Investigations into the genetic, morphogenetic and teratogenic factors that influence early mammalian development

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1984
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Abstract

In this thesis, the results of a considerable number of investigations into the genetic, morphogenetic and teratogenic factors that influence early mammalian development are presented. In virtually all of the investigations in which experimental procedures have been carried out, mouse embryos have been studied as embryos from this species have the singular advantage that all stages of gestation are easily accessible, and following their isolation and explantation into tissue culture may be maintained for lengthy periods of time in vitro with minimal obvious detrimental effect on their growth or development potential.

In the majority of the studies presented here, the principally genetic factors that influence the development of the pre-implantation conceptus have been analysed. In many of these studies the development potential of (diploid) fertilized embryos has been compared with that of parthenogenetically activated embryos which have been induced to develop under controlled experimental conditions in vitro. By definition, such embryos develop from a female gamete in the absence of any contribution from a male gamete. By comparing the development of appropriate groups of embryos, it has been possible to investigate the effect of homozygosity versus heterozygosity, and ploidy as well as analysing the influence of aneuploidy on early mouse embryonic development. In addition, this experimental approach has enabled, albeit indirectly, the influence of the fertilizing spermatozoon to be studied. Haploid and diploid parthenogenetically activated embryos are also capable of surviving into the early post-implantation period, and when combined in a chimaeric association with fertilized embryos,
adult mice are obtained in which the parthenogenetically-derived cells are capable of contributing to all the tissues of the body including the germ cells.

Studies are also described in which pluripotential cells have been established initially from fertilized embryos but subsequently from both haploid and diploid parthenogenones. Both fertilized- and parthenogenetically-derived cells have also been used to investigate the genetic and morphogenetic factors that influence early mammalian development.

Because the events that occur in the early post-implantation period are generally less well understood than those occurring during the pre-implantation period, various descriptive studies have been carried out which have sought to investigate the normal morphological changes that occur in the embryo at and shortly after implantation. More particularly, the events associated with the organogenesis of the neural tube and heart have been studied as these are the first major organ systems to develop within the embryo. In addition to these accounts of the normal development of these systems, studies are described in which embryos were exposed, either in vivo or in vitro, to a wide range of potentially teratogenic stimuli. Using this approach, a variety of studies have been carried out which have enabled the morphogenetic and to a lesser extent the genetic factors that influence early post-implantation mammalian development to be investigated. These studies clearly demonstrate that most of the agents tested appear to influence the cellular cytoskeletal system and in consequence interfere with the normal cell shape changes that should occur during organogenesis. Some of these agents are also capable of interfering with chromosome segregation during the meiotic divisions associated with oocyte maturation.
Bibliography

The numbers on the items submitted are closely related to the chronological sequence of their publication. A full list of these publications follows in addition to the items themselves.

The various publications presented here describe investigations into the genetic, morphogenetic and teratogenic factors that influence early mammalian development. These may be divided into 2 major categories each of which may be conveniently subdivided into several inter-related groups.

In the first category, the studies which constitute the largest individual group are those in which parthenogenetically activated eggs and embryos have been used as tools to investigate the principally genetic factors that influence early mammalian development. This group includes items:-

3, 5, 8-15, 19, 21-25, 28, 33, 37, 43-45, 48, 49, 51, 55. Item 51 being a recently published monograph on this topic.

In a second and closely related group, similar cytogenetic analyses on ovarian oocytes and early fertilized material are reported. This group includes items:-

2, 4, 7, 10, 16, 20, 46, 51, 56, 58.

In an equally related group, the establishment and subsequent analysis of pluripotential cells and cell lines derived from parthenogenetic and fertilized embryos is described. This group includes items:-

39, 45, 47-49, 53, 55, 57.

Several additional papers have looked at various aspects of ovarian follicular physiology and the fertilizability of oocytes under certain experimental conditions. This group includes items:-

1, 6, 29
The second major category includes investigations generally of a more morphological nature which have studied the factors that influence mammalian embryogenesis and organogenesis.

Of those concerned with normal morphogenesis, all are concerned either directly or indirectly with the development of the neural tube and/or heart. This group includes items:-

31, 40, 42, 50, 54.

Of those concerned with abnormal morphogenesis (i.e. teratogenesis, including the induction of chromosomal anomalies in oocytes and embryos - both fertilized and parthenogenetically-derived), a high proportion of these are also concerned with the factors that influence neural tube closure. This group includes items:-

17, 18, 20, 26, 27, 30, 32, 34-36, 38, 41, 44, 46, 52, 56, 58.

Please note that item 51, the monograph entitled:-

Early Mammalian Development: Parthenogenetic Studies

published by Cambridge University Press is appended as a separate volume.
As may be observed from this Bibliography, the research was entirely that of the candidate in 22 of the publications cited, namely in the following items:

2-5,7,12,13,16,18,20,24,25,28,31,37,38,43,44,46,50,51,54.
The candidate was the major contributor and first author in 16 of these publications in which one or more additional authors are present, namely in the following items:

1,8,9,11,14,15,17,19,23,26,40,41,45,55,56,58.
Out of a total of 12 publications with 2 authors in which the candidate was the second author, in 8 instances the first author was a Research Student under the supervision of the candidate. This was the situation in items:

27,30,32,34,36,42,52.
In the remaining 4 instances in which the candidate was the second author (out of 2), both authors made an approximately equal contribution. This situation occurred in the following items:

10,21,39,47.
In those publications in which the candidate was one of several authors but not the first author, he was a principal investigator in the following items:

6,22,29,33,48,49,53,57.
The above information accounts for all of the publications cited in the Bibliography.


VIABILITY OF MOUSE OOCYTES OVULATED WITHIN 14 HOURS OF AN INJECTION OF PREGNANT MARES' SERUM GONADOTROPHIN

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(Received 16th September 1971, accepted 20th October 1971)

Summary. Adult female mice ovulated within 14 hr following an injection of pmsg. The oocytes were fertilized in vitro. Zygotes developed to the blastocyst stage when cultured for 96 hr. Transfer of two- and four-cell embryos to foster mothers resulted in viable fetuses.

Sexually mature female mice will ovulate within 24 hr of an injection of PMSG, HCG, LH or FSH (Burdick & Whitney, 1941; Burdick, Watson, Ciampa & Ciampa, 1943; Saunders, 1947; Edwards & Fowler, 1960; Stern & Schuetz, 1970). The number of oocytes ovulated are similar to the number released at normal oestrus (Edwards & Fowler, 1960; Kaufman, unpublished data) but unless the mice are injected immediately before oestrus, they do not mate (Stern & Schuetz, 1970). In the present study, the viability and developmental capacity of oocytes ovulated within 13 to 14 hr of a single injection of PMSG were examined and compared with those of oocytes obtained by superovulation, by means of fertilization in vitro, embryo culture and transfer to foster mothers.

Adult F1 hybrid mice (C57B1 x A2G), selected at random with respect to their oestrous cycles, were divided into two groups and oocytes were obtained as follows.

(1) One group of mice received an intraperitoneal (i.p.) injection of 10 i.u. PMSG (Gestyl, Organon) and oocytes were recovered 13 to 14 hr later. Approximately 100% of the mice routinely ovulated in response to the injection (Kaufman, in preparation).

(2) Another group of mice received an i.p. injection of 10 i.u. HCG (Pregnyl, Organon) and oocytes were recovered following superovulation 13 to 14 hr later. This group received 10 i.u. PMSG i.p. 48 hr before administration of the HCG and the latter injection coincided with the PMSG injection of Group 1. The second group was used as the control group in each experiment since oocytes released after such treatment have previously been shown to be viable (Fowler & Edwards, 1957; Whittingham, 1968).

Uterine spermatozoa were collected from mice (CFLP strain, Carworth Europe) mated 1 to 2 hr previously with fertile males (CFLP strain, Carworth Europe). The females had been induced to mate synchronously by treatment with gonadotrophins (Biggers, Whitten & Whittingham, 1971). The contents of the uterine horns were expressed into 1 ml medium contained in an embryo-
logical watchglass. This was diluted by the addition of a further 3 ml medium, and droplets of approximately 30 to 50 µl sperm suspension were made under liquid paraffin contained in 60-mm diameter plastic Petri dishes (Falcon). The culture medium was the same as the modified Krebs-Ringer bicarbonate solution described for routine culture of mouse ova (Whittingham, 1971), except that the bovine serum albumin was increased from 4 mg/ml to 32 mg/ml (Cross & Brinster, 1970). The oviducts of the experimental groups were dissected out and the eggs from each ampulla released into separate droplets of medium containing spermatozoa by incising the dilated ampullar wall with fine watchmaker’s forceps; the empty ampulla was discarded. The dishes were incubated for 5 hr at 37°C in an atmosphere of 5% CO₂ in air. After incubation, the ova were washed in one change of medium (30 to 50 µl) containing 4 mg/ml bovine serum albumin (Whittingham, 1971, unmodified medium) to remove excess spermatozoa, and cultured in droplets of similar medium for a further 19 to 20 hr when the number of two-cell ova was recorded.

