979; Holinka et al., 1979; Miller and Riegle, 1980). Although corpora lutea form and function normally in these animals, those of cows tend to become cystic, leading to infertility late in the second decade of life (Erickson et al., 1976). It is important to consider whether hormonal changes preceding conception are altered by ageing because uterine receptivity depends on an appropriate strength, duration and sequence of stimulation by gonadal steroids (Finn and Martin, 1970). In middle-aged rats, the uterus is exposed to oestrogen for a longer period, corresponding to the extension of cycle length at this phase (Page and Butcher, 1982), and this condition has been held responsible for some of the subsequent embryonic mortality (Butcher and Pope, 1979; Page et al., 1983).

In climacteric women, menstrual cycles are often infertile owing to luteolysis within 10 days of ovulation (Table 4.1). A short luteal phase is likely to be of greater significance for fertility than are relatively small reductions in peak
amounts of progesterone in middle-aged women (Reyes et al., 1977) because an embryo may be unable to rescue the corpus luteum, and so perishes. In contrast to the situation in laboratory rodents, responsibility for producing most of the gestational steroids in human pregnancy is progressively transferred from the ovary to the placenta, which assumes primary responsibility at about 6 weeks of gestation (Csapo et al., 1974). It would be interesting to have more information about the function of the feto-placental unit in older mothers, in view of the higher incidence of large placental infarcts and other placental disorders in women over 40, especially among smokers (Naeye, 1979).

Following the demonstration of normal luteal activity in most ageing animals, attention was diverted to the measurement of hormone-binding capacity in target tissues in order to find alternative explanations for poor adaptation of the uterus to gestation. Most evidence obtained from human and rodent uteri suggests that neither concentrations nor physical characteristics of cytosolic receptors for oestrogen and progesterone are affected by ageing (Flickinger et al., 1977; Blaha and Leavitt, 1978; Belisle et al., 1982); discrepant results were obtained
only with animals of postreproductive age (Gesell and Roth, 1981). One study shows that a significant fraction of the oestrogen–receptor complexes in the postmenopausal uterus are unable to bind to nuclear acceptor sites and are therefore biologically inactive (Strathy et al., 1982). This result is probably secondary to a changing endocrine milieu, for there can be no doubt that the human endometrium is capable of responding to ovarian steroids long after menopause and that normal uterotrophic responses can be obtained in animals of advanced age (Holinka et al., 1977). Pregnancies are occasionally reported in middle-aged women after menopause, as conventionally defined, but these are invariably unsuccessful (Krohn, 1964). A well-documented case has now been described in a woman aged only 25 who had menopause of at least 5 years’ standing (Lutjen et al., 1984). After several attempts, an embryo derived by fertilization in vitro of a donor’s egg with her husband’s spermatozoan was successfully brought to term with the support of exogenous hormones. Similarly, the uterus of CBA mice of postreproductive age is occasionally capable of maintaining gestation when stimulated by ovarian grafts, confirming that atrophic changes can be reversed (Krohn, 1964; Felicio et al., 1984).

In ageing uteri, several notable cytological changes occur which could contribute to postimplantation death. Considerable attention has been focussed on the decidual cell reaction, not because any definite function can yet be assigned to it but because it is dramatically reduced (Finn, 1966; Maibenco and Krehbiel, 1973; Holinka and Finch, 1977). The reaction may be impaired because production of prostaglandin (particularly PGE-2) is reduced (Brown et al., 1984), this group of substances having been implicated in early postimplantation physiology, including hyperaemia and decidualization (Tobert, 1976; Kennedy, 1977).

Surprisingly little attention has been paid to the possibility that postimplantation conceptuses die because of inadequate vascular perfusion. It is well known that when blood flow to the placenta is restricted by ligating uterine vessels, fetal development is retarded, sometimes leading to resorption (Wigglesworth, 1964). During ageing in rabbits the rate of blood flow to the gravid uterus is halved when calculated per unit weight of tissue (Larson and Foote, 1972). This result could simply be due to lower respiratory demands, and so measurements of lactate and blood gases in uterine veins are needed to check this finding. These measurements of uterine blood flow also highlight a problem which is frequently encountered when comparing young and ageing tissues which have differing compositions. The greater proportion of collagen in old tissues leads to an underestimate of cellular perfusion when unit weights of tissue are compared. Underperfusion of placentas in old uteri would be expected to lead to smaller fetal size, but newborn pups are of normal weight for litter size (R. G. Gosden, unpublished observations), and in our own species birth weight increases with parity (Selvin and Janerich, 1971). The sclerotic changes which cause stenosis of
uterine vessels in multiparous rodents and women (Wexler, 1964; Naeye, 1983) may be of little consequence if there are compensatory changes involving more efficient oxygen extraction in the placenta or, in polytocous species, if death of some fetuses releases resources for remaining ones. Nevertheless, it seems prudent for older mothers to rest, control any hypertension and refrain from smoking in order to optimize the utero-placental circulation.

The ageing uterus of rodents steadily accumulates collagen (Schaub, 1964–1965), whereas the collagen content of human uteri has been found to be constant between 30 years of age and menopause (Woessner, 1963). The accumula-

Fig. 5.7. Transverse section of the uterine wall of a multiparous mouse, showing abundant phagocytes laden with haemosiderin in the muscle coat of the mesometrial side. L, lumen. Perl's stain and neutral red (X75). (From Gosden, 1979.)
tion of fibre can be attributed to less catabolism because of (1) greater physical and chemical stability conferred by covalent intermolecular bonding (Kao et al., 1976) and (2) less collagenase (Maurer and Foote, 1972). These changes could be adaptive, though most workers suspect that they lead to detrimental changes, perhaps affecting the compliance of the uterus and cervix during gestation and parturition.

Scar tissue accumulating at former sites of placentation is another factor which might affect subsequent implantations. It consists of macrophages replete with lipofuscin and haemosiderin pigments which are residues of "mopping-up" operations postpartum (Fig. 5.7). These pigments are responsible for the yellow-brown coloration of multiparous rodent uteri and should not be confused with vitamin E deficiency pigments, which have similar histochemical properties but are found in uterine smooth muscle cells. Vitamin E deficiency leads to dystrophy of uterine muscle and fetal death in rats (Kitabchi, 1980), but there is no general evidence, from either histochemistry or vitamin supplementation work, to show a deficiency during ageing, despite one claim that vitamin E requirements rise steeply with age (Ames, 1974). The most conspicuous cytological change affecting the human uterus is the accumulation of autofluorescent particles beginning at the time of puberty (Fig. 5.8). Though they are abundant by middle age, the significance of these particles is obscure.

Effects of Reproductive History

Since pregnancy and lactation impose enormous metabolic demands, high parity might be expected to cause adverse wear and tear on the maternal constitution and perhaps accentuate specific pathophysiological changes, such as sclerosis of blood vessel walls. This assumption is difficult to verify in our own species because superabundant reproduction is commonly associated with social deprivation and poor standards of obstetric care, diet and housing. At the opposite end of the spectrum of reproductive output, primigravidae aged more than 34 years take longer to establish a successful pregnancy (Guttmacher, 1956) and have a high age-specific rate of perinatal mortality (Israel and Deutschberger, 1964). Such findings ought not to be accepted uncritically as a biological effect because this group may be self-selected for poor fertility following earlier unsuccessful attempts to reproduce (see p. 98). This limitation on the interpretation of human fertility statistics can be avoided by studying animals for which some definite biological effects of parity have been found under the special conditions of intra-uterine fetal crowding.

First, it is necessary to emphasize that no simple wear and tear hypothesis can accommodate all the facts concerning ageing and litter size because animals are not protected from the normal age-dependent toll of fetal death when mating is delayed until mid-life. According to some studies, elderly primigravid rats and mice are rather less fertile than multiparous animals of similar age (Asdell et al.,
Fig. 5.8. Uterine smooth muscle cell from a 48-year-old multiparous woman. The cell contains membrane-bound particles of variable electron density, with satellite dense bodies (presumably lysosomes). Osmium tetroxide (X47,200). (From Gosden et al., 1978.)
1941; Nishimura and Shikata, 1960). The effects of parity have been tested experimentally in rodents following unilateral ovariectomy at young adult ages. The remaining ovary sheds double its normal quota of ova, which, if fertilized, will implant only in the ipsilateral uterine horn. Thus, one of each pair of horns carries double the normal load of fetuses, whereas the other one remains barren, though subject to the same systemic hormonal conditions. The total number of live offspring produced after unilateral ovariectomy was approximately halved compared with that of intact controls, though there were detailed differences in breeding patterns according to the rodent species tested (Jones and Krohn, 1960; Biggers et al., 1962; Adams, 1970). In rats, litter size fell sharply after the sixth delivery, though not to zero, as in some strains of mice (Fig. 5.9), whereas in rabbits it declined steadily throughout life (Adams, 1970). Since corpora lutea and conceptuses were still produced after delivery of the final litter, Biggers et al. (1962) postulated that accelerated uterine ageing of the functional horn was responsible for pregnancy failure. Confirmation was obtained when it was found

![Figure 5.9](image_url)

**Fig. 5.9.** Mean number of live young produced per functional uterine horn during the lifespan of intact (*) and unilaterally ovariectomized (o) CFLP mice. Animals with intact ovaries produced 96 live young in toto by 271 days of age (mean age at final parturition), whereas those having only one ovary from early adulthood delivered 48 young by 226 days. The opportunity for reproduction was maximized in both groups by the continuous presence of fertile males and the removal of pups at birth. (From Gosden, 1979.)
that fewer blastocysts survived after transfer from young donor mice to parous horns of unilaterally ovariectomized animals rather than to intact controls (Gosden, 1979). There may be other reasons besides uterine ageing for the early loss of fertility, such as the increasing incidence of aneuploid embryos which have been observed (Brook et al., 1984).

