THE BIOCHEMISTRY OF OVARIAN CELLS In Vitro

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STATEMENT AS TO THE AUTHOR'S PARTICIPATION IN THE WORK SUBMITTED.

I hereby declare that the data obtained in this thesis were obtained by myself in the course of a programme of research conducted at the MRC Unit of Reproductive Biology, Edinburgh, except where otherwise acknowledged.

Keith M. Henderson.

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The following publications have been produced in connection with this thesis:


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LIST OF ABBREVIATIONS AND TRIVIAL NAMES.

AMP  adenosine monophosphate
androstenedione  4-androstene-3, 17-dione
ATP  adenosine triphosphate
c-AMP  adenosine 3′:5′-cyclic monophosphate
DNC  dibutyryl adenosine 3′:5′-cyclic monophosphate
DNA  deoxyribonucleic acid
FSH  follicle-stimulating hormone
hCG or HCG  human chorionic gonadotrophin
LH  luteinizing hormone
NAD  nicotinamide-adenine dinucleotide (oxidized).
NADH  nicotinamide-adenine dinucleotide (reduced).
NADP  nicotinamide-adenine dinucleotide phosphate (oxidized)
NADPH  nicotinamide-adenine dinucleotide phosphate (reduced)
oestradiol  1,3,5(10) - oestratriene -3, 17β - diol
oestrone  3-hydroxy-1,3,5(10) - oestratrien - 17 - one
PG  prostaglandin
PGDII  prostaglandin 15-hydroxy dehydrogenase
progesterone  4-pregnene -3,20-dione
RNA  ribonucleic acid
testosterone  17β - hydroxy -4 - androsten - 3 - one.
ABSTRACT OF THESIS.

The major roles of prostaglandins of the E and F series in reproductive processes are reviewed. The structures and biosynthesis of the prostaglandins are described before discussing how prostaglandins may be involved in ovulation, luteal regression and uterine physiology in primates and non-primates. Finally the possible role(s) of prostaglandins in semen is discussed.

Granulosa cells obtained from human Graafian follicles can be induced to luteinize in tissue culture, if given optimum gonadotrophic support, and so provide a convenient in vitro model of the progesterone secreting cells of the human corpus luteum. The effects of several prostaglandins, including prostaglandin $F_{2 \alpha}$ (PGF$_{2 \alpha}$) and PGE$_2$, on progesterone production by these cells was investigated. PGF$_{2 \alpha}$ displayed luteolytic characteristics in this in vitro system by inhibiting both basal and gonadotrophin stimulated progesterone production. In contrast, PGE$_2$ stimulated production of progesterone by human granulosa cells, and this stimulation was not prevented by the addition of PGF$_{2 \alpha}$. It is suggested that PGF$_{2 \alpha}$ may initiate functional luteal regression in vivo by a biochemical action on the luteal cell to inhibit specifically LH activation of adenylate cyclase. This possibility was supported by in vivo studies using ewes bearing ovarian autotransplants since it was found that, just as in vitro, simultaneous administration of PGE$_2$ would antagonise the luteolytic action of PGF$_{2 \alpha}$. Corpora lutea were infused with either PGF$_{2 \alpha}$, PGE$_2$, PGF$_{2 \alpha}$ + PGE$_2$ or saline on Day 10 of the cycle. Ovarian venous blood obtained before, during and up to 12 hours after the infusion period was assayed for progesterone. PGF$_{2 \alpha}$ produced an immediate, rapid and sustained decline in progesterone secretion, but infusion of
PGE$_2$ together with PGF$_2\alpha$ prevented the decline until after the infusion. Progesterone secretion was unaffected by infusion of PGE$_2$ alone. Oestrous behaviour was observed in 4 out of 7 animals infused with PGF$_{2\alpha}$, but in only 1 out of 6 infused with PGF$_{2\alpha} + $PGE$_2$. None of the animals infused with PGE$_2$ alone or saline only came into heat.

Although the studies with luteinized human granulosa cells indicated that PGF$_{2\alpha}$ has the potential of being luteolytic in women, unacceptable side-effects arising from its concomitant action on smooth muscle limits its potential usefulness clinically as a "menstrual inducer". Some 16-aryloxy analogues of PGF$_{2\alpha}$ are potent luteolysins in laboratory and farm animals, and virtually devoid of side-effects on smooth muscle at luteolytic doses. When their effect on progesterone production by luteinized human granulosa cells in tissue culture was investigated, inhibition of both basal and gonadotrophin stimulated progesterone production was observed, so revealing characteristics expected of potential human luteolysins. The analogues were, however, unable to inhibit progesterone production stimulated by PGE$_2$, suggesting that like PGF$_{2\alpha}$ these compounds may act by specifically blocking LH-activated adenylate cyclase. These in vitro findings suggest clinical studies to assess the potential of 16-aryloxy analogues to act as "menstrual inducers" in women by causing luteal regression might be worthwhile.

The newly formed corpus luteum of many species is refractory to the lytic action of PGF$_{2\alpha}$. This phenomenon was studied utilizing luteinized porcine, bovine and human granulosa cells in tissue culture. PGF$_{2\alpha}$ readily inhibited progesterone production by cells producing low amounts of progesterone, but as the cellular capacity to synthesize progesterone rose, a decreasing ability to bind PGF$_{2\alpha}$ rendered the cells refractory.
Since the steroidogenic potential of the luteinized granulosa cells is related to the amount of LH bound to the cell, bound LH may protect the cell from the lytic action of PGF<sub>2α</sub>. A "see-saw" type of interaction between LH and PGF<sub>2α</sub> is postulated to account for the resistance of the newly formed corpus luteum to PGF<sub>2α</sub>

A relatively simple technique utilizing collagenase disaggregation was found to be effective in obtaining dissociated, isolated bovine luteal cells. PGF<sub>2α</sub> inhibited hCG-stimulated progesterone production by these cells in short-term cultures (18 hours) but failed to inhibit either PGE<sub>2</sub> or dibutyl cyclic AMP-stimulated progesterone production. Progesterone production by the isolated luteal cells could not be sustained in long-term cultures (>1 day), which therefore limits the usefulness of these cells in in vitro studies of corpus luteum function.

The ability of luteinized bovine granulosa cells in tissue culture to synthesize oestrogens was examined. The cells had an active 19-hydroxylase enzyme system which aromatized both exogenous androstenedione and testosterone to oestradiol. In the absence of exogenous androgens, however, the cells failed to synthesize oestrogens due to their limited capacity to synthesize androgen precursor. It is suggested that the function of the theca-lutein cells, present in those corpora lutea which synthesize oestrogens, may be to provide androgen precursor for aromatization to oestrogens by the granulosa-lutein cells.

Finally, luteal regression in primates and non-primates is compared, and a mechanism is proposed of how gonadotrophins and PGF<sub>2α</sub> between them, may regulate luteal function in non-pregnant and pregnant women.
CHAPTER 1.

LITERATURE REVIEW.
The Regulatory Role of Prostaglandins in Reproductive Processes

Introduction.

The prostaglandins are the most recently discovered class of hormones, and, in terms of their structure-function relationship are probably the least well understood. Among their many physiological functions, they play a key role in the hormonal control of reproduction in many species, including the human. In addition they exert marked pharmacological effects on reproductive tissue, quite distinct from their physiological functions. Consequently, research into both the physiological and pharmacological actions of prostaglandins is an area of considerable importance in the search for improved means of regulating human reproduction.

Historical Outline.

In 1930 Kurzrok and Lieb reported that human semen could alter the contractile state of the human myometrium both in vitro and in vivo (Kurzrok and Lieb, 1930). A few years later Goldblatt and Von Euler independently reported the presence of an active principle in human semen with both smooth-muscle-stimulating and vasodepressor properties (Goldblatt, 1933, 1935; Von Euler, 1934). Von Euler found that the active principle was a lipid soluble fatty acid belonging to a new group of naturally occurring substances, to which he gave the name prostaglandin (Von Euler, 1936). Twenty years later Bergström and his co-workers succeeded in isolating prostaglandins in a pure crystalline form for the first time from thousands of sheep seminal vesicles. (Bergström and Sjovall, 1957). Five years later they were able to determine the chemical structure of several prostaglandin compounds (Bergström, Ryhage, Samuelsson and Sjovall, 1962).
Extraction and purification of prostaglandins from sheep seminal vesicles yielded only small amounts that were both expensive and inadequate for extensive biological studies. However, elucidation of the prostaglandin structures provided the vital clue to the natural prostaglandin precursors utilised in vivo. This permitted the development of bio-conversion processes using the endogenous enzyme systems of sheep seminal vesicles (van Dorp, Beerthuis, Nugteren, Vonkeman, 1964; Bergström, Danielsson and Samuelsson, 1964). These bio-conversion techniques, although still cumbersome and expensive, enabled the Upjohn Company to provide sufficient prostaglandins to investigators to initiate the first significant physiological and pharmacological studies. Meanwhile, systems for the total chemical synthesis of prostaglandins were being developed by Corey and his associates, and by the Upjohn Company in an effort to provide a practical inexpensive source of prostaglandins (Corey, Arnold and Hutton 1970, Pike, 1972). This was greatly facilitated by the surprising discovery that large amounts of prostaglandin derivatives could be isolated from the gorgonian coral Plexaura hormonall found off the Florida coast (Weinheimer and Spraggins, 1969; Schneider, Hamilton and Rhuland, 1972). Today, supplies of prostaglandins for both research and clinical purposes are no longer a problem since total synthesis, and partial synthesis from the coral derivatives, enable the Upjohn Company to produce prostaglandins in kilogramme quantities.

Chemical Structure of the Prostaglandins.

The prostaglandins are unsaturated hydroxy fatty acids containing 20 carbon atoms arranged to form a cyclopentane ring with two adjacent carbon side chains. The most widely studied prostaglandins, in relation to productive physiology, are those of the E and F series, and in particular prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α),
whose structures are shown in Figure 1. (Page 4). The remainder of this review will be concerned mainly with these two compounds which, although only differing in their configuration about C-9, often behave quite differently (Kuehl, 1974).

Prostaglandin Biosynthesis.

Both PGF\(_{2\alpha}\) and PGE\(_2\) are synthesised from the essential dietary polyunsaturated fatty acid, arachidonic acid, by a complex series of microsomal enzymes, found in most animal tissues and collectively known as prostaglandin synthetase (Samuelsson, 1972). The major rate limiting step in prostaglandin biosynthesis is the availability of substrate.

Arachidonic acid exists, not in its free form, but esterified in the form of triglyceride esters, cholesterol esters and in phospholipids (Land and Samuelsson, 1968). It is, however, only free unesterified arachidonic acid which can be utilised by the prostaglandin synthetase system for conversion to PGE\(_2\) and PGF\(_{2\alpha}\) (Granström, Lands & Samuelsson, 1968). The phospholipids are the most important store of arachidonic acid. Being the principle component of most membranes, they are closely associated with the prostaglandin synthetase system. Release of arachidonic acid from phospholipids is achieved through the action of phospholipase A\(_2\). Thus, factors either activating phospholipase A\(_2\) or removing barriers preventing its access to phospholipids will, in turn stimulate prostaglandin synthesis (Kunze & Vogt, 1973). These factors are diverse, ranging from hormonal action to gentle mechanical manipulation of the tissue. However, one "common thread" running through the various ways of inducing prostaglandin synthesis is a disturbance of the plasmalemma (Piper & Vane, 1971). In this respect, activation of the plasma membrane bound adenylate cyclase is probably of prime importance. Studies in the ovary and
PROSTAGLANDIN STRUCTURES

Figure 1. Structures of PGE$_2$ and PGF$_{2\alpha}$
testis (Kuehl, Cirrillo, Ham and Humes, 1973) and thyroid (Burke, Chang and Szabo, 1973) indicates that adenosine 3':5' - cyclic monophosphate (C-AMP) plays a major role in controlling prostaglandin synthesis through influencing the liberation of precursor fatty acids.

Prostaglandin biosynthesis may also be limited, in certain instances, by the total amount of active prostaglandin synthetase (Poyser, 1973). Studies by Takeguchi, Kohno and Sih (1971), and Landis, Le Tellier and Vanderhoek, (1973) have shown the existence of an inhibitor substance capable of inactivating prostaglandin synthetase. The factors involved in controlling the synthesis and degradation of this inhibitor substance have not yet been elucidated. Thus its physiological significance, if any, must, at present remain questionable.

The overall sequence of reactions leading to the formation of PGE₂ and PGF₂α from free arachidonic acid is depicted in Figure (2). The short-lived endoperoxides PGG₂ and PGH₂ (t ½ ~ 5 mins. in aqueous soln. at 37° C) are the first relatively stable compounds formed from arachidonic acid (Hamberg and Samuelsson, 1973; Hamberg, Svensson, Wekabayashi and Samuelsson, 1974). Both PGG₂ and PGH₂ have been found to have very much greater biological potency than either PGE₂ or PGF₂α, in terms of their ability to stimulate smooth muscle (Hamberg, Hedqvist, Stradberg, Svensson and Samuelsson, 1975). Furthermore, PGH₂ can modify adenylate cyclase activity in adipocyte ghosts. (Gorman, Hamberg, and Samuelsson, 1975). It is conceivable that in some instances these prostaglandin endoperoxides may be biologically important, not only as precursors of PGE₂ and PGF₂α, but through their own effects. However, the prostaglandin endoperoxides are only capable of mediating "gross prostaglandin effects". Specific prostaglandin effects rely on the difference at C-9
Figure 2. Biosynthesis of PGE₂ and PGF₂α.
between PGE$_2$ and PGF$_{2\alpha}$ and therefore cannot be mediated through the endoperoxides. The very short half-life of the endoperoxides requires that they also must exert their effects very close to their site of synthesis, whereas PGE$_2$ and PGF$_{2\alpha}$, being very much more stable, can exert their effect more distantly.

Although the level of free arachidonic acid dictates the total amount of prostaglandin synthesised (PGE$_2$ plus PGF$_{2\alpha}$), the different biological capabilities of PGE$_2$ and PGF$_{2\alpha}$ requires that the relative amounts of each synthesised must also be regulated. The formation of PGF$_{2\alpha}$ from the endoperoxides PGE$_2$ and PGH$_2$ requires an additional reducing equivalent over that necessary for PGE$_2$ formation (See Figure 2.) The ratio of PGE$_2$ to PGF$_{2\alpha}$ may be regulated through the availability of this endoperoxide reductase, and/or available reductants. Indeed, although the natural reducing co-factors involved in PGF$_{2\alpha}$ production have not yet been identified, several co-factors including reduced glutathiones (Hamberg and Samuelsson, 1973), Cu$^{2+}$ (Maddox, 1973), dihydrolipoamide (Lands, Lee and Smith, 1971), and L-epinephrine (Takeguchi et al., 1971) have been reported to influence levels of PGF$_{2\alpha}$ formed by the prostaglandin synthetase system. Recently, it has been suggested that guanosine 3':5' cyclic monophosphate (c-GMP) may be involved in inducing changes in the composition of prostaglandin synthetase which results in an increase in the PGF to PGE ratio (Ham, Cirillo, Zanetti and Kuehl, 1975).

The ratio of PGE$_2$ to PGF$_{2\alpha}$ may also be regulated by the enzyme prostaglandin E 9-ketoreductase which converts PGE to PGF. This enzyme has been found in microsomal fractions (NADH-dependent) as well as cytoplasmic fractions (NADPH-dependent) of tissue homogenates (Lee & Levine, 1974a; Lee & Levine, 1975). The oxidised forms of the required co-factors inhibit the enzymes' activity. Thus the relative amounts
of PGE₂ and PGF₂α biosynthesised could be influenced by intracellular changes in the ratio of oxidised to reduced pyridine nucleotides.

Prostaglandin Metabolism.

Prostaglandins, once synthesised, are not stored intracellularly (Samuelson, Granström, Green, Hamberg & Hammarström, 1975). Their cellular concentration at any time therefore depends upon the relative rates of synthesis and degradation. The rate-limiting step in the metabolic degradation of prostaglandins is the initial oxidation of the C-15 alcohol group (Hamberg & Samuelsson, 1971) which results in substantial loss of biological activity (Samuelson, Granström, Green and Hamberg, 1971). This is followed by reduction of the Δ¹⁵ double bond by a prostaglandin reductase which requires a carbonyl group at C-15. Further catabolism by ρ-oxidation of the carboxyl side chain, and ω-oxidation of the alkyl side chain, following hydroxylation at C-19 and/or C-20, produces the dicarboxylic acids which are the major prostaglandin excretory products. The enzyme responsible for catalysing the initial, rate-limiting step in prostaglandin degradation is prostaglandin 15-hydroxy dehydrogenase (PGDH), a cytoplasmic enzyme found in all mammalian tissues. Recently two types of PGDH have been isolated which are distinguishable by their co-factor requirements and relative affinities for PGE₂ and PGF₂α (Lee and Levine, 1974b). Type I PGDH prefers NAD as co-factor, while Type II prefers NADP. Type I enzymes are strongly inhibited by NADH but not by NADPH, whereas Type II enzymes are inhibited by NADPH but not by NADH. Type II PGDH oxidises PGF₂α more effectively than PGE₂. Since the conversion of PGE₂ to PGF₂α by cytoplasmic PGE 9-ketoreductase requires NADPH, a co-factor that inhibits Type II PGDH, it has been suggested that coupling of the NADPH linked PGE 9-ketoreductase and the NADP linked PGDH activities may regulate the ratio of PGE₂ to PGF₂α and also their intracellular steady-state concentrations (Lee and Levine, 1974b). The activity of PGDH can
be influenced by both progesterone and oestradiol; progesterone increasing PGDH activity (Bedwani & Marley, 1975; Alan, Russell, Tabor & Moulton, 1976) while oestradiol decreases PGDH activity (Blackwell & Flower, 1976). Many of the actions of progesterone and oestradiol are mediated through alterations in cellular protein synthesis. Thus it is possible that these effects on PGDH activity occur as a result of changes in its rate of synthesis, rather than any direct action by the steroid on the enzyme itself. This is supported by the finding that PGDH is a very short lived enzyme \( t_{\frac{1}{2}} \sim 1 \text{ hour} \) whose replacement depends upon de novo protein synthesis (Blackwell, Flower and Vane, 1975).

**Site of Action of Prostaglandins.**

Rapid metabolism by the lungs rapidly removes prostaglandins of the E and F series from peripheral circulation (Piper, Vane and Wyllie, 1970; Granström, 1972). Consequently, PGE\(_2\) and PGF\(_{2\alpha}\) tend to be local hormones producing their effects mainly within, or immediately adjacent to the tissue in which they were synthesised. An exception to this, however, is the situation in many non-primate species occurring during luteolysis, whereby PGF\(_{2\alpha}\) is synthesised in the uterus, but acts on the corpus luteum to cause its regression. This occurrence is made possible by the existence of a direct anatomical route between the uterus and ovary which allows PGF\(_{2\alpha}\) to reach the corpus luteum from the uterus, without passing through the lungs (Goding, Cumming, Chamley, Brown, Cain, Cerini, Cerini, Findlay, O'Shea and Pemberton, 1972). In some species, notably the rabbit (Scott & Rennie, 1970) and horse (Ginther & First, 1971), the luteolytic effect of the uterus is mediated systemically. Although systemic administration of PGF\(_{2\alpha}\) will induce luteal regression in both the rabbit (Pharriss, 1970) and horse (Noden, Oxender and Hafs, 1974), it is not known whether PGF\(_{2\alpha}\) is in fact the natural uterine derived luteolysin in either of these species. It is possible, for instance, that the uterus releases a substance that triggers PGF\(_{2\alpha}\) synthesis...
within the corpus luteum itself; PGF\textsubscript{2α} then acting locally to cause luteolysis. However, if PGF\textsubscript{2α} is the natural uterine derived luteolysin in the rabbit and horse, this would require that the rate of peripheral metabolism in these species be very low in order that sufficient amounts of PGF\textsubscript{2α} reach the ovary to initiate luteal regression.

The cellular response to PGE\textsubscript{2} and PGF\textsubscript{2α} is mediated through interaction with separate specific plasma membrane receptors (Samuelsson et al., 1975). Occupancy of the E\textsubscript{2} and F\textsubscript{2α} receptors sets in motion different series of intra-cellular events which account for the different actions of PGF\textsubscript{2α} and PGE\textsubscript{2}. However, because PGE\textsubscript{2} and PGF\textsubscript{2α} differ only in their configuration about C-9, under high non-physiological levels of prostaglandins the discriminatory power of the receptors can break down (Kuehl, 1974). It is probably this more than anything else that has produced much of the confusion that exists in the literature today regarding the various physiological functions of PGE\textsubscript{2} and PGF\textsubscript{2α}.

**Prostaglandins and Ovulation.**

Systemic administration of indomethacin, a potent inhibitor of prostaglandin synthesis, (Vane, 1971), blocks spontaneous and LH induced ovulation in rats and rabbits (Armstrong and Grinwich, 1972; Orczyk and Behrman, 1972; Tsafriri, Lindner, Zor and Lamprecht, 1972). Several experiments have demonstrated that this block is exerted at the level of the follicle rather than at the hypothalamus or pituitary: -a) the plasma LH levels determined by radioimmunoassay show a normal normal proestrous peak in rats treated with indomethacin during the critical period (Tsafriri, Koch and Lindner, 1973). b) Systemic injection of indomethacin results in unruptured follicles containing histologically normal luteinised granulosa cells, and entrapped ova which have undergone maturation (Grinwich, Kennedy and Armstrong, 1972). c) Intrafollicular injection of indomethacin restricts ovulation
blockade and entrapment of ova to injected follicles (Armstrong, Grinwich, Moon and Zamecnik, 1974). It can be concluded from these studies that prostaglandins have an essential role in the mechanism by which LH brings about follicular rupture, but are not essential mediators of either LH induced ovum maturation, or granulosa cell luteinisation. However, because indomethacin acts to inhibit the initial reaction in the conversion of arachidonic acid to prostaglandins, it is not possible to determine from these studies which prostaglandins are involved in the ovulatory process. Direct measurements of the prostaglandin content of rat and rabbit Graafian follicles show that marked changes in the levels of PGE and PGF occur throughout the ovulatory process, thus implicating their involvement in follicular rupture (Yang, Marsh and LeMaire, 1974; Le Maire, Leidner and Marsh, 1975). Following the ovulatory LH surge, there is a progressive, parallel rise in the follicular content of both PGE and PGF in those follicles destined to ovulate; no such changes occur in non-ovulating follicles. Following ovulation, the levels of PGE slowly decline to baseline levels, while the levels of PGF decline rapidly. This difference in the time course of the changes in PGE and PGF suggests the possibility of a different source, and/or a different role for these two prostaglandin types. Attempts to determine the relative importance of PGE and PGF in follicular rupture have proved inconclusive. Armstrong et al (1974) found that intrafollicular injection of antiserum specific for F prostaglandins was very much more effective in blocking ovulation in the rabbit than antiserum specific for E prostaglandins. Conversely, Tsafriri et al (1974), found that administration of PGF2 was highly successful in overcoming indomethacin blocked ovulation in rats. Organ culture of isolated human and rabbit follicles has also been used to study the stimulation of PGE and PGF synthesis (Marsh, Yang and Le Maire, 1974; Moon, Zamecnik, and Armstrong, 1974; Plunkett, Moon, Zamecnik and Armstrong, 1975).
These studies demonstrate that the follicle synthesises the prostaglandins itself, rather than accumulating them from other sources. Furthermore the ability to stimulate synthesis of PGE and PGF is limited to LH or HCG and probably mediated through the action of cyclic AMP, as is so many of the other follicular actions of LH and HCG. This in vitro system could also prove useful for studying the factors regulating the relative amounts of PGE and PGF synthesised in response to LH.

The cell types within the follicle responsible for the synthesis of PGE and PGF have not yet been determined. Preliminary studies by Erickson, Challis and Ryan (1977) suggest that the granulosa cells may be the principle source of PGF. Cultures of isolated rabbit granulosa cells produced significantly more PGF than cultures of isolated thecal tissue. Furthermore, PGF production by the granulosa cells could be stimulated with oestradiol. This suggests that the observed changes in the oestradiol content of the rabbit follicle at coitus (Younglai, 1972) may be important in regulating the relative amount of PGF produced in response to the ovulatory surge of LH. As yet, PGE production by different compartments of the follicle has not been studied.

Although LH stimulation of prostaglandin synthesis is clearly required for follicular rupture, the precise nature of this prostaglandin participation remains obscure. However, recent studies suggest that prostaglandins may be involved through their ability to influence smooth muscle contractility (Sanner, Rozek and Cammarata, 1968). Examination by fluorescent microscopy of serial sections of rat follicles treated with specific antisera to smooth-muscle myosin or actin, and then treated with fluoresceinated anti γ-globulin, showed that the external theca contained several layers of elongated cells which reacted intensely with both the anti-actin and anti-myosin
(Amsterdam, Lindner and Groschel-Stewart, 1975, and in press). This, together with the fact that the theca cells of mature Graafian follicles are rich in microfilaments (Coutinho and Maia, 1972) indicates that the theca externa cells have the properties of smooth muscle cells. Moreover, the follicle wall is capable of a contractile response to prostaglandins which is consistent with this concept (Okamura, Okazaki and Nakajima, 1974).
Thus thecal contractions, stimulated by prostaglandins synthesised in response to the ovulatory surge of LH, could contribute to ovum extrusion by providing the final mechanical disruption necessary to rupture the follicle wall after it has already been considerably weakened by the action of proteolytic enzymes, synthesised under the control of follicular steroids (Epsey, 1974; Beer, Strickland and Reich, 1975).

Prostaglandins and Luteal Regression in Non-Primates.
Exogenous administration of PGF$_{2\alpha}$ has been shown to induce luteal regression in many non-primate species including the rat (Pharris and Wyngarden, 1969), rabbit (Gutknecht, Cornette and Pharriss, 1969), guinea pig (Blatchley and Donovan, 1969), hamster (Labhsetwar, 1971), mouse (Labhsetwar 1972), ewe (McCcracken, Glew and Scaramuzzi, 1970), cow (Liehr, Marion and Olson, 1972), sow (Gleeson, 1974) and mare (Noden et al., 1974).

In contrast to PGF$_{2\alpha}$, PGE$_2$ has no demonstrable luteolytic activity. On the contrary it has been shown to be antiluteolytic in the ewe (Henderson, Scaramuzzi and Baird, 1977). Extensive studies have been carried out by many workers in an attempt to determine whether this luteolytic action of PGF$_{2\alpha}$ is merely a pharmacological phenomenon or if it is also of physiological significance in the normal regression of the corpus luteum. As a result, there is now strong evidence, recently reviewed by Horton and Poyser, (1976) that PGF$_{2\alpha}$ is also the natural uterine luteolytic hormone responsible for normal regression of the corpus luteum.
luteum in many of the non-primates where it has a demonstrable luteolytic effect when administered exogenously.

Studies in the ewe (Umo, 1975; Gemmell, Stacy and Thorburn, 1976, Stacy, Gemmell and Thorburn, 1976) hamster (Giannina, Butler, Sawyer and Steinetz, 1973) rabbit (Koering, Kirton and Thor, 1973) and guinea pig (Bagwell, Davies and Ruby, 1975) indicate that both natural and PGF\textsubscript{2} induced luteal regression occurs in two stages. Firstly, there is a marked reduction in progesterone secretion by the luteal cell which occurs in the absence of any significant changes in cellular structure (functional regression). This is followed, considerably later, by progressive degenerative changes in the luteal cellular structure culminating in the final irreversible morphological deterioration of the corpus luteum (structural regression). Studies in the ewe by Baird and Scaramuzzi, (1975) demonstrate that functional regression commences on Day 12 of the oestrous cycle in response to small amounts of PGF\textsubscript{2} released from the uterus. The resulting fall in progesterone secretion can be halted by hysterectomy as late as Day 15, but on Days 15 and 16 there is a massive release of PGF\textsubscript{2} from the uterus which ensures the final irreversible structural regression of the corpus luteum. The mechanism(s) by which PGF\textsubscript{2} causes both functional and structural regression is obscure. It has been suggested that PGF\textsubscript{2} might exert its luteolytic effect by virtue of its vasoconstrictive properties (Du Charme, Weeks and Montgomery, 1963) reducing blood flow to the corpus luteum; luteolysis arising as a consequence of anoxia (Pharris, 1970; Thorburn and Hales, 1972; Niswender, Diekmann, Nett and Akbar, 1973; Novy & Cook, 1973; Bruce and Moor, 1975). However, such a mechanism cannot account for the initiation of functional regression since both Bruce and Hillier (1974) and Einer-Jensen and McCracken (1976) have demonstrated that this occurs in the absence of any reduced blood flow to the corpus
luteum. Furthermore, there is no evidence of anoxic damage to the luteal cell during functional regression. The onset of luteal regression is, however, associated with biochemical changes in the luteal cell, the most significant being a decline in adenylate cyclase activity and a loss of responsiveness to LH, both of which would cause a decrease in progesterone secretion (Andersen, Schwartz and Ulberg, 1974; Hichens, Grinwich and Behrman, 1974; Grinwich, Hichens and Behrman, 1976; Chatterjee and Greenwald, 1976). Thus, it seems likely that functional regression may be initiated by a direct biochemical action of PGF$_{2\alpha}$ on the luteal cell resulting in an inhibition of steroidogenesis. This concept is supported by the findings that PGF$_{2\alpha}$ can inhibit progesterone production by luteal tissue in vitro (O'Grady, Kahorn, Glass, Caldwell, Brock and Speroff, 1972; Demers, Behrman and Greep, 1973; McNatty, Henderson and Sawers, 1975). Furthermore this in vitro inhibition by PGF$_{2\alpha}$ appears to result from a direct specific inhibition of LH-activated adenylate cyclase (Henderson and McNatty, 1975; Lahav and Lindner, 1976) which is consistent with the biochemical changes in the luteal cell found in vivo at the onset of both natural and PGF$_{2\alpha}$ induced luteolysis.