### Table 1

**DEVELOPMENT OF MOUSE OOCYTES, FERTILIZED AND INCUBATED IN VITRO**

<table>
<thead>
<tr>
<th>Group</th>
<th>Exp. no.</th>
<th>No. of animals</th>
<th>No. of oocytes cultured</th>
<th>No. of two-cell ova at 24 hr (%)</th>
<th>No. of blastocysts at 96 hr (% two-cell ova developing into blastocysts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovulated following i.p. injection of pmsg alone</td>
<td>1</td>
<td>5</td>
<td>37</td>
<td>30 (81-1)</td>
<td>6* (35-3)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>42</td>
<td>8† (19-1)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>43</td>
<td>33‡ (76-7)</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>122</td>
<td>71 (58-2)</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Superovulated following i.p. injections of pmsg 48 hr before hCG</td>
<td>1</td>
<td>6</td>
<td>129</td>
<td>71 (55-0)</td>
<td>25 (35-2)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>107</td>
<td>27§ (25-2)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>41</td>
<td>29 (70-7)</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>277</td>
<td>127 (45-9)</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

* Thirteen ova transferred at four-cell stage. † Thirty-two ova transferred at two-cell stage. § Not cultured beyond two-cell stage.

The results of the three experiments designed to compare the fertilizability and subsequent developmental capacity of oocytes obtained following pmsg injection or superovulation are presented in Table 1. In the three experiments, a total of 71 out of 122 oocytes (58.2%) from the groups receiving pmsg alone proceeded through the first cleavage division compared with 127 out of 277 oocytes (45.9%) in the group undergoing superovulation. In the first experiment, there was a significantly higher proportion of two-cell ova 24 hr after insemination, from oocytes ovulated 13 to 14 hr after pmsg (30/37) than from oocytes produced by superovulatory treatment (71/129). This difference did not occur in Exps 2 and 3 and, in Exp. 1, it may have resulted from the very unequal sample sizes in each group producing greater variation in the number of oocytes introduced into each culture droplet. The occurrence of fertilized ova in all droplets of medium in the groups receiving pmsg alone eliminated the possibility that only those oocytes released from animals injected in pro-oestrus were fertilizable. The two-cell embryos in Exp. 1 were cultured for a further 72 hr
when 6/17 (35.3%) two-cell ova in the group receiving PMSG alone developed to the blastocyst stage (13/30 two-cell embryos had been removed for transfer to foster mothers at the four-cell stage) and 25/71 (35.2%) two-cell ova developed to blastocysts in the group undergoing superovulation. In the same group in Exp. 3, 23/29 two-cell ova (79.3%) developed into blastocysts.

To test the postimplantation viability and normality of the oocytes obtained following PMSG alone that had been fertilized in vitro, embryos were transferred at the two- and four-cell stages to the oviducts of suitably prepared recipient foster mothers. No superovulated oocytes fertilized in vitro were transferred in this experimental series since their viability has already been established (Whittingham, 1968). Six two-cell ova from Exp. 2 and 32 two-cell ova from Exp. 3 were transferred unilaterally to the left oviducts (5 to 7 ova/oviduct) of six pseudopregnant CFLP-strain females previously mated with vasectomized CFLP males, proven sterile, on Day 1 of pseudo-pregnancy (the day on which a copulation plug was found in the vagina) following the procedure described by Tarkowski (1959). In addition, 13 four-cell ova from Exp. 1 were transferred to the right oviducts of two CFLP mice on Day 1 of pregnancy (6 and 7 ova/oviduct, respectively). These females had been mated to fertile CFLP males.

On Day 15, the uterine contents of the ‘pseudopregnant’ group were examined. One female was found to be pregnant, and seven apparently normal fetuses were removed from the left uterine horn. Six of these had black eyes and one had pink eyes, the presence of black eyes excluding the possibility that they were native fetuses derived from a non-sterile mating. On Day 17, the uterine contents of the originally pregnant group were examined and one female was found to be pregnant. The left uterine horn contained four resorptions while the right horn (the side of egg transfer) contained one resorption, two apparently normal fetuses with black eyes and one with pink eyes. In this case, foreign fetuses with pink eyes would be indistinguishable from the native ones. These results demonstrate that oocytes released within 14 hr of an injection of PMSG can be fertilized in vitro and are capable of further development when transferred to suitably prepared recipient foster mothers even though the animals in which this ovulation has been induced will not mate unless injected at pro-oestrus (Stern & Schuetz, 1970).

The population of oocytes released within 14 hr of an exogenous gonadotrophic stimulus may be those destined either for ovulation at the next natural oestrus or for atresia if they remain unovulated. Since the number ovulated after injecting PMSG approximates to the number released at normal oestrus, the former would probably have been ovulated naturally at the next oestrus and not become atretic.

Further studies are in progress to determine the relationship between this population of oocytes induced to ovulate by the exogenous gonadotrophic stimulus and those destined for natural ovulation or atresia.

We thank Professor C. R. Austin for his help and criticism. The work was supported by a grant from the Ford Foundation. D.G.W. is a recipient of a Beit Memorial Fellowship. M.H.K. is a recipient of an M.R.C. Junior Research Fellowship.
REFERENCES


Non-Random Segregation during Mammalian Oogenesis

INVESTIGATIONS of the breeding performance and early embryogenesis of the XO female mouse by Cattanach and Morris were difficult to explain because the number of XO females born to XO mothers was considerably less than predicted. Cattanach suggested there was either a preferential loss of the chromosome sets lacking an X to the polar bodies during meiosis, or death during early embryonic development. Morris and others concluded that YO was inviable. The proportion of oocytes with and without an X chromosome was determined at metaphase II. The sex-linked Tabby gene was used as a marker in the XO mice, which were of similar stock to those used by Cattanach and Morris. XO females in succeeding generations were mated to +/y and Ta/y males. Ta/O females were superovulated and autopsied 16–20 h after the human chorionic gonadotrophin (HCG) injection. The freshly ovulated oocytes were liberated from the ampullae and ovarian oocytes from the larger intact follicles. Oocytes at the germinal vesicle stage were cultured for 18–24 h in modified Krebs-Ringer bicarbonate medium, after which most reached metaphase II. The metaphase II cells were placed in 1% sodium citrate solution for 10–20 min. Air-dried preparations were made by the method described by Tarkowski and stained with 2% Giemsa at pH 7.0 for 45 min. The slides were washed briefly with distilled water, allowed to dry and then dehydrated with xylol and mounted with 'Clearmount'.

Control female heterozygous Ta/+ mice were superovulated and autopsied 17–19 h after HCG. The oocytes were liberated and treated as described above.
The 145 preparations from XO mice and the 105 from the control mice were divided into three groups according to the source of the oocytes. (A) Ovulated oocytes removed from the ampullae. (B) Ovarian oocytes at metaphase II obtained by follicular puncture. (C) Germinal vesicle oocytes obtained by follicular puncture and cultured to metaphase II.

Table 1 shows the numbers of chromosomes present in each group. χ² analyses of numbers of oocytes with 19 or 20 chromosomes present in XO and control groups were carried out. Groups A and C showed significant differences (χ²(1) = 11.81, P<0.001; and χ²(1) = 8.14, P<0.005 respectively); B showed a trend in the same direction but not a significant difference. When the XO series was compared with a random distribution, A alone was significantly different (P<0.02—Fisher's exact test). The trend in groups B and C, although not statistically significant because of the small sample size, was also in the same direction. All three groups showed approximately twice as many oocytes with 20 chromosomes (X-bearing gametes) as those with 19 chromosomes (gametes without an X) in the XO series.

In XO female mice there is an unusually low (about 30%) segregation of gametes without an X chromosome in ovulated oocytes. This gives a considerably higher incidence of XX and XY than XO and YO offspring (approximately 2:2:1:1). This segregation gives ratios XO/XX = 0.5, and XO+XX/XY = 1.5. If YO leads to anomalous development then 1/6 or 16.7% of cleavage embryos should be grossly abnormal and inviable. (A YO karyotype has been seen at the first cleavage division (M. H. K., unpublished observations). Morris found XO/XX = 0.399 at weaning and XO+XX/XX = 1.45 at birth and 1.505 at weaning. He also described two types of abnormality in preimplantation 3.5 day embryos. One group

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of metaphase II preparations examined</th>
<th>Chromosomes &lt;16</th>
<th>Chromosome counts 17 18 19 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, Ovulated oocytes</td>
<td>90</td>
<td>24</td>
<td>3 1 2 19 41</td>
</tr>
<tr>
<td>B, Fresh ovarian oocytes</td>
<td>39</td>
<td>15</td>
<td>2 — 1 5 16</td>
</tr>
<tr>
<td>C, Cultured ovarian oocytes</td>
<td>16</td>
<td>1</td>
<td>1 — 1 5 8</td>
</tr>
<tr>
<td>Control group</td>
<td>105</td>
<td>34</td>
<td>3 — 3 4 61</td>
</tr>
</tbody>
</table>

* Preparations with inadequate separation of chromosomes.
occurred with equal incidence in the XO and XX litters, and probably accounted for the spontaneous level of preimplantation loss. The second group accounted for only 2% of the embryos from XX litters and 20% in the XO litters. If this group accounted for the whole class of YO zygotes and included a small loss of XO zygotes, the XO/XX ratio would increase from 0.399 at weaning to nearer the postulated 0.5 ratio occurring at fertilization. Cattanach suggested that non-random segregation of the chromosomes could account for the observations of the XO mouse.