An experiment carried out by the distinguished eighteenth-century anatomist and surgeon, John Hunter (1728–1793), is pertinent to discussions of parity. He compared the long-term breeding performance of two sows from the same farrow, one of which had been unilaterally ovariectomized. This experiment was designed to distinguish two opposing hypotheses: (1) that each ovary can produce only a fixed number of ova during the lifespan, in which case extirpation of one should halve the total number of offspring; (2) that breeding performance is limited by constitutional factors, in which case ovariectomy would not have a major effect. He reported that the ovariectomized sow bred for only 4 years and produced 76 piglets, whereas the control animal continued to breed for an additional 2 years, yielding a total of 162 piglets (Fig. 5.10). Hunter concluded, “It seems most probable that the ovaria are from the beginning destined to produce a fixed number, beyond which they cannot go, . . . but that the constitution at large has no power of giving to one ovarium the power of propagating equal to both; for in the present experiment the animal with one ovarium produced 10 pigs less than half the number brought forth by the sow with both ovaria” (Hunter, 1787). Since litter sizes were maintained in the animal with only one ovary, his experiment provides the first evidence that ovulation rate is controlled globally (p. 45). It cannot, however, be used to support the postulated effect of fetal crowding because in this species embryos can become distributed evenly between the two horns, irrespective of their origin, whereas, in rodents, intra-uterine migration is prevented by an anatomical barrier (Biggers et al., 1962). Thus, the poorer breeding performance of the ovariectomized sow may turn out to depend on ovarian (rather than uterine) ageing, vindicating Hunter’s conclusion. The experiment needs repeating, but modern biologists faced with the dual problems of time and expense will find sympathy with Hunter’s excuse: “It may be thought by some that I should have repeated this experiment, but an annual expense of £20 for 10 years, and the necessary attention to make the experiment complete, will be a sufficient reason for my not having done it”.

The underlying mechanisms responsible for the detrimental effects of fetal crowding in rodents have not yet been identified, nor is it clear whether they are significant under normal conditions of breeding. Poor uterine vascular supply and extensive scarring of the uterine wall after earlier implantation are hypotheses worth testing. The infertility of elderly primigravidae might be explained by the unnatural pattern of repeated cycles of oestrogenic stimulation of the endometrium or by earlier onset of irregular cycles, which heralds the end of cyclic life. Either of these hypotheses could explain why fertility is restored to long-

Biological Basis of Infertility in Middle Age
Fig. 5.10. Long-term reproductive performance of one intact ("perfect") and one unilaterally ovariectomized sow. (Adapted from Hunter, 1787.)

term castrated mice of post-reproductive age by transplanting ovaries from young donors (Felicio et al., 1984).

5.4. Contraception in Middle Age

Demographic statistics clearly show that older women in many countries have successfully regulated their fertility (Table 5.1). In recent years, the fertility rates of older women have fallen dramatically, especially in Western nations. For example, the live birth rate among Scottish mothers aged 40 and over fell by
79.9% between 1950 and 1980, whereas national fertility fell by only 8.6%. Undoubtedly, many social and economic factors have contributed to this shift. In addition, there is greater public awareness of the mortality and morbidity associated with late pregnancy, although the risks for healthy women enjoying good obstetric care have sometimes been exaggerated.

The natural fertility of women wanes from the third decade of life, but a residual capacity for pregnancy remains in some individuals until menopause. Therefore, precautions against pregnancy are required in middle age, and it is prudent to maintain them for at least 1–2 years after the last natural menses. Many couples who have completed their desired family choose sterilization of one partner, but other couples prefer the reversibility of contraception, in which case the method will be dictated by personal circumstances such as medical history, frequency of coitus and attitudes to unplanned pregnancy. Reliability is often an uppermost consideration when choosing contraceptives, and it is worth pointing out that a substantial margin of safety can be gained by combining the efficiency of a contraceptive with the decreased natural fertility of ageing. For example, if a contraceptive of only 90% efficiency in young fertile groups is combined with a loss of fertility of 90%, then according to a general probability model (Biggers, 1976), the overall degree of protection is $100 \times (1 - (1 - 0.90)^2) = 99\%$, assuming that they act independently. This consideration should, in theory, make the simple but less reliable methods of contraception more attractive for older couples, particularly where the more sophisticated ones carry an increased risk of disease. However, it has not been possible to quantify the natural loss of fertility for individuals. Clearly, there is a need for new contraceptives which can be prescribed safely and continuously for 2 or 3 decades until middle age, but for the present, we can only discuss the suitability of the familiar methods.

**Hormonal Methods**

Hormonal methods include some of the most effective reversible methods of contraception and are now employed by millions of women worldwide. However, clinical research and epidemiological surveys have shown that prolonged steroid contraception may increase the risk of a number of serious diseases, e.g. myocardial infarction, thromboembolism, liver and gallbladder disease and cancer of the cervix and breast (Royal College of General Practitioners’ Oral Contraception Study, 1977; Vessey et al., 1977, 1983; Jick et al., 1978a; Pike et al., 1983). Although the evidence for some of these diseases is not as well established as for others, the International Planned Parenthood Federation, the U.S. Food and Drug Administration and other authoritative bodies have advised women over 40 years of age to choose alternative methods of contraception. Some authorities recom-
mend an upper limit for oral contraceptive use of 35 or even 30 years, whereas others take a more circumspect view and emphasize the overriding importance of predisposing factors, such as smoking and medical history (Greenblatt et al., 1979). It is unlikely that early agreement will emerge concerning the absolute levels of risk incurred by prolonged steroid contraception because the technical difficulties of obtaining reliable and representative data are now compounded by the shift in recent years to lower-dose preparations (<50 μg ethinyl oestradiol or mestranol). The possible hazards of using preparations containing oestrogen are unlikely to be overcome by switching to ones containing only progestogen because, although the side effects on metabolism are minor (Howard et al., 1982), these contraceptives carry the important disadvantage of occasional breakthrough bleeding. All irregular bleeding in older women requires investigation because of the possibility of pelvic disorders, including malignant disease.

**Intra-Uterine Devices**

Intra-uterine devices (IUDs) are suitable for parous women who are free of menorrhagia, fibromyomata and anatomical deformations of the uterus or cervix.

**Barrier Methods**

Diaphragms, spermicidal jellies and condoms can be highly effective when used properly, and some carry the additional benefit of protection from sexually transmitted diseases. Such methods may be appropriate for older couples, especially where steroids or IUDs are contraindicated, but diaphragms and condoms cannot be worn on organs which have lost their tone, and the latter might exacerbate any loss of male potency.

**Coitus Interruptus**

Coitus interruptus, perhaps the most ancient method of birth control, is still widely practised despite its unreliability and tendency to reduce satisfaction.

**Rhythm Methods**

Rhythm methods depend on abstinence from coitus at the fertile time of the cycle, which is identified from calendar records of menstruation or analysis of basal body temperature rhythms or cervical mucus. Unless cycles are regular, these methods are not recommended, and during the climacteric women should be firmly discouraged from using them.
Induced Abortion

Although not strictly a contraceptive method, abortion is frequently used as a backup when contraception fails. The number of abortions among women aged 45–49 in England and Wales in 1980 almost equaled the number of live births for the same group (525 compared to 625), and the abortion rate was about 10 times that of women aged 25–29 years. Such statistics serve to highlight current attitudes and problems associated with late fertility.

6.4. Atrophy of Epithelium

Menopause, whether spontaneous or induced, is suspected to lead to atrophy of epithelium which are target tissues of estrogens and require hormonal stimulation for normal growth and differentiation. However, the distinction between atrophy arising from hormonal withdrawal, which is reversible, and that from continuous aging processes, which is irreversible, may sometimes be difficult to find. The most clear-cut examples of estrogen-dependent atrophy are found in the postmenopausal genital tract, in which the appearance of epithelia is a faithful, if imprecise, index of hormone concentrations.

Vagina

After withdrawal of estrogens as a consequence, changes in the vaginal lead gradually to atrophy of the epithelium and reduced compliance as applied force, perhaps because of fragmentation or vacuolization (Hicks, 1967). Epithelial and stromal cell volume decreases as atrophic processes predominate, and the vaginal epithelium at younger ages predominates. The thickness of the stratified epithelium is reduced from 8 to 10 cell layers before menopause to as few as 3 or 4 layers at menopausal times (1969). A post-menopausal vagina is vulnerable to mechanical injury and rapidly heals. This condition is aggravated by dryness caused by an impaired transudation of water to the vaginal walls and may cause pain at coitus (Sylla et al., 1977). The poor secretory capacity is necessary to lower vaginal blood flow after withdrawal of estrogens (Molander and Wagner, 1982). However, the strengthened vaginal tissues are unable to respond to estrogens without leading to hyperplasia, and in women with atrophic vaginitis, estrogen for the vulval vaginitis or those produced have been shown to improve mucus production rates. The epithelium is relatively thick and still contains cells with vacuolated superficial cells.