Whether a biochemical initiation of functional regression is sufficient to trigger the later degenerative changes in the luteal cells associated with structural regression is uncertain. The later stages of luteal regression are associated with a reduction in capillary blood flow to the corpus luteum (Thorburn and Hales, 1972; Niswender et al, 1973; Bruce and Moor, 1975), but this may be a consequence of structural regression rather than its cause. Thus, although PGF$_{2\alpha}$ likely initiates luteal regression by a direct biochemical action on the luteal cell resulting in an inhibition of progesterone production, the possibility that a vasoconstrictive action of PGF$_{2\alpha}$ may be involved in ensuring the
final irreversible morphological deterioration of the corpus luteum cannot be ruled out at present.

The ability of exogenously administered PGF$_{2\alpha}$ to induce luteal regression has already found a major application in the cattle industry. By administering PGF$_{2\alpha}$ the farmer can now artificially induce and synchronize oestrus in his herd of cattle. This allows higher pregnancy rates to be achieved, the routine use of artificial insemination in combination with "high quality" sperm, reduced labour costs and improved efficiency of both herd and farm management.

PGF$_{2\alpha}$ Release from the Non-Primate Uterus.

Hysterectomy prolongs the life of the corpus luteum in those non-primates in which the evidence suggests that PGF$_{2\alpha}$ is the natural luteolysin, indicating a uterine origin for the PGF$_{2\alpha}$. Although the physiological route by which PGF$_{2\alpha}$ travels directly from the uterus to the ovary has been well worked out, particularly in the sheep (see Goding et al., 1972; Horton and Poyser, 1976; Baird, 1977), the hormonal requirements for the synthesis and release of PGF$_{2\alpha}$ from the uterus are less clear.

Studies in the ovariectomized ewe (Caldwell, Tillson, Brock and Speroff, 1972; Scaramuzzi, Baird, Boyle, Land & Wheeler, 1977), guinea pig (Blatchley and Poyser, 1974) and ovariectomized mouse (Saksena and Lau, 1973) indicate that progesterone priming of the uterus, followed by oestradiol treatment alone provides the best stimulus for PGF$_{2\alpha}$ production. Progesterone and oestradiol, either alone or continuously together, are much less effective in inducing PGF$_{2\alpha}$ production. Thus, although progesterone is required to initially prime the uterus to respond to oestradiol, its continued presence actually inhibits the response to oestradiol.

This inhibitory action may arise from the stimulating effect progesterone has on 15-hydroxy prostaglandin dehydrogenase activity (Bedwani and Marley,
1975, Alan et al. 1976). The changes in progesterone, oestradiol and PGF$_{2\alpha}$ levels throughout the luteal phase have been most closely studied in the ewe (Scaramuzzi, Baird, Land and Wheeler, 1974). Functional regression in the ewe commences on Day 12-13 of the oestrous cycle when small amounts of PGF$_{2\alpha}$ are released from the uterus and start to inhibit progesterone production by the corpus luteum. This initiates a closed cycle of events in which falling levels of progesterone lead to increased amounts of PGF$_{2\alpha}$ being produced by the uterus, which in turn causes a more effective inhibition of progesterone production. This culminates on Day 15-16, by which time progesterone production by the corpus luteum is almost completely inhibited, in a massive release of PGF$_{2\alpha}$ which ensures the final irreversible morphological destruction of the corpus luteum. It was originally thought that the increased amounts of oestradiol secreted by the pre-ovulatory follicle in response to the falling levels of progesterone were also important in inducing the increased PGF$_{2\alpha}$ production by the uterus (Cox, Thorburn, Currie and Restall, 1974). However, when oestrogen antiserum is used to neutralise most of the oestrogen secreted by the pre-ovulatory follicle, normal regression still occurs (Fairclough, Smith and Petersen, 1976). It seems likely, therefore, that continual low levels of oestradiol, in combination with falling levels of progesterone is sufficient to ensure the maximum release of PGF$_{2\alpha}$ from the uterus. What actually triggers the initial release from the uterus of the small amounts of PGF$_{2\alpha}$ that initiates functional regression is not known. It may merely be an event occurring when the uterus has been sufficiently primed with luteal progesterone to respond to the small amounts of oestradiol which are continually being secreted by the Graafian follicles throughout the luteal phase (Baird, Land, Scaramuzzi and Wheeler, 1976).
It is likely that progesterone and oestradiol exert their effects on the uterus at the cellular level, through their ability to modify genome expression (O'Malley, MGuire, Kahler and KOrlman, 1969; De Angelo and Gorski, 1970), since intrauterine administration of Actinomycin D, an inhibitor of DNA transcription by RNA polymerase, prevents normal luteal regression (French and Casida, 1973). How progesterone exactly achieves its "priming effect" by modifying genome expression remains entirely speculative at present. The stimulatory action of oestradiol on PGF$_{2\alpha}$ production by the uterus can, however, be more easily explained. The release of PGF$_{2\alpha}$ from the uterus in vivo is immediately preceded by its rapid synthesis, since indomethacin abolishes oestradiol-stimulated PGF$_{2\alpha}$ production by the uterus (Barcikowski, Carlson, Wilson and McCracken, 1974). Thus oestradiol must act by stimulating prostaglandin synthesis, and oestradiol has indeed been shown to increase the prostaglandin F$_{2\alpha}$ synthesising capacity of the uterus in both rats (Ham et al., 1975) and guinea-pigs (Naylor and Poyser, 1975). This could be achieved through a number of the known actions of oestradiol, all of which could be mediated through modifying genome expression including, increasing the total amount of phospholipid precursor available for conversion to prostaglandin F$_{2\alpha}$ (Aizawa and Mueller, 1961); modifying prostaglandin synthetase activity to favour PGF$_{2\alpha}$ production (Ham et al., 1975) and by decreasing the activity of 15-hydroxy prostaglandin dehydrogenase (Blackwell and Flower, 1976). Increased amounts of phospholipase A$_2$, cholesterol esterase or triglyceride lipase, and an increase in the total amount of prostaglandin synthetase could also increase PGF$_{2\alpha}$ production by the uterus, but there is no evidence, at present, that oestradiol is capable of inducing these changes. Following a successful mating the corpus luteum must be maintained, since its progesterone secretion is essential for embryonic survival.
In these species in which PGF$_{2\alpha}$ is the uterine derived luteolysin, this can be achieved by either preventing the synthesis and/or release of PGF$_{2\alpha}$ from the uterus, or via a luteotrophin to block the luteolytic action of PGF$_{2\alpha}$ at the corpus luteum. These two possibilities have been most closely studied in the ewe. An inhibition of PGF$_{2\alpha}$ release from the uterus is supported by the findings of Thorburn, Cox, Currie, Restall and Schneider (1973) and Nett, Staigmiller, Akbar, Diekman, Ellinwood and Niswender (1976), who found a marked reduction in the release of PGF$_{2\alpha}$ into the uterine vein during the pregnant cycle, while Peterson, Tervit, Fairclough, Havik and Smith (1976) found that the presence of an embryo greatly suppressed jugular venous blood levels of 13,14-dihydro-15-keto-prostaglandin F. However, uterine venous (Mapleton, Del Campo and Ginther, 1975) and ovarian arterial blood (Mapleton, Lapin and Ginther, 1976) on the side of a gravid uterus in the sheep apparently have luteotrophic properties. It seems likely, therefore, that in sheep at least the corpus luteum of pregnancy is maintained by both blocking the release and/or synthesis of uterine PGF$_{2\alpha}$, and via a luteotrophin.

Prostaglandins and Luteal Regression in Primates.
The factor(s) provoking regression of the primate corpus luteum are poorly understood at present. Systemic administration of PGF$_{2\alpha}$ will induce premature luteal regression and menstruation in monkeys (Kirton, Gutknecht, Bergström, Wyngarden and Forbes, 1972; Russell, 1975) but all attempts to demonstrate a similar luteolytic effect in women have failed, the best achieved being a transient decline in circulating progesterone levels (Jewelewicz, Cantor, Dyrenfurth, Warren and Vande Wiele, 1972; Jones and Wentz, 1972; Sato, Ami, Shinada and Igarashi, 1973; Wentz and Jones, 1973; Coudert, Winter and Faiman, 1974). This lack of success in women may be due to insufficient amounts of PGF$_{2\alpha}$.
reaching the ovary. PGF$_{2\alpha}$ is rapidly inactivated on passage through the lungs (Piper et al., 1970; Granström, 1972), but limitations are imposed on the amount of PGF$_{2\alpha}$ that can be administered systemically to women because of unacceptable side-effects arising from its concomitant action on smooth muscle, e.g. diarrhoea, vomiting and nausea. However, future studies with 16-aryloxy analogues of PGF$_{2\alpha}$ which have a greater luteolytic activity and fewer side-effects (Dukes, Russell and Walpole, 1974) may allow a luteolytic action to be demonstrated in the human female.

In contrast to those non-primate species in which PGF$_{2\alpha}$ is thought to be the natural luteolysin, ovarian cyclicity in monkeys (Burford & Diddle, 1936; Neill, Johansson and Knobil, 1969) and humans (Beavis, Brown and Smith, 1969; Doyle, Barclay, Duncan and Kirton, 1971) is not altered by surgical removal of the uterus. Thus, if PGF$_{2\alpha}$ is the primate luteolysin, it must be synthesised within the ovary itself, and so exert a direct local effect. Recent studies indicate this could well be the case. Human luteal tissue has an active prostaglandin synthetase, and can synthesise large amounts of PGF$_{2\alpha}$ (Challis, Calder, Dilley, Forster, Hillier, Hunter, MacKenzie and Thorburn, 1976). Direct measurement of the PGF$_{2\alpha}$ content of human corpora lutea show the levels to be significantly higher during the late-luteal phase, coincident with luteolysis, than in the mid-luteal phase (Shutt, Clarke, Fraser, Goh, McMahon, Saunders & Shearman, 1976). Furthermore a specific membrane receptor for PGF$_{2\alpha}$ has been found in the human corpus luteum (Powell, Hammarström, Samuelsson and Sjoberg, 1974). A similar receptor has been found in the corpus luteum of the cow (Powell, Hammarström and Samuelsson, 1975) and sheep (Powell, Hammarström and Samuelsson, 1974).
two species in which PGF$_{2\alpha}$ is thought to be the natural luteolysin.

Oestradiol injected directly into the ovary containing the corpus luteum hastens the onset of luteal regression in women (Hoffmann, 1960) and monkeys (Karsch and Sutton, 1976). This effect appears to be mediated via prostaglandins since indomethacin abolishes estrogen-induced luteolysis in monkeys (Auletta, Caldwell, and Speroff, 1976). Whether PGF$_{2\alpha}$ is the actual prostaglandin involved is uncertain at present, but it does seem likely, particularly in view of the positive effect oestradiol has on PGF$_{2\alpha}$ synthesis by the uterus in non-primates (see Horton and Poyser, 1976). This luteolytic action of oestradiol may also be of physiological importance in inducing normal regression of the primate corpus luteum. Swanston, McNatty and Baird (1977) have recently shown there is a marked rise in the oestradiol content of human corpora lutea during the mid-luteal phase. It is possible that this rise in oestradiol stimulates the increase in PGF$_{2\alpha}$ observed in the late luteal phase by Shutt et al. (1976); the PGF$_{2\alpha}$ in turn being responsible for inducing luteolysis. A rise in the oestradiol content of the corpus luteum towards the end of the luteal phase has also been observed in the monkey (Knobil, 1973), which would allow for the possibility of a similar mechanism.

There is good evidence that the pregnant primate corpus luteum is maintained by a chorionic gonadotrophin secreted by the trophoblast (Knobil, 1973; Jones and Wetzl, 1976). How this prevents normal luteal regression is unknown, but, if PGF$_{2\alpha}$ is the primate luteolysin, the chorionic gonadotrophin may act by inhibiting PGF$_{2\alpha}$ synthesis since corpora lutea of early pregnancy contain very low levels of PGF$_{2\alpha}$ (Shutt et al, 1976).
Prostaglandins and Menstruation.

The possibility that prostaglandins synthesised by the endometrium might be involved in the menstrual process was first suggested by Pickles, Hall, Best and Smith (1965). These authors found large amounts of both PGF$_{2\alpha}$ and PGE$_2$ in menstrual fluid, the levels of PGF$_{2\alpha}$ being considerably higher than those of PGE$_2$. Endometrial curettings were also found to contain considerable amounts of PGF$_{2\alpha}$ and PGE$_2$, and it was noticed that the PGF/PGE ratio was higher in curettage obtained in the secretory phase than in the proliferative phase. This latter observation has been confirmed and extended by Downie, Poyser and Wunderlich (1974) and Levitt, Tobon and Josimovich (1975). These studies indicate that the endometrial levels of PGF$_{2\alpha}$ are 3-4 times higher during the mid and late luteal phases and at menstruation than at times earlier in the cycle. In contrast, while the PGE$_2$ levels are similar to PGF$_{2\alpha}$ during the proliferative and early luteal phase, they do not rise during the mid and late luteal phase, and only show a 2-3 fold increase at menstruation. This preferential stimulation of PGF$_{2\alpha}$ synthesis during the luteal phase is possibly controlled by oestradiol secreted by the corpus luteum, since oestrogen treatment of ovariectomized monkeys stimulates a marked increase in uterine fluid prostaglandin F levels (Demers, Yoshinaga and Greep, 1974). Furthermore, Ham et al (1975) have demonstrated in vitro that oestradiol increases the ratio of PGF:PGE produced by rat uteri. Circulating progesterone levels may also influence the total amount of PGF$_{2\alpha}$ produced by the human endometrium in vivo as progesterone has been shown to inhibit PGF synthesis by human endometrium in organ culture. (Cane and Villee, 1975).

Taken together, these findings can be incorporated into a possible mechanism of menstruation. Following ovulation, peripheral plasma levels of oestradiol and progesterone rise to a maximum in the mid-luteal phase (McNatty, Hunter, McNeilly and Sawers, 1975).
Rising oestradiol levels would stimulate preferential endometrial synthesis of PGF$_2\alpha$, but this would be antagonised by the rising progesterone levels. From the mid-luteal phase to menstruation, plasma progesterone levels steadily decline from 10-1 ng/ml but oestradiol levels fall only slightly from 125-75 pg/ml. The falling levels of progesterone would not only allow increased amounts of PGF$_2\alpha$ to be synthesised by the endometrium, under the influence of oestradiol, but would also make the myometrium more susceptible to contractile stimulation by PGF$_2\alpha$ (Porter and Behrman, 1971). The final shedding of the endometrium could then result from a combination of PGF$_2\alpha$ stimulated myometrial contractions, and the vasoconstrictive property of PGF$_2\alpha$ (Cséply and Csápo, 1972) causing the constriction of the endometrial spiral arteriols that immediately precedes menstruation (Markée, 1940). Prostaglandin synthesis stimulated by anoxic damage to the endometrial cells might also account for the increased PGE$_2$ levels seen at menstruation (Downie et al, 1974).

Pregnancy requires prevention of menstruation. How might this be achieved? During a pregnant cycle plasma progesterone levels do not decline from the mid-luteal phase onwards, but rather increase due to HCG stimulation of progesterone production by the corpus luteum (Johansson, 1969). These elevated levels of progesterone could then prevent menstruation in two ways (a) by inhibiting the synthesis of PGF$_2\alpha$ by the endometrium (Cane and Villee, 1975); (b) by substantially reducing the contractile response of the myometrium to any PGF$_2\alpha$ that might be synthesised (Porter and Behrman, 1971).

Prostaglandins might also be involved in menstrual disorders. It has been suggested that excessive production of PGF might be the cause of dysmenorrhoea in women. (Pickles et al, 1965). This is substantiated by the finding that the administration of flufenamic acid (a prostaglandin
prostaglandin synthetase inhibitor) provided relief to patients whose symptoms were refractory to conventional treatments (Lindner, Zor, Bauminger, Tsafiri, Lamprecht, Koch, Antebi and Schwartz, 1974).

Perhaps the most clinically useful pharmacological action of PGE and PGF is their ability to stimulate uterine contractions. As a consequence, PGE and PGF are widely used in inducing labour at term (Karim and Hillier, 1973). Moreover, prostaglandin treatment appears to be more satisfactory than other available methods for aborting pregnancies after the first trimester (Brenner, 1975). While PGE₂, PGF₂α and some of their 15-methyl analogues are effective first trimester abortificients, their use, at present, is less satisfactory than vacuum aspiration. However, recent studies suggest the use of vaginal suppositories may increase their usefulness in this respect (Bygdeman, Borell, Leader, Lundstrom and Martin, 1975; Robbins, 1976). Furthermore, the simplicity of vaginal administration raises the possibility of self-administration.

**Prostaglandin and Reproduction in the Male.**

Although the majority of research today concerning the role of prostaglandins in reproductive biology is being carried out in relation to female reproduction, the prostaglandins were first discovered in human semen (Kurzrok and Lieb, 1930). Since this initial discovery, much work has been done to characterise the prostaglandin content of the male sex accessory tissues and their secretions (see review by Cenedella, 1975). Of all animal tissues and secretions examined, the seminal plasma of man possesses the highest concentration of prostaglandins. Consequently, no other source has been studied more extensively. Until the recent discovery of the 19-OH E prostaglandins (Taylor and Kelly, 1974; Jonsson, Middleditch and Desiderio, 1975), it was thought that there were
13 prostaglandins in human semen, among which were the eight dehydrated prostaglandins of the A, B, 19-OH A and 19-OH B series (Hamberg and Samuelsson, 1966). However, after the finding of the 19-OH PGEs accompanied by little, if any, 19-OH PGAs or 19-OH PGBs, it was suggested that the 19-OH PGAs, 19-OH PGBs, PGAs and PGBs were artefacts (Middleditch, 1975). Recent studies indicate that the prostaglandin content of human semen is composed of predominantly 19-OH PGE1 and 19-OH PGE2 (approx. 270 μg/ml total) together with appreciable amounts of PGEs (approx. 70 μg/ml total) plus small amounts of 19-OH PGFs (approx. 20 μg/ml total) and PGFs (approx. 2 μg/ml total) (Templeton, Cooper and Kelly, 1977).

Production of the 19-OH PGEs is highly androgen dependent since their concentration in the ejaculate of hypogonadal individuals falls within 48 hours of stopping testosterone replacement therapy (Skakkebaek, Kelly and Corker, 1975). Studies in the stump-tailed macaque (Macaca arctoides) indicate that the 19-OH PGEs are stored in the seminal vesicles, and that this store can be replenished rapidly following ejaculation (Kelly, Taylor, Hearn, Short, Martin and Marston, 1976).

While human semen, and primate semen in general, contains these large amounts of 19-OH PGEs, they are undetectable in the semen of domestic and laboratory animals (Kelly et al, 1976). What the function(s) of the 19-OH PGEs and the other prostaglandins present in human semen is remains unknown at present. The very high prostaglandin content of human semen does not make the human male any more fertile than males of non-primate species whose semen prostaglandin content is very low (Cenedella, 1975). On the contrary, the human male is considerably less fertile. It has been reported intermittently over the past 25 years, that low levels of prostaglandins in semen, particularly PGEs are
associated with human male infertility (see Cenedella 1975 for references). However these reports were made before the discovery of the 19-OH PGEs, and it is possible that low PGE levels may be compensated for by high 19-OH PGE levels. It therefore is now necessary to re-examine the relative amounts of the different prostaglandins in semen in relation to fertility. The possibility that seminal prostaglandins might promote the longevity or the motility of the sperm seemed to be ruled out by the studies of Eliasson, Murdoch and White (1968). These workers found that PGE$_2$ had no effect on the oxygen uptake, fructose utilization or lactate production of suspensions of washed human sperm. However, these studies must also now be repeated using the 19-OH PGEs since they may have quite different effects. Both the 19-OH PGEs and the PGEs have been shown to relax spontaneously contracting non-pregnant human uterine muscle strips in vitro (Kelly et al., 1976), while PGE$_2$ has been shown to cause relaxation of the Fallopian tube isthmus (Coutinho and Maia, 1977). Seminal prostaglandins deposited intravaginally during coitus may therefore relax the uterus and Fallopian tubes, thereby aiding sperm transport and fertilisation. Seminal prostaglandins could also be involved in controlling testicular steroidogenesis (Bartke, Musto, Caldwell and Behrman, 1973; Sakena, El Safoury and Bartke, 1973) and ejaculation (Hedqvist and von Euler, 1972; Stahl, 1972).
CHAPTER 2.

INTRODUCTION TO PRESENT STUDY.
Background to Present Study.

During the normal human menstrual cycle, the corpus luteum starts to regress about 10 days after its formation, as indicated by falling levels of plasma progesterone (see Fig. 3). In a fertile cycle, the corpus luteum is maintained, almost certainly by the luteotrophic action of human chorionic gonadotrophin (hCG) secreted by the implanting blastocyst (Vaitukaitis, Bermudez, Cargille, Lipsett & Ross, 1971). Persistence of the corpus luteum is essential for foetal survival in the initial stages of pregnancy. If the corpus luteum is surgically enucleated within 4 - 5 weeks of the first missed menstruation, the foetus will be expelled (Csapo, Pulkkinen, Ruttner, Sauvage and Wiest, 1972). The importance of the secretion of progesterone by the corpus luteum is indicated by the fact that abortion following luteectomy in early pregnancy can be prevented by treatment with exogenous progesterone (Csapo, Pulkkinen and Wiest, 1973). After this initial critical 4-5 week period, the corpus luteum is no longer necessary for the maintenance of pregnancy since the placenta is, by this time, capable of producing adequate quantities of progesterone to support the pregnancy (Csapo et al, 1972). Clearly, therefore, a compound which would inhibit the secretion of progesterone by the corpus luteum before the "luteal-placental shift" could be used to induce abortion early in pregnancy by "menstrual induction". Such a "contraceptive" would have the advantage that, in contrast to regimes in current practice, e.g. the combined pill, it would only need to be taken in the event of a missed menstrual period.

In several species, the hormone responsible for the regression of the corpus luteum at the end of each non-fertile cycle is PGF_2α (see literature review). Although receptors for PGF_2α are present in the human corpus luteum (Powell et al, 1974), thus implicating PGF_2α involvement in luteolysis, attempts to induce premature menstruation in women by systemic administration of PGF_2α have failed (see literature review for reference).
Figure 3  Plasma progesterone levels during a normal and pregnant cycle. T.E. = total oestrogens (from Johansson, 1969).
However, because PGF$_{2\alpha}$ is rapidly metabolized by the lungs and liver (Piper et al., 1970; Granstrom, 1972), it is not known whether this failure to induce luteolysis in women is due to the unresponsiveness of the corpus luteum to PGF$_{2\alpha}$, or because insufficient amounts reach the corpus luteum due to the rapid peripheral metabolism of PGF$_{2\alpha}$. Moreover, due to its concomitant action on smooth muscle which causes nausea, cramp, vomiting and diarrhea, the amount of PGF$_{2\alpha}$ that can be administered systemically to women is limited. Thus, an in vitro test system which would permit determination of the ability of PGF$_{2\alpha}$ to inhibit the production of progesterone by human luteal tissue would be useful. If PGF$_{2\alpha}$ displayed luteolytic characteristics, such an in vitro system would be useful in testing the luteolytic activity of analogues of PGF$_{2\alpha}$, designed to be more resistant to peripheral metabolism, prior to their clinical use. Furthermore, the in vitro system could be used to investigate the mechanism by which PGF$_{2\alpha}$ inhibits progesterone production, in the hope that it might be possible to trigger the luteolytic process by some other means. The main scope of this study was therefore to investigate in vitro how production of progesterone by the human corpus luteum might be regulated by prostaglandins.

Choice of In Vitro System to Study the Human Corpus Luteum.

Perhaps the biggest problem facing any proposed in vitro study utilizing human material is obtaining sufficient amounts of tissue. Although ovariectomies may be carried out regularly, the number of corpora lutea obtainable is limited because (a) the corpus luteum is present for only half the menstrual cycle, (b) many patients use steroidal contraceptives, (c) many patients are menopausal, (d) ovariectomy is frequently performed on patients undergoing hysterectomy for anovulatory dysfunctional bleeding. Furthermore, although luteal slices have proven suitable for the study of
steroidogenic pathways and gonadotrophic action in the corpus luteum of several species, the requirement of relatively large amounts of tissue, and high between-slice and between-tissue variability (Seifert & Hansel, 1968; Le Maire, Rice and Savard, 1968), hinders the extensive utilization of such an in vitro system. These latter problems may be partially overcome by using a dispersed luteal cell preparation. However, at the commencement of this study no successful method for dispersing human luteal cells had been reported, while the methods for dispersing animal luteal tissue were complicated, requiring substantial amounts of luteal tissue and several enzymes (Sayers, Portanova, Beall, Seeling and Malamud, 1971; Gospodarowicz & Gospodarowicz, 1972). It was therefore felt that an attempt to find a suitable method to obtain dispersed human luteal cells with the limited amount of human luteal tissue that would be available at any one time would be impractical. An alternative in vitro system to investigate the human corpus luteum was sought.

Granulosa cells obtained from preovulatory follicles of the mare (Channing, 1966, 1969a; Channing & Grieves, 1969) human (Channing, 1969b; McNatty & Sawers, 1975), monkey (Channing 1970a) pig (Schomberg, 1969; Channing 1970b) and rat (Fischer & Kahn, 1972) "luteinize" spontaneously in tissue culture; luteinization being defined as an increased secretion of progesterone, and cellular hypertrophy accompanied by an increase in the cytoplasmic - nuclear ratio, together with an accumulation of cytoplasmic granules and lipid droplets (Channing, 1970c). Granulosa cells obtained from smaller, less mature follicles fail to luteinize spontaneously in vitro, but can be induced to luteinize if they are cultured in the presence of gonadotrophins (Channing & Ledwitz - Rigby, 1974). Ultrastructural studies on granulosa cells, obtained from monkey preovulatory follicles, and luteinized in tissue culture indicate they
are also similar in appearance to corpus luteum cells (Crisp & Channing, 1972). These findings, together with the relative ease with which human granulosa cells can be obtained from Graafian follicles and induced to luteinize in tissue culture (McNatty, 1975), would seem to make this a suitable in vitro system in which to study progesterone production by the human corpus luteum. However, there are several drawbacks that must be borne in mind. Firstly, although granulosa cells will luteinize in vitro, their morphological and fine structural appearance is not identical to granulosa cells which have luteinized in vivo. For instance, granulosa cells luteinized in vitro contain none of the yellow pigment characteristic of luteal tissue. Consequently, because of these morphological differences, one can never be sure that the behaviour of granulosa cells luteinized in vitro will mimic exactly the behaviour of cells that have luteinized in vivo. Some small cytoplasmic or nuclear difference in the in vitro luteinized cells could make their behaviour quite different. Secondly, the human corpus luteum does not consist solely of luteinized granulosa cells, but also contains large numbers of luteinized theca, or paralutein cells, together with small amounts of fibrous tissue (Jones & Wentz, 1976). Although there is histochemical (Goldberg, Jones & Turner, 1965), and biochemical (Savard, Le Maire & Kumari, 1969) evidence that the granulosa-lutein cells are the progesterone secreting cells of the human corpus luteum, their progesterone production could be influenced by factors secreted by the other cell types within the corpus luteum. Thirdly, results obtained utilizing luteinized granulosa cells in tissue culture only represent the behaviour of the cells in that particular culture system. Thus, any attempt to interpret results in relation to physiological events must be viewed with extreme caution. However, despite these drawbacks, it was felt that the tissue culture of luteinized human granulosa cells would be the most favourable system with which to study the human corpus luteum in vitro.
Studies with Luteinized Granulosa Cells.

Human luteinized granulosa cells in tissue culture were to be used primarily to study the effects of prostaglandins, particularly \( \text{PGF}_{2\alpha} \), on cellular progesterone production. The possible physiological relevance of results obtained with \( \text{PGF}_{2\alpha} \) in this system were to be assessed by comparative studies using bovine and porcine luteinized granulosa cells in tissue culture; the cow and pig being two species in which \( \text{PGF}_{2\alpha} \) is luteolytic \textit{in vivo} (Liehr et al., 1972; Gleeson, 1974). It was also hoped to use the tissue culture of human luteinized granulosa cells to screen other potential human luteolysins, like \( \text{PGF}_{2\alpha} \), as a preliminary to initiating clinical trials.

In some species such as the human (Guraya, 1971) and pig (Corner, 1971) the corpus luteum is composed of large numbers of paralutein cells as well as granulosa-lutein cells. Granulosa cells can be obtained as a homogeneous cell population and consequently when luteinized, they allow the steroidogenic potential of the granulosa-lutein cells to be studied. By comparing the biosynthetic capacity of luteinized granulosa cells with the known biosynthetic capacity of whole corpora lutea of different species, it was hoped to gain some information on what interrelationships might exist between the granulosa-lutein and other cell types found in corpora lutea.