Although there was no previous evidence in the Mammalia non-random segregation had been described in plants. Rhoades found that in Zea heterozygous for a terminal heterochromatic knob the homologue with the knob reached the functional embryo-sac in 70% of meioses. If the migration of the chromosome pair on the meiotic spindle had been random 50% would be expected. In flowering plants, meiotic preferential distribution of the B-chromosome occurs. These chromosomes appear as univalents and do not divide at anaphase but distribute themselves towards a privileged pole of accumulation. The male nucleus that receives the B-chromosome has the greater chance of fertilizing the egg. Seiler found that the behaviour of the X chromosome in Talaeoporia tubulosa was strongly influenced by the temperature level. With normal and subnormal temperatures the X most frequently passed into the polar body, while under high temperatures and in “over-ripe” eggs the X more often remained in the egg.

The XO mouse results seem to be unrelated to the phenomenon of “affinity” in which a linkage-like association between centromeres of different chromosomes results in their non-random segregation at meiosis causing chromosomes of the same ancestral origin to tend to travel to the same pole. The closeness of results to previously published breeding data suggests non-random segregation at the first meiotic division.

I thank Dr A. McLaren, Dr D. G. Whittingham, M. F. Lyon and B. M. Cattanach for discussion, and Professor C. R. Austin for his criticism of the manuscript. Dr W. K. Whitten provided the XO mice. The work was supported by a grant from the Ford Foundation. M. H. K. is an MRC junior research fellow.

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Received March 23, 1972.


10 Rhoades, M. M., Genetics, Princeton, 27, 395 (1942).
11 Battaglia, E., Caryologia, 17, 245 (1964).
12 Seiler, J., Arch. Zellforsch., 15, 249 (1921).
Parthenogenesis in the Mouse

In vitro treatment of mouse eggs with hyaluronidase can activate them to develop parthenogenetically as well as de-nuding them of cumulus cells. Activation and subsequent behaviour of the eggs depend on certain defined conditions. Szollosi observed morphological changes in unfertilized mouse eggs in vivo after approximately 14 h in the oviduct. Rotation and migration of the spindle towards the centre of the egg was the first and most obvious change. This prepares the egg for equal cleavage rather than the usual unequal division which results in the formation of the second polar body. Graham stimulated eggs 12-17 h after ovulation and obtained about 30% “immediate cleavage” in the activated group, whereas Tarkowski applied an electric shock to the exposed oviduct 2-4 h after ovulation and obtained activation in approximately 50% but a very low incidence of immediate cleavage. This suggested that the time of activation after ovulation might be important in the type of parthenogenones induced. Eggs were considered to have undergone immediate cleavage only if, at the time of examination, they were morphologically indistinguishable from a normal fertilized two-cell egg—that is, apart from the absence of a second polar body. They resulted from a biochemical or experimental stimulus other than that provided by fertilization. The few eggs with unequal blastomeres were therefore not included in this group.

(C57Bl x A2G)F1 females were superovulated and killed at intervals of 2 h, 14-20 h after injection of human chorionic gonadotrophin (HCG). (Ovulation occurs approximately 12 h after this injection.) Oocytes were released from the ampullae into a modified Krebs-Ringer bicarbonate culture medium containing 4 mg ml⁻¹ bovine serum albumin and 100 IU ml⁻¹ hyaluronidase (Koch-Light, ovine testes), and incubated at 37°C in 5% CO₂ in air. After 10 min the eggs were transferred to a hyaluronidase-free medium and culture continued for a further 6 h. Atretic and fragmented eggs were not transferred to the hyaluronidase-free medium as they were ovulated in this state, or resulting from the PMSG-induced ovulation.

Eggs were examined under the x50 magnification of a Wild dissecting microscope to determine the overall frequency and types of parthenogenones induced. These results are presented in Table 1 (groups 1 to 4). Results from a more extensive series in which mice were autopsied 18-20 h after injection of HCG.
are also presented in Table 1 (group 5). If the results of groups 1 to 5 are combined, 740/757 (97.8%) of the activated eggs had a single pronucleus and second polar body (the first very rarely persists), seven had two pronuclei (0.9%) and ten underwent immediate cleavage (1.3%). Further groups of mice were killed approximately 25 h after HCG and these results are presented in Table 1 (groups 6 and 7). Control eggs were cultured in hyaluronidase-free medium. No spontaneous activation was observed at the time of isolation of the eggs from the oviducts and only in the more aged groups was handling of the eggs sufficient to activate a proportion of the eggs in the control series. In the group 6 (where a fresh epididymal sperm suspension in the modified Krebs-Ringer bicarbonate culture medium described earlier was used as the stimulus) the overall frequency of activation was 73.2% (224/306), but the incidence of the various types of parthenogenones differed considerably from the previous series, with immediate cleavage in 77.7% (174/224). In group 7 (where hyaluronidase medium was used) a similar frequency of activation was obtained (75.4%, 288/382) and a slightly lower incidence of immediate cleavage (62.2%, 179/288).

These results place on a quantitative basis the ageing changes previously observed, and probably explain the high incidence of immediate cleavage observed in late insemination by Marston and Chang. This seems to be a more likely explanation than that spindle migration occurred as a direct result of the activation procedure as suggested by Braden and Austin, though this central migration might be accelerated by heat or other experimental stimuli. A low incidence of eggs with two haploid sets of chromosomes without extrusion of the second polar body was observed in this series. This may be explained by strain variation.

In another series of experiments where females were killed approximately 20 h after injection of HCG and eggs were added to a fresh epididymal sperm suspension (group 8), 774/1404 eggs were activated (55.1% overall activation frequency). The frequency of activation was 62.8% (558/888) when similar eggs were added to a sperm-free filtrate (group 9). When aged eggs were added to a sperm suspension (groups 6 and 8), certain factors in the suspension activated the eggs mechanically or biochemically. By filtering the sperm suspension through a 'Millipore' filter (group 9) I concluded that activation is not due to direct (mechanical) stimulation by sperm, but to a factor or factors released by sperm into the culture medium (for example hyaluronidase or other enzymes from the acrosomal region).

My results help to define the conditions needed for the experimental induction of different types of parthenogenones.
Table 1 Reaction of Eggs 6 h after Addition to Various Experimental Situations

<table>
<thead>
<tr>
<th>Group</th>
<th>Stimulating medium</th>
<th>Hours after HCG injection when females were killed</th>
<th>Total number of eggs</th>
<th>Activated eggs 1 pronucleus + 2nd polar body</th>
<th>Activated eggs 2 pronuclei</th>
<th>Immediate cleavage</th>
<th>Overall % activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hyaluronidase medium</td>
<td>14</td>
<td>172</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.0%</td>
</tr>
<tr>
<td>2.</td>
<td>Hyaluronidase medium</td>
<td>16</td>
<td>211</td>
<td>56</td>
<td>1</td>
<td>—</td>
<td>27.0%</td>
</tr>
<tr>
<td>3.</td>
<td>Hyaluronidase medium</td>
<td>18</td>
<td>117</td>
<td>89</td>
<td>—</td>
<td>—</td>
<td>76.1%</td>
</tr>
<tr>
<td>4.</td>
<td>Hyaluronidase medium</td>
<td>20</td>
<td>137</td>
<td>112</td>
<td>2</td>
<td>1</td>
<td>83.9%</td>
</tr>
<tr>
<td>5.</td>
<td>Hyaluronidase medium</td>
<td>18-21</td>
<td>689</td>
<td>483</td>
<td>4</td>
<td>9</td>
<td>72.0%</td>
</tr>
<tr>
<td>6.</td>
<td>Fresh sperm suspension</td>
<td>25</td>
<td>306*</td>
<td>—</td>
<td>50</td>
<td>(combined group)</td>
<td>73.2%</td>
</tr>
<tr>
<td>7.</td>
<td>Hyaluronidase medium</td>
<td>25</td>
<td>382*</td>
<td>96</td>
<td>13</td>
<td>179</td>
<td>75.4%</td>
</tr>
<tr>
<td>8.</td>
<td>Fresh sperm suspension (a)</td>
<td>18-20</td>
<td>1,404</td>
<td>756</td>
<td>34†</td>
<td>18</td>
<td>55.1%</td>
</tr>
<tr>
<td>9.</td>
<td>Sperm-free filtrate of (a)</td>
<td>18-20</td>
<td>888</td>
<td>537</td>
<td>9</td>
<td>12</td>
<td>62.8%</td>
</tr>
</tbody>
</table>

* Apparently normal one-cell eggs or immediately cleaved eggs as observed 6 h after addition to the stimulating medium. About 5% of eggs transferred to the hyaluronidase medium (and controls) in the 25 h group were fragmented at 6 h, and these are excluded from the total of group 7 to make the data comparable with those from group 6. No fragmentation was seen at 6 h after activation in the other time groups or in their controls.

† Possibly due to *in vitro* fertilization, so these eggs are not included in calculation of activation frequency.
The critical factor is clearly the age of the oocyte when the stimulus is applied.

Varying the osmolarity of the culture medium also results in different types of parthenogenone\(^3\),\(^4\), an effect that may possibly arise through changes resembling those that accompany ageing.