Significant changes of the vaginal wall occur also in menopause because the...
Somatic, Metabolic and Behavioural Consequences of Menopause

6.1. Atrophy of Epithelia

Menopause, whether spontaneous or iatrogenic, is expected to lead to atrophy of epithelia which are target tissues of oestrogen and require hormonal stimulation for normal growth and differentiation. However, the distinctions between atrophy arising from hormone withdrawal, which is reversible, and that from continuous ageing processes, which is irreversible, are sometimes difficult to find. The most clear-cut examples of oestrogen-dependent atrophy are found in the postmenopausal genital tract, in which the appearance of epithelia is a faithful, if imprecise, index of hormone concentrations.

Vagina

After withdrawal of oestrogen at menopause, atrophic changes in the vagina lead gradually to shortening of the organ and reduced compliance to applied force, perhaps because of fragmentation of elastic fibres (Lang and Aponte, 1967). Epithelial and stromal cell volumes decrease as autolytic processes predominate, and the rugae characteristic of younger ages disappear. The thickness of the stratified epithelium is reduced from 8 to 10 cell layers before menopause to as few as 3 or 4 layers afterwards (Hafez, 1982). A thin-walled vagina is vulnerable to mechanical injury and readily bleeds. This condition is aggravated by dryness caused by an impaired transudate response to erotic stimuli and may cause pain at coitus (dyspareunia). The poor response might be secondary to lower vaginal blood flow after withdrawal of oestrogen (Semmens and Wagner, 1982). However, the atrophic vagina retains an ability to respond to oestrogen without leading to hyperplasia, and in women receiving exogenous oestrogen for senile vaginitis or those producing large amounts at extra-glandular sites, the epithelium is relatively thick and well differentiated with cornified superficial cells.

Significant changes of the vaginal flora occur after menopause because the
amount of glycogen associated with thinner epithelia is diminished (compare Figs. 6.1 and 6.2). Glycogen liberated from moribund and exfoliated epithelial cells provides energy for the most abundant commensals, lactobacilli (Döderleins). Since lactic acid is the end product of their metabolism, the vaginal pH is 3.5–4.5 and moderately bacteriocidal, but after menopause the pH rises above 5 (Semmens and Wagner, 1982) and the microbial population often includes staphylococci and streptococci. Thus, lower oestrogenic stimulation of the vagina is associated with a higher risk of bacterial infections; nevertheless, postmenopausal vaginitis is mainly due to yeast infections, and antibiotics may encourage their growth and make matters worse (Ross, 1978).

**Urethra and Urinary Bladder**

Atrophic cystitis and urethritis, loss of tone of the urinary bladder and urinary incontinence occur in many women of postmenopausal age. The lower urinary tract is sensitive to the same hormonal changes as the vagina (Smith, 1972), and both structures have stratified squamous epithelia derived from the urogenital sinus of the embryo. It is not therefore surprising that they show parallel responses to oestrogen withdrawal and treatment.
Atrophy of Epithelia

Fig. 6.2. Atrophic vaginal epithelium of a postmenopausal woman. Periodic acid-Schiff reaction (X50).

Uterus and Cervix

The overall size and cytological appearance of the uterus and cervix are strongly influenced by the amount of circulating oestrogen. The postmenopausal uterus is slowly reduced in bulk, largely because of reduced muscle, until it is a small, wedge-shaped structure less than one-quarter the dimensions of the functioning organ. The vaginal portion of the cervix may disappear altogether. The postmenopausal endometrium is typically atrophic, with a flattened, non-proliferating epithelium and glands lined by only a single layer of cells (Fig. 6.3). Glands show no cyclical changes of activity and little sign of secretion, with comparable changes occurring in the cervix uteri.

In the absence of progesterone, oestrogen of endogenous or exogenous origin can stimulate atrophic endometrial glands to proliferate, which, if prolonged, can lead to cystic changes characterized by a "Swiss cheese" appearance (Fig. 6.4). At the same time, there is hypertrophy of stromal and epithelial cells, with increased density of microvilli and cilia and the reappearance of endometrial and cervical secretions. Glandular hyperplasia is also seen in some perimenopausal women having prolonged cycles and at middle age in anovulatory rodents pre-
Fig. 6.3. Atrophic endometrium of a postmenopausal woman. Haematoxylin and eosin (X50).

Fig. 6.4. Cystic glandular hyperplasia of the endometrium in a perimenopausal woman. Haematoxylin and eosin (X35).
Atrophy of Epithelia

senting persistent vaginal cornification. When maintained in breeding condition, virgin rabbits remain in persistent oestrus throughout most of their adult life. Chronic oestrogenic stimulation of the uterus without a luteal phase leads to hyperplasia and, hence, to adenomas and adenocarcinomas, which afflict the majority of animals at 5–6 years of age (Adams, 1970). It is a matter of concern that continuous exposure to oestrogen may cause or encourage similar diseases of the human endometrium.

Oviduct

The reduction in the length and thickness of the oviduct progresses slowly during the postmenopausal phase or following castration, but this condition can be ameliorated by administering oestrogen (Rumery and Eddy, 1974; Gaddum-Rosse et al., 1975). Smooth muscle becomes replaced by fibrous tissue, and mucosal rugae and cilia gradually disappear (Gaddum-Rosse et al., 1975; Hafez, 1982). Shortly after menopause the epithelium resembles that of cyclic women during the menstrual phase when plasma oestrogen levels reach their nadir, but after 20–30 years postmenopause substantial atrophy of ciliated and secretory cells will have taken place.

Skin

Skin is a target tissue for sex steroids. The most obvious signs of this are the sexually dimorphic plumage of birds, sex skin of primates and facial hair of men, but other widespread, if less obvious, signs are present. Human skin possesses specific cytosolic oestrogen receptors of high affinity and low capacity, but these are not uniformly distributed on the surfaces of the body (Hasselquist et al., 1980). It also contains 17α-hydroxylase, which catalyses oxidation of oestradiol to oestrone (Weinstein et al., 1968). The effects of ageing and low oestrogen status are more difficult to distinguish in skin than in the genital tract. There is no clear demonstration that exogenous oestrogens have a beneficial (i.e. cosmetic) effect on wrinkled facial skin or sagging breasts. The epidermis and dermis become thinner with increasing age after early adulthood in women and men (Punnonen, 1972; Marks and Shahrad, 1976). They lose water and elasticity and become more pigmented, and such changes are accelerated in skins regularly exposed to the ultraviolet rays in sunlight. Oestrogen seems to affect several parameters of ageing in skin, but the physiological significance of most observations is questionable because large doses have been used. In a study of ovariectomized mice, oestrone stimulated epidermal growth initially, but when administration continued, the effects were reversed and the rate of hair growth fell (Bullough, 1947). In ovariectomized women, large doses of conjugated oestrogen were found to stimulate epidermal growth but did not have the self-
limiting effect observed in mice (Punnonen, 1972). Besides the effects on cell numbers, oestrogen stimulated the synthesis, maturation and turnover of collagen in animals, but the net result of prolonged treatment was to decrease amounts overall (Smith and Allison, 1966; Henneman, 1968). After castration, animals deposit more fat in their subcutaneous tissues and deep body sites, and it is probable that human menopause is one factor which predisposes middle-aged women to obesity.

The skin of the vulva shows conspicuous changes after middle age which are independent of oestrogen levels. It becomes delicate and parchment-like, and may show hyperplastic and hyperkeratotic alternation ("kraurosis vulvae") (Lang and Aponte, 1967). Wrinkling occurs through loss of subcutaneous fat, and hair follicles become sparse. Hair is lost at many sites of the body, but at a few sites it grows more thickly (e.g. the chin and upper lip), which has been attributed to a higher androgen:oestrogen ratio after menopause.

6.2. Autonomic Nervous Disturbances

The major vasomotor disorders of the climacteric are hot flushes (flushes), palpitations and headaches. The first is probably the most common climacteric symptom, affecting three out of four women to some degree. Flushing episodes begin shortly before or after the last menses and persist for a few years, but there is considerable individual variation, and in exceptional cases these episodes continue for 20 or more years (Table 1.1). Their frequency varies from several each day to one or two per week.

The symptoms are well defined. There is a prodromal phase in which women describe sensations of pressure in the head (rather like a headache) which increase in intensity, and the flush is felt within the following 4 min. The flush itself is characterized by a feeling of heat commencing in the head and neck which then passes to the shoulders and chest region. Physiological recordings show cutaneous vasodilatation with sweating in most regions of the body, but subjects are not always aware how widespread these effects are. These symptoms are distressing, especially in social contexts, and are sometimes responsible for insomnia, which may benefit from oestrogen treatment of flushing (Kupperman et al., 1959; Hagen et al., 1982a). Similar symptoms commence within a few days of bilateral ovariectomy in women of premenopausal age (Aksel et al., 1976).