Studies with Dispersed Bovine Luteal Cells.

The validity of results obtained from studies using the tissue culture of human luteinized granulosa cells, as an \textit{in vitro} model of the corpus luteum, would be strengthened if it were possible to show that similar results could be obtained using dispersed luteal cells. Although only limited amounts of human corpora lutea could be obtained at any one time, large numbers of bovine corpora lutea were available from a local abattoir. It was therefore hoped to develop a simple technique for obtaining dispersed bovine luteal
cells. Comparative studies with luteinized bovine granulosa cells and dispersed bovine luteal cells should then give some indication of how good an in vitro model of the corpus luteum the tissue culture of luteinized granulosa cells is. Furthermore, if it were possible to obtain simply a good yield of viable, dispersed bovine luteal cells from relatively small amounts of tissue, the same technique might also prove applicable for obtaining dispersed human luteal cells.

Comparative In Vivo Studies.

One must always be extremely cautious in interpreting the results from in vitro studies since they may merely be an artefact of that particular in vitro system, and consequently have no physiological relevance whatsoever. The possible physiological relevance of results obtained from the in vitro studies with luteinized granulosa cells was therefore to be tested in vivo, using ewes with ovarian autotransplants (Goding, McCracken & Baird, 1967). In these animals the right ovary is removed, and the left ovary autotransplanted to a carotid-jugular skin loop in the neck. This preparation is particularly useful for studying luteal function in vivo since, not only is the corpus luteum maintained due to the absence of the luteolytic influence of an adjacent uterine horn (Baird, Goding, Ichikawa & McCracken, 1968), but the easy access to both the ovarion arterial and venous circulation allows the corpus luteum to be studied in a conscious, relatively unstressed animal.
CHAPTER 3.

MATERIALS AND METHODS.
Radioimmunoassay of Steroid Hormones.

The concentration of progesterone, oestradiol - 17β, oestrone testosterone and androstenedione in tissue culture media, and the concentration of progesterone in ovarian venous plasma was measured by specific radioimmunoassays.

(i) Solvents and reagents.

Analar petroleum spirit (40-60°C), diethylether and hexane (BDH) were purified by redistilling prior to use. Before being used in the oestradiol - 17β and oestrone assays, the diethylether was cleared of peroxide by washing with 50% (g/v) ferrous sulphate (BDH) in 5% sulphuric acid (BDH) and distilled water, and redistilled within 24 hours before use. Analytical grade ethanol (Burroughs Ltd.) was used without further purification.

The assay buffer was a 0.1M phosphate buffer, pH7 (8.6 anhydrous di-sodium hydrogen orthophosphate and 6.03g sodium dihydrogen orthophosphate, per litre distilled water) and contained 0.97° sodium chloride, 0.1% gelatin and 0.1% sodium azide (all reagents BDH, Analar grade). The assay buffer, henceforth referred to as PBSG, was stored at 4°C.

The dextran-coated charcoal mixture used for separation of free and antibody-bound hormone was prepared just prior to use by suspending 250 mg activated charcoal Norit A (Sigma Chemical Company) and 25 mg dextran T 70 (Pharmacia Fine Chemicals) in 100 ml of PBSG. The suspension was continuously stirred with a magnetic stirrer and kept in an ice bath while being used.

The scintillation fluid was prepared by dissolving 10g 2,5-diphenyloxazole (PPO, Nuclear Enterprises Ltd.) and 0.75g 1,4-di-2-(5-phenyloxazolyl) - benzene (POPOP, BDH) in 2.5 litres of sulphur-free toluene (A. & J. Beveridge Ltd.) which was then added to 1.25 litres of Triton X-100 (A. & J.
Beveridge Ltd.) and mixed thoroughly until a homogeneous solution was obtained.

(ii) Steroids

Non-radioactive steroids were purchased from Sigma Chemical Company, and stored at 4°C as 1μg/ml stock solutions in ethanol. Standard solutions for use in the assays, or for addition to cell cultures, were prepared from these stock solutions by further diluting with ethanol, and these were also kept at 4°C.

Radioactive progesterone - 1α-2α-3H (4.2 Ci/mM), testosterone - 1α - 2α-3H (58 Ci/mM) and oestrone - 2,4,6,7-3H (110 Ci/mM) were purchased from the Radiochemical Centre, Amersham. Radioactive oestradiol - 6-7-3H (62 Ci/mM) and androstenedione -1-2-3H (50 Ci/mM) were purchased from New England Nuclear. These radioactive tracers were diluted with ethanol to obtain stock solutions of approximately 20μCi/ml and stored at 4°C. The radiochemical purity of these tracers was checked periodically by Sephadex LH-20 column chromatography as described by Carr, Mikhail & Flickinger (1971). Purity was considered adequate when they were eluted as a single peak. Typical elution profiles are shown in Fig. 4. For the radioimmunoassays, an aliquot of the stock tracer solution was dried down under nitrogen stream, and redissolved in PBSG to give a solution containing the desired number of counts/minute in 0.1 ml of buffer. These buffer solutions were stored at 4°C and kept for a maximum of 4 weeks before being discarded.

(iii) Materials.

Seven ml. glass tubes were used in the extraction of the culture media and plasma samples and disposable glass test-tubes (7.6 X 0.9 cm. GWS)
Figure 4. Typical elution profiles of radioactive steroids eluted through 15cm Sephadex LH-20 columns.
2ml fractions were collected and counted.
for the assay. Organic solvents were evaporated on an electric Dri-Block DB-3 (Techne) with thermostatic control. Liquid scintillation counting was performed on a Packard Tri-carb 2450 (Packard) using glass counting vials of the low background type (Packard Instrument Company). In the system described the counting efficiency was ±35%. Samples were counted for sufficient time (4-10 minutes) so that the counting error was less than 2%, and background (15-20 cpm) was subtracted automatically.

**Progesterone Radioimmunoassay of Culture Medium.**

(i) **Progesterone antiserum.**

Radioimmunoassay of progesterone levels in tissue culture media was carried out using serum from the 8th bleed (R1-8) of a rabbit immunized against progesterone - 110Xhemisuccinate-bovine serum albumin conjugate, kindly supplied by Dr. K.K. Dighe of the Department of Pharmacology, University of Edinburgh. The preparation of the immunogen, and the immunization schedule employed have been reported elsewhere (Dighe & Hunter, 1974). Aliquots of the antiserum diluted with PBSG to 1/10C (v/v) were kept frozen at -20°C until required. The assay procedure was validated as described previously by Neal, Baker, McNatty & Scaramuzzi (1975).

(ii) **Selection of antibody dilution.**

Dilution and displacement curves for the antiserum were constructed by incubating (overnight at 4°C) triplicate 0.1 ml aliquots of serially diluted antiserum (in PBSG) with 0.1 ml of tritiated progesterone tracer (10,000 cpm) and 0.1 ml of either PBSG or non-radioactive progesterone (1 ng) in PBSG. In addition, 6 'total count' tubes containing 0.2 ml PBSG and
0.1 ml tracer were also incubated. These tubes allowed the average amount of radioactive progesterone added to each tube to be determined. The next day, separation of bound and free hormone was performed at 4°C by the addition of 1 ml of the dextran-coated charcoal suspension to the incubation tubes, except for the 6 'total count' tubes which received 1 ml PBSG instead. After mixing and standing for 15 minutes, all the tubes were centrifuged at 2,500 rpm for 15 minutes at 4°C on a Mistral 2L (MSE) centrifuge. The supernatant containing the antibody-bound steroid was decanted into scintillation vials and 10 ml of scintillator added. The vials were shaken and allowed to equilibrate at 4°C in the scintillation counter for 1-2 hours prior to counting.

The percentage of tracer bound was plotted against the logarithm of the antiserum dilution, and a displacement curve was constructed (Fig. 5). Maximum displacement was achieved with an antiserum dilution of 1/10,000 (1/30,000 final dilution). This dilution was therefore chosen for use in the assay.

(iii) Standard curve.

An aliquot of the stock μg/ml progesterone solution was serially diluted with ethanol to provide solutions ranging from 100 ng/ml to 100 pg/ml. Triplicate amounts corresponding to 20, 50, 100, 200, 500, 1000, 2000 and 5000 pg were pipetted with Eppendorf pipettes into assay tubes and dried under nitrogen. The residue was taken up in 0.1 ml PBSG, and the tubes mixed and left at room temperature for at least 1 hour. 0.1 ml of antiserum (1/10,000) and 0.1 ml tritiated progesterone tracer (10,000 cpm), both in PBSG, were then added to the tubes containing the progesterone standards. The tubes were mixed briefly and incubated overnight at 4°C. In addition 6 tubes containing 0.2 ml PBSG plus 0.1 ml tracer, and four 'zero' tubes containing 0.1 ml PBSG, 0.1 ml antiserum and 0.1 ml tracer
Figure 5  Progesterone antiserum dilution and displacement curves.
A  dilution curve in the absence of non-radioactive progesterone.
B  dilution curve in the presence of 1 ng non-radioactive progesterone per tube.
C  displacement curve $\frac{A - B}{A} \times 100$
were also incubated overnight at 4°C. Next day, 1 ml of the dextran-coated charcoal suspension was added to the progesterone standards and zero tubes; the tubes mixed, allowed to stand for 15 minutes, centrifuged and the supernatant counted. Three of the 6 tubes containing 0.2 ml PBSG and 0.1 ml tracer were treated with 1 ml dextran-coated charcoal as above, and these served as checks that the dextran-coated charcoal was absorbing satisfactorily the unbound steroid. The other 3 tubes were treated with 1 ml PBSG instead of dextran-coated charcoal, and these enabled the average amount of radioactive progesterone added to each tube to be checked at the end of the assay.

A standard curve was constructed by plotting the percent cpm of bound progesterone against the logarithm of the amount of progesterone added. The cpm bound when no progesterone was added i.e. in the 'zero' tubes was defined as 100%. At the 95% confidence limit 50 pg was significantly different from zero pg. The coefficient of variation at each point of the standard curve was less than 5% and the sensitivity varied from 20-50 pg of progesterone. A typical standard curve is shown in Fig. 6.

(iv) Effect of the dextran-coated charcoal suspension on the dissociation of antibody-antigen complex. ("stripping")

This was evaluated by measuring the percentage of radioactive progesterone remaining in the supernatant i.e. antibody bound progesterone at various time intervals (2-60 minutes) after addition of the dextran-coated charcoal suspension. The study was performed at 4°C at 3 different concentrations of antibody-tracer complex; maximal tracer binding (zero tubes without non-radioactive progesterone), intermediate tracer binding (tubes containing 250 pg non-radioactive progesterone) and absent tracer binding (tubes without antiserum). Results are shown in Fig. 7. The
Figure 6  Standard curve for progesterone.

Each point is the mean of triplicate determinations.
Figure 7. Effect of "stripping" with dextran-coated charcoal.

**0** Incubation tubes contained 0.1 ml PBSG, 0.1 ml antiserum (1/10,000) and 0.1 ml tritiated progesterone (10,000 cpm).

**250** Incubation tubes contained 250 pg non-radioactive progesterone in 0.1 ml PBSG, 0.1 ml antiserum (1/10,000) and 0.1 ml tritiated progesterone (10,000 cpm).

**PBSG** Incubation tubes contained 0.2 ml PBSG and 0.1 ml tritiated progesterone (10,000 cpm).
"stripping" rate i.e. the percentage decrease in antibody-bound tracer per unit of time, was \( \leq 0.4\% \) within 15 minutes of addition of the dextran-coated charcoal suspension to the maximal and intermediate tracer binding tubes. By 15 minutes the dextran-coated charcoal had also bound 97\% of the free tracer in the tubes without antiserum. A delay period of 15 minutes between addition of the dextran-coated charcoal suspension and centrifugation was therefore sufficient to ensure full absorption of unbound tracer and reduce "stripping" to a negligible rate.

(v) **Effect of duration of incubation on tracer-binding.**

The percentage of tracer bound to the antibody in the absence of non-radioactive progesterone was determined for various periods of incubation (1-24 hours) at 4°C. No significant increase in binding was obtained after four hours of incubation at 4°C. However, for convenience incubations were always performed overnight at 4°C.

(vi) **Specificity of progesterone antiserum.**

The specificity of a radioimmunoassay depends on the ability of the antiserum to respond only to the compound which the assay is intended to quantify. The specificity of the progesterone antiserum was tested by cross-reaction studies with various steroids (Table 1). Percentage cross reaction was defined according to Abraham, Odell, Edwards and Purdy (1970) as \( \frac{x}{y} \times 100 \), where \( x \) is the mass of unlabelled progesterone and \( y \) is the mass of the heterologous compound required to produce 50\% inhibition of the binding of trace amounts of tritiated progesterone (10,000 cpm) by the antibody. Apart from the hapten components of the antigen used to raise the antiserum (i.e. \( 11\alpha^- \)-hydroxy progesterone and \( 11\alpha^- \)-hydroxy progesterone hemisuccinate) only 4 other steroids showed appreciable cross-reaction (see Table 1). However, the available evidence indicates that luteinised human granulosa cells in tissue culture secrete
Table 1  Cross-reactions of various steroids with progesterone antiserum R1 - 8

<table>
<thead>
<tr>
<th>Steroid</th>
<th>% Cross-Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>C₂₁ Steroids</td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>100</td>
</tr>
<tr>
<td>11α - Hydroxyprogesterone</td>
<td>78</td>
</tr>
<tr>
<td>Hemisuccinate</td>
<td></td>
</tr>
<tr>
<td>11α - Hydroxyprogesterone</td>
<td>35</td>
</tr>
<tr>
<td>11β - Hydroxyprogesterone</td>
<td>12</td>
</tr>
<tr>
<td>11 - OXoprogestosterone</td>
<td>15</td>
</tr>
<tr>
<td>6β - Hydroxyprogesterone</td>
<td>0.8</td>
</tr>
<tr>
<td>20α - Hydroxypregn-4-ene-3-one</td>
<td>1.0</td>
</tr>
<tr>
<td>20β - Hydroxypregn-4-ene-3-one</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>5α - Pregnane - 3, 20 - dione</td>
<td>23</td>
</tr>
<tr>
<td>5β - Pregnane - 3, 20 - dione</td>
<td>5</td>
</tr>
<tr>
<td>17 - Hydroxyprogesterone</td>
<td>1.2</td>
</tr>
<tr>
<td>5α - Pregnane - 3, 20 - diol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>5β - Pregnane - 3, 20 - diol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>11 - Deoxycorticosterone</td>
<td>0.7</td>
</tr>
<tr>
<td>11 - Deoxycortisol (Compound S)</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>C₁₉ Steroids</td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>C₁₈ Steroids</td>
<td></td>
</tr>
<tr>
<td>Oestradiol - 17β</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Oestrone</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Oestriol</td>
<td>&lt; 0.1</td>
</tr>
</tbody>
</table>
predominately progesterone and pregnenolone and little, if any, of the potentially cross-reacting steroids (Channing, 1969b; McNatty, 1975). Thus, since pregnenolone has a very low cross-reaction with this progesterone antiserum, progesterone levels in the culture medium can be determined without a chromatographic purification step.

(vii) Validation of the assay for culture medium.

The accuracy of the assay was determined by measuring progesterone levels in a pool of culture medium to which known amounts of progesterone had been added. Replicate amounts of progesterone corresponding to 50, 100, 200, 500, 1000, 2000 and 5000 pg, together with 2,000 cpm tritiated progesterone were added in ethanol to extraction tubes. The ethanol was evaporated off under nitrogen, the residue taken up in 0.1 ml culture medium and the tubes left at room temperature for 2 hours to equilibrate. 0.1 ml PBSG and 20 μl ethanol was then added, and progesterone extracted with 2 ml distilled petroleum spirit (40-60°C) by mixing thoroughly on a vortex whirlmixer for 1 minute. After clear separation of the 2 phases, the lower aqueous layer was quick frozen in a methanol/dry ice bath, and the upper ether layer was decanted into an assay tube. The ether was then evaporated to dryness under a stream of nitrogen and the dried residue taken up in 0.2 ml PBSG. The tubes were mixed briefly and left to stand for 1 hour at room temperature. 50 μl was then removed and counted to provide an estimate of the amount of progesterone recovered, while 0.1 ml was removed and assayed together with a standard curve as described previously. The amount of progesterone in the "unknowns" was calculated by interpolation on the standard curve. The results were then corrected for procedural losses. Results are shown in Table 2. Regression analysis of the means gave the equation

\[ Y = 0.85X + 97.68, \quad r = 0.99 \]

where

\[ X = \text{mass progesterone added in pg}, \quad Y = \text{mass progesterone} \]
Table 2. Recovery of known amounts of progesterone added to 0.1 ml aliquots of pooled culture medium. Values are the means of 5 replicate determinations. Mean procedural recovery was 78%.

<table>
<thead>
<tr>
<th>MASS ADDED (pg)</th>
<th>MASS RECOVERED (pg)</th>
<th>RECOVERY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt; 20</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>77</td>
<td>154</td>
</tr>
<tr>
<td>100</td>
<td>127</td>
<td>127</td>
</tr>
<tr>
<td>200</td>
<td>232</td>
<td>116</td>
</tr>
<tr>
<td>500</td>
<td>514</td>
<td>103</td>
</tr>
<tr>
<td>1000</td>
<td>914</td>
<td>95</td>
</tr>
<tr>
<td>2000</td>
<td>2180</td>
<td>109</td>
</tr>
<tr>
<td>5000</td>
<td>4200</td>
<td>84</td>
</tr>
</tbody>
</table>
recovered in pg and \( r = \) coefficient of linear correlation (\( n = 8 \)).

From the percentage recoveries the assay is most accurate when measuring progesterone levels between 100 and 1000 pg progesterone.

In order to simplify determination of progesterone levels in the cell culture medium, it was desirable to dispense with the petroleum extraction step. The validity of omitting this extraction step was therefore checked by comparing progesterone levels in extracted and unextracted culture medium (Fig. 8). Regression analysis of the results gave the equation \( Y = 0.91 X + 14.23 \), \( r = 0.99 \), where \( X \) = amount of progesterone in 0.1 ml unextracted medium, \( Y \) = amount obtained after extraction and \( r = \) coefficient of linear correlation (\( n = 30 \)). Clearly the extraction step can be omitted.

(viii) Assay precision.

The within and between assay variance was evaluated by duplicate measurements of the same samples in the same assay and in 2 different assays. The coefficient of variation (CV) of the results of duplicate determinations from their means was estimated by the following formulae (Snedecor 1952).

\[
CV = \sqrt{\frac{\sum d^2}{2n}}
\]

where \( d = \left[ \frac{\text{highest value of each duplicate}}{\text{lowest value of same duplicate}} - 1 \right] \times 100 \)

\( n = \) number of duplicate determinations.

On 28 duplicate determinations performed in the same assay with 0.1 ml aliquot of culture medium containing 100 - 2500 pg progesterone, the coefficient of variation was 8%. In 20 duplicate determinations in 2 different assays, the coefficient of variation was 12%.
Figure 8. Comparison of the levels of progesterone in 0.1 ml aliquots of unextracted culture medium and culture medium extracted with petroleum spirit (20/1, v/v).
(ix) Measurement of progesterone in ovarian venous plasma.

The concentration of progesterone in ovarian venous plasma was measured by the radioimmunoassay previously described and validated by Scaramuzzi, Corker, Young & Baird (1975). Replicate 0.02 - 0.1 ml amounts of ovarian venous plasma, made up to 0.2 ml with PBSG and containing 2,000 cpm tritiated progesterone (added in 0.02 ml ethanol) were extracted, after equilibrating for 30 minutes, with 2 ml distilled petroleum spirit (40 - 60°C) by mixing thoroughly on a vortex whirlmixer for at least 1 minute. After separation of the 2 phases the lower plasma layer was quick frozen in a dry ice/methanol bath, the petroleum spirit decanted into incubation tubes and dried down under nitrogen. The dried residue was taken up in 0.5 ml PBSG and left to equilibrate for at least 1 hour. Duplicate 0.1 ml and 0.05 ml aliquots were removed and assayed for progesterone, together with a standard curve, as described for the assay of progesterone in culture medium, except that the antiserum described by Scaramuzzi et al (1975) was used. A further 0.1 ml aliquot was also removed, and counted to provide an estimate of the amount of progesterone recovered by the extraction procedure. In 32 duplicate determinations performed in the same assay, the coefficient of variation, calculated by the method of Snedecor (1952) was 13%. In 15 duplicate determinations in 2 different assays, the coefficient of variation was 18%.

Measurement of oestrogens and androgens in culture medium.

(i) Oestrogens.

Oestradiol and oestrone were extracted from culture medium and assayed by specific radioimmunoassays using antisera previously described and validated for the determination of plasma oestradiol (Van Look, 1976) and plasma oestrone (Rowe, Cook & Dean, 1973). 0.2 or 0.5 ml aliquots of culture medium were extracted with 2 ml purified diethylether by thoroughly mixing on a vortex whirlmixer for 1 minute. The plasma layer
was quick frozen in a dry ice/methanol bath, the ether decanted into assay tubes and dried down under nitrogen. The dried residue was taken up in 0.1 ml PBSG, the tubes mixed briefly and left to stand at room-temperature for at least 1 hour. Standard curves for oestradiol and oestrone were prepared as described for the progesterone standard curve, except that the oestradiol and oestrone standards ranged from 2-1000 pg. 0.1 ml of oestradiol or oestrone antiserum, and 0.1 ml of tritiated oestradiol or oestrone (5000 cpm), each in PBSG, were then added to the assay and standard tubes. The tubes were mixed and incubated overnight at 4°C. The next day, separation of bound and free hormone was carried out using dextran-coated charcoal as described for the progesterone assay.

In each assay an additional 8 tubes containing 0.2 or 0.5 ml of pooled culture medium and 1000 cpm tritiated oestradiol or oestrone (added in 0.02 ml PBSG) were extracted with diethylether as above. However after decanting and evaporating the ether, the residue was taken up in 0.3 ml PBSG. The tubes were mixed, and left at room-temperature until transferred with the other assay tubes to the fridge for overnight incubation at 4°C. Analysis of duplicate 0.1 ml aliquots of these samples provided an estimate of the mean procedural loss of the extraction procedure. Although between assays the mean recovery of tritiated oestradiol and oestrone from culture medium ranged from 75 - 85%, within each assay the extraction procedure was highly reproducible, the within-assay coefficient of variation for tritiated oestradiol and oestrone recovery being < 6%. This high reproducibility permitted recovery determinations on individual samples to be omitted, the mean value for recovery of tritiated oestrogen from the 8 separate samples being sufficient to estimate the procedural loss in each assay.
Intra and inter assay precision was estimated by including in each assay replicate 0.5 ml aliquots of culture medium taken from two pools containing approximately 150 pg/ml of either oestradiol or oestrone. The intra-assay coefficient of variation was <10% for both assays while the inter-assay coefficient of variation was 15% for the oestradiol assay and <10% for the oestrone assay.

(ii) Androgens.
Testosterone and androstenedione were extracted from culture medium and assayed by specific radioimmunoassays using antisera previously described and validated for the determination of plasma testosterone (Corker & Davidson, 1977) and plasma androstenedione (Baird, Burger, Heavon-Jones & Scaramuzzi, 1974). The only difference between the androgen assay procedure and that described for the oestrogens was that 0.1 ml aliquots of culture medium were extracted with 2 ml hexane/diethylether (4:1 ratio). The rest of the assay procedure was identical. The amount of androgen extracted from the culture medium was estimated, as in the oestrogen assays, by extracting 8 X 0.1 ml aliquots of pooled culture medium containing 1000 cpm of tritiated androgen. Greater than 90% of tritiated androgen was consistently recovered in each assay, the within-assay recovery coefficient of variation being <4%. Intra and inter assay variations, estimated as in the oestrogen assays were each <12% for both the testosterone and androstenedione assay.

Tissue Culture Techniques.

(i) Culture of granulosa cells.
Human ovaries were obtained from patients undergoing ovariectomy for various gynaecological disorders. Bovine and porcine ovaries were obtained from animals within 1 hour of their slaughter at a local abattoir. The ovaries were collected into chilled Medium 199 containing
Hanks' salts, HEPES buffer* (20mM/l) and supplemented with glutamine (2mM/l) amphotericin B (2.5μg/ml) and gentamicin (50μg/ml) - all reagents obtained from Flow Laboratories. The techniques for obtaining dispersed granulosa cell suspensions and the method of granulosa cell culture were as previously described by McNatty & Sawers (1975). Antral follicles were dissected out of the ovaries and the follicular fluid aspirated by syringe. The collapsed follicles were slit open and the granulosa cells scraped gently into chilled Medium 199, supplemented as above, using a platinum loop. A section of the remaining follicle wall was fixed in Bouin's Fluid for subsequent histological examination to ensure that the basement membrane was still intact (and thus that the culture was free of theca cells). An aliquot of the harvested granulosa cells was removed to determine the cell concentration by haemocytometer counting and the cell viability was determined using nigroin vital stain (Kaltenbach, Kaltenbach & Lyons, 1958). The remaining cells were then diluted to a minimum concentration of 5 X 10⁵ "live" cells per ml with culture medium consisting of 20% donor calf serum and 80% Medium 199 containing Hanks' salts and HEPES buffer, and supplemented with glutamine and antibiotics as above. 18mm² glass coverslips, previously washed in acetone, dried with lens tissue and sterilized at 200°C, were placed in individual 19mm² compartments of a tissue culture box containing twenty-one such compartments, obtained from Flow Laboratories. 0.1 ml aliquots of the diluted granulosa cell suspension containing a minimum of 5 X 10⁶ "live" cells were then layered onto the coverslips. A further 0.9 ml culture medium with or without added gonadotrophins was added. Prostaglandins and steroids were added

* HEPES buffer: N-2-hydroxyethyl - piperazine - N-2-ethanesulphonic acid (see Good, Winget, Winter, Connolly, Izawa & Singh, 1966)
to the test cultures in 5 or 10 μl ethanol, control cultures receiving 5 or 10 μl ethanol only. All cultures were then incubated at 37°C using air as the gas phase. The culture medium was replaced daily, and stored at -20°C until assayed for steroids. At the end of the culture period, the coverslips were removed, washed extensively with Medium without serum and the cells fixed with 'Smearfix' (Raymond Lamb). After staining with haematoxylin/eosin (Paul, 1975), the number of cells on the coverslips was estimated. This was achieved by counting the number of cells within a 0.16 mm² area at 16 uniformly spaced sampling points on the coverslip, the total number of cells then being estimated by extrapolation. When facilities allowed, cell counting was aided by projecting an amplified image of the sampling point onto a television screen (Green 1974).

The typical appearance of haematoxylin/eosin stained human granulosa cells before and after culture is shown in Plate 1.

(ii) Culture of isolated bovine luteal cells.

The only methods described at the start of these studies for the isolation of bovine corpus luteum cells employed a number of enzymes in a rather complex procedure (Sayers et al 1971; Gospodarowicz & Gospodarowicz, 1972). A simpler method was desired. Crude bacterial collagenase is a particularly useful enzyme for the disaggregation of tissues, especially adult tissue, and has been successfully used alone to isolate cells of the pancreas (Lacy & Kostianovsky, 1967), adrenal (Richardson & Schulster, 1972) testis (Boyle & Ramachandran, 1973) and ovary (Bajpai, Dash, Lidgley & Reichert, 1974). The effectiveness of crude collagenase in dissociating bovine luteal tissue was therefore studied, and the following relatively simple method, summarised in Fig. 9, was found to be quite satisfactory. Bovine corpora lutea were obtained from cattle within 1 hour of their
Plate 1. Appearance of haematoxylin/eosin stained human granulosa cells before and after culture (Mag. X 300)

(a) human granulosa cells harvested from small antral follicles and incubated overnight at 37°C on a glass coverslip in Medium 199 to obtain a monolayer suitable for staining.