I thank Professor C. R. Austin for his criticism of the manuscript. The work was supported by a grant from the Ford Foundation. M. H. K. is an MRC junior research fellow.

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Received December 4, 1972; revised January 9, 1973.

TIMING OF THE FIRST CLEAVAGE DIVISION OF THE MOUSE AND THE DURATION OF ITS COMPONENT STAGES: A STUDY OF LIVING AND FIXED EGGS

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SUMMARY

Fertilized mouse eggs were examined between 27 and 34 h after the superovulating injection of human chorionic gonadotrophin (HCG). Out of 1334 eggs examined, 432 were already at the 2-cell stage; the remaining 902 at the 1-cell stage were examined in detail. All chromosome preparations of the first cleavage mitosis were classified into groups corresponding with the stages of prometaphase (early and late), metaphase (early or 'prechromatid', 'chromatid' and 'late chromatid') and anaphase.

An indirect estimate was made of the duration of the first cleavage mitosis and of its component stages from the incidence of stages observed at different time intervals after the HCG injection. Fertilized eggs were also observed at 37 °C by time-lapse cine-photography and the interval between the disappearance of the pronuclei and the beginning of telophase of the first cleavage division was determined. The progress of eggs fertilized in vitro was also observed under normal culture conditions. A close correlation was observed between the indirect method of assessing the mitotic time and the direct values obtained from the studies on time-lapse and in vitro culture. The effect of temperature on the mitotic time was also examined by the time-lapse method.

INTRODUCTION

Analysis of the chromosomal events of the first cleavage division of the mouse (Tarkowski, 1966; McGaughey & Chang, 1969, 1971; Donahue, 1972a, b) has demonstrated its accessibility to study and its variations from subsequent mitotic divisions (Hughes, 1952; Mazia, 1961). Descriptions of the events occurring during this mitotic division in the mouse are as yet rather incomplete though the general morphology has recently been demonstrated by the examination of whole-mount preparations (Donahue, 1972b). This method has certain advantages over the various air-drying procedures available, which are usually only minor modifications of Tarkowski's (1966) technique involving hypotonic pre-treatment of eggs prior to their fixation. Air-drying, however, allows a much more detailed analysis of the stages of the mitotic cycle and is invaluable for any detailed assessment of the events occurring during metaphase of the first cleavage division. This investigation was therefore carried out with the air-drying technique to examine the time course in vivo of the mitotic stage of the first cleavage division in CFLP (Carworth, Europe) mice. A further indirect estimate of the total duration of the first cleavage mitosis was made in $F_1$ hybrid females ($(C_{57} Bl \times A_2 G) F_1$) mated to $F_1$ males. Observations were made
on eggs flushed from the oviducts of females killed between 28 and 31 h after the superovulating injection of HCG. The disappearance of the pronuclei prior to syngamy and the progress of eggs through the first cleavage division were also examined by time-lapse photography at 37 °C with mice of the same strain.

Further eggs were examined by the time-lapse method when the temperature of the system was varied. In this series of investigations CFLP females were mated with F1 males.

A further series of experiments was carried out with the technique of in vitro fertilization, where the time of addition of eggs to the sperm suspension gives an exact starting-point for the purpose of timing studies. In these experiments F1 eggs were fertilized in vitro by F1 sperm and observations made on the time of disappearance of the pronuclei. The values obtained from the various methods of investigation are compared.

**Materials and Methods**

**Fixed preparations**

Female CFLP mice 8–10 weeks old were superovulated with 10 I.U. pregnant mares’ serum gonadotrophin (PMSG) followed at a 48-h interval by 10 I.U. of HCG, and paired with fertile CFLP males shortly after the HCG injection. Females that had mated were killed between 27 and 34 h after the HCG injection, and the eggs flushed from the oviducts. Air-dried chromosome preparations of all 1-cell eggs were made by the method described by Tarkowski (1966) and stained with 2% Giemsa (Giemsa stain R 66, G. T. Gurr) at pH 7.0 for 1 h. The number of 2-cell eggs present was also recorded. Slides were then dehydrated and mounted with Clearmount (E. Gurr Ltd.). Each chromosome preparation was classified according to its mitotic stage. A brief description of the classification used follows this section. Fig. 2 (p. 824) presents the regressions of percentage of eggs with pronuclear breakdown (1-cell eggs in mitosis, plus 2-cell eggs) and 2-cell eggs observed in this series against various times after the injection of HCG.

**In vitro fertilization**

F1 mice 8–10 weeks old were superovulated as described above and killed between 13 and 14 h after the HCG injection. The oviducts were removed and the eggs released by tearing open the wall of the dilated ampullar region with fine watchmaker’s forceps. The eggs were released directly into 0.5 ml of a freshly prepared suspension of F1 epididymal spermatozoa containing approximately 1500–3000 sperm per µl under light liquid paraffin in solid watchglasses, and incubated at 37 °C in a continuous flow of humidified 5% CO2 in air. This is similar to the method of Whittingham (1968) except that epididymal instead of uterine spermatozoa were used. The medium used for the sperm suspension was a modified Krebs-Ringer bicarbonate medium (Whittingham, 1971) containing crystalline bovine serum albumin at a concentration of 32 mg per ml, while that used for washing the eggs and their continued culture was similar in composition but contained 4 mg of bovine serum albumin per ml.

After approximately 6 h the eggs were removed from the sperm suspension, washed in fresh medium, and eggs with 2 obvious pronuclei separated into batches of 10 in 30–50 µl droplets of medium under light liquid paraffin in plastic Petri dishes, to facilitate further observation. The Petri dishes were returned to the incubator, and culture continued. Eggs were examined at ×50 magnification with a Wild dissecting microscope at hourly intervals from 15 h onwards after the addition of eggs to the sperm suspension. After disappearance of the pronuclei eggs were removed from the droplets and examined by the air-drying technique to confirm their diploid status. In this series the fertilization rate was 85±1% and observations were made on 97 diploid eggs (obtained from 4 females).
Timing of the first cleavage mitosis

Indirect estimate of mitotic time in eggs from F₁ females mated to F₁ males

F₁ female mice 8-10 weeks old were superovulated as described above and paired with F₁ males shortly after the HCG injection. Females that had mated were killed between 28 and 31 h after the HCG injection and the eggs flushed from the oviducts. The total number of eggs ovulated and the number of 1-cell eggs with and without pronuclei and those at the 2-cell stage were recorded. Fig. 3 (p. 805) represents the percentage of total eggs showing stages later than pronuclear breakdown (1-cell eggs in mitosis, plus 2-cell eggs) and of those at the 2-cell stage, observed in this series at various times after the injection of HCG. In this series 21 females were killed, and observations made on 739 eggs.

Time-lapse analysis of living eggs

A further group of mice (prepared as described in the first paragraph of Materials and Methods) was killed between 27 and 28 h after the HCG injection. The eggs were flushed from the oviducts and those with 2 obvious pronuclei were rapidly transferred into a micro-drop of modified Krebs-Ringer bicarbonate medium (Whittingham, 1971) under 4 ml of light liquid paraffin in a plastic Petri dish. The dish rested on a Leitz heating stage and was gassed with a prewarmed and humidified mixture of 95% air and 5% CO₂. The heating stage could be controlled to within ±0.25 °C. A Bolex 16-mm camera linked to a Paillard-Wild time-lapse unit and Kodak 16-mm high-contrast film gave excellent results with an exposure duration of 0.4 s and a 30-s interval between frames, and all filming was carried out with phase-contrast optics. Fig. 1 illustrates the apparatus used.

The progressive changes in eggs were checked every 1-2 h against a control dish kept under similar conditions of gassing in an incubator at 37 °C. All eggs passing to the 2-cell stage were noted, and the film inspected under a Wild dissecting microscope (M 3) at low magnification. Further groups of eggs but from CFLP females mated to F₁ males were observed under similar conditions of continuous gassing, with the heating stage equilibrated at 34.25, 37 and 39 °C (±0.25 °C in each case).
Table 1. Analysis of mitosis in eggs undergoing fertilization and observed at various times between 27 and 34 h after the superovulating injections of HCG

<table>
<thead>
<tr>
<th>Hours after HCG</th>
<th>I</th>
<th>IIa</th>
<th>IIb</th>
<th>III</th>
<th>IV</th>
<th>Va</th>
<th>Vb</th>
<th>V (combined)</th>
<th>2-cell</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>27°0</td>
<td>49</td>
<td>2</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>2</td>
<td>56</td>
</tr>
<tr>
<td>28°0</td>
<td>92</td>
<td>5</td>
<td>15</td>
<td>13</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>104</td>
</tr>
<tr>
<td>28.75</td>
<td>52</td>
<td>6</td>
<td>10</td>
<td>32</td>
<td>8</td>
<td>4</td>
<td>5</td>
<td>42</td>
<td>22</td>
<td>281</td>
</tr>
<tr>
<td>29.25</td>
<td>98</td>
<td>15</td>
<td>27</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>8</td>
<td>29</td>
<td>154</td>
<td>374</td>
</tr>
<tr>
<td>30.5</td>
<td>128</td>
<td>51</td>
<td>65</td>
<td>18</td>
<td>18</td>
<td>25</td>
<td>83</td>
<td>374</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>32.0</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>10</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>80</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>33.0</td>
<td>3</td>
<td>1</td>
<td>8</td>
<td>16</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>96</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>34.0</td>
<td>—</td>
<td>—</td>
<td>5</td>
<td>2</td>
<td>—</td>
<td>1</td>
<td>2</td>
<td>95</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>491</td>
<td>35</td>
<td>118</td>
<td>167</td>
<td>26</td>
<td>45</td>
<td>20</td>
<td>65</td>
<td>432</td>
<td>1334</td>
</tr>
</tbody>
</table>

Classification of the stages in the first cleavage mitosis

In this classification of the stages in the first cleavage mitosis (based on that of McGaughey & Chang, 1971), each stage is fairly precisely delineated, allowing analysis of observations derived from air-dried chromosome preparations. This procedure permits a clearer understanding of the more crowded events occurring during metaphase, namely the early (III), intermediate (IV) and late (Va) stages. From an analysis of the data presented in Tables 1 and 2 the time relations of the various events have been examined.