Physiological studies have countered earlier claims that hot flushes are entirely psychosomatic. It is surprising how few measurements have been made in view of the number of women affected and the interesting biological nature of the phenomenon. Molnar (1975) was the first to carry out physiological measurements of hot flushes, but he studied only one subject. Most observers agree that
the first objective sign of a flushing episode is a rise in skin conductance (a function of the rate of sweating) beginning about 45 sec after the subjective premonition (Sturdee et al., 1978; Tataryn et al., 1980). Conductance is maximal about 4 min later and declines to baseline in a further 14 min (Fig. 6.5). The concomitant rise of skin temperature is of longer duration and is more variable in extent, being highest in the toes and fingers (c. 4°C), perhaps because of less cooling by evaporation of sweat. Skin temperature changes are generally less reliable as indicators of the time course of the flushing episode than changes in conductance (Tataryn et al., 1980).

During climacteric flushing the rate of blood flowing to the hand rises three–fourfold in the first 2–3 min and is maintained at the higher level for a few minutes longer before returning rapidly to baseline (Fig. 6.6). The time course of similar changes in forearm and finger blood flows are slightly different. Since skeletal muscle and skin each receive their distinctive types of vasomotor innervation, it is probable that these haemodynamic changes are confined to cutaneous vessels. The flush is accompanied by a rising heart rate of 20% over resting values, but there are no changes in blood pressure or circulating catecholamines or irregularities of the electrocardiogram (Sturdee et al., 1978; Casper et al., 1979; Ginsburg et al., 1981). The sweating and peripheral vasodilatation lead to an increased heat loss by evaporation and radiation and, hence, to a fall in core body temperature by about 0.2°C (Fig. 6.5).

Since flushing and sweating are associated with ovarian failure and are usually alleviated by exogenous oestrogens, they have been assumed to be consequences of oestrogen deficiency, either directly or indirectly. In some studies, symptomatic women had lower levels of oestradiol and/or oestrone than asymptomatic individuals at perimenopausal ages (Campbell, 1976; Chakravarti et al., 1979;
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Fig. 6.6. Mean hand and forearm blood flow and pulse rate in six women before, during and after a perimenopausal hot flush. (From Ginsburg et al., 1981; reprinted with permission.)
Hagen et al., 1982b), but in other studies no distinction was seen (Aksel et al., 1976; Chakravarti et al., 1977; Badawy et al., 1979). The vasomotor disorders can be attributed to habituation of neurons to oestrogen after puberty. When hormone levels fall profoundly in mid-life a period of metabolic adjustment to the new internal environment is required. If there is a critical period for habituation it must be brief because teenage girls have reported flushing after ovarian failure or castration. These observations seem to be at variance with findings from hypogonadal women with Turner’s syndrome, in whom symptoms are totally lacking despite the fact that 30% of them experience some menstrual activity and many receive exogenous oestrogens (W. H. Price, personal communication).

There are strong indications that climacteric flushing is neither a direct nor a specific effect of oestrogen withdrawal. Flushing has been recorded after hypothalamic damage in a small number of girls and boys at early stages of puberty, i.e. without exposure to adult female levels of oestrogens (Witt and Blethen, 1983). In double-blind trials, adult symptoms have been alleviated significantly by treatment with placebos and progestogens, and the benefits of the latter have sometimes approached those obtained with oestrogens (Dennerstein et al., 1978; Paterson, 1982; Lobo et al., 1984). Also, non-hormonal substances such as clonidine and naloxone have been reported to be effective (Clayden et al., 1974; Lightman et al., 1981), but negative results have been obtained more recently (Lindsay and Hart, 1978; DeFazio et al., 1984). Flushing is frequently experienced by hypogonadal men soon after castration or treatment with LHRH agonists, the peripheral physiological changes being similar to those of climacteric women (Ginsburg and O’Reilly, 1983). These observations imply that withdrawal of androgens or possibly other non-oestrogenic gonadal secretions can trigger vasomotor disorders.

Although pituitary gonadotrophins rise during the climacteric, they cannot be held responsible for these symptoms. Neither exogenous gonadotrophins nor LHRH trigger symptoms in young women. Moreover, hot flushes and sweating occur after hypophysectomy when levels of gonadotrophins are lowered (Mulley et al., 1977; Meldrum et al., 1981b). A notable synchrony between flushing episodes, identified by rising finger temperature, and pulsatile release of LH has been found (Tataryn et al., 1979; Casper et al., 1979). Because FSH pulses are less clearly defined, it is not certain whether these are also synchronized (Fig. 6.7). No such relationship has been found with oestrogen levels, although plasma dehydroepiandrosterone levels are elevated during flushing and may be secondary to stressful effects of symptoms (Meldrum et al., 1980). That the pulses of LH are not responsible for flushes has been demonstrated by two types of experiments, each using superactive LHRH analogues to inhibit pulsatile release of LH by down-regulating pituitary LHRH receptors. The analogues produced flushing in premenopausal women by lowering gonadotrophins and, hence, go-
Fig. 6.7. Simultaneous measurements in a climacteric woman of hot flushes (onset marked by arrows which indicate increased finger temperature), serum FSH, LH, oestrone (E₁) and oestradiol (E₂). (From Meldrum et al., 1980; reprinted with permission.)

nadial secretion (DeFazio et al., 1983) and were unable to relieve spontaneous symptoms in climacteric women (Casper and Yen, 1981).

Since each pulse of LH corresponds to a pulse of LHRH neurosecretion (Clarke and Cummins, 1982), flushing symptoms would seem to have a central origin. This hypothesis is strengthened by the following points: (1) Objective signs of flushing are often preceded by premonitions. (2) There is the theoretical difficulty of explaining how vasodilatation, involving adrenergic fibres, and sweat gland excitation, involving sympathetic cholinergic fibres, could both be triggered by a common peripheral factor carried in the circulation. On the other hand, this dual activity is wholly consistent with the physiological property of increasing sympathetic drive centrally under circumstances that require the core temperature to be lowered. (3) The fall in core temperature during the flush is itself an indication of a central disorder since peripheral cooling would be expected to lead to thermoregulatory adjustments to abrogate any fall in core temperature (Judd and Korenman, 1982). There is therefore good reason to believe that vasomotor symptoms are the result of inappropriate activity of central thermoregulatory mechanisms (Tataryn et al., 1980).

Central thermoreceptors are located in the posterior preoptic–anterior hypothalamic areas, not far from the hypothalamic LHRH-containing neurons which are influenced during flushing. The initiating event of the hot flush seems to cause a transient fall of the set point of the central thermostat. A set point in a regulatory system is that value of a controlled variable at which control action is
zero. A fall therefore indicates the need to dissipate heat and brings about peripheral changes leading to lower core temperature.

The cellular mechanism by which withdrawal of oestrogen affects the central thermoregulatory set point is not known. One possibility which is currently under study is that decreasing levels of catechol oestrogen, which correspond to the postmenopausal withdrawal of classical oestrogens, lead to thermoregulatory instability by affecting monoaminergic synapses. The catechol oestrogens, which are products of metabolizing oestradiol and oestrone (see Section 2.4), are cleared so rapidly from blood that circulating amounts are unlikely to be high enough to have physiological effects (Merriam et al., 1980), but those formed in the hypothalamus might be sufficient to exert a localized influence on neurones (Fishman and Norton, 1975). Because of their structural similarity, the 2-hydroxyoestrogens are potent competitive inhibitors of the metabolism of catecholamines by catechol-o-methyltransferase (Ball et al., 1972; Lloyd et al., 1978). On the other hand, they displace catecholamines from cellular binding sites, at least in non-neural tissue (Schaeffer and Hsueh, 1979), and affect catecholamine synthesis by inhibiting the rate-limiting enzyme, tyrosine hydroxylase (Lloyd and Weisz, 1978). The net effect of these opposing actions of catechol oestrogens on synaptic activity is not predictable, though the findings provide grounds for suspecting their involvement in climacteric flushing.

6.3. Demineralization of Bone

Demineralization of bone is potentially the most serious long-term consequence of human menopause. Not only does it cause immense suffering to individuals, but it is also costly to the community. Excessive loss of bone leads to clinical osteoporosis, which is responsible for an increasing incidence of bone fractures among older women (Nordin et al., 1980a). All parts of the axial and appendicular skeleton become progressively rarefied after menopause, but it is the distal forearm, neck of the femur and spine that have the greatest risk of fracture. Spinal fractures are diagnostic of osteoporosis in old age because they occur spontaneously when there is insufficient trabecular bone to act as supporting cross braces, leading to compression damage (Twomey et al., 1983). Limb bones are fractured by traumatic accident, the femoral neck being the most important site because it is a significant cause of death in elderly women. The incidence of this fracture rises exponentially after age 50; by the ninth decade the cumulative incidence in an American population was 32% in women but only 17% in men (Gallagher et al., 1980a). Thus, there is good reason for the current attention on the pathophysiology of bone loss in postmenopause.

Albright et al. (1941) were first to recognize a higher frequency of osteoporotic patients amongst postmenopausal women, but the importance of their discov-
ery was insufficiently appreciated at the time. Meema (1966) and Nordin et al. (1966) showed that commencement of bone loss depended on postmenopausal status rather than chronological age, and it soon became apparent that this is not due to the presence of diseases which are recognised causes of bone deterioration, viz. renal failure, hyperparathyroidism, thyrotoxicosis and Cushing’s syndrome. On the basis of the epidemiology of osteoporosis and the pattern of cortical and trabecular bone loss, two distinct syndromes of differing aetiology have been identified. Type I osteoporosis is associated with oestrogen deficiency after the menopause. Type II (“senile osteoporosis”) affects a proportion of much older women (>75 years) and involves accelerated bone loss with poor absorption of calcium (Riggs and Melton, 1983). Unfortunately, this distinction has not always been made, which could explain some conflicting data.