(b) human granulosa cells harvested from small antral follicles and cultured for 8 days in the presence of human LH and FSH (30 mu./ml of each)
ISOLATION OF BOVINE LUTEAL CELLS

MINCED CORPUS LUTEUM

ENZYME TREATMENT
(collagenase 2000 U/g tissue)

20 mins at 37°C

CELLS SPUN DOWN AND RESUSPENDED

TISSUE FRAGMENTS FURTHER ENZYME TREATMENT

FILTER THROUGH STERILE GAUZE

WASH BY CENTRIFUGATION X5

CELL PREPARATION SUSPENDED IN MEDIUM 199

HAEMOCYTMETER COUNT

+ NIGROSIN EXCLUSION TEST

SHORT TERM INCUBATION
4 HOURS

LONG TERM CULTURE
8 DAYS

Figure 9. Flow diagram of enzymatic dispersal of bovine luteal tissue.
slaughter at a local abattoir, and transported to the laboratory in chilled Medium 199. The Medium 199 was supplemented for this and the following procedures with Hanks' salts, HEPES buffer, glutamine and antibiotics as described previously. The corpus luteum was freed of adherent connective tissue, a section was taken for histology, and the remainder chopped into small pieces, about 2 mm in diameter, with scalpel blades and fine micro-dissecting scissors. The chopped tissue was washed twice with chilled Medium 199 and transferred to a conical flask containing a teflon coated magnetic stirrer. Medium 199 (5 ml/g tissue), preheated to 37°C, was added and the contents stirred for 10 minutes at 37°C. The medium was then decanted and fresh Medium 199, preheated to 37°C, containing collagenase (2.5 - 3mg/ml, Sigma Type II) added and the incubation with stirring continued. At 20 minute intervals, the medium containing released cells was decanted off and replaced with fresh Medium 199 containing collagenase, and the dissociation continued. The released cells were collected by low speed centrifugation, taken up in chilled Medium 199 and stored at 4°C. When the remaining incubating tissue fragments became fragile, dissociation was further aided by drawing through a 2 ml syringe barrel. After 4 - 5 medium renewals the dispersion process was discontinued, with only fibrous material remaining undissociated. The freed cells were then pooled together, filtered through sterile gauze, washed 5 times with chilled Medium 199 to remove all traces of collagenase and finally resuspended in chilled Medium 199. An aliquot of the cell suspension was removed to determine the cell concentration by haemocytometer counting, and cell viability was determined by their ability to exclude nigrosin dye. The typical appearance of the dispersed bovine luteal cells is shown in plate 2. Their appearance is very similar
Plate 2 Haematoxylin/eosin stained smear of isolated bovine luteal cells (Mag. x 250).
to those described by Gospodarowicz & Gospodarowicz (1972). The cell yield with this procedure was in the range 10-20 \times 10^6 "live" cells per gram of corpus luteum; cell viability being >80%. Recently, a similar procedure involving collagenase disaggregation has been described for obtaining dispersed Rhesus monkey corpus luteum cells (Stouffer, Nixon, Gulyas, Johnson & Hodgson, 1976). This suggests this simple procedure may be directly applicable to obtaining dispersed human luteal cells.

Progesterone production by the isolated bovine luteal cells was studied in long-term cultures of 8 days, and in short-term incubations of 4 hours duration. The long-term cultures were set up identically as described for granulosa cells. The short-term incubations were carried out in glass scintillation vials. \(1 \times 10^5\) large "live" cells were incubated in 2 ml K medium 199, with or without added hormones, for 4 hours at 37°C. The incubations were terminated by transferring the vials to a 60°C water bath for 20 minutes to kill the cells and denature any steroid enzyme systems. The medium was then stored at -20°C until assayed for progesterone by radioimmunoassay.

Prostaglandin Infusion of the Ovary of Ewes Bearing Ovarian Autotransplants.

Eleven ewes bearing ovarian autotransplants were used to study the effects of prostaglandins on progesterone production by the corpus luteum in vivo. The ovarian transplant preparation used has been described in detail by Goding et al (1967). In this procedure the right ovary is removed, and the left ovary with its vascular pedicle is transplanted with vascular anastomosis to the carotid artery and the jugular vein contained in a pre-fashioned skin loop in the neck (Fig. 10). This preparation allows easy access to both the ovarian arterial and venous circulation, thereby enabling in vivo studies of ovarian function to be carried out in conscious, relatively unstressed animals. When the ovary is autotransplanted to the neck, the corpus luteum persists due to the absence of the luteolytic influence of an adjacent uterine horn (Baird et al, 1963).
Figure 10. Diagram of the ovarian transplant preparation
(from Goding et al., 1967)
For the purposes of this study it was necessary, however, to use corpora lutea of the same age. The persisting corpora lutea were therefore caused to regress, and the ovarian cycles of the animals synchronized, by administering a single intramuscular injection of ICI 80,996 (50 μg), a potent luteolytic 16-aryloxy derivative of PGF₂α. This induced oestrous behaviour within 2 or 3 days; the infusion experiments being carried out 10 days after the onset of oestrus.

On the day of the infusion experiment, the left jugular vein was cannulated for the collection of ovarian venous blood as previously described (Goding et al., 1967). A polyethylene catheter was inserted into the jugular vein so that the tip came to lie opposite the orifice of the ovarian vein (see Fig. 11). The left carotid artery was then cannulated on the cardiac side of the transplanted ovary using an intra-arterial needle. The animal was given 5000 units heparin through the venous catheter, and rested for at least 2 hours before further experimental procedures were carried out. Before commencing the infusions, a pneumatic cuff was placed around the upper part of the loop and inflated to a pressure of 200 mm Hg. Since this pressure is higher than the carotid arterial pressure of the sheep, carotid arterial blood containing the infusate exclusively supplies the transplanted ovary. Prior to infusing prostaglandins, the ovary was infused for 2 hours with 0.9% physiological saline (10 ml/hour) through the intra-arterial needle using a constant infusion pump. During this period, 2 "control" samples of ovarian venous blood were obtained 30 and 90 minutes after commencement of the infusion. The ovarian venous blood was collected by means of the catheter inserted into the jugular vein (Fig. 11). Immediately before the collection period, the catheter was flushed out with heparin (250 units/ml) in saline. The jugular vein below the ovary was obstructed manually in order to prevent any retrograde dilution of ovarian venous blood by systemic venous blood. Blood was allowed to flow from the catheter for approximately 15 seconds before it was collected in order to allow any peripheral venous blood to be flushed out of the jugular vein. An
Figure 11. Techniques for the collection of ovarian venous blood and for the constant intra-arterial infusion into the transplanted ovary (from McCracken, Uno, Goding, Ichikawa & Baird, 1969).
accurately timed sample of ovarian venous blood was then collected, the plasma obtained by centrifugation and stored at -20°C until assayed for progesterone. The secretion of progesterone from the ovary was calculated from the plasma concentration, the blood haematocrit and blood flow rate. Following the infusion of saline, the ovary was infused for a further 4 hours with either PGF2α, PGE2, PGF2α + PGE2 or saline only; 10 µg prostaglandin in 10 ml saline being delivered at a constant rate each hour. Timed collections of ovarian venous blood were made at hourly intervals for 6 hours, commencing 30 minutes after the start of the test period. Each animal was given a further 5000 units of heparin after the first collection of ovarian venous blood. At the end of the 6 hour sampling period, the arterial cannula was removed. A final sample of ovarian venous blood was taken the next day, approximately 18 hours after the start of the infusion. The venous catheter was removed and the animals allowed to run with a raddled vasectomized ram for 4 days; they being inspected twice daily for signs of mating (Radford, Watson & Wood, 1960).

**Uptake of Tritiated Prostaglandin F2α**

The uptake studies referred to in Chapter 7 were carried out as follows. Human and bovine granulosa cells were cultured in the presence of human LH and FSH (36 μg/ml and 24 μg/ml respectively) for 2 - 10 days. By varying the length of time in culture, it was possible to obtain granulosa - luteal cells which were secreting varying amounts of progesterone ranging from \(< 0.5 - 7 \text{ pg/cell/day}\). At the end of each culture period, the coverslips were thoroughly washed with Medium 199 (supplemented as before), and incubated for 1 hour at 37°C in 1 ml Medium 199 containing either 5 ng \(^{3}H\)-PGF2α (25 X 10^4 cpm) or 1 ng \(^{3}H\)-PGF2α (5 X 10^4 cpm). The Medium was then decanted into a counting vial, and the cells were washed twice with Medium 199, each washing being transferred separately into counting vials, and counted. The cells were then re-incubated for 1 hour in the same manner but with 10 μg unlabelled PGF2α.
Since this procedure did not cause a significant release of radioactive 
$\text{PGF}_2\alpha$, the percentage uptake of $^3\text{H}-\text{PGF}_2\alpha$ was expressed as follows:

$$\% \text{ uptake} = \frac{A - P}{A} \times 100$$

where $A$ is the total number of counts remaining after incubating $^3\text{H}-\text{PGF}_2\alpha$ for 1 hour in the absence of granulosa cells, and $P$ is the total number of counts remaining after incubating $^3\text{H}-\text{PGF}_2\alpha$ for 1 hour in the presence of granulosa cells. The coverslips were then stained, and the cells remaining counted. From a knowledge of the cell number and specific activity of $^3\text{H}-\text{PGF}_2\alpha$ (42 mCi/mg), the amount of $^3\text{H}-\text{PGF}_2\alpha$ retained by the cells was expressed finally as ng$^3\text{H}-\text{PGF}_2\alpha/10^6$ cells.

**Gonadotrophin and Prostaglandin Preparations.**

(i) **Gonadotrophins.**

Ovine luteinizing hormone (NIH-LH-S12) and ovine follicle-stimulating hormone (NIH-FSH-S4) were supplied by the National Institute of Health, Bethesda, Maryland, U.S.A. Human luteinizing hormone (LH-DEAE-1) containing 7550 units LH/mg and 25 units FSH/mg was kindly supplied by Dr. A. Stockell-Hartree of the Dept. of Biochemistry, University of Cambridge. Human follicle-stimulating hormone (FSH-73/519) containing 2200 units FSH/mg and 8.8 units LH/mg was supplied by the MRC National Institute for Biological Standards and Control.

All the gonadotrophins were diluted with culture medium and stored at $-20^\circ\text{C}$ until added to granulosa cell cultures in 0.1 ml aliquots.

(ii) **Prostaglandins.**

All the non-radioactive prostaglandins, except for the 16-aryloxy analogues of $\text{PGF}_2\alpha$, were generously supplied by Dr. J. Pike of the Upjohn Company, Kalamazoo, Michigan, U.S.A. Radioactive prostaglandin $\text{F}_2\alpha - 9^{-3}\text{H}$ (42 mCi/mg) was obtained from the Radiochemical Centre, Amersham. These
prostaglandins were diluted with 100% ethanol and stored at 4°C until added to the cell cultures in 5 or 10 μl aliquots. Control cultures received appropriate amounts of 100% ethanol only. The 16-aryloxy analogues of PGE2α (ICI 80,996, ICI 79,939 and ICI 81,008) were donated by Dr. M. Dukes, ICI Pharmaceuticals Division, Alderley Edge, Cheshire, England. They were diluted in phosphate-buffered saline (Flow Laboratories) and stored at 4°C until added to the cell cultures in 0.1 ml aliquots; control cultures receiving 0.1 ml phosphate-buffered saline only.
CHAPTER 4.

EFFECTS OF PROSTAGLANDINS

ON THE PRODUCTION OF PROGESTERONE

BY HUMAN GRANULOSA CELLS IN TISSUE CULTURE.
As discussed previously, granulosa cells obtained from human Graafian follicles and induced to luteinize in tissue culture under optimum gonadotrophic support provide a convenient \textit{in vitro} model of the corpus luteum. This system was used to investigate the possible effects of prostaglandins, particularly \( \text{PGF}_2 \), on the human corpus luteum.

Figure 12 shows the effect of daily addition of \( \text{PGF}_2 \) on basal and gonadotrophin stimulated progesterone production by human granulosa cells during 10 days of tissue culture. The low basal secretion of progesterone by the control cultures probably reflects low endogenous gonadotrophic activity in the culture medium, which contains 20\% donor calf-serum. Addition of \( \text{PGF}_2 \) significantly inhibited this basal progesterone production (\( P < 0.02 \)). Progesterone production by the cell cultures was increased twofold by the daily addition of human luteinizing and follicle stimulating hormone (LH + FSH): FSH acting to stimulate LH binding to the granulosa cells (Zeleznik, Midgley & Reichert, 1974; Channing, 1975; Nimrod, Tsafriri & Lindner, 1977), while LH stimulates steroidogenesis through activating adenylate cyclase (Marsh, 1970). The amount of gonadotrophin added (30 \mu g/ml each of LH and FSH) has previously been shown to be sufficient to stimulate maximum production of progesterone by human granulosa cells in tissue culture (McNatty & Sawers, 1975). This gonadotrophin - induced stimulation of progesterone production was abolished when \( \text{PGF}_2 \) was added together with the gonadotrophins, though the extent of the inhibition was not as great as that observed in the absence of added LH + FSH. A dose - response study showed that as little as 10 ng/ml \( \text{PGF}_2 \) was sufficient to produce maximum inhibition of gonadotrophin stimulated progesterone production (Table 3).

This inhibition of progesterone production by \( \text{PGF}_2 \) would appear to result from a direct biochemical action of \( \text{PGF}_2 \). Examination by light microscopy
Figure 12. Mean daily production of progesterone by human granulosa cells in vitro and exposed daily to PGF$_{2\alpha}$ (50 ng/ml) with or without added human LH and FSH (both 30 mu./ml). Numbers in parentheses refer to the number of replicate cultures. Precision between replicate cultures in relation to the production of progesterone for all treatments was $<10\%$. PGF$_{2\alpha}$ treated cultures produced significantly less progesterone than their respective controls (P $<0.02$, paired t-test).

Cross, control; open triangle, LH + FSH; solid circle, PGF$_{2\alpha}$; solid triangle, LH + FSH + PGF$_{2\alpha}$

(from McNatty, Henderson & Sawers, 1975).
Table 3  Effect of prostaglandin F<sub>2α</sub> on the total production of progesterone by human granulosa cells cultured for 10 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Progesterone (μg/10&lt;sup&gt;6&lt;/sup&gt; cells; mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.2 (2)</td>
</tr>
<tr>
<td>LH + FSH</td>
<td>38.8 ± 4.6 (4)</td>
</tr>
<tr>
<td>LH + FSH + 10 ng PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>17.0 ± 1.8 (4)</td>
</tr>
<tr>
<td>LH + FSH + 50 ng PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>15.5 ± 2.9 (3)</td>
</tr>
<tr>
<td>LH + FSH + 100 ng PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>22.1 ± 1.3 (3)</td>
</tr>
<tr>
<td>LH + FSH + 500 ng PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>18.7 ± 3.3 (4)</td>
</tr>
</tbody>
</table>

Gonadotrophins (30 μu./ml each of human LH and FSH) and PGF<sub>2α</sub> were added daily from the start of the culture period. Numbers in parentheses represent the number of replicate cultures. Cultures treated with gonadotrophins and PGF<sub>2α</sub> together produced significantly less progesterone than those cultures treated with gonadotrophins alone. (P<0.01, Student's t-test).
of the haematoxylin/eosin stained cells at the end of the culture period revealed no apparent morphological differences between the PGF$_{2\alpha}$ treated and control groups, nor was there any difference in the number of cells remaining at the end of the culture period between the two groups.

Present evidence (see literature review) indicates that PGF$_{2\alpha}$ induced luteal regression in vivo is probably a two stage process; firstly a biochemical inhibition of progesterone production (functional regression), followed considerably later by the morphological deterioration of the luteal cell (structural regression). The inhibition of progesterone production by PGF$_{2\alpha}$ observed in this in vitro system is analogous to functional regression in vivo, in that it occurs in the absence of any cellular deterioration. The failure of PGF$_{2\alpha}$ to cause cell death in vitro suggests that other PGF$_{2\alpha}$ induced mechanisms, possibly dependent on the presence of a vascular system, are required to bring about structural regression of the corpus luteum.

The fact that low amounts of PGF$_{2\alpha}$ can inhibit progesterone production by human granulosa cells in tissue culture supports the possibility that the failure to induce luteolysis in women, with systemically administered PGF$_{2\alpha}$, may be because rapid peripheral metabolism results in insufficient amounts of PGF$_{2\alpha}$ reaching the ovary. Moreover, these in vitro studies with PGF$_{2\alpha}$ demonstrate the usefulness of human granulosa cells in tissue culture as a system not only for studying the possible luteolytic mechanism(s) of PGF$_{2\alpha}$ but also for screening possible human luteolysins prior to initiating clinical trials.

In contrast to the inhibitory action of PGF$_{2\alpha}$ on progesterone production PGE$_2$, which differs from PGF$_{2\alpha}$ only in configuration about C-9 (Fig. 1), stimulates progesterone production by human granulosa cells in tissue culture (Table 4). This structure-activity relationship of the prostaglandins is demonstrated further in Figure 13. Removal of the carbonyl group at C-9, the hydroxyl group at C-11, or reversal of the configuration of
Table 4. Effect of prostaglandin E\(_2\) on the total production of progesterone by human granulosa cells cultured for 6 days.

<table>
<thead>
<tr>
<th>PGE(_2) (ng/ml)</th>
<th>Progesterone (μg/10(^6) cells; mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>19.5 ± 1.7 *</td>
</tr>
<tr>
<td>10</td>
<td>16.2 ± 1.2 *</td>
</tr>
<tr>
<td>50</td>
<td>20.7 ± 1.1 *</td>
</tr>
<tr>
<td>500</td>
<td>15.7 ± 0.9 *</td>
</tr>
<tr>
<td>2000</td>
<td>18.3 ± 1.5 *</td>
</tr>
</tbody>
</table>

Each value is the mean of 4 replicate cultures.

* Significantly different from control value
(P<0.01, Student's t-test).
Figure 13. Stimulatory and inhibitory effect of various prostaglandins on progesterone production by human granulosa cells cultured for 8 days. Stimulation of progesterone production is expressed as a percentage of that achieved with PGE₂. All prostaglandins were tested at a concentration of 50 ng/ml in a minimum of 3 replicate cultures and tested against 3 control and 3 PGE₂ (50 ng/ml) treated cultures.
the hydroxyl group at C-15 from S to R completely abolishes the stimulatory action of PGE₂, while the inhibitory action of PGF₂α requires an α positioned hydroxyl group at C-9. It is interesting to note that 19-OH PGE₂, which is the principle prostaglandin component of human semen (Kelly et al., 1976; Templeton et al., 1977) mimics the stimulatory action of PGE₂. Whether semen 19-OH PGE₂ might have a luteotrophic function must, however, remain entirely speculative at present. While the polar groups at C-9, C-11 and C-15 are clearly essential for prostaglandin activity, the carbon side-chains appear to have a less well defined role. The C-5, 6 double bond can be reduced without affecting the activity of either PGE₂ or PGF₂α, while the rather bulky aryloxy group can be attached to the alkyl side-chain of PGF₂α without affecting its inhibitory action. Thus, it is possible that a main function of the carbon side-chain is to confer membrane solubility on the prostaglandin molecules, while the polar hydroxyl and carbonyl groups at C-9, C-11 and C-15 may be required to attach to specific membrane receptors for either PGE₂ or PGF₂α, the cellular response then being triggered by occupancy of these receptors.

Although PGF₂α readily inhibits both basal and gonadotrophin stimulated progesterone production by granulosa cells (Fig. 12, Table 3), the inhibitory action of PGF₂α is masked in the presence of PGE₂ (Table 5). This provides a clue to the possible site of action of PGF₂α. There is substantial evidence that both LH (Gospodarowicz, 1973; Han, Rajaniemi, Cho, Hirshfield & Midgley, 1974) and PGE₂ (Rao, 1973, 74) interact with respective, specific receptors situated on the plasma membrane to stimulate progesterone production through activation of the adenylate cyclase enzyme system (Marsh, 1970a,b, 1971, 1976). Adenosine 3':5' - cyclic monophosphate (c-AMP) is produced in response to activation of adenylate cyclase, and this "2nd messenger" initiates a series of complex intracellular events, recently
Table 5  Effect of adding prostaglandins $\text{P}_2\alpha$ and $\text{E}_2$
alone and together on the production of progesterone
by human granulosa cells.

<table>
<thead>
<tr>
<th>Prostaglandin added</th>
<th>Concentration (ng/ml)</th>
<th>Total production of progesterone during 10 days of culture ($\mu$g progesterone / $10^6$ cells; mean $\pm$ S.E.M.)</th>
<th>Number of separate cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>51.8 $\pm$ 4.6</td>
<td>5</td>
</tr>
<tr>
<td>$\text{PGE}_2$</td>
<td>10</td>
<td>60.3 $\pm$ 1.9</td>
<td>5</td>
</tr>
<tr>
<td>$\text{PGF}_2\alpha$</td>
<td>500</td>
<td>61.9 $\pm$ 2.3</td>
<td>5</td>
</tr>
<tr>
<td>$\text{PGF}_2\alpha$</td>
<td>10</td>
<td>23.2 $\pm$ 3.7</td>
<td>5</td>
</tr>
<tr>
<td>$\text{PGF}_2\alpha$</td>
<td>500</td>
<td>17.5 $\pm$ 2.1</td>
<td>5</td>
</tr>
<tr>
<td>$\text{PGE}_2$</td>
<td>10 (10)</td>
<td>49.9 $\pm$ 4.9</td>
<td>3</td>
</tr>
<tr>
<td>$\text{PGF}_2\alpha$</td>
<td>50 (50)</td>
<td>48.8 $\pm$ 5.1</td>
<td>3</td>
</tr>
<tr>
<td>$\text{PGE}_2$</td>
<td>100 (100)</td>
<td>57.6 $\pm$ 4.3</td>
<td>3</td>
</tr>
<tr>
<td>$\text{PGF}_2\alpha$</td>
<td>500 (500)</td>
<td>57.2 $\pm$ 6.6</td>
<td>3</td>
</tr>
</tbody>
</table>

(from McNatty, Henderson & Sawers, 1975)
comprehensively reviewed by Marsh (1976) and summarised in Fig. 14.

The overall effect is stimulation of progesterone synthesis, chiefly through an enhancement of the conversion of cholesterol to pregnenolone, the rate-limiting step in the biosynthesis of progesterone. Prolonged stimulation of progesterone synthesis is dependent on elevated levels of c-AMP being maintained. This, however, requires the constant synthesis of c-AMP, since once formed, it is rapidly inactivated by conversion to adenosine monophosphate (AMP) by the enzyme phosphodiesterase. Thus, removal or inhibition of the hormonal stimulus activating adenylate cyclase will result in a rapid depletion of intracellular c-AMP with a consequent reduction in synthesis of progesterone. How do LH and PGE\textsubscript{2} bring about their activation of adenylate cyclase? The receptors for LH and PGE\textsubscript{2}, and the adenylate cyclase (AC) enzymes are best regarded as discrete and separate structures located in the plasma membrane (Cuatrecasas, 1974). The hormone binding sites of the receptors are orientated towards the outside of the cell, while the catalytic sites of the AC enzymes are orientated towards the inside of the cell. Until the AC enzymes are coupled with the hormone receptors they remain inactive and unable to convert adenosine triphosphate (ATP) to c-AMP. The plasma membrane is not a rigid static structure but has dynamic properties, and is best described as a fluid mosaic (Singer & Nicholson, 1972; Cuatrecasas, 1974). This fluidity of the membrane allows lateral diffusion of the receptors and AC enzymes along the plane of the membrane, thereby allowing the receptors and AC enzymes to interact by random encounters (Fig. 15). However, only when they are coupled to their respective hormones can the receptors for LH and PGE\textsubscript{2} activate the AC enzymes to produce c-AMP. Since both LH and PGE\textsubscript{2} stimulate progesterone production through this activation of adenylate cyclase, but PGE\textsubscript{2\alpha} only inhibits stimulation of progesterone production by LH, it seems likely that PGE\textsubscript{2\alpha} acts to inhibit specifically LH activation of adenylate cyclase. Other more recent findings support this proposed mechanism.
Figure 14. Mechanism by which LH and PGE$_2$ stimulate progesterone production via c-AMP. Solid arrows indicate biochemical reactions. Large dashed arrows indicate sites of action of c-AMP on the steroidogenic process. Small dashed arrows indicate transport of a substance through cellular membranes.

(Modified from Marsh, 1976).
Two-step fluidity model for LH and PGE$_2$ activation of adenylate cyclase.

(Modified from Cuatrecasas, 1974)
of action of PGF$_{2\alpha}$. Lahav et al (1976) and Green and Wakeling (1977, personal communication) have demonstrated directly PGF$_{2\alpha}$ inhibition of LH-stimulated c-AMP production by corpora lutea in vitro, while the work of Behrman & Hichens (1976) and Grinwich et al (1976) shows that this inhibition may arise as a consequence of PGF$_{2\alpha}$ causing a rapid and sustained loss of LH binding by the luteal cell, thereby preventing LH from activating adenylate cyclase.
CHAPTER 5.

SIMULTANEOUS INFUSION OF PROSTAGLANDIN E\_2

ANTAGONIZES THE LUTEOLYTIC ACTION OF

PROSTAGLANDIN F\_2\_\_ IN VIVO.
Since PGF$_{2\alpha}$ can inhibit progesterone production in vitro by directly blocking the action of LH, it is possible that PGF$_{2\alpha}$ may initiate luteal regression in vivo by the same mechanism. This possibility is supported by the fact that the onset of luteal regression is associated with biochemical changes in the luteal cell consistent with the biochemical action of PGF$_{2\alpha}$, i.e. a decline in adenylate cyclase activity and loss of responsiveness to LH (Andersen et al., 1974; Hichens et al., 1974; Behrman & Hichens, 1976; Grinwich et al., 1976). The "luteolytic" action of PGF$_{2\alpha}$ in vitro can be overcome by simultaneous treatment with PGE$_2$ (Table 5). Thus if PGF$_{2\alpha}$ initiates luteolysis in vivo as it does in vitro by interfering specifically with the coupling of LH and adenylate cyclase, the action of PGF$_{2\alpha}$ should be overridden by simultaneous administration of PGE$_2$. This possibility was tested using ewes in which the ovary had been autotransplanted to the neck.

Effect of prostaglandin infusions on progesterone secretion.

Figure 16 shows the effect of infusion into the ovarian artery of saline, PGE$_2$, PGF$_{2\alpha}$ and PGE$_2$ + PGF$_{2\alpha}$ on progesterone secretion by corpora lutea of ewes bearing ovarian autotransplants. The results are expressed as a percentage of the mean progesterone secretion rate obtained from the two control ovarian venous blood samples taken prior to the start of the infusions (see Methods section). The overall rates of progesterone secretion during the control infusion period for each of the experimental groups were not significantly different from each other (P > 0.05), the values being: saline, 6.8 ± 1.3 (S.E.M.) μg/min; PGF$_{2\alpha}$, 8.4 ± 0.8; PGE$_2$, 9.2 ± 1.3; PGF$_{2\alpha}$ + PGE$_2$, 8.0 ± 1.1.

Infusion of PGF$_{2\alpha}$ alone produced an immediate, rapid and sustained fall in progesterone secretion, while infusion of PGE$_2$ alone had no significant
Figure 16. Mean progesterone secretion rate during and after infusion of saline and prostaglandins (PG) in ewes with ovarian autotransplants. The results are expressed as a percentage of the values obtained during the control period (see Methods section). Numbers in parentheses refer to the number of animals in each group.

* P < 0.05: significantly different from the corresponding points of the control animals. Closed square, saline; open triangle, PGF$_2$- (10 μg/h); closed circle; PGE$_2$ (10 μg/h); closed triangle, PGF$_2$ + PGE$_2$ (10 μg/h of each).
effect. When \( \text{PGF}_2 \alpha \) was infused together with \( \text{PGE}_2 \), the secretion of progesterone remained unaltered until the infusion period had finished, but declined rapidly thereafter. By 18 hours there was no significant difference in the rate of progesterone secretion resulting from either \( \text{PGF}_2 \alpha \) or \( \text{PGF}_2 \alpha + \text{PGE}_2 \) infusion (\( P > 0.05 \)).

Ovarian venous blood flow was significantly increased by \( 13.5 \pm 1.8\% \) (\( P < 0.05 \)) during infusion of \( \text{PGE}_2 \) alone, but fell to control levels at the end of infusion. Infusion of \( \text{PGF}_2 \alpha \) or \( \text{PGF}_2 \alpha + \text{PGE}_2 \) did not alter blood flow (\( P > 0.05 \)).

Effect of prostaglandin infusions on oestrous behaviour.

A higher proportion of animals showed oestrous behaviour following infusion of \( \text{PGF}_2 \alpha \) alone (four out of seven) than of those animals infused with \( \text{PGF}_2 \alpha + \text{PGE}_2 \) (one out of six). None of the animals infused with \( \text{PGE}_2 \) alone (7) or saline only (6) was marked by the ram.

Discussion.

These results demonstrate that \( \text{PGE}_2 \) when infused simultaneously with \( \text{PGF}_2 \alpha \) can antagonize the luteolytic action of \( \text{PGF}_2 \alpha \) and are consistent with the concept that \( \text{PGF}_2 \alpha \) initiates luteolysis through a direct action on the luteal cell to inhibit specifically LH-activated adenylyl cyclase (Hichens et al, 1974; Henderson & McNatty, 1975; Behrman & Hichens, 1976; Grinwich et al, 1976; Lahav et al, 1976). However, in view of the observed increase in blood flow during infusion of \( \text{PGE}_2 \), it could also be argued that the vasodilator properties of this prostaglandin counteract a vasoconstrictive action of \( \text{PGF}_2 \alpha \) on the corpus luteum (Thorburn & Hales, 1972; Niswender et al, 1973; Bruce & Moor, 1975). However, Bruce & Hillier (1974) and Einer-Jensen & McCracken (1976) have demonstrated that \( \text{PGF}_2 \alpha \) induced inhibition of progesterone secretion can occur
without depression of corpus luteal blood flow, and there is little evidence that anoxic damage causes a reduction in progesterone secretion in the early stages of PGF$_2\alpha$ induced luteal regression (Umo, 1975; Stacy et al, 1976). Furthermore, effects on corpus luteal blood flow would not explain why, in those animals infused with PGF$_2\alpha$ + PGE$_2$, progesterone levels declined after the infusion was terminated.