Stage I included eggs with lightly staining pronuclei where no condensed chromatin was demonstrable.

Stage IIa included eggs at the late pronuclear or early prometaphase stage where stainable chromatin fibres were present though filamentous, and it was generally impossible to distinguish between the individual elements.

Stage IIb or late prometaphase included eggs where 2 individual haploid groups of chromosomes could be identified. These groups were usually slightly asynchronous in their degree of chromosomal condensation.

Stage III early metaphase or 'syngamy' where the 2 haploid groups have united on the spindle equator and have achieved a synchronous degree of chromosomal condensation.

Stage IV the 'chromatid' stage of metaphase where all the chromosomes were divided longitudinally into 2 morphologically identical chromatids.

Stage Va or late metaphase included eggs where the 2 chromatids were seen to be separating or had completely separated. The chromatids were probably still present on the spindle equator, as all preparations at this stage appeared as a single group of 80 chromatids.

Stage Vb or anaphase included eggs where the group of 80 chromosomes had separated into 2 groups of 40 chromatids, and were observed to be migrating towards or had reached the poles of the spindle.

RESULTS

Duration of the first cleavage mitosis from observations on fixed preparations

An analysis of stages of mitosis in eggs undergoing fertilization from CFLP females mated to CFLP males, observed at various times between 27 and 34 h after the superovulating injection of HCG, is presented in Table 1. When these data are re-expressed
Timing of the first cleavage mitosis

Table 2. Data from Table 1 rewritten to show the proportionate incidence of the various stages in each time group

<table>
<thead>
<tr>
<th>Hours after HCG</th>
<th>Stages of the first cleavage division</th>
<th>No. of females per group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>IIa</td>
</tr>
<tr>
<td>27:0</td>
<td>0:875</td>
<td>0:036</td>
</tr>
<tr>
<td>28:0</td>
<td>0:885</td>
<td>—</td>
</tr>
<tr>
<td>28:75</td>
<td>0:565</td>
<td>0:054</td>
</tr>
<tr>
<td>29:25</td>
<td>0:488</td>
<td>0:030</td>
</tr>
<tr>
<td>29:75</td>
<td>0:442</td>
<td>0:032</td>
</tr>
<tr>
<td>30:5</td>
<td>0:342</td>
<td>0:040</td>
</tr>
<tr>
<td>32:0</td>
<td>0:010</td>
<td>0:010</td>
</tr>
<tr>
<td>33:0</td>
<td>0:022</td>
<td>0:007</td>
</tr>
<tr>
<td>34:0</td>
<td>—</td>
<td>0:048</td>
</tr>
<tr>
<td>Total</td>
<td>3:629</td>
<td>0:209</td>
</tr>
</tbody>
</table>

Proportionate length of each stage, and its duration in min, as derived from these data:

- 0:089 | 0:302 | 0:381 | 0:062 | 0:114 | 0:051 | 0:999
- 10:4 | 35:3 | 44:6 | 7:3  | 13:3 | 5:9  | 116:8 min

* A, prometaphase; † B, stages at which chromosomes lie on equator of metaphase spindle; ‡ C, anaphase.

The total duration of the first cleavage mitosis (M) was calculated to be 117 min by the method described in the Appendix (p. 807), from which may be derived the duration of prometaphase (45.7 min), the duration of the stages when the chromosomes are present on the equator of the spindle (65.2 min) and time elapsed from anaphase to early telophase (5.9 min). These values represent the mean duration from an assessment of 902 1-cell preparations. The 50% point for eggs entering the first cleavage mitosis (29.56 h after the HCG injection) and the 2-cell stage (31.51 h after the HCG injection) in this series has been calculated from Fig. 2.

In vitro fertilization

Data from observations on eggs fertilized in vitro (F1 × F1) enabled the 50% point for eggs entering the first cleavage mitosis to be calculated. This point was achieved 17:48 h after the eggs were added to a sperm suspension. The comparable value obtained from the in vivo series was 17:56 h after ovulation (taken as HCG + 12 h) when CFLP females were mated to CFLP males (see first section of Results).
Fig. 2. Regressions of percentage of CFLP x CFLP eggs with pronuclear breakdown, and at the 2-cell stage, at various times after the injection of HCG.

**Indirect estimate of duration of mitosis from data on eggs fertilized in vivo**

Data from observations on eggs fertilized *in vivo* \((F_1 \times F_1)\), presented in graphical form in Fig. 3, enabled the 50% points to be calculated for eggs entering the first cleavage mitosis \((28-16\;\text{h after the HCG injection})\) and the 2-cell stage \((29-81\;\text{h after the HCG injection})\).

**Duration of the first cleavage mitosis as derived from analyses of time-lapse cinemomicrographic records**

Fertilized eggs from CFLP females mated to CFLP males were observed over a period of 8-10 h at \(37 \pm 0.25\;\text{°C}\). As the interval between frames was 30 s the starting-point of mitosis (when the 2 pronuclei disappeared from view) and the end-point (the beginning of telophase) could be assessed with considerable accuracy, namely to within 1 or 2 frames in nearly all cases. In a few eggs it was technically impossible to determine either the beginning or end-point (because the pronuclei were out of the plane of focus or because the cleaving egg was viewed end-on and the cleavage furrow could not be observed). These were excluded from the series. Twelve eggs progressed to the 2-cell stage in \(114.9 \pm 2.3\;\text{min}\).

**Time-lapse observations on the effect of temperature**

The duration of mitosis was calculated in a similar manner when fertilized eggs from CFLP females mated to \(F_1\) males were observed at \(34.25, 37\) and \(39\;\text{°C} \left(\pm 0.25\;\text{°C}\right)\).
Timing of the first cleavage mitosis

Fig. 3. Regressions of percentage of \( F_1 \times F_1 \) eggs with pronuclear breakdown, and at the 2-cell stage, at various times after the injection of HCG.

Fig. 4. The effect of temperature on the duration of the first cleavage mitosis.

Table 3. A comparison between the time of entry into mitosis and its duration in fertilized eggs of CFLP and \( F_1 \) mice

<table>
<thead>
<tr>
<th>Strains examined</th>
<th>Time of entry into mitosis, 50% time after HCG injection, h</th>
<th>Time of cleavage, 50% time after HCG injection, h</th>
<th>Total duration of mitosis, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFLP ( \times ) CFLP</td>
<td>Indirect estimate 29.56 -</td>
<td>Indirect estimate 31.51 -</td>
<td>Indirect estimate 117 114.9 ± 2.3</td>
</tr>
<tr>
<td>CFLP ( \times F_1 )</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>( F_1 \times F_1 )</td>
<td>Indirect estimate 28.16 17.48* -</td>
<td>Indirect estimate 29.81 -</td>
<td>Indirect estimate 99 100.4 ± 1.8</td>
</tr>
</tbody>
</table>

* 50% time (h) after eggs added to sperm suspension (in vitro fertilization).
The duration of mitosis was shown to be temperature dependent. At 34.25 °C the duration was 153.5 ± 4.3 min (14 eggs); at 37 °C, 109.4 ± 1.8 min (7 eggs); and at 39 °C, 101.4 ± 3.5 min (8 eggs). This information is presented in graphical form in Fig. 4.

Table 3 summarizes all our results at 37 °C, and provides a comparison between the time of entry into mitosis and its duration in fertilized eggs of CFLP and F1 mice.

**DISCUSSION**

A detailed classification of the various stages of the first cleavage mitosis has allowed an estimate to be made of their individual duration. As no comparable data were available on the exact timing of this division, time-lapse analysis of similar material allowed a direct estimate to be made of the total duration of this mitosis as it progresses *in vitro*.

Evidence from fixed material in the first part of this investigation suggested that the disappearance of the male and female pronuclei was closely correlated with the earliest stage of prometaphase, where the chromosomes first become apparent as elongated filamentous strands.