Several methods for measuring bone mass are available. X-ray film densitometry and γ-ray and X-ray absorptiometry are commonly used for measuring cortical bone thickness of the appendages, the second metacarpal of the right hand being the site favoured by many research groups. Trabecular bone can be assessed simply by an X-ray film of the proximal femur, but for quantitative information biopsies of the iliac crest are taken for histomorphometry. Progressive changes in bone density can be monitored readily in longitudinal studies of individual women. In cross-sectional studies, values obtained with these methods must be corrected for total bone volume because osteoporosis is defined according to the relative amounts of bone lost (mass : volume ratio); otherwise all women with small bones would be assigned to this category.

These methods have provided unequivocal evidence that both cortical and trabecular bone are lost in postmenopausal women. During the early postmenopausal phase, the rate of trabecular bone loss is the greater of the two, but because this component represents only 20% of the skeleton at most, the rate falls after age 60 because of the limited amounts remaining (Crilly et al., 1981). Hence, the incidence of spinal fractures does not rise continuously in old age, in contrast to that of hip fractures, which depend on loss of both cortical and trabecular bone. The rate of cortical bone loss is greater during the first 3 years of postmenopause than afterwards (−2.7% compared to −0.7% annually in the study of Lindsay et al., 1976), which, in a general way, parallels the slow metabolic adjustment of the thermoregulatory apparatus to lower levels of oestrogen. The rates of bone loss might seem small, but since losses are cumulative, they are highly significant in the long run. Bone is also lost in ageing men, though a later onset and a lower rate of loss afford them greater protection against fractures in old age (Dequecker et al., 1971).

Since 99% of body calcium is located in the skeleton and all bones tend to become porous in advancing years, measurements of total body calcium (TbCa) using neutron activation analysis provide a valuable guide to global changes of bone density. In this method a beam of partially moderated, fast neutrons from a
cyclotron or other source is directed towards the subject, in whom the reaction $^{48}\text{Ca}(n,\gamma)^{49}\text{Ca}$ is induced, besides other elemental changes (Cohn, 1980). The dose of radiation absorbed is small (c. 0.3 rem) and can therefore be repeated for longitudinal studies. Radioisotopes generated in tissue emit a spectrum of $\gamma$ radiation which is specific for each element and can be detected in a whole body counter. Such methods are readily standardized and are less dependent on subjective criteria than those mentioned above. The TbCa of normal, healthy white Americans is shown in Fig. 6.8. Individual values are highly variable; nevertheless, two findings are outstanding: (1) Those of women are 20–40% lower than those of men of the same age, reflecting differences in body size. (2) The TbCa declines from middle age in women, but this decline is less marked in men. Cohn et al. (1976) divided the data for women into two sets by imposing an arbitrary age of 55 to separate pre- and postmenopausal groups. The rate of loss of TbCa was estimated to be 0.38% annually in those aged 35–54, rising to

![Diagram of TbCa variations during aging](image-url)
1.08% in women aged 55–79. These figures imply that 95 mmoles (3.8 g) and 190 mmoles (7.6 g) of calcium are lost annually in pre- and postmenopausal women, respectively. Thus, by age 79, 6.25 mmoles (250 g) of calcium will have been lost, which amounts to about 28% of the TbCa of women aged 30. The amounts of phosphorus lost during life are in close accord with theoretical predictions based on the stoichiometric relationship to calcium. These important data are marred only by the assumption of the late menopausal age of 55. The rate of bone loss in young women may, therefore, have been seriously overestimated by combining a low (if existent) premenopausal value with higher values in postmenopausal women. Such doubts have been strengthened by Kennedy et al. (1982), who found that the TbCa was stable in premenopausal women but subsequently declined at an annual rate of 1.5%. Neither study was able to verify whether postmenopausal bone loss commences at a high rate and falls later; longitudinal studies of individual women will be required to test this hypothesis. The extent to which ageing men become demineralized also requires further study. Cohn et al. (1976) estimated the annual rate of loss of calcium was 0.7%, whereas Kennedy et al. (1982) were unable to find any significant change during ageing.

There are surprisingly large ethnic and national differences in the age-specific incidence of bone fractures. Anecdotal evidence suggests that old women in some parts of the developing world are less vulnerable to fractures than those elsewhere (World Health Organization, 1981). There is little doubt that in this regard older American black women have a strong advantage over American whites (Iskrant, 1968). The greater total body levels of calcium and phosphorus in these black women at most ages, even after adjusting for differences in height and weight, would appear to explain these differences (Cohn et al., 1977). Progressive demineralization during ageing might be a general phenomenon, but whether it leads to fragile bones will depend on life expectancy after menopause, mineralization in early adulthood, diet and other factors.

Albright et al. (1941) assumed that postmenopausal osteoporosis is caused by diminished formation of new bone, but this has turned out to be incorrect. Most evidence indicates that bone remodelling is increased during ageing, the rate of resorption exceeding that of new bone formation (Riggs et al., 1969; Heaney et al., 1978b; Delmas et al., 1983). After menopause, fasting levels of urinary hydroxyproline and plasma alkaline phosphatase rise, these being non-specific indicators of bone resorption (Crilly et al., 1981). The process of resorption is responsible for higher plasma levels of calcium and phosphate in fasting postmenopausal and castrated women (Young and Nordin, 1967; Gallagher et al., 1972), but these levels rarely exceed the upper limit of the normal range and do not therefore lead to clinical hypercalcaemia. The effects of ageing and menopause on phosphate excretion are more controversial and depend on the circulating amounts of parathyroid hormone, which are affected, in turn, by blood
calcium levels (see below). Many of the changes associated with postmenopausal bone loss are small, subtle deviations within the normal physiological range. If considered singly these changes might be overlooked, but they are significant over long periods of time.

Many clinical studies have shown that exogenous oestrogens inhibit bone loss in postmenopausal women (World Health Organization, 1981). This treatment can even lead to a net increase in bone material if sufficiently large doses are given (>25μg ethinyl oestradiol/day) (Christiansen et al., 1982; Horsman et al., 1983). Most oestrogens can prevent bone loss, but oestradiol requires continuous administration in large doses (Lindsay et al., 1979). Conservation of bone reduces the risk of fractures later in life. In an American study, the risks of fracturing either the distal forearm or the hip were reduced 50–60% when oestrogen treatment had continued for at least 6 years (Weiss et al., 1980). To be fully effective, treatment should commence at menopause and continue indefinitely. In one study, withdrawal of treatment led to a high rate of bone loss which cancelled out benefits accruing from earlier conservation (Lindsay et al., 1978), but some other studies have shown that long-term protection is afforded even by limited treatment with oestrogens (Christiansen et al., 1981).

The prophylactic effects of exogenous oestrogens suggest, but do not prove, that the loss of bone mineral content in postmenopausal women is due to a deficiency of follicular oestrogen. Some studies show plasma oestrogen levels are related inversely to fasting urinary levels of calcium, hydroxyproline and the degree of metacarpal bone loss; these elements being of a more physiological nature, provide firmer support of the hypothesis (Frumar et al., 1980; Crilly et al., 1981). However, a relationship between oestrogen levels and clinical osteoporosis has not been established. Marshall et al. (1977) found osteoporotic women had lower circulating levels of oestrone than age-matched controls. Davidson et al. (1982, 1983) were unable to confirm this result in patients with hip fractures, although sex steroid-binding globulin activity was raised. A higher incidence of postmenopausal osteoporosis among non-obese than obese women (Daniell, 1976; Davidson et al., 1982) might be accounted for by lower production of oestrogen and/or less biologically active circulating oestrogen (see Section 2.3). If women require oestrogen to maintain bone mineral content at young adult levels, young hypogonadal women would be expected to be particularly vulnerable to fractures and so would be expected to require hormonal therapy throughout life. But, paradoxically, women with Turner's syndrome do not become rapidly demineralized, nor do they have a high risk of fractures late in life (Smith et al., 1982). Presumably, they are either protected by a special feature of their constitution or they have not been exposed to sufficient oestrogen for a withdrawal response to occur.

Many explanations have been offered for the protective effects of oestrogen on bone mineral status in postmenopausal women. The effects may turn out to be
indirect for, despite a thorough search, high-affinity cytosolic receptors for oestrogen have not been detected in bone (Chen and Feldman, 1978; van Paassen et al., 1978). The significance, if any, of aromatase activity in this tissue is therefore obscure (Vittek et al., 1974; Frisch et al., 1980). Consequently, more attention is now being focussed on possible mediating effects of other hormones involved in calcium homeostasis.