Inhibition of LH-activated adenylate cyclase by PGF$_2\alpha$ infusion would result in rapid depletion of the pool of LH-induced c-AMP through the action of phosphodiesterase; thereby causing a reduction in steroid synthesis, and depressing progesterone secretion. The continuing decline in progesterone secretion following termination of PGF$_2\alpha$ infusion probably results from the sustained action of PGF$_2\alpha$ continuing to prevent interaction of LH with its receptor (Behrman & Hichens, 1976; Grinwich et al, 1976). When PGE$_2$ is infused together with PGF$_2\alpha$, this action of PGF$_2\alpha$ is masked due to the ability of PGE$_2$ to stimulate the adenylate cyclase enzyme system through its own separate receptor and coupling units. Hence, as long as the intracellular production of c-AMP is maintained, the secretion of progesterone remains unaffected. On termination of the infusion, this compensatory action of PGE$_2$ is lost since, in the absence of PGE$_2$, the pool of PGE$_2$ - induced c-AMP will also be rapidly depleted by phosphodiesterase, so resulting in the observed fall in the rate of progesterone secretion.

In contrast to the findings utilising human granulosa cells in tissue culture, infusion of PGE$_2$ alone had no stimulatory effect on the secretion of progesterone, relative to the controls. However, it is likely that the corpus luteum on Day 10 is already secreting maximally, since infusion of massive amounts of LH on Day 10 of the cycle fails to stimulate an increased secretion of progesterone (Land, Collett & Baird, 1974).
The studies of Baird & Scaramuzzi (1975) and Gemmel et al (1976) indicate that luteal regression in the ewe is a two stage process consisting of functional regression followed by morphological regression (see literature review). The results of this study not only support the concept that PGF$_{2\alpha}$ initiates functional regression by specifically inhibiting LH-activation of adenylate cyclase, but may also provide a clue to the mechanism involved in morphological regression. Not only does simultaneous infusion of PGE$_2$ override the action of PGF$_{2\alpha}$ for the duration of the infusion, but a much lower proportion of animals infused with PGF$_{2\alpha}$ + PGE$_2$ go on to show oestrous behaviour compared to those infused with PGF$_{2\alpha}$ alone. Thus, PGE$_2$ also confers on the corpus luteum an increased chance of recovering from the lytic action of PGF$_{2\alpha}$. How PGE$_2$ does this remains speculative, though it is conceivable that if PGF$_{2\alpha}$ inhibits adenylate cyclase, the falling levels of c-AMP may trigger off further biochemical processes necessary for the morphological deterioration of the corpus luteum, such as the release of lysosomal enzymes (Kaley & Weiner, 1975; Weiner & Kaley, 1975). By simultaneously infusing PGE$_2$ and so maintaining elevated levels of c-AMP for a much longer period, these processes may not be adequately triggered.

In summary: these findings support the concept that PGF$_{2\alpha}$ may initiate functional regression of the corpus luteum by inhibiting specifically LH activation of adenylate cyclase, a process which can be overridden by the simultaneous administration of PGE$_2$. The ultimate morphological regression of the corpus luteum may also arise through further cellular processes being triggered by this initial biochemical action of PGF$_{2\alpha}$. 
CHAPTER 6.

INFLUENCE OF 16-ARYLOXYPROSTAGLANDINS ON THE PRODUCTION OF PROGESTERONE BY HUMAN GRANULOSA CELLS IN VITRO.
The ability of \( \text{PGF}_{2\alpha} \) to inhibit progesterone production by luteinized human granulosa cells \textit{in vitro} suggests that \( \text{PGF}_{2\alpha} \) has the potential of being luteolytic in women. Consequently, the failure to demonstrate any luteolytic activity in women with \( \text{PGF}_{2\alpha} \) (see literature review) may be due to difficulty in delivering adequate amounts to the ovary via the arterial supply, since \( \text{PGF}_{2\alpha} \) is rapidly inactivated by metabolism on passage through the lungs and liver (Piper et al., 1970; Granström, 1972). However, even if \( \text{PGF}_{2\alpha} \) is ultimately shown to be luteolytic in women, its use as a contraceptive by "menstrual induction" would be limited by unacceptable side effects such as diarrhea, vomiting and nausea which arise from its concomitant action on smooth muscle.

Some 16-aryloxy analogues of \( \text{PGF}_{2\alpha} \), namely ICI 79,939, ICI 80,996 and ICI 81,008 (see Fig. 17 for structures) have been shown in some species of laboratory animals, to be very much more potent luteolysins than \( \text{PGF}_{2\alpha} \) without having a correspondingly increased activity on smooth muscle (Binder, Bowler, Brown, Crossley, Hutton, Senior, Slater, Wilkinson & Wright, 1974). Consequently, low doses will induce luteolysis in laboratory and domestic animals without side-effects (Dukes et al., 1974).

It is not known, however, whether the increased potency of these compounds is due to the fact that they are metabolized more slowly than the parent substance or whether they are intrinsically more luteolytic. As a preliminary to initiating clinical trials in women, the luteolytic properties of the three 16-aryloxy derivatives were tested using human granulosa luteal cells in tissue culture.

Table 6 shows the effect of the three 16-aryloxy prostaglandins on the total production of progesterone by human granulosa cells cultured \textit{in vitro}. All three analogues markedly inhibited the basal secretion of progesterone. This effect, like that obtained with \( \text{PGF}_{2\alpha} \), would
ICI 79,939 \( R_1=F; R_2=H \) (racemic)
ICI 80,996 \( R_1=H; R_2=Cl \) (racemic)
ICI 81,008 \( R_1=H; R_2=CF_3 \) (racemic)

Figure 17. Structures of the 3 test 16-aryloxy prostaglandins.
Table 6. The effect of 16-aryloxy prostaglandins on the total production of progesterone by human granulosa cells cultured for 8 days in vitro.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Progesterone (μg/10^6 cells; mean + S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.2 ± 2.3 (3)</td>
</tr>
<tr>
<td>ICI 79,939 (50 ng/ml)</td>
<td>1.6 ± 0.5 (3)</td>
</tr>
<tr>
<td>Control</td>
<td>3.7 ± 0.8 (4)</td>
</tr>
<tr>
<td>ICI 80,996 (50 ng/ml)</td>
<td>0.4 ± 0.1 (4)</td>
</tr>
<tr>
<td>Control</td>
<td>4.4 ± 0.14 (3)</td>
</tr>
<tr>
<td>ICI 81,008 (50 ng/ml)</td>
<td>1.1 ± 0.07 (4)</td>
</tr>
</tbody>
</table>

The 16-aryloxy prostaglandins were added daily from the start of the culture period.

Numbers in parentheses refer to the number of replicate cultures. Prostaglandin treated cultures produced significantly less progesterone than control cultures (P < 0.01, Student's t-test)
appear to be a direct biochemical inhibition of steroidogenesis since the analogues did not affect the number of cells remaining at the end of the culture period, relative to the control cultures, nor was there any observable morphological difference between the two groups on examination by light microscopy.

The limited number of human granulosa cells available at any one time makes it difficult to study the effects on steroidogenesis of differing amounts of analogue. However, large numbers of viable porcine granulosa cells could be obtained from the follicles of pigs slaughtered at a nearby abattoir. Utilizing these cells, it was found that as little as 50 pg/ml of analogue was equally as effective as 50 ng/ml in inhibiting progesterone production (Table 7).

Figure 13 shows the effect of ICI 80,996 on progesterone production by human granulosa cells simultaneously treated with either gonadotrophin or PGE₂. Although the analogue effectively inhibited progesterone production stimulated by gonadotrophins (P<0.01 paired t-test), it had no effect on steroidogenesis stimulated by PGE₂, as was also observed previously with PGE₂(α) (Table 5). Similar results were obtained with the other analogues suggesting that, like PGE₂(α), these compounds may act by specifically inhibiting adenylate cyclase activation by LH.

These results demonstrate that analogous with PGE₂(α), the three 16-aryloxy prostaglandins tested inhibit both basal and gonadotrophin stimulated progesterone production by human granulosa cells in tissue culture, thus displaying in vitro characteristics expected of potential human luteolyins.

The possible pharmacological significance of these findings is strengthened by the ability of the 16-aryloxy analogues to inhibit steroidogenesis by granulosa cells obtained from the pig, a species in which these drugs are luteolytic (Ash & Heap, 1973; Guthrie & Polge, 1976a,b). Although there
Table 7. The effect of 16-aryloxyprostaglandins on the total production of progesterone by porcine granulosa cells cultured for 8 days in vitro (ng progesterone/10^6 cells; mean ± S.E.M.).

<table>
<thead>
<tr>
<th></th>
<th>ICI 80,996</th>
<th>ICI 79,939</th>
<th>ICI 81,008</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>(6)</td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>50 pg/ml</td>
<td>157 ± 42</td>
<td>133 ± 27</td>
<td>92 ± 10</td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>1340 ± 280</td>
<td>120 ± 35</td>
<td>85 ± 12</td>
</tr>
<tr>
<td>50 pg/ml</td>
<td>108 ± 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The 16-aryloxyprostaglandins were added daily from the start of the culture period.

Numbers in parentheses refer to the number of replicate cultures.

Prostaglandin treated cultures produced significantly less progesterone than the control cultures (P<0.01, Student's t-test)
Figure 13. The effect of daily addition of ICI 80,996 on progesterone production by human granulosa cells simultaneously treated with either gonadotrophins (A) or with PGE₂ (B).

Numbers in parentheses represent the numbers of replicate cultures. Gonadotrophin treated cultures were exposed daily to 50 ng ovine LH (NIH-LH-S12) and 50 ng ovine FSH (NIH-FSH-S4). Vertical lines represent ± S.E.M.
are differences in the control of function of the corpus luteum between pigs and women, it is likely that the biochemistry of the progesterone-secreting cells is similar. Therefore, the ability of 16-aryloxy prostaglandins to inhibit progesterone production by human granulosa cells in vitro is perhaps a good indication of their potential in vivo, providing that sufficient analogue can reach and interact with the receptor for PGF$_{2\alpha}$ located on the corpus luteum (Powell et al, 1974). Since 16-aryloxy prostaglandins are very much more potent luteolysins than PGF$_{2\alpha}$ without being correspondingly more toxic, it may be possible to deliver to the corpus luteum an amount of analogue sufficient to initiate luteolysis, but without producing the undesirable side-effects associated with infusion of PGF$_{2\alpha}$.

Recently, preliminary studies have shown that either intra-uterine administration (Csapo & Mocsary, 1976a), vaginal gel application (Csapo & Mocsary, 1976b) or vaginal infusion (Mocsary, 1977) ofICI 81,008 will effectively induce menstruation in women during early pregnancy. However, like the abortificant action of PGF$_{2\alpha}$ and PGE$_2$, this is due to a direct effect on uterine muscle, and not the result of a luteolytic action. In view of the above in vitro findings, detailed clinical studies to assess the potential of these 16-aryloxy prostaglandins to act as "menstrual inducers" by interacting directly with the corpus luteum to inhibit progesterone production would seem worthwhile.
CHAPTER 7.

A POSSIBLE INTERRELATIONSHIP BETWEEN
GONADOTROPHIN STIMULATION AND PROSTAGLANDIN F\(_{2\alpha}\)
INHIBITION OF STEROIDGENESIS BY GRANULOSA-LUTEAL
CELLS IN VITRO
Despite the luteolytic characteristics displayed by the 16-aryloxy prostaglandins in vitro (chapter 6), intramuscular injection of ICI 80,996 or ICI 81,008 failed to induce either premature menstruation in non-pregnant women, or abortion in women in the first 4 - 5 weeks of pregnancy (Wrixon & Baird, 1976 - personal communication). In these studies it was unlikely that this failure was due to insufficient amounts of the prostaglandin analogues reaching the ovary, since while up to 250 µg of analogue was administered to the women without success, a single intramuscular injection of 50 µg ICI 80,996 is quite sufficient to induce luteolysis in sheep (Baird & Scaramuzzi, 1975). It would seem, rather, that the human corpus luteum is unresponsive to the analogues. This is somewhat reminiscent of the situation found in many non-primates during the early luteal phase. While PGF$_2\alpha$ can induce luteal regression in laboratory and domestic animals (Weeks, 1972, Inskeep, 1973), the newly formed corpus luteum of several species shows considerable resistance to PGF$_2\alpha$. In the horse (Allen & Rowson, 1973), sheep (Kearnshaw, Restall & Gleeson, 1973) and cow (Rowson, Tervit & Brand, 1972; Henricks, Long, Hill & Dickey, 1974) the corpus luteum is refractory to PGF$_2\alpha$ for the first 4 days after its formation, while in the pig, this refractory period is 12 to 14 days (Koeljono, Bazer & Thatcher, 1976). This unresponsiveness of the newly formed corpus luteum could arise because of a lack of receptors for PGF$_2\alpha$, or because PGF$_2\alpha$ fails in some way to interact with its receptors.

Granulosa cells harvested from Graafian follicles and induced to luteinize in tissue culture provide a convenient model of the newly formed corpus luteum. The tissue culture of porcine, bovine and human granulosa-luteal cells was therefore used in an attempt to gain some insight into why the newly formed corpus luteum should be refractory to the lytic action of PGF$_2\alpha$. 

Effect of Pgf\textsubscript{2\alpha} on progesterone production by porcine granulosa-luteal cells.

Porcine granulosa-luteal cells when cultured in Medium containing 20% calf-serum secreted approximately 0.2 pg progesterone/cell/day. Steroidogenesis could be stimulated by the daily addition of ovine LH and ovine FSH, each to a final concentration of 100 ng/ml; progesterone production then increasing to a maximum of 2.5 pg/cell/day within 8 days of culture.

The effect of various concentrations of Pgf\textsubscript{2\alpha}, added daily from the start of the culture period, on the total production of progesterone by porcine granulosa-luteal cells cultured with or without added gonadotrophins is shown in Table 8. As little as 50 pg/ml Pgf\textsubscript{2\alpha} significantly inhibited progesterone secretion in the presence or absence of added gonadotrophins. Furthermore, from Fig. 19, it can be seen that when the addition of Pgf\textsubscript{2\alpha} to porcine cells treated with LH and FSH was delayed until day 6, 50 pg Pgf\textsubscript{2\alpha} still inhibited steroidogenesis.

Effect of Pgf\textsubscript{2\alpha} on progesterone production by bovine granulosa-luteal cells.

The daily production of progesterone by bovine granulosa-luteal cells cultured in Medium containing 20% calf-serum was very much greater than that of the porcine cells, progesterone production gradually increasing from approximately 1-3 pg/cell/day to a maximum of 6-7 pg/cell/day within 7 days of culture. This was probably due to the bovine granulosa-luteal cells being capable of responding maximally to the endogenous gonadotrophins present in the culture medium (50 ng FSH/ml; 4 ng LH/ml and 9 ng prolactin/ml, as assayed by specific radioimmunoassays for ovine gonadotrophins) which originate from the
Table 8. The effect of PGF$_2\alpha$ on the total production of progesterone by porcine granulosa-luteal cells cultured for 9 days.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Total production of progesterone during 9 days of culture (pg/cell; mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.9 ± 0.5 (4)</td>
</tr>
<tr>
<td>50 pg PGF$_2\alpha$</td>
<td>0.7 ± 0.2 (4)  P&lt;0.02</td>
</tr>
<tr>
<td>500 pg PGF$_2\alpha$</td>
<td>0.8 ± 0.1 (4)  P&lt;0.02</td>
</tr>
<tr>
<td>50 ng PGF$_2\alpha$</td>
<td>0.6 ± 0.1 (4)  P&lt;0.02</td>
</tr>
<tr>
<td>500 ng PGF$_2\alpha$</td>
<td>0.9 ± 0.3 (4)  P&lt;0.02</td>
</tr>
<tr>
<td>LH + FSH</td>
<td>10 ± 1.5 (4)</td>
</tr>
<tr>
<td>LH + FSH + 50 pg PGF$_2\alpha$</td>
<td>2.2 ± 0.9 (3)  P&lt;0.01</td>
</tr>
<tr>
<td>LH + FSH + 500 pg PGF$_2\alpha$</td>
<td>3.7 ± 1.7 (3)  P&lt;0.02</td>
</tr>
<tr>
<td>LH + FSH + 10 ng PGF$_2\alpha$</td>
<td>5.7 ± 2.0 (3)  P&lt;0.05</td>
</tr>
<tr>
<td>LH + FSH + 100 ng PGF$_2\alpha$</td>
<td>4.0 ± 0.6 (3)  P&lt;0.05</td>
</tr>
</tbody>
</table>

PGF$_2\alpha$ was added daily from the start of the culture period.

Gonadotrophin treated cultures were exposed to 100 ng LH (NIH-LH-S12) + 100 ng FSH (NIH-FSH-S4).

Numbers in parentheses represent the number of replicate cultures.

Levels of significance were calculated using Student's t-test.
Figure 19. Effect of PGF\textsubscript{2\alpha} on the daily production of progesterone by porcine granulosa-luteal cells stimulated daily for 6 days with ovine gonadotrophins (100 ng NIH-LH-S12 + 100 ng NIH-FSH-S4) before addition of PGF\textsubscript{2\alpha}. Numbers in parentheses represent the number of replicate cultures and vertical lines represent + S.E.M. All PGF\textsubscript{2\alpha} treated cultures produced significantly less progesterone from day 8 onwards than those treated with gonadotrophins alone (P<0.02, paired, t-test). Crosses, LH + FSH; open circle, PGF\textsubscript{2\alpha} added together with LH + FSH from day 6.

PROGESTERONE (pg/cell/day)

DAYS IN CULTURE
added calf-serum. This is supported by the finding that unlike the porcine cells, progesterone production by the bovine cells could not be further increased by the daily addition of ovine LH and ovine FSH, each to a final concentration of 100 ng/ml.

Table 9 and Fig. 20 show that the daily addition of PGF$_{2\alpha}$ from the start of the culture period at concentrations of $\geq$ 500 pg/ml significantly inhibited progesterone production by bovine granulosa-luteal cells. However, although PGF$_{2\alpha}$ could inhibit steroidogenesis if added from the start of the culture period, when progesterone production was relatively low, it was not possible to inhibit progesterone production with any amount of PGF$_{2\alpha}$ (up to 5 $\mu$g/ml tested) if the cells were cultured for 6-8 days, so that they were secreting maximally (6-7 pg progesterone/cell/day) before the addition of PGF$_{2\alpha}$. While being refractory to PGF$_{2\alpha}$, progesterone production by bovine cells secreting maximally could, however, be inhibited with a "classical" type of inhibitor of steroidogenesis namely anapalon, an inhibitor of $3\beta$-hydroxysteroid dehydrogenase obtained from ICI Pharmaceuticals Ltd. (Fig. 21). This unresponsiveness to PGF$_{2\alpha}$ of the maximally secreting bovine cells is similar to an observation made using human granulosa-luteal cells. While 50 ng/ml PGF$_{2\alpha}$ had been found to be sufficient to inhibit progesterone production when added from the start of the culture period (Fig. 12, table 3), a higher dose was required if the cells were cultured for several days and were producing increased amounts of progesterone prior to the addition of PGF$_{2\alpha}$ (Fig. 22).

**Uptake of $^{3}\text{H}-\text{PGF}_{2\alpha}$**

Taking the above porcine, bovine and human findings together, it would seem that while those cells producing only low amounts of progesterone (<2 pg/cell/day) are readily susceptible to the lytic action of PGF$_{2\alpha}$, as the 'steroidogenic potential' of the cells increase, they become
Table 9. The effect of PGF2α on the total production of progesterone by bovine granulosa-luteal cells cultured for 9 days.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Total production of progesterone during 9 days of culture (pg/cell; mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.2 ± 2.6 (3)</td>
</tr>
<tr>
<td>50 pg PGF2α</td>
<td>21.6 ± 6.8 (3) P&gt;0.05</td>
</tr>
<tr>
<td>500 pg PGF2α</td>
<td>15.8 ± 5.0 (3) P&gt;0.05</td>
</tr>
<tr>
<td>50 ng PGF2α</td>
<td>10.4 ± 1.3 (3) P&lt;0.01</td>
</tr>
<tr>
<td>500 ng PGF2α</td>
<td>14.6 ± 7.1 (3) P&lt;0.05</td>
</tr>
</tbody>
</table>

PGF2α was added daily from the start of the culture period.

Numbers in parentheses represent the number of replicate cultures.

Levels of significance were calculated using Student's t-test.
Figure 20. Effect of PGF$_{2\alpha}$ (50 ng/ml) on the daily production of progesterone by bovine granulosa-luteal cells cultured for 10 days. PGF$_{2\alpha}$ was added daily from the start of the culture period.

Numbers in parentheses represent the number of replicate cultures.

Vertical lines represent ± S.E.M. PGF$_{2\alpha}$ treated cultures produced significantly less progesterone than control cultures ($P<0.02$, paired t-test).
Figure 21. Effect of PGF\textsubscript{2\alpha} and anapalon, added daily from day 6, on progesterone production by bovine granulosa-luteal cells. Numbers in parentheses represent the number of replicate cultures. Progesterone production by the 8 control and 6 PGF\textsubscript{2\alpha} treated cultures were not significantly different from each other and the results have been grouped together. Vertical lines represent ± S.E.M. Anapalon treatment significantly inhibited progesterone production ($P \leq 0.01$, paired t-test).
Figure 22. Effect of delaying the addition of PGF$_{2\alpha}$ on the daily production of progesterone by human granulosa-luteal cells in vitro. Values are mean daily production of progesterone by cells exposed to either 50 or 1000 ng PGF$_{2\alpha}$/ml daily from day 6. Numbers in parentheses refer to number of experiments. Cross, control; solid circle, 50 ng/ml PGF$_{2\alpha}$; open circle 1000 ng/ml PGF$_{2\alpha}$.

(from McNatty, Henderson & Sawers, 1974).
increasingly refractory to \( \text{PGF}_{2\alpha} \). To determine whether the ability of \( \text{PGF}_{2\alpha} \) to bind to granulosa-luteal cells might be inversely related to their steroidogenic potential, uptake studies using \( ^3\text{H-PGF}_{2\alpha} \) were carried out on bovine and human granulosa-luteal cells producing varying amounts of progesterone (\(<0.5-7\) pg/cell/day). Uptake studies were not carried out on porcine granulosa-luteal cells since in this culture system, even in the presence of added gonadotrophins, they produced relatively low amounts of progesterone (\(<0.2 - 2.5\) pg/cell/day) and consequently as seen in Table 8 and Fig. 19 steroidogenesis could always be inhibited by low amounts of \( \text{PGF}_{2\alpha} \).

The relationship between \( \text{PGF}_{2\alpha} \) uptake and steroidogenesis is shown in Fig. 23. As progesterone production increased, so the ability of the cells to retain \( ^3\text{H-PGF}_{2\alpha} \) decreased.

**Discussion.**

The effects of \( \text{PGF}_{2\alpha} \) on progesterone production by porcine and bovine granulosa-luteal cells in tissue culture, as observed in this study, appear quite contrary to the luteolytic effect of \( \text{PGF}_{2\alpha} \) on the porcine and bovine corpus luteum in vivo. The pig corpus luteum in vivo is refractory to \( \text{PGF}_{2\alpha} \) for 12-14 days after its formation (Moeljono et al., 1976) and yet very low concentrations of \( \text{PGF}_{2\alpha} \) (50 pg/ml) readily inhibited both basal and gonadotrophin stimulated progesterone production by porcine granulosa-luteal cells in vitro; \( \text{PGF}_{2\alpha} \) being equally as effective when added from the start, or from day 6 of the culture period. In contrast, the bovine corpus luteum in vivo is more susceptible to the lytic action of \( \text{PGF}_{2\alpha} \) than the pig corpus luteum, it being refractory to \( \text{PGF}_{2\alpha} \) for only 4 days after its formation (Rowson et al., 1972; Henricks et al., 1974). However, \( \text{PGF}_{2\alpha} \) was much less effective in inhibiting progesterone production by bovine granulosa-luteal cells in tissue culture than porcine cells, it requiring a higher minimum concentration
Figure 23. Uptake of $^3$H-PGF$_{2\alpha}$ by human and bovine granulosa-luteal cells of differing steroidogenic potential. The number of uptake studies is given in parentheses. The vertical lines represent + S.E.M.
of PGF₂α (500 pg/ml), and PGF₂α being effective only when added from the start of the culture period. This difference in behaviour between the porcine and bovine granulosa-luteal cells is probably due to their different steroidogenic capacities in this particular in vitro system, as reflected in the different amounts of progesterone the porcine and bovine granulosa-luteal cells were each capable of producing. The porcine granulosa-luteal cells in tissue culture, even when exposed to exogenous ovine gonadotrophins (100 ng each of LH and FSH per day), produced considerably less progesterone than the bovine granulosa-luteal cells which responded maximally to the endogenous gonadotrophic activity of the culture medium. This lack of response by the porcine granulosa-luteal cells to either the endogenous bovine gonadotrophins in the culture medium, or to the exogenous gonadotrophins may be due to the gonadotrophin receptors on the porcine granulosa-luteal cells being specific for porcine gonadotrophins. Possibly, therefore, to achieve a progesterone production by the porcine granulosa-luteal cells comparable to the maximum amounts of progesterone produced by the bovine cells (6-7 pg/cell/day) it may be necessary to culture the porcine granulosa-luteal cells in the presence of porcine LH and FSH. Perhaps then, if capable of producing 6-7 pg progesterone/cell/day, the porcine granulosa-luteal cells would also, like the bovine cells, be refractory in vitro to PGF₂α. Unfortunately, porcine gonadotrophins were not available throughout the course of this study, consequently this possibility could not be tested.

However, taking together the results of the above studies utilizing porcine and bovine granulosa-luteal cells in tissue culture, it would seem that while, in vivo, the age of the corpus luteum is crucial in determining its
response to PGF\textsubscript{2α} in vitro the most critical factor in determining the response of the granulosa-luteal cells to PGF\textsubscript{2α} is the steroidogenic potential of the cells. Those cells producing only low amounts of progesterone (<\sim 2 \text{ pg/cell/day}) are readily susceptible to the lytic action of PGF\textsubscript{2α}, but as the cellular capacity to produce progesterone rises, the cells become increasingly refractory to PGF\textsubscript{2α}. The results obtained with human granulosa-luteal cells are also consistent with this concept. An explanation for this is provided by the results of the uptake study where it was found that the ability of \textsuperscript{3}H-PGF\textsubscript{2α} to bind to granulosa-luteal cells was inversely related to their steroidogenic potential. Thus, as the steroidogenic potential of the cell rises, a decreased ability to bind PGF\textsubscript{2α} renders it refractory to the lytic action of PGF\textsubscript{2α}.

Why should those granulosa-luteal cells producing maximum amounts of progesterone be refractory to PGF\textsubscript{2α}? The daily production of progesterone by granulosa-luteal cells in vitro is related to the cellular response to gonadotrophin present in the culture medium (Channing & Ledwitz-Rigby, 1974; McNatty & Sawers, 1975). In the absence of gonadotrophin, granulosa cells secrete minimal amounts of progesterone (McNatty, Bennie, Hunter & McNeilly, 1975), whilst the addition of gonadotrophin stimulates production of progesterone; FSH acting to stimulate LH binding to the granulosa cells (Zeleznik et al., 1974; Channing, 1975; Nimrod et al., 1977), while LH, on binding to its specific plasma membrane receptors (Gospodarowicz, 1973; Han et al., 1974), stimulates progesterone production by activating adenylate cyclase (Marsh, 1970). Those granulosa-luteal cells secreting maximum amounts of progesterone will therefore have more LH bound to the plasma membrane than those cells producing minimal amounts of progesterone. Since cells producing high levels of progesterone retain only low amounts of PGF\textsubscript{2α}, it is possible that receptor-bound LH antagonizes the uptake of PGF\textsubscript{2α}. Interaction between the LH and PGF\textsubscript{2α}.
receptors is conceptually possible since both receptor types are located in the plasma membrane (Gospodarowicz, 1973; Han et al, 1974; Powell et al, 1974, 1975). Moreover, this interpretation of an antagonistic action of receptor bound LH on PGF$_{2\alpha}$ uptake is consistent with in vivo observations. The luteolytic action of exogenous PGF$_{2\alpha}$ in rats can be prevented by administration of LH (Behrman, Yoshinaga & Greep, 1971; Fuchs, Nok & Sundaram, 1974) or hCG (Chatterjee, 1976).

Similarly, in the ewe intravenous infusion of LH prolongs the lifespan of the corpus luteum (Karsch, 1970; Karsch, Roche, Noveroske, Foster, Norton & Malbandov, 1971), while in the cow the same effect is achieved with single injections of LH (Donaldson & Hansel, 1965); the ewe and cow both being species in which PGF$_{2\alpha}$ is thought to be the natural uterine derived luteolysin.