Under normal circumstances sperm penetration and egg activation probably occurs quite soon after ovulation. If the span of ovulation is taken into account, the time from sperm penetration to the first cleavage mitosis in mouse eggs is remarkably constant, with only slight inter-strain variation. Once mitosis has begun there seems to be little variation in its overall duration between eggs at this stage of development, and this degree of synchrony is maintained through the second and third cleavage divisions. Later in development intermitotic times vary considerably between cells of the same and different tissues, whereas the mitotic time probably remains fairly constant. In mouse spleen cells in culture, for example, the nuclear structure was beautifully clear in those cells that were expanded flat on the coverglass, and the overall duration of mitosis was found to be 43–90 min. The duration of the mitotic stages was also recorded, namely prophase 20–35 min, metaphase 6–15 min, anaphase 8–14 min and telophase 9–26 min (Hughes, 1952).

The intermitotic intervals were also recorded in similar cells in culture and these ranged from 8 to 18 h, with a mode of 9 h (Fell & Hughes, 1949).

While the papers by Fell & Hughes (1949) and Hughes (1952) and the present report all deal with mitosis in the mouse, the subject material of the previous authors was a somatic mitosis, and the present investigation was concerned with the first cleavage mitosis, which is in many respects atypical even when compared to subsequent cleavages in preimplantation embryos. Differences in the durations of component stages might well, therefore, be expected.

The duration of the first cleavage mitosis of approximately 2 h has been demonstrated by several means, from indirect *in vivo* and direct *in vitro* estimates, the *in vitro* work involving time-lapse and culture studies. The agreement was very close, and the duration of mitosis in the 2 strains examined is also very similar. Since the time of ovulation after HCG is similar in both strains (onset approximately 11–12 h
and completion approximately 13 h after HCG) the difference in time of onset of mitosis probably corresponds to the faster penetration of the $F_i$ spermatozoon compared to the CFLP spermatozoon, while the in vitro fertilization data with epididymal sperm suggest a delay for capacitation.

When eggs were examined by time-lapse at temperatures above and below 37 °C, the duration of mitosis was shown to be temperature dependent within the range 34.25–39 °C ($\pm 0.25 °C$), and the regression (Fig. 4) of mitotic time against temperature is very close to that expected from simple chemical kinetics, where a fall of 10 °C results approximately in a doubling of the reaction time.

An indirect assessment of the duration of each stage in the first cleavage mitosis is presented in this paper. It is not at present practicable to obtain a direct estimate in the living mouse egg. To do this would necessitate a more sophisticated optical system than standard phase-contrast microscopy, and other refinements designed to ensure a final resolution comparable to that obtained with fixed or flattened living cells; these things depend upon technical advances that are yet to be made. Observations at higher magnification than used in this analysis will probably require continuous monitoring to maintain the spindle apparatus in focus throughout the period of filming and this may prove to be technically difficult, and the final resolution only fractionally as good as observation on flattened cells in tissue culture.

**APPENDIX**

The total duration of the first cleavage mitosis ($M$) was calculated from the data presented in Table 2 (p. 803) by determining firstly the time from the HCG injection to the mid-point of stage IIa by the following means:

$$\frac{27.0 (0.036) + 28.0 (0) + 28.75 (0.054) \ldots}{(0.036) + (0) + (0.054) \ldots} = 29.31 \text{ h.}$$

By similar means, the mid-point of stage V (combined) = 31.01 h after the HCG injection. The difference of 1.70 h or 102 min represents

$$(IIa/2) + IIb + III + IV + (V/2),$$

since the number of preparations of the various stages of mitosis observed in the whole series is directly proportional to the mean duration of each stage. It follows that:

$$M[(0.089/2) + 0.302 + 0.321 + 0.062 + (0.165/2)] = 102 \text{ min},$$

i.e.

$$M \times 0.872 = 102, \text{ therefore } M = 102/0.872 = 117 \text{ min.}$$

However, stage V is further divided into 2 substages Va and Vb in the proportion of 45:20, so that the duration in minutes of each stage is that shown in Table 2 (p. 803).

I would like to thank Professor C. R. Austin for his criticism of the manuscript and Dr D. E. Walters of the A.R.C. Statistics Group for statistical advice. The work was supported by a grant from the Ford Foundation. The author is a recipient of an M.R.C. Junior Research Fellowship.
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(Received 12 October 1972)
TIMING OF THE FIRST CLEAVAGE DIVISION OF HAPLOID MOUSE EGGS, AND THE DURATION OF ITS COMPONENT STAGES

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SUMMARY

Mouse eggs were activated by treatment with hyaluronidase which removed the follicle cells, followed by culture in vitro, and examined at the first cleavage mitosis. Second polar body extrusion usually occurred and haploid parthenogenesis was initiated. Air-dried chromosome preparations were made between 11 and 15.5 h after activation. Out of the 308 eggs examined 74 had already progressed to the 2-cell stage; the remaining 234 at the 1-cell stage were examined in detail. All chromosome preparations of the first cleavage mitosis were classified into groups corresponding with the stages of prometaphase, metaphase (early or 'pre-chromatid', 'chromatid' and 'late chromatid') and anaphase.

An indirect estimate was made of the duration of the first cleavage mitosis and of its component stages from the incidence of stages observed at different time intervals after activation. Similar eggs were also observed at 37 °C by time-lapse cine-photography and the interval between the disappearance of the pronucleus to the beginning of telophase of the first cleavage division was determined. The results of timing studies on the haploid eggs were compared with results obtained from similar observations on the first cleavage division of fertilized eggs which would of course normally be diploid.

Artificially activated eggs with 2 pronuclei, resulting from second polar body suppression, were also examined, and serial chromosome preparations during mitosis showed that the 2 pronuclear chromosome groups unite on the first cleavage spindle and divide to give a heterozygous diploid 2-cell embryo.

INTRODUCTION

It is possible to activate mouse eggs by various experimental means, for example, by the electrical stimulation of the ampullar region of the oviduct containing ova (Tarkowski, Witkowska & Nowicka, 1970), and by the removal of the cumulus cells with hyaluronidase, followed by culture in vitro (Graham, 1970, 1971, 1972; Kaufman, 1973b). The advantages and disadvantages of these methods have recently been reviewed by Tarkowski (1971); previous work on vertebrates has been critically reviewed by Beatty (1957, 1967) and mammalian work by Austin (1961) and Fechheimer (1968) and some of the mechanisms leading to parthenogenetic and heteroploid development have also recently been discussed (Beatty, 1972).

The development and cell numbers of diploid and polyploid embryos at day 3.5 were investigated by Beatty & Fischberg (1951), and their general conclusion was that the mean number of cells in polyploid embryos relative to diploids was approximately inversely proportional to the ploidy of the embryo. Edwards (1958) repeated this work but considered the cleavage number to be a more useful guide for comparing develop-
mental stages. However, in Edwards's analysis haploids as well as diploids were examined. Gynogenetic haploids recovered after irradiation or nitrogen mustard treatment of the sperm were all very retarded when compared to diploids, while very few haploids recovered after colchicine treatment of the eggs prior to insemination were observed to have cleaved at all. All of the gynogenetic haploids showed signs of abnormal cleavage, so that the relationship between ploidy and cleavage rate did not appear to relate to haploids. Edwards suggested that these differences might be due to a delay in the onset of the first cleavage division rather than different rates of cleavage between haploids (and triploids) when their development at day 3.5 was compared to diploids.

Tarkowski et al. (1970) found that the development of parthenogenetic embryos was retarded in the preimplantation period, and that the normal appearance of the embryos did not seem to be related to their degree of ploidy. The causes of the apparent failure of development of parthenogenetic embryos would seem to be multifactorial (Tarkowski, 1971; Graham, 1971) but it is possible that asynchrony with the uterus at the time of, or just after, implantation may be a very important component. It was with this in mind that the present investigation was initiated, to compare the time course of events at the first cleavage division of the haploid parthenogenone to confirm or deny that this division is delayed compared to similar events in fertilized eggs.

During the course of this and previous investigations on the activation of mouse eggs (Kaufman, 1973b) large numbers of haploid chromosome preparations were examined by the air-drying technique at all stages of the first cleavage mitosis. As a result of this it was possible to make observations on the normal chromosomal morphology in well spread preparations. The main stages of this mitosis are described and illustrated as an aid to those who are unfamiliar with the morphology of this atypical mitotic division.

In addition to the haploid parthenogenones, which constituted about 98% of the activated eggs (of those activated between 18 and 21 h after the HCG injection of superovulation), other types of parthenogenones were also obtained. About 1% of the activated eggs had 2 pronuclei resulting from second polar body suppression or failure of its extrusion, and these eggs were examined in detail prior to their first cleavage division. Because of the controversial nature of their fate, this aspect is discussed in some detail, and evidence presented to suggest that they develop into heterozygous diploid parthenogenones instead of undergoing a form of 'delayed immediate cleavage' to give haploid 2-cell embryos as suggested by Graham (1971, 1972).