The thyroid hormone, calcitonin, has been considered as a possible mediator of the protective effects of oestrogen on bone minerals. Calcitonin conserves bone calcium and opposes the resorptive activities of parathyroid hormone and vitamin D. The suspicion that calcitonin is deficient after menopause is reinforced by circumstantial evidence that plasma levels are lower in women than in men and are increased by exogenous oestrogen (Morimoto et al., 1980; Stevenson et al., 1981). Moreover, ovariectomy in rats impairs the normal release of calcitonin by calcium salts, this effect being reversed by oestrogen treatment (Catherwood et al., 1983). In women this response to calcium wanes slowly throughout adult life without an abrupt change at menopausal age, and so oestrogen withdrawal is not likely to be a prime cause of lower calcitonin (Shamonki et al., 1980). Conflicting results have been obtained when blood calcitonin levels were analysed in osteoporotic women. In one study levels were sub-normal (Milhaud et al., 1978), but in another study they were not related to the severity of the disease (Chestnut et al., 1980). Firm conclusions cannot be drawn at this stage, but it is unlikely that insufficient calcitonin is entirely responsible for demineralization after menopause.

Parathyroid hormone has also been considered to be a potential agent of postmenopausal bone loss because it is a hypercalcaemic hormone which can withdraw mineral stored in the skeleton, besides promoting calcium retention by acting on the kidneys and gut. Organ culture studies have shown that oestrogen inhibits the ability of parathyroid hormone to release calcium and phosphate from mouse calvaria (Atkins et al., 1972), but the interpretation of these data is made difficult by the studies which failed to find cytosolic receptors in other bones. The circulating levels of parathyroid hormone have been found to be slightly elevated during normal ageing as well as in osteoporotic patients (Insogna et al., 1981; Delmas et al., 1983; Marcus et al., 1984). These levels could be a physiological response to poor absorption of calcium. Oestrogen might affect the actions of other hormones, leading to a negative calcium balance, e.g. by affecting actions of growth hormone (Schwartz et al., 1969), or by some undefined mechanism leading to greater catabolism of the collagenous matrix of bone (Smith and Allison, 1966; Henneman, 1968).

Bone mineral loss is aggravated by an inadequate intake of calcium, which may partly explain why the rates of loss are so variable between individuals. Calcium deficiency is attributed to a poor diet (low calcium content and/or bound to phytate) or an impaired ability of the gut to absorb available calcium, a
condition which is prevalent among ageing women, especially those with severe osteoporosis (Ireland and Fordtran, 1973; Gallagher et al., 1979; Crilly et al., 1981). Poor absorption could arise from vitamin D deficiency because vitamin levels are often marginal or inadequate in elderly women, especially during the winter months at higher latitudes. However, this should not imply that histological features of osteoporosis and osteomalacia are necessarily concurrent; indeed, in an English study the histological features of these distinct conditions were combined in only 10% of cases (Nordin et al., 1980a). Small doses of vitamin D improved the absorption of calcium in short-term studies of osteoporotic women (Gallagher et al., 1982), but primary failure of the absorptive mechanism in the small intestine was important in some cases (Francis et al., 1984). Oestrogen may help to preserve the status of vitamin D by directly or indirectly stimulating renal 1α-hydroxylase activity to bring about formation of the active metabolite, 1,25-dihydroxycholecalciferol. Of these two alternatives, the indirect pathway is more probable, namely, that oestrogen inhibits the transfer of calcium from bone to blood (by some elusive mechanism), allowing levels of parathyroid hormone to rise, which then acts on the kidney (Gallagher et al., 1980b). Low calcium intake is an important factor contributing to demineralization of the skeleton, but the greater urinary excretion of calcium indicates that its role is secondary.

To sum up, it has not been possible to demonstrate a simple causal nexus between lower oestrogen levels and demineralization after menopause. Nevertheless, oestrogen is suspected of having some kind of protective effect on bones of normal women. Human biologists will have to surmount many technical problems, as well as uncover interactive factors in clarifying the causes of osteoporosis. Besides the widespread and complex metabolic changes after menopause, there are continuously changing patterns of life-style which could be as relevant. The significance of vitamin D status and calcium content of the diet has already been stressed, and to it must be added the detrimental effects of physical inactivity on bone condition. Further reference to these factors is made in the next chapter because small changes in life-style might have lasting benefits.

6.4. Intermediary Metabolism

The effects of oestrogen on cellular metabolism and growth and the consequences of oestrogen deficiency after menopause have been discussed in many sections of this book. But, so far, there has been no discussion of whether metabolic changes arising from oestrogen withdrawal promote major degenerative diseases of middle and old age. Loss of ovarian activity does not appear to affect the metabolism of carbohydrates in normal healthy women (Notelovitz,
6.5. Sexual Behaviour

Circulating levels of sex steroids fall after menopause, but it is doubtful whether they can fully account for the reputed decline in sexual interest and activity of ageing. Human sexual behaviour is the most complex variable mentioned so far and is affected by biological, psychological and sociological factors. Only for animals can it be stated with confidence that hypogonadism abolishes the sexual response.

Female rodents are sexually receptive for only a few hours around the time of ovulation. During this brief period of "oestrus" they adopt a "lordotic" posture (Gk. lordosis: bent backwards) to allow mounting and penetration by the male. Synchrony of sexual behavior and ovulation is achieved by the same internal signals, namely, exposure of the brain to gonadal steroids, especially oestrogen. Progesterone has a potentiating effect in animals primed with oestrogen, but it inhibits lordosis under other circumstances. Although details differ between species, the ovarian steroids are essential for receptivity in all higher animals, including sub-human primates (Michael and Bonsall, 1977).

In old age, when production of sex steroids falls because of a dwindling store of ovarian follicles, rodents become sexually unreceptive (anoestrus). However, most strains do not reach this phase, but instead enter one of persistent vaginal...
cornification, with moderately elevated levels of oestrogen remaining for much of postcyclic life (p. 71). During this anovulatory phase, multiparous rats aged 19 months old were frequently, but not uniformly, receptive to males. Coitus temporarily interrupted their acyclical vaginal smear pattern, suggesting that neuroendocrine responses had been elicited (Cooper and Linnoila, 1977; Hendricks et al., 1979). Individual differences in receptivity probably depended on oestrogen levels because rats having vaginal smears containing a mixture of cornified cells and leucocytes (probably indicative of less oestrogenic stimulation) rarely allowed mounting to occur. On the other hand, when ageing animals presented short cycles their lordosis behaviour was indistinguishable from that of young animals, as was that of ovariectomized rats receiving gonadal steroid replacement treatment at ages 20–30 months (Peng et al., 1977). Therefore, the brain and motor systems of old rats with breeding experience are capable of normal sexual responses given appropriate priming by hormones.

These findings should not be extrapolated to our own species because of the strong influence of non-biological factors on human sexuality. Valid comparisons cannot be made because our sexuality is generally measured as "libido", a vague subjective measure of sex drive, whereas, in rodents, a specific response (lordosis) is measured. It is still not clear to what extent physiological levels of sex steroids affect libido in women because few rigorous double-blind experiments with ovariectomized subjects have been undertaken. There is tentative evidence that a small quantity of oestrogen is required for libido, but a dose–response relationship does not exist above a low threshold (Sanders and Bancroft, 1982). Since human sexual activity is maximal at both pre- and postmenstrual stages of the cycle, oestrogen does not have such a dominating influence on behaviour as it does in animals (Bancroft, 1983). The role of other gonadal steroids in human libido is even more obscure. Testosterone has been shown to be proceptive, at least in pharmacological doses, but might require aromatization in the brain (Naftolin et al., 1975). And in the case of progesterone, equivocal results have been obtained. These major uncertainties about the normal role of sex steroids inevitably limit our understanding of sexuality during ageing.

The effects of age on human sexual behaviour were studied extensively by Kinsey et al. (1953) in the United States. Marital coital rates at 40 years of age were half those at age 20. Sexual interest and activity in men declined continuously from puberty, whereas solitary sexual activities of unmarried women did not fall until age 55–60. The authors concluded that women do not age in their sexual capacities until late in life, and they blamed husbands for declining marital coitus. Similar conclusions were reached by James (1974b) in an English survey. Coital rates were correlated more closely with the age of the husband than with that of the wife. There was a fall by 0.8 coitons per month for every 5 additional years of the husband’s age; the corresponding value for wives was
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only 0.1 coition when other variables were controlled. Other studies carried out in the somewhat artificial environment of the laboratory showed that postmeno-
pausal women retained a potential for effective sexual responses, especially when regularly stimulated, although the intensity and duration at each phase were reduced (Masters and Johnson, 1966).

Some studies have found a reduction in sex drive and capacity for orgasm during the climacteric. Such findings are not necessarily in conflict with the conclusions of Kinsey et al. (1953) and James (1974b), who were mainly concerned with long-term changes, but they definitely refute the old notion of a flareup of sex drive in middle age (Hallström, 1979). Waning libido in climac-
teric women may be secondary to the physical discomfort of hot flushes, sweating and dyspareunia or to insomnia and anxiety, which are common at these ages. Sex steroid therapy might, therefore, benefit sexual pleasure indirectly by relieving these symptoms (Masters and Johnson, 1966; Morrell et al., 1984).