While it is quite possible that receptor bound LH antagonizes the luteolytic action of PGF$_{2\alpha}$ by preventing PGF$_{2\alpha}$ binding to the PGF$_{2\alpha}$ receptors, several studies show that the reverse situation also occurs, in that PGF$_{2\alpha}$ antagonizes LH binding to the LH receptors (Hichens et al, 1974; Grinwich et al, 1976; Behrman & Hichens, 1976). This suggests the existence of a "see-saw" interrelationship between the receptors for LH and PGF$_{2\alpha}$. The filling of the LH receptors may prevent any interaction of PGF$_{2\alpha}$ with the PGF$_{2\alpha}$ receptors, while conversely, occupancy of the PGF$_{2\alpha}$ receptors may inhibit any interaction of LH with the LH receptors. This would offer an intrinsic mechanism to account for the resistance of the newly formed corpus luteum to the lytic action of PGF$_{2\alpha}$. Following the ovulatory "surge" of gonadotrophins, the receptors for LH on the newly formed corpus luteum would be saturated with LH, thus causing the receptors for PGF$_{2\alpha}$ to be effectively masked. Consequently, the newly formed corpus luteum would be refractory to PGF$_{2\alpha}$. The levels of LH found in peripheral plasma during the luteal phase are very low, relative to the levels occurring at the time of the
preovulatory surge. A concentration gradient will therefore exist between LH bound to the corpus luteum at ovulation (high LH concentration) and luteal phase plasma levels of LH (low LH concentration). This concentration gradient will encourage the gradual dissociation of LH from its receptors on the corpus luteum, the dissociation continuing throughout the luteal phase until equilibrium is reached between the amount of LH bound to the corpus luteum and the levels occurring in plasma. Progesterone production by the corpus luteum will be unaffected by this dissociation of LH since only a fraction of the LH-receptor sites need be occupied to ensure maximum stimulation of adenylate cyclase (Koch, Zor, Chobsieng, Lamprecht, Pomerantz & Lindner, 1974). Thus, the amount of LH remaining bound to the corpus luteum, once equilibrium has been established with the low LH levels in plasma, should still be sufficient to maintain maximum progesterone production by the corpus luteum. However, gradual vacation of the LH receptors would also result in the gradual unmasking of the receptors for PGF$_{2\alpha}$, so causing the corpus luteum to become increasingly susceptible to the lytic action of PGF$_{2\alpha}$.

On the basis of this "see-saw" type of interaction between LH and PGF$_{2\alpha}$, the different refractory periods to PGF$_{2\alpha}$ between the corpus luteum of the cow or sheep (4 days) and pig (12-14 days) would be due, principally, to differences in the rates of dissociation of LH from its luteal cell receptors; LH being bound more strongly to the pig corpus luteum than to that of the sheep or cow. Available evidence supports this concept. LH would appear to be relatively weakly bound to the sheep corpus luteum since hypophysectomy of cyclic or pregnant sheep causes immediate and complete degeneration of the corpus luteum (Kaltenbach, Graber, Niswender & Nalbandov, 1968a, b). Luteal regression following hypophysectomy can,
however, be prevented by continuous infusion of LH (Kaltenbach et al., 1968b). In contrast, while LH is luteotrophic in pigs (Anderson & Melampy, 1967) the studies with hypophysectomized pigs by du Meanil, du Buisson & Leglise (1963) show that porcine corpora lutea can function independent of pituitary support for 12-14 days following the ovulatory LH surge, indicating that LH is tightly bound and dissociates only very slowly from porcine luteal cell receptors. This higher affinity of LH for the pig corpus luteum is also apparent from studies using antiserum to LH. While administration of LH antiserum causes premature luteal regression in cycling cows (Snook, Brunner, Saatman & Hansel, 1969) and sheep (Fuller & Hansel, 1970) it does not do so when administered to cycling pigs, though it does cause luteolysis in pregnant pigs (Spies, Slyter & Quadri, 1967). These findings, taken together, indicate that LH does appear to be bound more tightly to the LH receptors on the pig corpus luteum than to those on the cow or sheep corpus luteum. Thus it is quite possible that the different refractory periods shown to PGF\textsubscript{2α} by the bovine or ovine (4 days) and porcine (12-14 days) corpus luteum may be due to differences in the rates of dissociation of LH from its receptors on the corpus luteum; receptor bound LH acting to prevent PGF\textsubscript{2α} interacting with the PGF\textsubscript{2α} receptor.

The inability of the 16-aryloxy analogues of PGF\textsubscript{2α} to induce premature menstruation in women could therefore be due to the human corpus luteum being similar to the pig corpus luteum, in that the receptors for PGF\textsubscript{2α} only become fully unmasked close to the time the corpus luteum normally regresses. Similarly, the failure of the 16-aryloxy prostaglandins to cause abortion in women in early pregnancy (first 4-5 weeks) could be due to the hCG secreted by the implanting blastocyst occupying vacant LH receptors on the corpus luteum and so ensuring that the receptors for PGF\textsubscript{2α} remain fully masked.
CHAPTER 8.

STUDIES WITH DISPERSED BOVINE LUTEAL CELLS.
While the tissue culture of luteinized granulosa cells provide a simple, convenient model of the corpus luteum, it would be more satisfactory if it were possible to carry out tissue culture studies using actual luteal cells. The limited amount of human luteal tissue available at any one time made it impractical to develop a method for obtaining dispersed human luteal cells. Large numbers of bovine corpora lutea could, however, be obtained from a local abattoir. These were therefore used to investigate the feasibility of obtaining and using dispersed luteal cells in tissue culture as an in vitro model of the corpus luteum.

**Morphological appearance of dispersed bovine-luteal cells.**

Dispersed bovine luteal cells were obtained by collagenase disaggregation as described in the Methods section, and summarised in Fig. 9. The histological appearance of the isolated cells is shown in plates 2 and 3. At least 2 different cell types are present; i.e. large spherical cells having a granular, highly eosinophilic cytoplasm, small prominent nuclei and diameters in the range 10-30μ (examples marked L in plate 3) and small epithelial type cells with no distinguishing morphological characteristics and <5μ in diameter (examples marked S in plate 3).

**Long term cultures.**

Long term cultures were set up using the dispersed bovine luteal cells without separating out the different cell types present. Aliquots of the dispersed cells corresponding to a total of 150 X 10^3 'live' cells were cultured for 8 days exactly as described for the tissue culture of granulosa cells, the culture medium being replaced daily and stored at -20°C until assayed for progesterone. The appearance of the cells at the end of the 8 days is shown in Plate 4. The cells appear morphologically healthy, and in fact very similar in appearance to the luteinized human
Plate 3. Haematoxylin/eosin stained smear of dispersed bovine luteal cells. (Mag. X 250)

L - large luteal cells 10-30μ in diameter.

S - small luteal cells <5μ in diameter.
Plate 4. Histology of haematoxylin/eosin stained bovine luteal cells after 8 days of culture. (Mag. X 300).
granulosa cells (plate 1b). However, in contrast to the studies with luteinized granulosa cells in tissue culture, progesterone production by the bovine luteal cells steadily declined throughout the culture period (Fig. 24). This fall in progesterone production did not correlate with changes in cell number since, while cell numbers dropped by 2/3 over the first 48 hours, they then started to rise again (Fig. 24). However, when the cell types present throughout the 8 day culture period were examined by light microscopy, it appeared that the large luteal cells did not survive but died, as indicated by the presence of large luteal cells with broken plasma membranes, within the first 48-72 hours of culture. Most of the small luteal cells, however, seemed to undergo hyperplasia and hypertrophy, and this would account for the rise in cell numbers seen after the first 48 hours. If the large luteal cells are the principal source of progesterone synthesis, which would be consistent with their morphological appearance, then the decline in progesterone production would be compatible with their death. Although the small luteal cells undergo hyperplasia and hypertrophy, they possibly have only a limited capacity to produce progesterone, and so cannot compensate for the death of the large luteal cells.

Why should the large luteal cells fail to survive in tissue culture? Histological examination of the sections of luteal tissue taken prior to disaggregation show that the death of the large cells is not due to the corpora lutea already undergoing morphological regression. It is also unlikely to be due to lack of gonadotrophic support for the cells since the culture medium contains considerable amounts of endogenous gonadotrophins (50 ng FSH/ml, 4 ng LH/ml and 9 ng prolactin/ml - as assayed by specific radioimmunoassays for ovine gonadotrophins) originating from the added calf-serum. Moreover, the daily addition of NIH ovine gonadotrophins (50 ng each of LH and FSH) had no effect on progesterone production, relative to control cultures. It might be argued that because no attempt
PROGESTERONE PRODUCTION BY ISOLATED BOVINE LUTEAL CELLS IN TISSUE CULTURE

Figure 24. Changes in cell numbers and the progesterone content of culture medium during the long term tissue culture of bovine luteal cells. Numbers in parentheses represent the number of replicate studies. Vertical lines represent + S.D.
was made to separate out the large and small luteal cells, hyperplasia and hypertrophy of the small cells in effect "suffocated" the non-dividing large cells. However, Lemon & Loir (1977), on separating out the large and small luteal cells obtained on disaggregation of porcine corpora lutea, could not maintain progesterone production by the large cells, cultured on their own, for longer than 8-10 hours. More likely the large bovine luteal cells die because, being well differentiated, they are merely less capable of surviving in tissue culture than the much less specialized, more adaptable small luteal cells. It may be possible to maintain the large bovine luteal cells for a longer period if they could be cultured in conditions more analogous to those in tissues (Knazek, Gullino, Kohler & Dedrick, 1972).

The maximum production of progesterone by luteinized bovine granulosa cells in tissue culture (6-7 pg/cell/day), is less than that of isolated bovine luteal cells. Thus, although the bovine granulosa cells do luteinize in tissue culture and secrete increased amounts of progesterone, their lower progesterone production, compared to the isolated luteal cells, indicate that they do not luteinize fully in vitro. Luteinized bovine granulosa cells are perhaps intermediate between the small luteal cells which survive in tissue culture but do not secrete much progesterone, and the large luteal cells which do not survive in tissue culture but secrete elevated amounts of progesterone.

**Short-term incubation studies.**

Although progesterone production by the isolated bovine luteal cells declined steadily over 8 days of culture, progesterone production over the first 24 hours was considerable. It was therefore decided to use the isolated bovine luteal cells in short-term incubation studies. Fig.25 shows the effect of HCG, PGF, and dibutyryl c-AMP (DBC) with and without PGF on progesterone production by isolated bovine luteal cells during 4
Figure 25. Effect of various hormones on progesterone production by isolated bovine luteal cells during 4 hour incubation periods. Numbers in parentheses represent number of replicate determinations. Vertical lines represent + S.E.M.
hour incubation periods. Progesterone production was stimulated 2-4 fold by HCG, PGE\textsubscript{2} and DBC. However, only HCG stimulated progesterone production was inhibited by PGF\textsubscript{2}\(\alpha\) (P<0.02, Student's t-test), PGF\textsubscript{2}\(\alpha\) failing to inhibit either PGE\textsubscript{2} or DBC stimulated progesterone production (P>0.05, Student's t-test). These results therefore support the concept that PGF\textsubscript{2}\(\alpha\) inhibits progesterone production by specifically blocking LH activation of adenylate cyclase. Moreover these results are entirely compatible with those obtained previously using luteinized granulosa cells in tissue culture, and those obtained on infusing sheep corpora lutea in vivo.

While their failure to sustain progesterone production limits the usefulness of dispersed bovine luteal cells in long term in vitro studies of the corpus luteum, they are highly satisfactory for use in short-term incubation studies. There are several advantages in using dispersed cells rather than tissue slices in short-term incubation studies: a) Considerably less tissue is required for studies utilizing dispersed cells. b) Standardized conditions are more easily obtained using dispersed cells, so reducing experimental variability. c) Dispersed cell systems usually require less hormone to produce a response. This not only allows economy in the use of rare substances, but often allows studies to be carried out with physiological, rather than pharmacological amounts of hormone. d) Dispersed cell systems allow added hormone to interact with all the cells. In studies with tissue slices, added hormone often cannot penetrate into the inner layer(s) of cells, therefore these cells may behave quite differently from the cells exposed to hormone, so confusing the results. Furthermore, lack of nutrient may cause abnormal behaviour by the inner layer(s) of cells in tissue slices.

The relative ease with which dispersed bovine luteal cells could be obtained by collagenase disaggregation suggests this technique may prove useful in obtaining dispersed human luteal cells. Indeed, Stouffer et al. (1976) have recently obtained viable luteal cells of the Rhesus monkey suitable for short-
term incubation studies using collagenase disaggregation. However, while dispersed luteal cells may be useful for studies involving short-term incubations, their failure to sustain progesterone production makes them less satisfactory for long-term in vitro studies of the corpus luteum. Luteinized granulosa cells in tissue culture are probably the most suitable system, at present, for long-term in vitro studies of the progesterone secreting cells of the corpus luteum. Although granulosa cells perhaps do not fully luteinize in vitro their progesterone production can be sustained for several days, so mimicking in vitro the behaviour of the corpus luteum in vivo. Furthermore, our studies with prostaglandins showing that results obtained with luteinized granulosa cells in tissue culture can also be obtained both in in vivo studies of the corpus luteum, and with dispersed luteal cells, justifies the use of luteinized granulosa cells in tissue culture as an in vitro model of the corpus luteum.
CHAPTER 9.

ANDROGEN AROMATIZATION BY LUTEINIZED

BOVINE GRANULOSA CELLS IN TISSUE CULTURE.
From studies involving short-term incubations with $^{14}$C-labelled radioactive precursors, Savard & Telegdy (1965) concluded that the bovine corpus luteum could not synthesize oestrogens because it lacked the 17-hydroxylase, 17, 20-lyase and 19-hydroxylase aromatase enzyme systems necessary for androgen synthesis and aromatization. By using a sensitive, specific radioimmunoassay for testosterone, Shemesh, Hansel & Concannon (1975) observed that the bovine corpus luteum did have the enzymatic capacity to synthesize small amounts of testosterone. However, these authors did not re-examine the capacity of the bovine corpus luteum to aromatize androgens to oestrogen. Bovine granulosa cells obtained from Graafian follicles and induced to luteinize in tissue culture provide a convenient system in which to study the bovine corpus luteum which is composed mainly of granulosa-lutein cells (Gier & Marion, 1961). This system was therefore used to study aromatization of androgens to oestrogen, and the relative capacity of the cells to synthesize progesterone, androgens and oestrogens.

**Androgen Aromatization.**

Figure 26 shows the total amount of oestradiol produced during 6 days of culture in response to exogenous testosterone and androstenedione (1-500 ng). The results have been corrected for any cross-reaction the androgens had with the oestradiol antiserum. Cross-reaction was determined using control cultures without cells but containing added amounts of testosterone or androstenedione (1-500 ng). A dose response was obtained for testosterone with maximum oestradiol production being achieved with a concentration of >100 ng/ml. Maximum response to androstenedione was obtained with a concentration of >10 ng/ml, but the maximum amount of oestradiol produced in response to androstenedione was significantly less ($P<0.01$, Student's t-test) than that produced in response to >100 ng/ml testosterone.

Significantly more oestradiol was produced in response to testosterone at
Figure 26. Total production of oestradiol by luteinized bovine granulosa cells during 6 days of culture. Androstenedione and testosterone were added daily from the start of the culture period. Each point is the mean of 4 replicate cultures. Vertical lines represent ± S.D. Means with the same superscript are not significantly different from each other. (P > 0.05, Student's t-test).
Solid bars, testosterone; hatched bars, androstenedione.
concentrations \( \leq 500 \rightarrow 50 \) ng/ml than to androstenedione over the same concentration range (P<0.025 Student's t - test). Androstenedione at concentrations \( \leq 10 \) ng/ml caused significantly more oestradiol production than the addition of \( \leq 10 \) ng/ml testosterone (P<0.01 Student's t - test). The oestradiol content of the control culture medium was not significantly different from the assay blank value of \( \leq 10 \) pg/ml. None of the control or androgen treated cultures produced any detectable amounts of oestrone; the limit of sensitivity being 5 pg.

**Production of progesterone, oestrogens and androgens.**

**Progesterone and oestrogens.**

Figure 27 (a & b) shows the daily production of progesterone and oestradiol by cultures treated daily with testosterone (100 ng/ml) or androstenedione (100 ng/ml). Standard deviations have been omitted from the progesterone results for clarity, but there was no significant differences in progesterone production between the control and androgen treated cultures (P>0.05, Student's t - test). Oestradiol production by the androgen treated culture was 200 - 400 fold less than their progesterone production. As in Fig. 26 testosterone treatment produced significantly more oestradiol than androstenedione treatment (P<0.01, paired t - test). Also, no significant amounts of oestradiol were produced by the control cultures, and none of the cultures produced any detectable oestrone.

**Androgens.**

Figure 27(c) shows the daily production of androstenedione by testosterone (100 ng/ml) treated cultures, and daily production of testosterone by androstenedione (100 ng/ml) treated cultures. Whereas androstenedione production steadily increased, testosterone production steadily declined throughout the culture period. The control cultures produced small, but significant amounts of both androstenedione and testosterone in the range 20 - 50 pg/10^5 cells/day.
Figure 27. Daily production of progesterone, oestradiol and androgens by luteinized bovine granulosa cells in tissue culture. Each point is the mean of 8 replicate cultures. Vertical lines represent ± S.D. Androstenedione (100 ng/ml) or testosterone (100 ng/ml) were added daily to the cultures from the start of the culture period. open triangle, control; closed triangle, androstenedione treated; closed square, testosterone treated.
Discussion.

These findings demonstrate that luteinized bovine granulosa cells contain an active 19-hydroxylase aromatase enzyme system which catalyses the conversion of both androstenedione and testosterone to oestradiol. Why there should be different dose-responses to androstenedione and testosterone is unclear. One possible explanation might be that the rates of penetration into the cell, a concentration dependent phenomenon, of androstenedione and testosterone may be quite different; the more polar testosterone perhaps penetrating the plasmalemma less easily than androstenedione. The different dose-responses would then be due to differences in the availability of substrate at the site of conversion. Bovine plasma contains high affinity binding proteins for testosterone, but none for androstenedione (Martin, Fouchet & Thibier, 1976). Thus, it might also be possible that testosterone penetration into the cell could be aggravated by the presence of high affinity binding proteins for testosterone in the culture medium, originating from the added calf-serum. However, when substrate concentrations were not limiting and maximum amounts of oestradiol could be produced, more oestradiol was produced in response to testosterone than to androstenedione. This is consistent with the findings of Oakey & Stitch (1967) using slices of rat ovary in short-term incubations. This finding, together with the fact that none of the cultures produced any measurable amounts of oestrone, would suggest that the most favourable metabolic route to oestradiol was via testosterone rather than oestrone.

The capacity of the luteinized bovine granulosa cells to synthesize progesterone was approximately 200 - fold greater than their capacity to aromatize androgens to oestradiol. The rate-limiting steps in the conversion of cholesterol to progesterone and testosterone to oestradiol are the hydroxylations at C-20 and C-19 respectively. Hydroxylation of
cholesterol at C-20 is catalysed by a mitochondrial mixed function oxidase, while hydroxylation of testosterone at C-19 is catalysed by a microsomal mixed function oxidase. Both these reactions require the participation of cytochrome P-450 (Savard, 1973). The mitochondrial cytochrome P-450 content of the bovine corpus luteum greatly exceeds its microsomal cytochrome P-450 content (Yohro & Horie, 1967; McIntosh, Uzgiris, Alonso & Salhanick, 1971). It is likely, therefore, that a similar situation could also occur in the luteinized bovine granulosa cells; thereby accounting for the different amounts of progesterone and oestradiol the cells are capable of producing.

The small amounts of androstenedione and testosterone (20 - 50 pg/10^5 cells/day) produced by the control cultures supports the finding of Shemesh et al., (1975) that the bovine corpus luteum is not entirely devoid of 17-hydroxylase and 17, 20-lyase enzymes. The rise in androstenedione production and fall in testosterone production by the androgen treated cultures throughout the culture period, Fig. 27(c) is possibly due to changes in the relative amounts of oxidised and reduced pyridine nucleotides. The interconversion of androstenedione and testosterone is a freely reversible reaction catalysed by a 17-keto-reductase which required NADPH as its co-factor. The biosynthesis of progesterone from cholesterol is NADPH dependent. Thus, as the cellular capacity to produce progesterone rises, the ratio of NADPH to NADP will fall, this in turn shifting the androstenedione-testosterone equilibrium in favour of androstenedione formation.

Several studies of follicular development indicate that maximum oestradiol production by the follicle requires a co-operative interaction between the follicular theca and granulosa cells (Falck, 1959; Ryan, Petro & Kaiser, 1968; Makris & Ryan, 1975; Channing, Wentz & Jones, 1976; Baird, 1977; Moor, 1977). As the follicle matures increasing amounts of androgen, together with small amounts of oestrogen, are secreted by the theca cells
which have a relatively low capacity to aromatize androgens to oestrogens. The granulosa cells, although lacking the enzymatic capacity to synthesize androgens do, however, under the influence of FSH, have the enzymatic capacity to aromatize androgens to oestrogens (Dorrington, Moon & Armstrong, 1975). Thus, androgens derived from the theca cells, on passing through the basement membrane, may be aromatized to oestradiol by the maturing granulosa cells.

The results of this present study show that luteinized, progesterone secreting bovine granulosa cells, although having an active 19-hydroxylase aromatase enzyme system, fail to synthesize oestradiol because of a very limited capacity to synthesize androgens. Thus, oestradiol production by the corpus luteum may, similar to the follicle, involve a co-operative interaction between theca-lutein and granulosa-lutein cells. In those species such as the human (Hammerstein, Rice & Savard, 1974) and pig (Watson & Leask, 1975) whose corpora lutea do synthesize oestradiol, granulosa-lutein and theca-lutein cells are easily distinguishable in the mature corpus luteum (Corner, 1919; Gureya, 1971). The theca-lutein might, therefore, provide androgen precursor for aromatization to oestradiol by the granulosa-lutein cells. In those species such as the cow and ewe, whose corpora lutea do not synthesize oestradiol (Savard, 1973), there are relatively few distinguishable theca-lutein cells present in their respective corpora lutea which are composed predominately of luteinized granulosa cells (Harrison, 1945; Grier & Marion, 1961). Thus, the failure of these corpora lutea to synthesize oestradiol may be because although the granulosa-lutein cells contain an active 19-hydroxylase aromatase enzyme system it receives an inadequate supply of androgen; the granulosa-lutein cells themselves having only a very limited capacity to synthesize androgens, and there being few androgen secreting theca-lutein cells present in these corpora lutea.
CHAPTER 10.

GENERAL DISCUSSION.
Since these studies commenced in 1974 there have been considerable advances in our understanding of the luteolytic action of PGF\textsubscript{2\alpha} in the non-primate. From an initial belief that PGF\textsubscript{2\alpha} might cause luteolysis by virtue of its vasoconstrictive properties, there is now accumulating evidence that PGF\textsubscript{2\alpha} may initiate luteolysis through a direct biochemical action on the luteal cell to inhibit specifically LH-activated adenylate cyclase; a vascular effect of PGF\textsubscript{2\alpha} possibly only occurring during the final stages of luteal regression (see literature review). The physiological relevance of the luteolytic action of PGF\textsubscript{2\alpha} is also now firmly established. Evidence that PGF\textsubscript{2\alpha} is the natural luteolysin in sheep and guinea-pigs is almost overwhelming (see Horton & Poyser, 1976; Baird, 1977) and it seems likely that PGF\textsubscript{2\alpha} will prove to be the natural luteolysin in many of the other non-primates in which it has a demonstrable luteolytic action. However, the involvement of PGF\textsubscript{2\alpha} in primate luteal regression still remains relatively obscure. Although PGF\textsubscript{2\alpha} has been shown to be luteolytic in the Rhesus monkey (Kirton et al, 1972; Russell, 1975) it has not yet been possible to induce luteolysis in women with either PGF\textsubscript{2\alpha} or analogues of PGF\textsubscript{2\alpha} (see literature review). However, the presence of a receptor for PGF\textsubscript{2\alpha} in the human corpus luteum (Powell et al, 1974) together with the fact that the human corpus luteum both contains and can synthesize PGF\textsubscript{2\alpha} suggests that PGF\textsubscript{2\alpha} could regulate luteal function in women (Challis et al, 1976; Shutt et al, 1976; Swanston et al, 1977). This is supported by the findings presented in this thesis that PGF\textsubscript{2\alpha} displays luteolytic properties when added to luteinized human granulosa cells in tissue culture. The results of this study also offers a possible mechanism of how PGF\textsubscript{2\alpha} may be involved in normal luteal regression in women and why the human corpus luteum should be so refractory to exogenously administered PGF\textsubscript{2\alpha}. 
Figure 28 shows the changes in progesterone, oestradiol and PGF$_{2\alpha}$ content of the human corpus luteum throughout the luteal phase (data taken from Swanston et al., 1977). The progesterone content of the corpus luteum declines progressively from its maximum value in the early luteal phase to its minimum value in the late luteal phase, and in corpora albicantia. Oestradiol content rises to a maximum in the mid-luteal phase and then progressively declines. The highest concentrations of PGF$_{2\alpha}$ occur in the postovulatory corpus luteum and in corpora albicantia, with low, relatively constant levels throughout the rest of the luteal phase. Studies in several species, including the human (see literature review) have demonstrated that the ovulatory process is associated with elevated follicular levels of PGF. Thus, the high levels of PGF$_{2\alpha}$ in the corpus luteum immediately following ovulation may be a "carry over" from the ovulatory process. The low, relatively constant levels of PGF$_{2\alpha}$ occurring throughout the remainder of the luteal phase might, however, be unexpected and appear contrary to a luteolytic role for PGF$_{2\alpha}$. If PGF$_{2\alpha}$ is involved in normal luteal regression in women, one might have expected a rise in PGF$_{2\alpha}$ levels accompanying the decline in progesterone content. However, these results are entirely compatible with the concept of a "see-saw" interrelationship occurring between LH and PGF$_{2\alpha}$, as proposed in Chapter 7.

Following the ovulatory "surge" of LH the receptors for LH on the corpus luteum will be saturated with LH. This will not only stimulate production of the elevated levels of progesterone found in the early luteal phase corpus luteum, but will also, as discussed in chapter 7, effectively mask any receptors for PGF$_{2\alpha}$. Thus, even although high levels of PGF$_{2\alpha}$ do occur in the newly formed corpus luteum they will not affect progesterone production, since the PGF$_{2\alpha}$ will be unable to interact with its receptors which are
Figure 28. Concentration of progesterone, oestradiol - 17β and prostaglandin $P_{2\alpha}$ (means ± S.E.M.) in human corpora lutea at various stages of the menstrual cycle.

RO, recent ovulation; EL, early luteal;
ML, mid-luteal; LL, late luteal; CA, Corpus albicans.

(data taken from Swanston et al, 1977)
masked by the bound LH. It is likely that the LH receptors on the human luteal cell have a relatively weak affinity for LH since in addition to the ovulatory 'surge' of LH, the human corpus luteum requires a small but constant supply of LH to continue producing progesterone throughout the whole luteal phase (Vande Wiele, Bogumil, Dyrenfurth, Ferin, Jewelewicz, Warren, Razkallah & Mikhail, 1970). The relatively low concentrations of LH in plasma during the luteal phase will, therefore, encourage the gradual dissociation of LH from its receptors. Not all the LH receptor sites will, however, be vacated since the low plasma LH levels will ensure that equilibrium between plasma and luteal LH levels will eventually be reached. Since only a fraction of the LH receptor sites need be occupied to ensure maximum stimulation of adenylate cyclase (Koch et al, 1974) the vacation of the LH receptors should not, in itself, cause a decline in progesterone levels. However, according to the "see-saw" concept, vacation of the LH receptors will cause unmasking of the receptors for PGF₂α. This will then allow any PGF₂α present in the corpus luteum to interact with these unmasked receptors and bring about the observed fall in progesterone levels by inhibiting activation of adenylate cyclase by those LH receptors still coupled to adenylate cyclase, as described previously. The studies with granulosa cells in tissue culture demonstrated that very low levels of PGF₂α were sufficient to inhibit progesterone production. Thus, the small amounts of PGF₂α continuously present in the human corpus luteum from the early to late luteal phase could be sufficient to interact with the receptors for PGF₂α as they are gradually unmasked and inhibit progesterone production. The critical factor therefore may not be the amount of PGF₂α present, but the availability of receptors for PGF₂α. This would explain why the human corpus luteum is so resistant to exogenously administered PGF₂α or the 16-aryloxy analogues of PGF₂α. Since the
receptors for PGF$_{2\alpha}$ might only be unmasked gradually, the endogenous PGF$_{2\alpha}$ content of the corpus luteum could be enough to occupy fully the receptors as they become available. Thus, even if large amounts of exogenously administered PGF$_{2\alpha}$ did reach the corpus luteum, there would be few receptors for PGF$_{2\alpha}$ available for it to occupy, consequently it would have little or no effect. It is possibly only very close to menstruation that there may be excess receptors available for occupancy by exogenous PGF$_{2\alpha}$. However, by that time it will be difficult to demonstrate clearly menstrual induction resulting from PGF$_{2\alpha}$ administration, it being so close to the normal expected time of menstruation.

The studies in the ewe by Baird & Scaramuzzi (1975) demonstrate that although the small amounts of PGF$_{2\alpha}$ (<5 ng/ml) released from the uterus on Days 12-15 are sufficient to cause functional luteal regression, they fail to cause structural regression, since the life-span of the corpus luteum can be prolonged by hysterectomy as late as Day 15 (Noor, Hay, Short & Rowson, 1970). Structural regression of the sheep corpus luteum is probably dependent on the elevated amounts of PGF$_{2\alpha}$ (>20 ng/ml) released from the uterus on Days 15-16 (Baird & Scaramuzzi, 1975). The human corpus luteum may be regulated in a similar fashion.