MATERIALS AND METHODS

Activation of eggs

Female (C57Bl × A2G)F1 mice 8–10 weeks old were superovulated with 10 I.U. pregnant mare's serum gonadotrophin (PMSG) followed at a 48-h interval by 10 I.U. of human chorionic gonadotrophin (HCG), and killed between 19 and 21 h after the HCG injection (ovulation occurs approximately 12 h after the HCG). Oocytes were liberated from the ampullae into a modified Krebs-Ringer bicarbonate culture medium containing 4 mg/ml bovine serum albumin (Whittingham, 1971) and 100 I.U./ml hyaluronidase (Koch-Light, ovine testes), and incubated at 37 °C in 5% CO2 in air. After 10 min the eggs were isolated from this medium and
transferred to hyaluronidase-free medium and culture continued for a further 6 h. Eggs were examined under the ×50 magnification of a Wild dissecting microscope to determine the overall frequency and types of parthenogenones induced. Approximately 98% of the activated eggs in this time group had a single pronucleus and second polar body (Route 1 a, haploid parthenogenesis, Beatty, 1957) and the overall activation frequency was in the region of 75–80% (Kaufman, 1973 b).

All eggs with a single pronucleus and second polar body were washed in fresh medium and separated into 8 approximately equal batches. Each batch of eggs was transferred to a 30–50 μl droplet of medium under light liquid paraffin in separate plastic Petri dishes to facilitate further observation. The Petri dishes were returned to the incubator and culture continued. Single Petri dishes were then removed from the incubator at regular intervals from 11 to 15 h after the time of addition of eggs to the hyaluronidase medium (taken as time zero) and the eggs examined under the × 50 magnification of the dissecting microscope to determine the number of 1-cell and 2-cell eggs present. On average 8–10 eggs were present in each droplet for each experiment, the whole experiment was repeated on 4 occasions, and the results from each time interval were pooled.

Fixed preparations. All 1-cell eggs were isolated from the droplets of medium, washed in fresh medium and air-dried chromosome preparations made by the method described by Tarkowski (1966) and stained with 2% Giemsa (Giemsa stain R66, G. T. Gurr) at pH 7.0 for 1 h. Each chromosome preparation was classified according to its mitotic stage. A brief description of the classification used follows this section, and typical chromosome preparations appropriate to these stages are illustrated (Figs. 2–6).

In vitro fertilization and analysis by time-lapse cine-photomicrography of living eggs. Detailed descriptions of the methods involved in these 2 operations have been published elsewhere (Kaufman, 1973 a). However, only one aspect of the in vitro fertilization results has been reported previously, namely the 50% point for their entry into mitosis, which occurred at 17.48 h after the addition of eggs to the sperm suspension. The regression line based on the data for rate of entry of these eggs into mitosis is given here for the first time, and the comparable line for the haploid is presented in Fig. 1 (p. 557), and allows a comparison to be made between the timing of the 2 in vitro systems.

Classification of the stages in the first cleavage mitosis

This classification is based on that of McGaughey & Chang (1971), and has been reported in Kaufman (1973 a). Because each stage is fairly precisely delineated, it allowed detailed analysis of observations derived from air-dried preparations. Figs. 2–8 illustrate some of the stages.

RESULTS

Duration of the haploid first cleavage mitosis from observations on fixed preparations

An analysis of the stages of mitosis in 308 eggs progressing through the first cleavage mitosis to the 2-cell stage observed at various times between 11 and 15.5 h after their addition to the hyaluronidase medium is presented in Table 1. When these data are re-expressed to show the proportion of eggs at the various stages in each time interval, they provide the information given in Table 2.

The total duration of the first cleavage mitosis was calculated to be 155.7 min by the method described previously (Kaufman, 1973 a). From the overall duration the proportionate lengths of each stage can be derived, so that the duration of prometaphase was calculated to be 10.3 min, the early stage of metaphase (stage III), 77.9, the 'chromatid' or intermediate stage of metaphase (IV), 36.5, the 'late chromatid' stage (Va), 10.3, and anaphase, 20.7 min.
Table 1. Numbers of eggs at different stages of mitosis when observed at various times between 11 and 15.5 h after activation

<table>
<thead>
<tr>
<th>Hours after induction</th>
<th>Stage of the first cleavage mitosis</th>
<th>Total eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>IIa</td>
</tr>
<tr>
<td>11</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>11.75</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>12.25</td>
<td>23</td>
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</tr>
<tr>
<td>13</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
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<td>1</td>
</tr>
<tr>
<td>14.5</td>
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<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>85</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 2. Data from Table 1 rewritten to show the proportionate incidence of the various stages in each time group

<table>
<thead>
<tr>
<th>Hours after induction</th>
<th>Stage of the first cleavage mitosis</th>
<th>Total eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>IIa</td>
</tr>
<tr>
<td>11</td>
<td>1.000</td>
<td>0</td>
</tr>
<tr>
<td>11.75</td>
<td>0.861</td>
<td>0.111</td>
</tr>
<tr>
<td>12.25</td>
<td>0.590</td>
<td>0.077</td>
</tr>
<tr>
<td>13</td>
<td>0.250</td>
<td>0.021</td>
</tr>
<tr>
<td>14</td>
<td>0.047</td>
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<td>14.5</td>
<td>0</td>
<td>0</td>
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<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>0.232</td>
</tr>
<tr>
<td>Proportionate length of each stage, and its duration, min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.3</td>
<td>77.9</td>
</tr>
<tr>
<td>Duration of comparable stages in fertilized eggs</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.4</td>
<td>79.9</td>
</tr>
</tbody>
</table>

* Data in Kaufman (1973a).

Duration of the haploid first cleavage mitosis from analyses of time-lapse cine-photomicrographic records

Activated haploid eggs were observed over a period of 8-10 h at 37 ± 0.25 °C from about 10 h after the inducing stimulus. As the interval between frames was 30 s the starting point of mitosis (when the pronuclear outline disappeared from view), and the end point (the beginning of telophase) could be assessed with considerable accuracy,
namely to within 1–2 frames in nearly all cases. In a few eggs it was technically impossible to determine either the beginning or end point (because the pronucleus was out of the plane of focus or because the cleaving egg was viewed end-on and the cleavage furrow could not be observed). These were excluded from the series.

Twenty-four eggs progressed to the 2-cell stage in a mean time of 145.7 ± 1.8 min.

**Timing studies on in vitro fertilization**

Previously published data (Kaufman, 1973a) from observations on (C57Bl × A2G)F1 eggs fertilized in vitro by (C57Bl × A2G)F1 sperm enabled the 50% point for eggs entering the first cleavage mitosis to be calculated. This point was achieved 17.48 h after the eggs were added to the sperm suspension. The comparable data for the activated group (see the first section, above) was 12.5 h after activation. The regression lines for the rate of entry into mitosis in the 2 groups are shown in Fig. 1.

**The fate of parthenogenetic eggs with 2 pronuclei**

In addition to the haploid parthenogenones already described, eggs with 2 pronuclei were also encountered (these resulted from second polar body suppression after anaphase) and about 20 examined by the air-drying technique at the first cleavage mitosis.

To avoid confusion it is necessary to subdivide Beatty’s (1957) route 1 c, leading to diploid parthenogenesis, into 2 groups. In group 1 c i, a single diploid female pronucleus is formed as a result of suppression of the 2nd polar body, and this was the pathway taken by rabbit eggs subjected to a temperature of 10 °C for 24 h (Chang, 1954). In
the second group, $1c\, ii$, suppression of the second polar body results in the formation of 2 haploid pronuclei, and the fate of these pronuclei is now discussed. Observation showed that these haploid groups act in the same way as the male and female pronuclear groups at fertilization by uniting on the first cleavage spindle, subsequently dividing at about the same time as in haploid parthenogenones to give diploid heterozygous embryos.

Graham (1971) isolated 18 of type $1c\, ii$ parthenogenones 8 h after in vitro activation and made 2-hourly observations on them. He noted that the 2 pronuclei came together but did not form a common metaphase plate and that the 2 pronuclei eventually moved apart before cleavage occurred. Chromosome analysis on similar eggs (Graham, 1972) after their overnight culture in colcemid further suggested that haploid embryos resulted from this abnormal division as all 6 eggs examined had 2 groups of 20 chromosomes each (in 2 eggs one group contained 20 chromosomes while the other had 19 chromosomes). Such a result would, however, be expected even if fertilized eggs were subjected to colcemid treatment before the first cleavage spindle was allowed to form, as was the case with these eggs, and a colcemid environment maintained prior to chromosome analysis.

A few eggs of this type were obtained in addition to the haploid parthenogenones which were the main products of activation in the present series (Kaufman, 1973b) and half-hourly observations made on them. Air-dried preparations were made on these eggs at varying time intervals after the 2 pronuclear outlines were observed to disappear. In this way serial chromosome preparations confirmed that the 2 pronuclear chromosome groups unite on the first cleavage spindle, and divide to give a diploid 2-cell embryo. Chromosome preparations of the critical stages of prometaphase and the 'chromatid' stage of metaphase in these embryos are illustrated in Figs. 7 and 8, respectively.