Patterns of sexual interest and activity during and following the climacteric are highly complex and variable, and it is unwise to make sweeping conclusions. In some couples the major factors might be the attitude of the male partner or cultural restraints on sexuality, whereas in others the biological effects of meno-
pause may be uppermost. Withdrawal of sex steroids would not appear to di-
rectly affect libido, and any benefits obtained with exogenous oestrogens are attributable to the reversal of genital atrophy and amelioration of climacteric symptoms.
The Hormone Replacement Therapy (HRT) Controversy

In recent years, the failure of postmenopausal human ovaries to produce oestrogen has probably been responsible for more attention on the ageing reproductive system than any other factor. This is not surprising since this failure is marked by the important landmark of menopause and is associated with unpleasant symptoms and long-term metabolic changes which extend beyond the redundancy of reproductive organs. Public interest in the subject has been heightened by the widespread use of oestrogen for "hormone replacement therapy (HRT)" in Western countries, in which a large proportion of women are of postmenopausal age. Such treatment is undoubtedly effective in alleviating those symptoms of middle age which arise from the biological effects of oestrogen withdrawal. However, the use of "replacement" hormones remains controversial because of fears of promoting serious diseases, and for individual cases the decision whether to prescribe them is not a clear-cut scientific one but requires the craft of the experienced practitioner. Nevertheless, knowledge of human biological factors forms a basis for the practical problems of management. In this concluding chapter, these factors are revisited as an introduction for readers with clinical and pharmacological interests.

First, it is necessary to consider whether postmenopause is a natural condition since the answer to this question will affect attitudes to HRT. Apart from embarking on a purely semantic argument, this question can be tackled in the following way. If "natural" implies a physiological state, the answer must be affirmative because all women reaching their seventh decade will have experienced menopause. However, the observation that menopause is universal does not lead to any fresh insight unless the phenomenon can be shown to be an evolutionary adaptation. Little evidence has been produced in support of this view. Menopause evidently is a spurious phenomenon, an inevitable consequence of lifespan extension, and a postreproductive phase of life occurs in other species under conditions which favour longevity (see Chapter 1). Apart from avoiding the higher risks associated with maternity at advanced age, individual women gain no biological advantage in postmenopause; indeed, they appear to
be physiologically ill-adapted for it. The use of HRT cannot, therefore, be ruled out on the grounds that it is unnatural, but it can be justified in certain cases on pragmatic grounds.

7.1. Who Requires HRT?

It is generally agreed that HRT is not required by all women and is positively not recommended for some. Most surveys show a small proportion of women are asymptomatic during the perimenopause, though it remains to be shown whether these individuals are also relatively protected from insidious development of osteoporosis and atherosclerosis at later ages. Only weak or insignificant correlations have been found between circulating oestrogen levels and the signs and symptoms associated with menopause. Moreover, cross-cultural surveys indicate that social factors influence the appearance and intensity of climacteric symptoms (p. 6). Since the levels of oestrogen in postmenopausal women are highly variable and a sub-population of hormone-deficient individuals cannot be delineated, it is difficult to predict which women will benefit from HRT. A secondary problem is the question of whether for a given individual, treatment should be confined to symptoms during the climacteric or prolonged indefinitely for prophylaxis against osteoporosis.

Bone demineralization, which proceeds at an annual rate of about 1%, is the most serious consequence of menopause because it increases the risk of fractures, which can be fatal in the elderly woman. Exogenous oestrogen counters this tendency by inhibiting or even reversing the loss of bone substance (see Section 6.3). The risk of fractures late in life is highly variable. Caucasian women seem to be more vulnerable than those of other races, and the risk is increased for the slender and for the smoker (Daniell, 1976). A low level of oestrogen (principally oestrone) circulates in postmenopausal women, but even this may afford some protection against the loss of mineral and damage to bones. Most of this oestrogen is derived from peripheral aromatization of androstene-dione of adrenal origin. Therefore, the natural tendency to lose bone is likely to be exacerbated under conditions of impaired adrenocortical function (e.g. corticosteroid therapy, adrenal disease). In contrast, the ovaries are no longer a significant source of oestrogen and only a minor source of its precursors, so their removal is unlikely to be significant. Since the extent to which the skeleton is demineralized depends on the number of years after menopause rather than on chronological age per se, precocious menopause, whether spontaneous or surgical, is expected to raise the risk of bone injury late in life. In addition, there is a greater possibility (though admittedly remote) of developing rare gonadotrophin-producing adenomas in the pituitary gland (Trouillas et al., 1981) and, more important, coronary artery disease (p. 138). Therefore, it would seem necessary
for women who have precocious menopause, but who are otherwise healthy, to begin HRT immediately after the last menses and continue medication at least until the normal age of menopause. Unfortunately, there is at present no generally agreed-upon normal age range for menopause so precocity cannot be defined by strict statistical criteria (see Section 3.7).

Another indication for HRT is for some cases of atrophy of the genito-urinary tract. These patients are likely to present with symptoms of dyspareunia and reduced interest in sexual union. Whereas these effects develop slowly and persist, vasomotor disturbances (hot flushes and sweating) tend to be confined to a few years before and after menopause. Nevertheless, it should be recognized that HRT may postpone the natural processes of physiological adaptation to lower levels of oestrogen, and symptoms may reappear when treatment is withdrawn. A gradual attenuation of dosage may avoid this effect.

Other benefits may accrue from long-term HRT, but these require closer study and are definitely not yet indications for treatment. There is some evidence of protection from ischaemic heart disease (Hammond et al., 1979a; Ross et al., 1981). This could be attributed to a restoration of the blood lipid pattern of premenopause with lower serum cholesterol, of which a higher proportion is carried in the high-density lipoprotein fraction (Barrett-Connor et al., 1979; Nikkilä, 1978) (p. 138). Such findings may seem to conflict with the higher incidence of myocardial infarction among younger women using oral contraceptives (p. 117) and among men treated with oestrogen (Coronary Drug Project Research Group, 1970), although the preparations used are frequently different and of greater oestrogenic potency than those used in HRT. Oral contraceptives have been found to confer protection against rheumatoid arthritis (Royal College of General Practitioners’ Oral Contraception Study, 1978), but it is doubtful whether this very minor effect is also conferred by HRT (World Health Organization, 1981).

7.2. How Is HRT Administered?

Natural oestrogens are most commonly used in administering HRT (e.g. oestradiol, oestrone, conjugated oestrogens), although synthetic forms are also available (e.g. ethinyl oestradiol), and even non-steroidal agents have been used (e.g. diethylstilboestrol). A detailed discussion of the clinical pharmacology of HRT is beyond the scope of this chapter, but a few pertinent biological facts will be outlined. The natural unconjugated steroid, oestradiol, is relatively inactive when administered orally, although activity is improved with “micronized” preparations. Oestrogen esters, such as those obtained from urine of pregnant
mares (p. 18), are suitable alternatives as they are absorbed effectively from the gut. The oral route is preferred for administration of HRT but other routes are used, thus raising the oestriadiol:oestrone ratio and minimizing effects on lipoprotein metabolism by avoiding the route involving the portal circulation and liver (Farish et al., 1984). Oestrogen can be implanted or injected (e.g. oestradiol valerate), but these methods are time consuming and more difficult to control. Recently, oestradiol has been administered percutaneously in an ointment (Nichols et al., 1984). The practice of using creams containing a natural oestrogen such as oestrone for topical application to the vaginal epithelium is well established. A smaller amount of oestrogen is then required than by the oral route, although a portion of it will be absorbed into the systemic circulation in sufficient quantities to have biological effects in other parts of the body (Punnonen et al., 1980).

When protection against bone loss is required, oestrogen is frequently given in combination with other substances. The use of calcium and vitamin D will be mentioned shortly. Supplementary fluoride has also been found to be beneficial (Harrison et al., 1981; Riggs et al., 1982). This has the pharmacological effect of stimulating osteoblast activity rather than inhibiting bone resorption (Farley et al., 1983).

The initiation and duration of treatment are matters requiring careful judgment and monitoring. Since the potential risks of therapy are likely to be dose related, it is prudent to use only the minimum dose required and for the shortest possible period for effective treatment and prophylaxis. As a further precaution, treatment is generally given cyclically to reduce undesirable stimulation of the endometrium. Interestingly, there is no biological reason a priori why treatment should mimic the 28-day cycle of premenopausal women, this being relatively rare among "primitive" hunter-gatherer societies, which have patterns of almost continuous pregnancy alternating with lactational amenorrhoea (Short, 1976).

7.3. What are the Risks of HRT?

A number of clinical conditions are aggravated by oestrogen treatment and so contraindicate the use of HRT, viz. history of thromboembolism, chronic liver disease, neuro-ophthalmological vascular disease, prophyria, endometriosis, oestrogen-dependent carcinoma of the genital tract or breast and undiagnosed vaginal bleeding (Edman, 1983). In addition, there is widespread concern that other women receiving HRT may have a higher risk of developing cardiovascular disease and cancer. Besides these fears, there is evidence of increased gallbladder disease (Boston Collaborative Drug Surveillance Program, 1974), but this has not been found in all surveys.