Although the small amounts of PGF$_{2\alpha}$ present in the human corpus luteum from the early to late luteal phase (Fig. 28) may be sufficient to cause the steady fall in progesterone production, they might be insufficient to cause the final structural deterioration of the corpus luteum. Indeed there is little morphological degeneration of the human corpus luteum until shortly before the onset of menstruation (Corner, 1956). Structural deterioration of the human corpus luteum may, as in the sheep, require elevated levels of PGF$_{2\alpha}$. The continuous presence of oestradiol in combination with falling levels of progesterone is required to cause the elevated amounts of PGF$_{2\alpha}$ that are released from the sheep uterus on
Days 15-16 (see literature review). However, although progesterone levels steadily decline, and oestradiol is continually present in the human corpus luteum (Fig. 28), Swanston et al., (1977) failed to observe any significant increase in PGF\(_{2\alpha}\) content of the human corpus luteum during the late luteal phase. This, however, is contrary to the findings of Shutt et al., (1976) who did find a rise in PGF\(_{2\alpha}\) levels during the late luteal phase. This discrepancy between the data of Swanston et al., (1977) and Shutt et al., (1976) may be due to subjective differences in their classification of corpora lutea as late luteal or as newly formed corpora albicantia. However, taking the data of Swanston et al., (1977) and Shutt et al., (1976) together, it does appear that the period of morphological degeneration of the corpus luteum is associated with elevated levels of PGF\(_{2\alpha}\). Thus, although the physiological control of luteal regression in sheep (a typical non-primate) and women is somewhat different, the actual mechanism of luteal regression in non-primates and primates may in fact be quite similar. In both PGF\(_{2\alpha}\) may be the natural luteolysin and in both luteolysis may be a two stage process consisting of functional regression followed subsequently by morphological regression. Functional regression in each may be initiated in response to small amounts of PGF\(_{2\alpha}\) producing a biochemical inhibition of progesterone production by inhibiting LH-activation of adenylate cyclase. The final morphological deterioration of the corpus luteum may require elevated amounts of PGF\(_{2\alpha}\) which are synthesised in response to oestradiol and to the declining levels of progesterone caused by functional regression.

The studies of Shutt et al., (1976) and Swanston et al., (1977) do not include any data on PGE\(_2\) content of the human corpus luteum throughout the menstrual cycle. Although Challis et al., (1976) did measure PGE\(_2\) levels in human corpora lutea, the number of patients was too few to determine whether there were any significant changes in PGE\(_2\) levels at various stages of the cycle, though these authors found the levels of PGE\(_2\) generally exceeded
those of PGF$_{2\alpha}$. This lack of data on PGE$_2$ is perhaps unfortunate in view of the findings of our studies that PGE$_2$ can override the luteolytic action of PGF$_{2\alpha}$. Thus, one might anticipate a change in the ratio of PGF$_{2\alpha}$ to PGE$_2$ during the luteal phase to favour PGF$_{2\alpha}$ formation as menstruation approaches. The rise in the oestradiol content of the human corpus luteum from the early to mid luteal phase would be consistent with this view since studies in the uterus show that oestradiol can modify prostaglandin production to favour PGF$_{2\alpha}$ formation at the expense of PGE$_2$ formation (see literature review). Furthermore the decline in progesterone production may also favour PGF$_{2\alpha}$ formation by an increased availability of reduced co-factors. Since LH activation of adenylate cyclase can stimulate prostaglandin synthesis by increasing the availability of the fatty acid precursors necessary for prostaglandin biosynthesis (Kuehl, 1974), PGF$_{2\alpha}$ induced inhibition of LH-activated adenylate cyclase would cause a reduction in prostaglandin synthesis. However, although total prostaglandin synthesis might be reduced, PGF$_{2\alpha}$ levels could remain constant or be elevated by a modification of the prostaglandin synthetase activity to favour PGF$_{2\alpha}$ production while reducing PGE$_2$ formation. This would not only ensure sufficient PGF$_{2\alpha}$ was available to continue luteolysis but would remove the "luteotrophic" effect of PGE$_2$. Thus, it would clearly be interesting to measure changes of both PGF$_{2\alpha}$ and PGE$_2$ levels in the human corpus luteum throughout the luteal phase. Furthermore, collagenase disaggregation of human corpora lutea obtained at various stages of the cycle would allow one to examine how prostaglandin production by dispersed cells might be modified by various hormonal treatments.

The concept of a "see-saw" interrelationship between LH and PGF$_{2\alpha}$ not only provides a mechanism for normal luteal regression in women, but also provides an explanation of how the corpus luteum is maintained in the pregnant cycle. During pregnancy the increase in serum progesterone occurring 8-10 days post-ovulation (Fig. 3) is closely associated with the appearance of human chorionic gonadotrophin (hCG) in the serum (Vaitukaitis et al., 1971). The luteal cell
LH-receptors can not only bind hCG, but have a very much higher affinity for hCG than they have for LH (Beals & Midgley, 1969; Koch et al, 1974). By the "see-saw" concept, therefore, the function of hCG would be to ensure that the LH receptors on the corpus luteum remain fully occupied by gonadotrophin, so ensuring that the receptors for PGF$_{2\alpha}$ remain masked. Thus any PGF$_{2\alpha}$ present in the corpus luteum will be unable to interact with its receptors and so fail to cause luteolysis. Consequently, by this mechanism, it is the view of this author that while PGF$_{2\alpha}$ may be the natural luteolysin in women, it is unlikely that PGF$_{2\alpha}$ or any long acting derivative of PGF$_{2\alpha}$ will ever be useful as abortificients in women by inducing luteal regression in early pregnancy. By the time a pregnancy is suspected owing to a missed menstruation, the blastocyst will already be secreting hCG, which will not only protect the corpus luteum from any endogenous PGF$_{2\alpha}$, but hCG induced masking of PGF$_{2\alpha}$ receptors will also serve to protect the corpus luteum from exogenously administered PGF$_{2\alpha}$. It is felt therefore that the future of prostaglandins as abortificients in early pregnancy lies, not in their luteolytic properties, but in their ability to influence uterine smooth muscle contractility. Current studies suggest that prostaglandins self-administered in vaginal suppositories may be effective abortificients in early pregnancy by virtue of this property (see literature review).

While there is strong evidence, supported by the findings of this study, that PGF$_{2\alpha}$ may initiate functional luteolysis by a biochemical action on the luteal cell to inhibit progesterone production by blocking specifically LH-activation of adenylate cyclase, the mechanism by which PGF$_{2\alpha}$ can induce the final, irreversible structural deterioration of the corpus luteum remains uncertain. There is evidence that structural regression could arise from a biochemical action of PGF$_{2\alpha}$ on the luteal cell causing lysosomal activation (Dott, 1973; Kaley & Weiner, 1975; Weiner & Kaley, 1975). However, in our studies it was found that although as little as 50 pg PGF$_{2\alpha}$ could inhibit progesterone production by granulosa-luteal cells, it was not possible to observe any
morphological damage to the cell, on examination by light microscopy, with up to 5 μg PGF$_{2α}$. Our studies would therefore suggest structural regression required the presence of a vascular system, thereby implicating the vasoconstrictive properties of PGF$_{2α}$ in structural regression. On this basis it could be argued that while small amounts of PGF$_{2α}$ may be sufficient to cause a biochemical inhibition of progesterone production, the vasoconstrictive action of PGF$_{2α}$ to be effective, may require the presence of much larger amounts of PGF$_{2α}$ such as those released from the sheep uterus at Days 15-16 just prior to the onset of structural regression. However, although structural regression is accompanied by a reduction in capillary blood flow to the corpus luteum, consistent with a vasoconstrictive action of PGF$_{2α}$ (see literature review for references), it is not known whether this reduction in blood flow is a cause or consequence of structural regression. Moreover, if structural regression of the corpus luteum arises from the rather non-specific vasoconstrictive property of PGF$_{2α}$ causing a reduction in blood flow to the corpus luteum, how can one account for the resistance of the newly formed corpus luteum to the lytic action of PGF$_{2α}$? This resistance of the newly formed corpus luteum would suggest that before PGF$_{2α}$ can initiate either functional or structural regression it must be able to interact with its receptors. This therefore favours the concept that PGF$_{2α}$ may cause structural regression through a biochemical action, possibly by causing lysosomal activation. Thus, it is conceivable that while a low amount of PGF$_{2α}$ may be sufficient to initiate functional regression it may provoke only partial activation of lysosomes. Full activation of the lysosomes may require the receptors for PGF$_{2α}$ to be fully occupied which would necessitate all the receptors for PGF$_{2α}$ to be unmasked and the presence of large amounts of PGF$_{2α}$. This, also, would explain why the major degenerative changes in the sheep corpus luteum do not occur before the release of large quantities of PGF$_{2α}$ from the uterus on Days 15-16. How PGF$_{2α}$ might cause lysosomal
activation is obscure. However, our finding, in the study with ewes bearing ovarian autotransplants, that simultaneous infusion with PGE₂ antagonized the ability of PGF₂α to cause structural regression (as indicated by the number of animals returning to oestrous) suggests that intracellular levels of c-AMP could be important in regulating lysosomal activity. The possibility that PGF₂α causes structural regression through a biochemical action, rather than by its vasoconstrictive properties can also be argued from a theoretical standpoint. In the sheep, for example, PGF₂α normally has to travel from the uterus to the ovary to exert its luteolytic effect. If PGF₂α were to cause structural regression by a vasoconstrictive action then any reduction in blood flow to the corpus luteum would also result in it becoming increasingly more difficult for PGF₂α to actually reach the corpus luteum from the uterus. This would be undesirable, and contrary to ensuring full and complete luteolysis. If PGF₂α does cause structural regression by a biochemical activation of lysosomes, why did we fail to observe any morphological deterioration of the granulosa-luteal cells on exposure to PGF₂α? This may perhaps be due to inadequacies in our in vitro system. While luteinized granulosa cells in tissue culture may be a suitable system in which to study progesterone production by the corpus luteum, it is perhaps a completely inadequate system for any morphological study of the corpus luteum due to the apparent failure of granulosa cells to luteinize fully in tissue culture (see Chapter 8).

While our finding that PGF₂α could inhibit progesterone production by granulosa cells in tissue culture is consistent with the in vitro findings of O'Grady et al., 1972; Demers et al., 1973; Grinwich, Ham, Hichens & Behrman, 1975 and Lahav et al., 1976, it is contrary to the findings of other groups of workers who found that PGF₂α stimulated progesterone production in vitro (Speroff & Ramwell, 1970; Behrman, Yoshinaga, Wyman & Creep, 1971; Santos, Hermier & Netter, 1973). Why PGF₂α should inhibit progesterone production in some in vitro studies but stimulate progesterone
production in others is difficult to explain. Perhaps the most likely explanation lies in the similarity in the structures of PGF$_{2\alpha}$ and PGE$_2$ (Fig. 1). Normally, when exposed to physiological concentrations of prostaglandins, the prostaglandin receptors on the luteal cell can discriminate between the prostaglandin types. However, under high, non-physiological concentrations of PGF$_{2\alpha}$ this discriminating power of the receptors may break down, and PGF$_{2\alpha}$ as well as occupying its own receptor may also bind and activate some of the PGE$_2$ receptors. Our findings showed that stimulation of adenylate cyclase by PGE$_2$ was not inhibited by PGF$_{2\alpha}$; PGF$_{2\alpha}$ only inhibiting LH activation of adenylate cyclase. Thus, stimulation of progesterone production by PGF$_{2\alpha}$ could be explained by activation of adenylate cyclase by PGE$_2$ receptors occupied by PGF$_{2\alpha}$ as a consequence of using high non-physiological concentrations of PGF$_{2\alpha}$.

Consequently any inhibition of progesterone production arising from occupancy of the PGF$_{2\alpha}$ receptors inhibiting LH-activation of adenylate cyclase would be overridden by occupancy of the PGE$_2$ receptors stimulating adenylate cyclase. Thus, dose-response studies for each particular in vitro system are essential when carrying out studies with PGF$_{2\alpha}$.

Furthermore, as discussed previously, tissue with a high LH content will probably be refractory to the lytic action of PGF$_{2\alpha}$ both in vitro and in vivo due to the LH masking any receptors for PGF$_{2\alpha}$. Consequently low doses of PGF$_{2\alpha}$ would fail to inhibit progesterone production in any in vitro study. In the absence of any inhibition, one might be inclined to increase the concentration of PGF$_{2\alpha}$. However, if the receptors for PGF$_{2\alpha}$ were masked this would have no effect, but if the concentration of PGF$_{2\alpha}$ was sufficiently high then PGF$_{2\alpha}$ might occupy some of the PGE$_2$ receptors and progesterone production may be stimulated. Thus, not only is the concentration of PGF$_{2\alpha}$ important, but the choice of tissue is also critical, it being important to use luteal tissue which is unlikely to be refractory to PGF$_{2\alpha}$.
Finally, prostaglandin involvement in reproductive physiology consists of basically two activities, (1) modulation of steroidogenesis by influencing adenylate cyclase activity and (2) alteration of smooth muscle contractility. Although both these activities are quite separate and distinct, it is theoretically possible that they could both originate from a single biochemical action. Changes in intracellular concentrations of c-AMP are frequently accompanied by parallel changes in intracellular calcium ion (Ca$^{2+}$) concentrations (see review by Rasmussen, Jensen, Lake, Friedman & Goodman, 1975). Consequently it has been suggested that the initial event in adenylate cyclase activation may in fact be Ca$^{2+}$ displacement from the plasma membrane and that phosphodiesterase activity might also be regulated by Ca$^{2+}$ ions (Rasmussen & Tenenhouse, 1968; Kretsinger, 1976). PGE$_4$ has been shown to release Ca$^{2+}$ from plasma membrane (Ramwell & Shaw, 1970) and PGE$_4$ can also activate adenylate cyclase (Marsh, 1971). Thus it is conceivable that PGE$_4$ on interacting with its plasma membrane receptor could stimulate adenylate cyclase through the release of Ca$^{2+}$; Ca$^{2+}$ in effect acting as the coupler between the hormone receptor and adenylate cyclase. In view of our limited understanding of the exact mechanism by which adenylate cyclase is activated, this possibility is entirely speculative, as is the possibility that prostaglandin induced inhibition of adenylate cyclase activity might also be mediated by Ca$^{2+}$. However, since smooth muscle contractility and relaxation is also dependent on intracellular changes in Ca$^{2+}$ concentration (Lehninger, 1972), Ca$^{2+}$ mediated modulation of adenylate cyclase activity would at least provide a common intermediate for the two main activities of prostaglandins in reproduction.


von Euler, U.S. (1936). On the specific vaso-dilating and plain
muscle stimulating substances from accessory genital glands in man and certain animals (prostaglandin and vesiglandin).


EFFECTS OF PROSTAGLANDIN F\textsubscript{2a} AND E\textsubscript{2} ON THE PRODUCTION OF PROGESTERONE BY HUMAN GRANULOSA CELLS IN TISSUE CULTURE

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SUMMARY

Human granulosa cells with differing steroidogenic potentials were cultured in vitro. The effects of prostaglandin F\textsubscript{2a} (PGF\textsubscript{2a}) and PGE\textsubscript{2} on the progesterone output and viability of these cells were investigated.

Prostaglandin F\textsubscript{2a} either alone or in combination with LH and FSH inhibited the production of progesterone over a wide range of concentrations (1-8000 ng/ml). However, the inhibitory effect of PGF\textsubscript{2a} was 200 times less effective when the cells were exposed to LH and FSH for 6 days before the addition of the prostaglandin. By contrast PGE\textsubscript{2}, at concentrations from 1 to 500 ng/ml, markedly stimulated the production of progesterone by granulosa cells, and this was not prevented by the addition of PGF\textsubscript{2a}. The degree of inhibition by PGF\textsubscript{2a} or stimulation by PGE\textsubscript{2} was related to the biosynthetic capacity of the cells.

These studies suggest that PGF\textsubscript{2a} may act directly on the adenylate cyclase system of human granulosa cells by blocking its activation by LH, and they demonstrate that functional regression of the luteal cell can be induced independently of the blood vascular system.

INTRODUCTION

The luteolytic action of exogenous prostaglandin F\textsubscript{2a} (PGF\textsubscript{2a}) has been demonstrated in the rat (Pharriss & Wyngarden, 1969), hamster (Gutknecht, Wyngarden & Pharriss, 1971), rabbit (Pharriss, 1970), cow (Rowson, Tervit & Brand, 1972), pig (C. Polge, personal communication), guinea-pig (Blatchley & Donovan, 1969) and ewe (McCracken, Glew & Scaramuzza, 1970) – in which its physiological significance as the luteolytic hormone is well established (McCracken, Carlson, Glew, Baird, Green & Samuelsson, 1972; Scaramuzza, Baird, Wheeler & Land, 1973; Goding, 1974). However, the mechanism(s) by which PGF\textsubscript{2a} induces functional and structural luteolysis is unknown; ovarian blood flow is not substantially altered during luteolysis (Baird, 1974; Bruce & Hillier, 1974; Jansen, Albrecht & Ahren, 1975), although a redistribution in capillary flow within the ovary may occur at this time (Jansen et al. 1975). The possibility that PGF\textsubscript{2a} may act directly by interfering with cholesterol ester synthetase activity (Behrman, MacDonald & Greep, 1971) or by decreasing the binding capacity of luteal tissue for human chorionic gonadotrophin (HCG) or luteinizing hormone (LH) (Hichens, Grinwich & Behrman, 1974) remains to be confirmed.

Attempts to induce luteal regression in women by short-term i.v. infusions of PGF\textsubscript{2a} have been unsuccessful, and there is only preliminary evidence in the monkey of a decline in
plasma levels of progesterone after similar prostaglandin treatment (Jewelewicz, Cantor, Dyrenfurth, Warren & Vande Wiele, 1972; Jones & Wentz, 1972; Lehmann, Peters, Breckwoldt & Bettendorf, 1972; LeMaire & Shapiro, 1972; Auletta, Speroff & Caldwell, 1973; Wentz & Jones, 1973). However, a specific PGF$_{2\alpha}$ receptor was recently demonstrated in the human corpus luteum (Powell, Hammarström, Samuelsson & Sjöberg, 1974), so that the infusion experiments may have failed because rapid peripheral metabolism allowed insufficient PGF$_{2\alpha}$ to reach the ovary (Piper, Vane & Wyllie, 1970; Granström, 1972) or because the human corpus luteum may be relatively refractory to the luteolytic effects of PGF$_{2\alpha}$ during much of its life.

Prostaglandin E$_{2}$, in contrast to PGF$_{2\alpha}$, mimics the actions of trophic hormones on both primate and non-primate ovarian tissue since it stimulates the production of progesterone by the human corpus luteum (Marsh & LeMaire, 1974), bovine corpus luteum (Speroff & Ramwell, 1970), rat follicle (Lindner, Tsafriri, Lieberman, Zor, Koch, Bauminger & Barnea, 1973), mouse follicle (Neal, Baker, McNatty & Searamuzzi, 1975), and monkey and prcine granulosa cells (Channing & Crisp, 1972; Kolena & Channing, 1972).

Human granulosa cells cultured in vitro provide a convenient biochemical model system for studying some of the possible effects of prostaglandins on the human corpus luteum (McNatty & Sawers, 1975). Granulosa cells harvested from large Graafian follicles in the mid- or late follicular phase of the menstrual cycle (i.e. ‘active follicles’) secrete maximum amounts of progesterone within 2–6 days in culture, probably because they have been exposed to high concentrations of follicle-stimulating hormone (FSH) and oestradiol in follicular fluid (McNatty, Hunter, McNeilly & Sawers, 1975; McNatty & Sawers, 1975). By contrast cells harvested from follicles containing little or no FSH and oestradiol (i.e. ‘inactive follicles’) do not attain their maximum biosynthetic capacity for at least 10 days in vitro (McNatty & Sawers, 1975). We have therefore used human granulosa cells of differing steroidogenic potentials to investigate the possible effects of PGE$_{2}$ and PGF$_{2\alpha}$ on the human corpus luteum.

**MATERIALS AND METHODS**

**Patients**

Ovaries were obtained at various stages of the menstrual cycle from 12 women (aged 34–42 years) who were undergoing hysterectomy for menorrhagia due to endometriosis (2) and dysmenorrhoea or chronic pelvic pain (10). The previous menstrual cycles of these women varied in length from 20 to 29 days and five had ovulated during the cycle under study. The remaining seven women were in the follicular phase as assessed from endometrial histology and date of the last menstrual period.

**Collection of follicular fluid and the culture of granulosa cells**

Thirty-five Graafian follicles of 4–15 mm diameter were dissected from the above ovaries. Follicular fluid was aspirated from each follicle through a 27 gauge needle into a 1 ml syringe and stored at $-20\,^\circ\text{C}$ until assayed for FSH, LH and oestradiol by radioimmunoassay (McNatty et al. 1975). The relationship between hormone concentrations of follicular fluid and steroidogenic potential of human granulosa cells has been described by McNatty & Sawers (1975).

Granulosa cells were scraped from the collapsed follicle into Medium 199 containing Hanks’ salts, HEPES buffer (20 mmol/l) with gentamicin (50 µg/ml), amphotericin-B (2-5 µg/ml) and 1% glutamine (Flow Laboratories, Irvine, Scotland).

The technique of culturing granulosa cells was identical to that previously described (McNatty & Sawers, 1975). The cells were grown for 10–12 days in 1 ml culture medium
containing 20% calf serum (v/v). The endogenous gonadotrophic activity of the culture medium was 1.7 mu. LH/ml and 1.8 mu. FSH/ml (McNatty & Sawers, 1975) (1 mu. LH = 11.6 ng LER 907 and 1 mu. FSH = 44.6 ng LER 907). The endogenous level of PGF<sub>2α</sub> in the culture medium was 36 pg/ml and PGE<sub>2</sub> < 200 pg/ml (as determined by radioimmunoassay and g.l.c.–mass spectrometry). The culture medium was replaced each day and stored at −20 °C until assayed for progesterone by radioimmunoassay (Neal et al. 1975). Triplicate cultures for each experiment were carried out whenever possible although the low viabilities (< 10%) of some cell preparations meant that a number of studies had to be made on individual cultures. The precision attained with replicate cultures in relation to the production of progesterone for all treatments was 7.6 ± 0.9% (S.E.M.) (n = 68) which was similar to that reported previously (McNatty & Sawers, 1975). The number of ‘live’ cells at the commencement of culture together with the number remaining after 10 days of culture were determined by the technique of McNatty & Sawers (1975).

**Prostaglandin F<sub>2α</sub> and E<sub>2</sub>**

Prostaglandin F<sub>2α</sub> and E<sub>2</sub> (Upjohn Company, Kalamazoo, U.S.A.) were each dissolved in 70% aqueous ethanol and stored at 4 °C in ampoules until added directly to the cell cultures in a volume of 5 or 10 µl. A similar volume of 70% aqueous ethanol without prostaglandin was added to the control cultures.

**Luteinizing hormone and follicle-stimulating hormone**

The gonadotrophins which were added to the cultures were: human LH (Stockell Hartree IRC-2, 24.6.69) containing 7550 u. LH/mg and < 25 u. FSH/mg; and human FSH (MRC 73/519) containing 2200 u. FSH/mg and 8.8 u. LH/mg. These immunological potencies were assessed using the following standards: LH, MRC 68/40 assumed 77 u./ampoule; FSH, MRC 68/39, 32.8 u./ampoule (MRC National Institute for Biological Standards and Control). The concentrations of LH and FSH are expressed as mu./ml. The gonadotrophins were added in culture medium at a concentration of 300 mu. LH or FSH/ml. The medium was stored at −20 °C in ampoules and 0.1 ml samples were added to the cell cultures.

**RESULTS**

**Effect of prostaglandins on viability and mitotic activity of granulosa cells in vitro**

When cells were exposed to PGF<sub>2α</sub> and PGE<sub>2</sub> alone or in combination there was no significant increase or decrease in cell numbers during 10 days of culture when compared with untreated control cultures with or without gonadotrophins except when PGE<sub>2</sub> was added to culture medium containing cells harvested from inactive follicles. In the latter experiments a twofold increase in cell numbers occurred (r = 1.91 ± 0.22 (S.E.M.; n = 4)) when compared with the control cultures (r = 1.08 ± 0.11, n = 4) (r = no. of cells after 10 days of culture divided by the number of live cells at the start of culture).

**Effect of differing doses of prostaglandin F<sub>2α</sub> on the total production of progesterone by granulosa cells in culture**

Table 1 shows the total production of progesterone in 10 days by granulosa cells exposed daily to PGF<sub>2α</sub> in culture medium at concentrations between 1 and 8000 ng/ml medium. At all dose levels PGF<sub>2α</sub> lowered the total production of progesterone by 60% or more when added to cells harvested from ‘active’ follicles. By contrast, a much smaller reduction in total progesterone output was observed by cells harvested from ‘inactive’ follicles. In both cases the higher the concentration of PGF<sub>2α</sub> the greater the inhibition in progesterone output.
Effect of prostaglandin $F_{2\alpha}$ and gonadotrophins on the daily production of progesterone by granulosa cells

The daily production of progesterone by granulosa cells harvested from 'active' follicles and exposed daily to PGF$_{2\alpha}$ with or without LH and FSH was markedly inhibited (60%) compared with the control cells (Fig. 1). Although LH + FSH induced a twofold increase in progesterone secretion, the addition of 50 ng PGF$_{2\alpha}$/ml medium markedly inhibited this stimulatory effect. In contrast (Fig. 2) the daily production of progesterone by granulosa cells harvested from 'inactive' follicles and treated daily with PGF$_{2\alpha}$ with or without LH and FSH was only slightly decreased (10%). Although LH + FSH induced a three- to sixfold

Table 1. Total production of progesterone by human granulosa cells after 10 days of culture in varying concentrations of prostaglandin $F_{2\alpha}$. (Values are means ± S.E.M. expressed as a percentage of controls)

<table>
<thead>
<tr>
<th>Source of granulosa cells</th>
<th>Prostaglandin $F_{2\alpha}$ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>'Inactive' follicles</td>
<td>93-5</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>'Active' follicles</td>
<td>39-3</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
</tr>
</tbody>
</table>

*Inactive* follicles contained < 1.3 mu. FSH/ml and < 250 ng oestradiol/ml follicular fluid. 'Active' follicles contained > 1.3 mu. FSH/ml and > 250 ng oestradiol/ml. The numbers in parentheses refer to the number of experiments on individual follicles.

Fig. 1. Mean daily production of progesterone by human granulosa cells in vitro and exposed to prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) (50 ng/ml) with or without added LH and FSH (both 30 mu./ml). Cells were harvested from follicles containing FSH and oestradiol (E$_2$): FSH, > 1.3 mu./ml; E$_2$, > 250 ng/ml. Numbers in parentheses refer to number of experiments. Cross, control; open triangle, LH + FSH; solid circle, PGF$_{2\alpha}$; solid triangle, LH + FSH + PGF$_{2\alpha}$.
Fig. 2. Mean daily production of progesterone by human granulosa cells in vitro and exposed to prostaglandin F₂α (PGF₂α) (50 ng/ml) with or without added LH and FSH (both 30 μg/ml). Cells were harvested from follicles containing low concentrations of FSH and oestradiol (E₂): FSH, < 1.3 μg/ml; E₂, < 250 ng/ml. Numbers in parentheses refer to number of experiments. Cross, control; open triangle, LH + FSH; solid circle, PGF₂α; solid triangle, LH + FSH + PGF₂α.

Fig. 3. Effect of delaying the addition of prostaglandin F₂α (PGF₂α) on the daily production of progesterone by human granulosa cells in vitro. Values are mean daily production of progesterone by cells exposed to either 50 or 1000 ng PGF₂α/ml daily from day 6. Cells were harvested from a follicle containing FSH and oestradiol. Numbers in parentheses refer to number of experiments. Cross, control; solid circle, 50 ng PGF₂α/ml; open circle, 1000 ng PGF₂α/ml.
increase in progesterone secretion the addition of 50 ng PGF$_{2\alpha}$/ml medium almost totally inhibited this stimulatory effect.

The effect of delaying the addition of PGF$_{2\alpha}$ is shown in Fig. 3. The daily addition of 50 ng PGF$_{2\alpha}$ from Day 6 had no effect on the production of progesterone. However, when 1000 ng PGF$_{2\alpha}$ were added daily from Day 6 there was a progressive decrease in progesterone output, so that by Day 12 it was only one-third of that achieved by the control cells.

![Graph](image_url)

**Fig. 4.** Mean daily production of progesterone by human granulosa cells *in vitro* exposed to prostaglandin E$_2$ (PGE$_2$) (50 ng/ml) with or without added LH and FSH (both 30 mu./ml). The cells were harvested from follicles containing FSH and oestradiol. Numbers in parentheses refer to number of experiments. Cross, control; open triangle, LH + FSH; open square, LH + FSH + PGE$_2$ (4) and PGE$_2$ only (12); solid square, PGE$_2$ + PGF$_{2\alpha}$ (50 ng/ml).

**Effect of prostaglandin E$_2$ and gonadotrophins on the daily production of progesterone by granulosa cells**

The daily production of progesterone by granulosa cells harvested from ‘active’ follicles and exposed daily to PGE$_2$ with or without LH + FSH is shown in Fig. 4. LH + FSH only stimulated a twofold increase in the production of progesterone, whilst the daily addition of 50 ng PGE$_2$/ml medium stimulated a three- to fourfold increase, so that the maximum secretion of about 5 pg/cell/day (McNatty & Sawers, 1975) was reached within 3–4 days in culture. The addition of LH + FSH together with 50 ng PGE$_2$/ml medium did not increase the daily production of progesterone above that achieved by PGE$_2$ alone. Furthermore, there was no dose-related increase in the daily production of progesterone if the cells were exposed to PGE$_2$ at concentrations between 1 and 500 ng/ml medium.
The daily production of progesterone by granulosa cells harvested from ‘inactive’ follicles and exposed to PGE₂ with or without LH + FSH is shown in Fig. 5. The addition of between 1 and 500 ng PGE₂/ml medium to cells harvested from ‘inactive’ follicles failed to stimulate the production of progesterone more than LH + FSH. Furthermore, the addition of PGE₂ together with LH + FSH failed to produce any further stimulation.

![Graph showing daily production of progesterone by human granulosa cells in vitro exposed to prostaglandin E₂ (PGE₂) (50 ng/ml) with or without added LH and FSH (both 30 µu./ml). The cells were harvested from follicles containing low concentrations of FSH and oestradiol. Numbers in parentheses refer to number of experiments. Cross, control; open triangle, LH + FSH + PGE₂ (3), PGE₂ alone (4) and LH + FSH (3).](image_url)

**Table 2. Effect of adding prostaglandins F₂α (PGF₂α) and E₂ (PGE₂) on the production of progesterone by human granulosa cells.**

<table>
<thead>
<tr>
<th>Prostaglandin added</th>
<th>Concentration (ng/ml)</th>
<th>Total production of progesterone during 10 days of culture (µg progesterone/10⁶ cells; mean ± S.E.M.)</th>
<th>Number of separate cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;0·04</td>
<td>51·8 ± 4·6</td>
<td>5</td>
</tr>
<tr>
<td>PGE₂</td>
<td>10</td>
<td>60·3 ± 1·9</td>
<td>5</td>
</tr>
<tr>
<td>PGE₂</td>
<td>500</td>
<td>61·9 ± 2·3</td>
<td>5</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>10</td>
<td>23·2 ± 3·7</td>
<td>5</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>500</td>
<td>17·5 ± 2·1</td>
<td>5</td>
</tr>
<tr>
<td>PGE₂</td>
<td>10</td>
<td>49·9 ± 4·9</td>
<td>3</td>
</tr>
<tr>
<td>PGE₂</td>
<td>50</td>
<td>48·8 ± 5·1</td>
<td>3</td>
</tr>
<tr>
<td>PGE₂</td>
<td>50</td>
<td>57·6 ± 4·3</td>
<td>3</td>
</tr>
<tr>
<td>PGE₂</td>
<td>100</td>
<td>57·2 ± 6·6</td>
<td>3</td>
</tr>
</tbody>
</table>

Cells were harvested from a single 15 mm late-follicular-phase follicle with detectable levels of LH (4·8 µu./ml), FSH (3·3 µu./ml) and oestradiol (2100 ng/ml) in the follicular fluid. The concentration refers to the final concentration of prostaglandin in the medium.
**Effect of adding prostaglandins F2α and E2 on the production of progesterone by granulosa cells**

The effects of adding 50 ng of each prostaglandin to granulosa cells harvested from 'active' follicles are shown in Fig. 4; the daily production of progesterone was similar to that achieved by adding PGE2 alone. The total production of progesterone by granulosa cells harvested from a prevoluntary follicle after 10 days of culture when exposed to varying doses of PGF2α, PGE2 or PGF2α + PGE2 is shown in Table 2. The addition of 10 to 500 ng PGF2α lowered the total production of progesterone by at least 60%. The addition of 10–500 ng PGE2/ml medium with PGF2α over the same dose range did not alter the total production of progesterone achieved by adding PGE2 alone.

**DISCUSSION**

These results show clearly that PGF2α inhibits the production of progesterone by human granulosa cells in vitro without affecting cell viability. They also show that provided PGF2α is added at the commencement of culture it is equally effective in inhibiting the daily production of progesterone by granulosa cells from active or inactive follicles, although the former have a greater biosynthetic potential to secrete progesterone (McNatty & Sawers, 1975). Furthermore, when PGF2α was added at the commencement of culture it blocked the stimulatory effect of LH + FSH on granulosa cells at all stages of their development. By contrast, when granulosa cells were exposed to high concentrations of LH + FSH for several days before the addition of 50 ng PGF2α, the production of progesterone was not inhibited. Although a detailed dose-relationship was not established, a much higher concentration of PGF2α (1000 ng/ml medium) was required to inhibit the production of progesterone.

The addition of PGE2 to human granulosa cells in vitro stimulated the secretion of progesterone at a greater rate than any concomitant increase in cell numbers. These experiments also showed that PGE2 was far more effective than LH + FSH in stimulating progesterone secretion by cells from actively developing follicles. By contrast, the addition of PGE2 to cells from 'inactive' follicles was no more effective than LH + FSH in stimulating the production of progesterone, emphasizing once more the differing biosynthetic potential of granulosa cells harvested from differing hormonal environments in follicular fluid (McNatty & Sawers, 1975). There was no evidence of any synergism between PGE2 and LH + FSH.

The present studies indicate that extremely low levels (1–50 ng/ml) of both prostaglandins can cause dramatic alterations in progesterone production under in-vitro conditions. Similar concentrations of PGF2α are present in the ovarian artery of the sheep during luteal regression (Baird & Scaramuzzi, 1975), and preliminary studies indicate that comparable levels are also present in the human corpus luteum (I. Swanston, D. T. Baird, R. W. Kelly and K. P. McNatty, unpublished data). Prostaglandins found within the human follicle and corpus luteum could therefore have a major controlling influence on steroid secretion by these structures. Although PGF2α was extremely effective (~ 60%) in blocking the stimulatory effect of LH + FSH on progesterone secretion, it was totally ineffective in blocking the stimulatory effects of PGE2. These studies provide some clues as to the site of action of PGF2α. Both LH (Channing, 1975) and PGE2 (Rao, 1973) have specific receptors on the membrane, and their steroidogenic response is mediated by activation of the adenylate cyclase system (Savard, Marsh & Rice, 1965; Dorrington & Kilpatrick, 1967; Robison, Butcher & Sutherland, 1971). This suggests that PGF2α acts directly on the adenylate cyclase system by blocking its activation by LH. Similar data have been obtained in vitro using porcine and bovine granulosa cells (K. M. Henderson & K. P. McNatty, unpublished) suggesting that the interaction of PGF2α with this cell type is species-independent. These studies,
however, may not be consistent with the hypothesis proposed by Kuehl (1974), since in his model PGE₂ is unlikely to overcome the inhibitory effect of PGF₂α. Such differences as exist between Kuehl's hypothesis and the present study may depend on the rates at which both PGE₂ and PGF₂α act on the biochemical pathway to progesterone synthesis. Since continued high levels of LH and FSH protect the granulosa cells against subsequent inhibition by PGF₂α, it seems likely that the inhibitory effect of PGF₂α is inversely related to the amount of gonadotrophin bound to its receptor; PGF₂α was 200 times less effective when granulosa cells had been exposed to high concentrations of LH + FSH for 6 days before its addition. These findings are in agreement with those of Hichens et al. (1974), who showed that PGF₂α decreased the binding capacity of luteal tissue to HCG.

In conclusion these data suggest that functional luteal regression of the human corpus luteum could occur by a biochemical mechanism independent of the vascular system. However, the lack of cell death in vitro after exposure to PGF₂α suggests that other mechanisms in addition to the inhibition of steroidogenesis may be necessary to bring about complete structural and functional regression of the gland.

We are indebted to Dr J. Pike (Upjohn Company, Kalamazoo, Michigan) for the generous supply of prostaglandins, Dr A. Stockell Hartree and the Medical Research Council for the purified human gonadotrophin preparations LH, IRC-69 and FSH, MRC 73/519. We gratefully acknowledge the help of Mrs E. Hunter and Mr D. Love (MRC Radioimmunoassay Team, Edinburgh) for the radioimmunoassays of oestradiol, LH and FSH in follicular fluid, Mr L. Mackenzie for his assistance in dating the endometria, and the gynaecological consultants of the Royal Infirmary, Edinburgh for arranging the supply of human ovaries. Mr I. Swanston and Dr R. W. Kelly kindly carried out the measurement of PGF₂α and PGE₂ in the culture media. K. P. McN. is a recipient of a New Zealand N.R.A.C. Fellowship, and K. M. H. of an M.R.C. Research Fellowship.

REFERENCES


INFLUENCE OF 16-ARYLOXYPROSTAGLANDINS ON THE PRODUCTION OF PROGESTERONE BY HUMAN GRANULOSA CELLS IN VITRO

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SUMMARY

16-Aryloxy analogues of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) are potent luteolysins in laboratory and farm animals. When their effect on progesterone production by luteinized human granulosa cells in tissue culture was investigated inhibition of both basal and gonadotrophin-stimulated progesterone production was observed, so revealing characteristics expected of potential human luteolysins. The analogues were, however, unable to inhibit progesterone production stimulated by PGE$_2$, suggesting that like PGF$_{2\alpha}$ these compounds may act by specifically blocking LH-activated adenylate cyclase.

The 16-aryloxyprostaglandins similarly inhibited progesterone production by porcine granulosa cells, so that the effects observed with the 16-aryloxyprostaglandins in vitro may be indicative of their potential in vivo.

INTRODUCTION

Although prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) is luteolytic in many laboratory and domestic animals (Weeks, 1972; Inskeep, 1973), attempts to demonstrate that it is luteolytic in women have so far met with little success (Coudert, Winter & Faiman, 1974; Pharriss & Shaw, 1974). Recently, we have shown that PGF$_{2\alpha}$ will inhibit the production of progesterone by luteinized human granulosa cells cultured in vitro (Henderson & McNatty, 1975; McNatty, Henderson & Sawers, 1975), and it is possible, therefore, that the relative ineffectiveness of PGF$_{2\alpha}$ in vivo in women may be due to difficulty in delivering adequate amounts to the ovary via the arterial supply, since PGF$_{2\alpha}$ is rapidly metabolized by the lungs and liver (Piper, Vane & Wyllie, 1970). However, even if PGF$_{2\alpha}$ should ultimately prove to be luteolytic in women, its use as a contraceptive would be limited by unacceptable side-effects such as the diarrhoea, vomiting and nausea arising from its concomitant action on smooth muscle.

Some 16-aryloxy analogues of PGF$_{2\alpha}$, namely ICI 79,939, ICI 80,996 and ICI 81,008, have been shown to be very much more potent luteolysins than PGF$_{2\alpha}$ without having a correspondingly increased activity on smooth muscle (Binder, Bowler, Brown, Crossley, Hutton, Senior, Slater, Wilkinson & Wright, 1974). Consequently, low doses will induce luteolysis in laboratory and domestic animals without side-effects (Dukes, Russell & Walpole, 1974). However, it is not known whether the increased potency of these compounds is due to the fact that they are metabolized more slowly than the parent substance or whether they are intrinsically more luteolytic.

The purpose of the present study was to investigate the luteolytic properties of three 16-aryloxy derivatives upon human granulosa luteal cells in tissue culture.
MATERIALS AND METHODS

Gonadotrophins

Ovine luteinizing hormone (NIH-LH-S12) and ovine follicle-stimulating hormone (NIH-FSH-S4) were supplied by the National Institutes of Health, Bethesda, Maryland, U.S.A. The gonadotrophins were diluted to 500 ng/ml with culture medium, and stored in ampoules at −20 °C. Samples of each (0-1 ml) were added to the cell cultures.

Prostaglandins

16-Aryloxyprostaglandins, ICI 80,996, ICI 81,008 and ICI 79,939, were obtained from ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire. Each was dissolved in phosphate-buffered saline (Flow Laboratories, Irvine, Scotland) and stored at 4 °C until added to the cell cultures in 0-1 ml aliquots.

Prostaglandin E₂ (PGE₂), obtained from the Upjohn Company, Kalamazoo, U.S.A., was dissolved in 100 % ethanol and stored at 4 °C until added to the cell cultures in 10 μl aliquots. Control cultures received appropriate volumes of either phosphate-buffered saline and/or 100 % ethanol.

Culture of granulosa cells

Porcine ovaries were obtained from pigs within 1 h of slaughter at a local abattoir. Human ovaries were obtained from patients undergoing ovariectomy for various gynaecological disorders.

The techniques for obtaining dispersed granulosa cell suspensions and the method of tissue culture of the porcine and human granulosa cells were as previously described (McNatty & Sawers, 1975). Briefly, a minimum of 5 × 10⁴ granulosa cells harvested from small antral follicles were cultured in 1 ml culture medium, consisting of 20 % calf serum and 80 % Medium 199 containing HEPES buffer (20 mmol/l) and supplemented with gentamicin (50 μg/ml), amphotericin-B (2.5 μg/ml) and 1 % glutamine (Flow Laboratories, Irvine, Scotland). Culture medium was replaced daily and stored at −20 °C until assayed for progesterone by radioimmunoassay (Neal, Baker, McNatty & Scaramuzzi, 1975). At the end of the culture period the coverslips were removed, washed extensively with Medium 199, stained with haematoxylin/eosin and the cells remaining counted.

Small antral follicles were chosen since it has previously been demonstrated that granulosa cells harvested from these follicles secrete relatively low amounts of progesterone (McNatty & Sawers, 1975). However, steroidogenesis can be readily stimulated by the daily addition of gonadotrophins to the culture medium.

The endogenous level of PGF₁₂ in the culture medium was 36 pg/ml, and of PGE₂ < 200 pg/ml (as determined by radioimmunoassay and g.l.c.-mass spectrometry).

RESULTS

Table 1 shows the effect of three 16-aryloxyprostaglandins on the total production of progesterone by human granulosa-luteal cells cultured in vitro. All three analogues markedly inhibited the basal secretion of progesterone. This effect would appear to be a direct biochemical inhibition of steroidogenesis since the analogues did not affect the number of cells remaining at the end of the culture period, relative to the control cultures, nor was there any observable morphological difference between the two groups on examination by light microscopy.

The limited number of human granulosa cells available at any one time makes it difficult to study the effects on steroidogenesis of differing amounts of analogue. However, large
numbers of viable porcine granulosa cells can be easily obtained. Utilizing these cells, it was found that as little as 50 pg/ml of analogue was as effective as 50 ng/ml (Table 2) a result very similar to that obtained with PGF$_{2\alpha}$ (Henderson & McNatty, 1977).

Figure 1 shows the effect of ICI 80,996 on progesterone production by human granulosa cells simultaneously treated with either gonadotrophins or PGE$_2$. Although the analogue effectively inhibited progesterone production stimulated by gonadotrophins ($P < 0.01$), it had no effect on steroidogenesis stimulated by PGE$_2$, as also observed with PGF$_{2\alpha}$ (Henderson & McNatty, 1975). Similar results were obtained with the other analogues, suggesting that, like PGF$_{2\alpha}$, these compounds may act by specifically inhibiting adenylate cyclase activation by LH.

Table 1. The effect of 16-aryloxyprostaglandins on the total production of progesterone by human granulosa cells cultured for 8 days in vitro (means ± S.E.M.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Progesterone (ng/10$^6$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.2 ± 2.3 (3)</td>
</tr>
<tr>
<td>ICI 79,939 (50 ng/ml)</td>
<td>1.6 ± 0.5 (3)</td>
</tr>
<tr>
<td>Control</td>
<td>3.7 ± 0.8 (4)</td>
</tr>
<tr>
<td>ICI 80,996 (50 ng/ml)</td>
<td>0.4 ± 0.1 (4)</td>
</tr>
<tr>
<td>Control</td>
<td>4.4 ± 0.1 (3)</td>
</tr>
<tr>
<td>ICI 81,008 (50 ng/ml)</td>
<td>1.1 ± 0.1 (4)</td>
</tr>
</tbody>
</table>

The numbers in parentheses refer to the number of replicate cultures. Prostaglandin-treated cultures produced significantly less progesterone than control cultures ($P < 0.01$).

Table 2. The effect of 16-aryloxyprostaglandins on the total production of progesterone by porcine granulosa cells cultured for 8 days in vitro (ng progesterone/10$^6$ cells; means ± S.E.M.)

<table>
<thead>
<tr>
<th>ICI 80,996</th>
<th>ICI 79,939</th>
<th>ICI 81,008</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (6)</td>
<td>50 pg/ml (6)</td>
<td>50 ng/ml (6)</td>
</tr>
<tr>
<td>1340 ± 280</td>
<td>157 ± 42</td>
<td>133 ± 27</td>
</tr>
</tbody>
</table>

The numbers in parentheses refer to the number of replicate cultures. Prostaglandin-treated cultures produced significantly less progesterone than the control cultures ($P < 0.01$).

**DISCUSSION**

These results demonstrate that analogous with PGF$_{2\alpha}$, 16-aryloxyprostaglandins inhibit both basal and gonadotrophin-stimulated progesterone production by human granulosa cells in tissue culture, thus displaying in-vitro characteristics expected of potential human luteolysins.

The possible pharmacological significance of these findings is strengthened by the ability of the 16-aryloxy analogues to inhibit steroidogenesis by porcine granulosa cells; these drugs are luteolytic in the pig (Ash & Heap, 1973). Although there are differences in the control of corpus luteum function between pigs and women, it is likely that the biochemistry of the progesterone-secreting cells is similar. Therefore, the ability of 16-aryloxyprostaglandins to inhibit progesterone production by human granulosa cells in vitro is perhaps a good indication of their potential in vivo, providing that sufficient analogue can reach and interact with the receptor for PGF$_{2\alpha}$ located on the corpus luteum (Powell, Hammarström, Samuelsson & Sjoberg, 1974). Since 16-aryloxyprostaglandins are very much more potent luteolysins than PGF$_{2\alpha}$, without being correspondingly more toxic, it may be possible to deliver to
the corpus luteum an amount of analogue sufficient to initiate luteolysis, but without producing the undesirable side-effects associated with infusion of PGF$_{2\alpha}$.

Recently, preliminary studies by Csapo & Mocsary (1976) have shown that intra-uterine administration of ICI 81,008 will effectively induce menstruation in women during early pregnancy. However, like PGF$_{2\alpha}$ and PGE$_2$, this effect depends on the vasoconstrictive properties of ICI 81,008 inducing uterine contracture, and not on its luteolytic properties. In view of our in-vitro findings, further detailed clinical studies to assess the potential of 16-aryloxyprostaglandins to act as 'menstrual inducers' by interacting directly with the corpus luteum to inhibit progesterone production seem worthwhile.

Fig. 1. The effect of ICI 80,996 (100 ng) on the production of progesterone by human granulosa cells simultaneously treated with either gonadotrophins (A) or with PGE$_2$ (B). Numbers in parentheses refer to the number of replicate cultures. Gonadotrophin-treated cultures were exposed to 50 ng ovine LH (NIH-LH-S12) and 50 ng ovine FSH (NIH-FSH-S4). Vertical lines represent ± s.e.m. ×, control; △, LH + FSH; △, LH + FSH + ICI 80,996; ●, PGE$_2$ (50 ng); ○, PGE$_2$ (50 ng) + ICI 80,996.

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SIMULTANEOUS INFUSION OF PROSTAGLANDIN E₂ ANTAGONIZES THE LUTEOLYTIC ACTION OF PROSTAGLANDIN F₂α IN VIVO

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SUMMARY

Corpora lutea of ewes bearing ovarian autotransplants were infused for 4 h with prostaglandin F₂α (PGF₂α) (10 μg/h), PGF₂α + PGE₂ (10 μg/h of each), PGE₂ (10 μg/h) or saline on day 10 of the cycle. Ovarian venous blood obtained before, during, and up to 12 h after the infusion period, was assayed for progesterone. Prostaglandin F₂α produced an immediate, rapid and sustained decline in progesterone secretion, but infusion of PGE₂ together with PGF₂α prevented the decline until after the infusion. Progesterone secretion was unaffected by infusion of PGE₂ alone. Oestrous behaviour was observed in four out of seven animals infused with PGF₂α but in only one out of six infused with PGF₂α + PGE₂. None of the animals infused with PGE₂ alone or saline only came into heat.

INTRODUCTION

Although prostaglandin F₂α (PGF₂α) is luteolytic in many laboratory and domestic animals (Weeks, 1972; Inskeep, 1973) its mechanism of action remains obscure. Several studies have suggested that PGF₂α may initiate luteolysis through a biochemical action on the luteal cell. Prostaglandin F₂α has been shown to inhibit progesterone production by ovarian tissue in vitro (Demers, Behrman & Greep, 1972; O'Grady, Kohorn, Glass, Caldwell, Brock & Speroff, 1972) and the onset of luteal regression is associated with biochemical changes in the luteal cell, namely a decline in adenyl cyclase activity and loss of responsiveness to luteinizing hormone (LH) (Andersen, Schwartz & Ulberg, 1974; Hichens, Grinwich & Behrman, 1974; Grinwich, Hichens & Behrman, 1976). Previous results from our laboratory also supported this concept, and provided a clue to the possible site of action of PGF₂α. Utilizing granulosa cells in tissue culture we found that although PGF₂α could inhibit the stimulatory effect of LH on progesterone production, it did not inhibit the stimulatory effect of PGE₂ (Henderson & McNatty, 1975; McNatty, Henderson & Sawers, 1975). There is substantial evidence that both LH and PGE₂ interact with respective specific membrane receptors (Rao, 1973; Han, Rajaniemi, Cho, Hirshfield & Midgley, 1974) to stimulate steroidogenesis through activation of the adenyl cyclase enzyme system (Marsh, 1970a, b, 1971, 1976), and so it seemed likely that PGF₂α inhibits steroidogenesis by specifically inhibiting LH activation of adenyl cyclase. More recent studies have shown directly that PGF₂α can indeed inhibit LH stimulation of cyclic AMP production in vitro (Lahav, Freud & Lindner, 1976).

If PGF₂α initiates luteolysis in vivo by specifically interfering with the coupling of LH and

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adenyl cyclase, the action of PGF\textsubscript{2\alpha} should be overridden by simultaneous administration of PGE\textsubscript{2}. This possibility has been tested in the present work.

**MATERIALS AND METHODS**

*Experimental animals*

Eleven ewes with the left ovary autotransplanted to a carotid–jugular skin loop as described by Goding, McCracken & Baird (1967) were used. In this preparation the corpus luteum persists due to physical separation of the ovary from the uterus, but as it was necessary to study corpora lutea of the same age, the persisting corpora lutea were caused to regress, and the ovarian cycles of the animals synchronized, by administering a single intramuscular injection of ICI 80,996 (50 µg), a potent analogue of PGF\textsubscript{2\alpha}. This induced oestrous behaviour within 2 or 3 days; the infusion experiments being carried out 10 days after the onset of oestrus.

*Infusion of the ovary*

The procedures associated with cannulation of the ovary, infusion through the ovarian artery, and the timed collection of ovarian venous blood samples have been described previously (McCracken, Uno, Goding, Ichikawa & Baird, 1969).

Before the infusion of prostaglandins, an infusion of 0·9% physiological saline (10 ml/h) was maintained during which time two ‘control’ samples of ovarian venous blood were obtained 30 and 90 min after commencement of infusion. Following this control period the ovary was infused for 4 h with PGF\textsubscript{2\alpha}, PGE\textsubscript{2}, PGF\textsubscript{2\alpha} + PGE\textsubscript{2} or saline only; 10 µg prostaglandin in 10 ml saline being delivered at a constant rate each hour. Timed collections of ovarian venous blood were made at hourly intervals for 6 h commencing 30 min after the start of the test period. A final sample was taken next day, approximately 18 h after the start of the infusion. At the end of the experiment the animals were run with a raddled, vasectomized ram for 4 days, and they were inspected twice daily for signs of mating (Radford, Watson & Wood, 1960).

*Progesterone assay*

The concentration of progesterone in ovarian venous plasma was measured by radioimmunoassay (Scaramuzzi, Corker, Young & Baird, 1975). The secretion of progesterone was calculated from the plasma concentration, the haematocrit and the blood flow.

In 32 duplicate determinations performed in the same assay, the coefficient of variation, calculated by the method of Snedecor (1956), was 13%. In 15 duplicate determinations in two different assays, the coefficient of variation was 18%.

**RESULTS**

*Effect of prostaglandin infusions on progesterone secretion*

The results shown in Fig. 1 are expressed as a percentage of the mean progesterone secretion rate obtained from the two control ovarian venous blood samples. The overall rates of progesterone secretion during the control infusion period for each of the experimental groups were not significantly different from each other (\(P > 0.05\)), the values being: saline, \(6.8 \pm 1.3\) (s.e.m.) µg/min; PGF\textsubscript{2\alpha}, \(8.4 \pm 0.8\); PGE\textsubscript{2}, \(9.2 \pm 1.3\); PGF\textsubscript{2\alpha} + PGE\textsubscript{2}, \(8.0 \pm 1.1\).

Infusion of PGF\textsubscript{2\alpha} alone produced an immediate, rapid and sustained fall in progesterone secretion while infusion of PGE\textsubscript{2} alone had no significant effect. When PGF\textsubscript{2\alpha} was infused together with PGE\textsubscript{2}, the secretion of progesterone remained unaltered until the infusion period had finished but declined rapidly thereafter. By 18 h there was no significant difference in the rate of progesterone secretion resulting from either PGF\textsubscript{2\alpha} or PGF\textsubscript{2\alpha} + PGE\textsubscript{2} infusion (\(P > 0.05\)).
Fig. 1. Mean progesterone secretion rate during and after infusion of prostaglandins (PG) in ewes with ovarian autotransplants. The results are expressed as a percentage of the values obtained during the control period (see text). Numbers in parentheses refer to the number of animals in each group. *P < 0.05: significantly different from the corresponding points of the control animals. ■, Saline; Δ, PGF₂α (10 μg/h); ●, PGE₂ (10 μg/h); ▲, PGF₂α + PGE₂ (10 μg/h of each).

Ovarian venous blood flow was significantly increased by 13.5 ± 1.8% (P < 0.05) during infusion of PGE₂ alone, but fell to control levels at the end of infusion. Infusion of PGF₂α or PGF₂α + PGE₂ did not alter blood flow (P > 0.05).

**Effect of prostaglandin infusions on oestrous behaviour**

A higher proportion of animals showed oestrous behaviour following infusion of PGF₂α alone (four out of seven) than of those animals infused with PGF₂α + PGE₂ (one out of six). None of the animals infused with PGE₂ alone (7) or saline only (6) was marked by the ram.

**DISCUSSION**

These results demonstrate that PGE₂ when infused simultaneously with PGF₂α can antagonize the luteolytic action of PGF₂α, and are also consistent with the concept that PGF₂α initiates luteolysis through a direct action on the luteal cell to inhibit specifically LH-activated adenyl cyclase (Hichens et al. 1974; Henderson & McNatty, 1975; Grinwich et al. 1976; Lahav et al. 1976). However, in view of the observed increase in blood flow during infusion of PGE₂, it could also be argued that the vasodilator properties of this prostaglandin counteract a vasoconstrictive action of PGF₂α on the corpus luteum (Thorburn & Hales,
1972; Niswender, Diekmann, Nett & Akbar, 1973; Bruce & Moor, 1975). However, Bruce & Hillier (1974) have demonstrated that PGF<sub>2α</sub>-induced inhibition of progesterone secretion can occur without depression of corpus luteal blood flow, and there is little evidence that anoxic damage causes a reduction in progesterone secretion in the early stages of luteal regression (Umo, 1975; Gemmell, Stacy & Thorburn, 1976).

Inhibition of LH activated adenyl cyclase by PGF<sub>2α</sub> infusion would cause the pool of LH-induced cyclic AMP to be depleted through the action of phosphodiesterase, so reducing steroid synthesis and depressing progesterone secretion. Infusion of PGE<sub>2</sub> together with PGF<sub>2α</sub> masks this effect of PGF<sub>2α</sub> through the ability of PGE<sub>2</sub> to stimulate adenyl cyclase through separate PGE<sub>2</sub>-sensitive receptor and coupling units, so sustaining increased levels of cyclic AMP. Consequently the rate of progesterone secretion is unaffected. On terminating the infusion, however, this pool of cyclic AMP would also be diminished by the action of phosphodiesterase, so causing a fall in the rate of progesterone secretion.

Infusion of PGE<sub>2</sub> alone had no significant effect on the secretion of progesterone. However, it is likely that the corpus luteum on day 10 is already secreting maximally since infusion of massive amounts of LH on day 10 of the cycle fails to stimulate an increased secretion of progesterone (Land, Collett & Baird, 1974). Studies with granulosa cells in tissue culture support this view, for although both PGE<sub>2</sub> and LH + follicle-stimulating hormone could stimulate progesterone production, cells which had been fully stimulated with gonadotrophins could not be further stimulated with PGE<sub>2</sub> (McNatty et al. 1975).

In conclusion, it seems likely that PGF<sub>2α</sub> may initiate functional regression of the corpus luteum by specifically inhibiting LH activation of adenyl cyclase, a process which can be overridden by the simultaneous administration of PGE<sub>2</sub>. The ultimate morphological regression of the corpus luteum may also arise through further cellular processes being triggered by this initial action of PGF<sub>2α</sub>.

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