At present, the risks are mainly highlighted by epidemiological studies of
younger women using oral contraceptives. However, it is not possible to ascertain precise risks because samples are not random and detection bias can arise (MacRae, 1981); besides, the doses used for older women are usually much lower than those used for contraception (e.g. 5–20μg cf. 30μg ethinyl oestradiol). Non-contraceptive oestrogens have been reported to increase the incidence of non-fatal myocardial infarction (Jick et al., 1978b), but this has not been confirmed (Boston Collaborative Drug Surveillance Program, 1974; Pfeffer et al., 1978). Synthetic hormones used in oral contraceptives affect blood-clotting factors (Meade, 1981), and, although treatment with natural oestrogen may not affect the coagulation–fibrinolysis system in the short-term (Notelovitz et al., 1984), it is still a matter of concern whether HRT increases the risk of thrombosis. Whether natural oestrogens have the same undesirable effects as synthetic ones will not be discussed here, though it is worth reiterating that both desirable and undesirable effects are likely to be dose dependent (Hunter et al., 1979). The risk of encouraging endometrial cancer cannot be estimated precisely for the same reasons as those given above for cardiovascular disease. However, it is recognized that continuous exposure to oestrogen unopposed is a factor and should be avoided if possible (British Gynaecological Cancer Group, 1981). Some workers suspect that the risk of breast cancer is also heightened by oestrogen exposure, but further evaluation is required before such claims are established (Hammond and Maxson, 1982; World Health Organization, 1981). For these reasons, it is not advisable to use HRT overactively or indiscriminately. Most authorities now recommend that oestrogen treatment be combined with a progestogen for at least 10 days during the second half of a treatment cycle, except in hysterectomized women in whom combined treatment is not indicated (British Gynaecological Cancer Group, 1981). The addition of a progestogen does not seem to oppose the beneficial effects of oestrogen and may even reinforce them (Hammond et al., 1979b). This can, of course, lead to regular vaginal bleeding, but it is a reassuring indication that any premalignant changes are being eliminated. It has been recommended that postmenopausal women, irrespective of whether they are receiving HRT, have an annual “progestogen challenge test” whilst risks of developing endometrial carcinoma from unopposed stimulation by oestrogen remain (Gambrell et al., 1980). Withdrawal bleeding signals the need to continue providing progestogen during each cycle until this response disappears.

Despite the uncertain degree of risk associated with the use of HRT, it would seem prudent at the present time to minimize it by careful selection and monitoring of patients for the presence of cardiovascular disease and cancer. Progress towards identifying such risks by epidemiological studies is likely to be slow and controversial because of inherent limitations of methodology. A better understanding is likely to be obtained in the longer term by basic research into the biological mechanisms of oestrogen action.
7.4. Are There Alternatives to HRT?

Alternative forms of treatment for climacteric and postmenopausal complaints should be sought because exogenous oestrogen is contraindicated in some women and adverse publicity has raised concern about risks for others. Not all symptoms associated with menopause are related directly to withdrawal of oestrogen (e.g. depression), and other types of medication and/or counselling may be more appropriate. There are also prospects of eventually finding alternative treatments for vasomotor disturbances and postmenopausal bone loss, since the effects of oestrogen withdrawal on non-genital tissues appear to be indirect. However, at the present time alternatives to oestrogen for the treatment of these effects are generally less effective (e.g. calcitonin, clonidine) or may have undesirable side effects (e.g. androgens, progestogens). In the case of atrophy of the genito-urinary tract, it is unlikely that alternatives will be found since for epithelial growth it is necessary for cytosolic oestrogen receptors to be activated.

It is worth considering whether endogenous oestrogen levels can be bolstered to avoid the need for exogenous hormones altogether. White adipose tissue is capable of increasing the production of oestrogen from androstenedione, and a larger proportion of the product circulates in the unbound (biologically active) form in obese women (p. 33). Severe weight loss aggravates a low oestrogen status (O’Dea et al., 1979). However, it would be unwise to advocate excessive weight gain in order to improve oestrogen status because the risks of a number of other diseases would be raised correspondingly. Moreover, it does not necessarily follow that obese women have fewer or less-severe climacteric symptoms (Hagen et al., 1982b).

In principle, it should be possible to raise endogenous oestrogen levels by reducing the amount excreted in faeces. The intestinal microflora evidently play a major role in the entero-hepatic circulation by hydrolysing steroid conjugates, for when they are affected by administering antibiotics, less oestrogen is reabsorbed (Aldercreutz et al., 1977; Back et al., 1981) (p. 34). When rats were switched from a diet of grain to one containing meat, the levels of faecal β-glucuronidase were raised (Goldin and Gorbach, 1976). This effect, which probably reflects a changing microbial population in the gut, would be expected to increase the hydrolysis and reabsorption of oestrogen glucuronides arriving via the bile duct. In women on a vegetarian diet, the amounts of oestrogen eliminated in the stool were increased threefold and the amounts of circulating oestrogen were reduced compared with omnivores, who lost more oestrogen in urine (Goldin et al., 1981). In another study, there was no change in peripheral blood levels of oestrogen when postmenopausal women switched diets (Hill et al., 1980). At present, there is insufficient evidence on which to base dietary
control of hormone levels, and further research is needed to clarify these conflicting claims.

In contrast to these uncertainties, dietary calcium is a factor of major significance for the prophylaxis of bone loss. Calcium requirements rise with age, and a strong case has been made for increasing its intake in postmenopausal women who are in negative calcium balance (Dixon, 1983; Parfitt, 1983). The daily calcium requirement is the lowest intake necessary to maintain zero calcium balance in the majority of women (>95%). The requirement of premenopausal women aged 35–50 is 25 mmoles (1 g), whereas in older women it rises to 37.5 mmoles (1.5 g) (Heaney et al., 1978a). These figures are considerably higher than the currently recommended dietary allowances in the United Kingdom and the United States of 12.5 and 20 mmoles, respectively. Therefore, a supplement of at least 1 g of elemental calcium per day is likely to benefit women of postmenopausal age without significant risks of side effects. Supplementary calcium probably protects bones by a different mechanism to oestrogen. Increased absorption will reduce the levels of parathyroid hormone and 1,25-dihydroxycholecalciferol, in contrast to oestrogen, which increases production of the latter (p. 137). Calcium is frequently recommended in combination with either or both vitamin D and oestrogen, depending on whether the hormone is contraindicated and the severity of actual or potential bone loss (Nordin et al., 1980b; Gallagher et al., 1982). However, supplements of calcium with vitamin D may not conserve bone quite as effectively as oestrogen (Recker et al., 1977), and vitamin D should not be used alone because of the possibility of accelerating the demineralization of bone (Nordin et al., 1980a).

Physical exercise is another strategy for maintaining bone condition. When the normal stresses on the skeleton are removed, as in patients confined to bed and in astronauts during space flight, bone mineral is lost at a greater rate than in postmenopause (Bortz, 1982; Krølner and Toft, 1983). Resumption of normal regular activity reverses these changes, and even among animals exercise has been found to promote deposition of calcium in bone (LeBlanc et al., 1983). It is likely, therefore, that postmenopausal women would benefit from regular light exercise, which might not only reduce the risk of fractures by strengthening bone but also improve muscle tone and increase neuromuscular coordination (Twomey and Taylor, 1983). Exercise can also be recommended on other grounds, such as the raising of high density lipoprotein cholesterol, which may reduce atherogenesis (Cauley et al., 1982).

Vitamin E has sometimes been used for treating climacteric symptoms. This practice may have arisen from the early discovery that this vitamin is required for fertility in rats; indeed, its chemical name (d-α-tocopherol) means “oil of fertility” (Kitabchi, 1980). However, it is not known to be required for fertility in our own species, and a clinical deficiency state is not recognised. Vitamin E is
known mainly by its anti-oxidant effects, and like other vitamins, it lacks oestrogenic activity. When the effects of vitamin E on climacteric symptoms were tested, the benefits were not significantly greater than those of placebos (Blatt et al., 1953; Kupperman et al., 1959), and current interest has now turned to the question of whether supplements can improve blood circulation (Hermann, 1982).

Finally, the fact that many women of middle age seek relief from their symptoms from sources outside medical and scientific orthodoxy should not be overlooked. In 1984, medical herbalists in Edinburgh enjoyed an active trade in remedies for the climacteric, but their customers may not have been aware that many preparations have attributes suggesting a connection with the ancient and discredited Doctrine of Signatures. This doctrine, which was raised to the status of an accepted science by Paracelsus (1493–1541), teaches that sympathetic interactions occur between substances and that, since plants were designed by their Creator for man, each carries a character or sign pointing to a special use. This would seem to explain why many contemporary herbal remedies for climacteric complaints are obtained from plants characterized either by late fragrance or red petals: oil of evening primrose (Oenothera spp.), centaury (Centaurium spp.), vervain and other Verbenaceae. Such remedies should not, however, be dismissed lightly, and careful, controlled studies of their properties might be worthwhile. Whilst their effects may turn out to be no better than those of placebos, they are nevertheless valuable, as are the laxative and carminative properties, vitamins and trace elements that herbs can provide. In other parts of the world, the use of herbal remedies is even more widespread. In the Peoples’ Republic of China, herbal medicine is dispensed side by side with modern drugs, and is presently under laboratory investigation at the respected Research Institute of Traditional Medicine in Beijing and elsewhere. The natural materials are obtained from seeds, flowers and roots, which are infused to make a drink (Xiao Bilian, personal communication). Whilst opinions of the efficacy of these materials must be reserved until further information is available, the use of alternative treatments for the climacteric in different parts of the world serves to illustrate an almost universal interest in the manifestations of the ageing reproductive system.
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