**DISCUSSION**

The timing of the stages of the first cleavage mitosis in fertilized eggs of superovulated and naturally mated mice has recently been reported (Kaufman, 1973a). A comparison was made between the results obtained from direct analysis (by time-lapse cine-photomicrography) and indirect analyses of the mitotic events occurring after the breakdown of the male and female pronuclear membranes to the onset of cytokinesis. Mouse eggs which have been activated and induced to develop parthenogenetically have also been analysed by the use of similar direct and indirect approaches. With the data from this investigation it is now possible to compare the duration of the individual stages of the first cleavage mitosis and determine why the duration of mitosis in the haploid takes approximately 30 min longer than in the fertilized egg. Before this comparison can be made, however, one important morphological characteristic of the haploid mitosis must be considered. At fertilization chromosomal condensation takes place in the 2 pronuclei prior to syngamy, and 2 stages of prometaphase can be distinguished. After the brief initial stage (IIa) when filamentous chromatin fibres become apparent in both haploid and diploid systems, a second and later stage of
prometaphase (IIb) can be distinguished only in eggs with 2 or more pronuclei. At this stage considerable condensation of the chromosomes of the 2 groups has occurred, though the 2 pronuclear groups are normally slightly asynchronous prior to the synchrony which is observed at syngamy or early metaphase (stage III) (McGaughey & Chang, 1971; Donahue, 1972), except in activated eggs with 2 pronuclei where the 2 groups seemed always to be synchronous (Fig. 7). In the haploid it is impossible to distinguish between the late pronuclear or condensed phase of prometaphase (IIb) and early metaphase (III), and for convenience in this context all haploids at these morphological stages have been classified as stage III. The direct analyses served to confirm the indirect data on the total duration of mitosis in these eggs. In haploid and diploid (fertilized) eggs early prometaphase (stage IIa) was of similar duration being 10-3 min in the haploid and 10-4 min in fertilized eggs. Similarly stage III in the haploid (77-9 min) was of approximately equal duration in the diploid (79-9 min) when for the reasons explained earlier, the duration of stages IIb and III are combined. For as yet unexplained reasons the duration of the intermediate (IV) and late (Va) stages of metaphase and the duration of anaphase (Vb) are quite different in the haploid compared to the diploid; the overall duration in the haploid was 67.5 min compared to 26.5 min in the diploid. The late stage of metaphase where chromatid separation probably takes place on the equator of the spindle (Va) prior to anaphase movement is slightly longer in the diploid (13-3 min compared to 10-3 min in the haploid) whereas the chromatid stage of metaphase (IV) and the stage of anaphase (Vb) take between 3 and 5 times as long in the haploid compared to the diploid. Stage IV in the haploid took 36-5 min compared to 7-3 min in the diploid, and stage Vb took 20-7 min in the haploid compared to 5-9 min in the diploid.

It remains to be seen whether diploid parthenogenones follow the timing schedule of fertilized eggs or parthenogenetic haploid eggs despite their difference in ploidy (manuscript in preparation).

The onset of mitosis in the haploid was compared to that of the diploid, using in vitro fertilization as the nearest comparable model. In both cases the starting point for timing studies was the time of addition of eggs to the stimulating medium—hyaluronidase medium for the induction of haploids, and a fresh epididymal sperm suspension for the diploids. The major difference between the 2 groups of eggs was in the time after superovulation that the females were killed. Recently ovulated eggs (HCG plus 14 h) were exclusively used for in vitro fertilization studies and relatively aged eggs (HCG plus 18–21 h) for the haploid timing studies. Freshly ovulated eggs could not be activated by simply removing the cumulus cells with hyaluronidase and placing them in culture (Kaufman, 1973b), and aged eggs (HCG plus 18–21 h) were often activated rather than fertilized when added to an epididymal sperm suspension. The difference in the time of onset of mitosis in these 2 groups is quite considerable, the 50% point for haploids entering mitosis being approximately 12.5 h after activation, almost exactly 5 h before the diploids in the in vitro fertilization system.

The pronuclei of parthenogenones appeared between 3 and 4 h after activation, while the male and female pronuclei in the fertilized eggs were usually seen between 5.5 and 7.5 h after the addition of eggs to the sperm suspension. The time of penetration
of spermatozoa from $F_1$ mice (from 2 inbred strains) tends to be considerably faster than when spermatozoa are obtained from inbred or crossbred males (Krzanowska, 1966), and for all the present studies both eggs and spermatozoa were obtained from (C57Bl × A2G)$F_1$ hybrid mice. Apart from the delay for capacitation, and this may be of quite short duration, the timing of fertilization in vitro may not be very different from that occurring in vivo. In a previous analysis (Kaufman, 1973a) the 50% point for eggs entering mitosis in (C57Bl × A2G)$F_1$ females mated to (C57Bl × A2G)$F_1$ males was 28.16 h after the HCG injection of superovulation, and 29.56 h after the HCG injection in CFLP females (Carworth, Europe) mated to CFLP males.

Due to ageing changes in the zona, this becomes permeable to certain stimulating substances including hyaluronidase, and a high proportion of these eggs are activated. This effect could not be reproduced in fresh eggs (Kaufman, 1973b). This contrasts markedly with the fertilization situation where eggs respond to the stimulus of sperm which have penetrated the zona and interacted with the vitelline membrane of the egg. In vivo in particular the level of sperm hyaluronidase is likely to be very low as are the levels of other acrosomal enzymes which may be released in the region of the egg. This is due mainly to the small number of sperm reaching the site of fertilization (Zamboni, 1972) but it is impossible to exclude a similar but more specifically local action once the sperm has reached the perivitelline space.

Having observed that the initial cleavage division in haploid eggs is not delayed compared to diploids it is possible that the comparable situation in vivo may arise only under conditions of delayed mating when other anomalies such as polyspermy are also more likely to arise (Austin, 1969). The duration of the first cleavage mitosis in the haploid has been shown to be only slightly longer than that of the diploid, so that the pattern of preimplantation development now remains to be investigated to determine whether the apparent retardation of these embryos by the normal time of implantation is a mitotic or intermitotic problem, or due to a more general nuclear and cytoplasmic deficiency state.

I would like to thank Professor C. R. Austin for his interest and criticism of the manuscript. The work was supported by a grant from the Ford Foundation. The author is a recipient of an M.R.C. Junior Research Fellowship.

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**Haploid first cleavage mitosis in mouse**


(Received 26 January 1973)
Scale for all figures: bar represents 5 μm.

Fig. 2. Haploid mouse egg at the 'pre-chromatid' or early stage of metaphase of the first cleavage mitosis (stage III).

Fig. 3. Haploid mouse egg at the 'chromatid' stage of the first cleavage mitosis (stage IV).

Fig. 4. Haploid mouse egg at late metaphase (an early example of stage Va) where the 2 chromatids are seen to be separating.
Haploid first cleavage mitosis in mouse
Fig. 5. Haploid mouse egg at late metaphase (a later example of stage Va than in Fig. 4) where complete chromatid separation has occurred, and a single group of 40 chromatids is present at the equator of the first cleavage spindle.

Fig. 6. Haploid mouse egg at anaphase of the first cleavage mitosis (stage Vb).
Haploid first cleavage mitosis in mouse
Fig. 7. Parthenogenetic mouse egg with 2 pronuclei (due to suppression of the second polar body) at the late prometaphase stage (an early example of stage IIb) where the 2 groups of chromosomes appear to be synchronous.

Fig. 8. Parthenogenetic mouse egg with two pronuclei (due to suppression of the second polar body) at the 'chromatid' stage of metaphase (stage IV) after the two haploid chromosome groups have united to give a diploid group of 40 chromosomes.
PREVENTION OF FERTILIZATION IN VITRO BY AN ACROSIN INHIBITOR FROM RETE TESTIS FLUID OF THE RAM

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(Received 21st March 1973)

One of the enzymes probably involved in sperm penetration through the outer layers of the ovum is a trypsin-like enzyme, called acrosin because of its location in the acrosome. This enzyme is inhibited by substances found in semen and in the secretion of seminal vesicles of several mammalian species (see Werle & Zickgraf-Rüdel, 1972). It is generally thought that the spermatozoa mix with these inhibitors during ejaculation, and a complex between the inhibitors and the acrosin is formed at that time (Zaneveld, Polakoski, Robertson & Williams, 1971; Werle & Zickgraf-Rüdel, 1972). Removal of these inhibitors from the acrosome is supposed to be an essential part of the process of capacitation and the acrosome reaction (see Bedford, 1970). Several attempts have been made to prevent fertilization with synthetic or natural trypsin inhibitors, and most of these experiments have been successful (Stambaugh, Brackett & Mastroianni, 1969; Zaneveld, Polakoski, Robertson & Williams, 1971; Zaneveld, Robertson, Kessler & Williams, 1971).

We have found a high concentration of trypsin inhibitor in the fluid collected from the rete testis of rams and boars (Suominen & Setchell, 1972), and it would appear therefore that the spermatozoa are exposed to trypsin inhibitors from the moment they leave the testis. This inhibitor from the rete testis fluid (RTF) of the ram has been purified and characterized; it can be separated into three fractions (I, II and III) by CM-cellulose chromatography, but all three inhibitor fractions have similar characteristics. They do not inhibit any other common proteolytic enzyme apart from trypsin (i.e. chymotrypsin, plasmin, kallikrein, thrombin or urokinase), their molecular weight is about 6500, they are very stable and they are probably polypeptides (Suominen & Setchell, in preparation).

The present experiments were carried out to see whether these inhibitors were able to prevent fertilization in vitro. The inhibitors were purified from the RTF of the ram as described by Suominen & Setchell (in preparation). The effect of the RTF inhibitors on fertilization was tested in vitro using mouse ova and epididymal spermatozoa (Kaufman, 1973) incubated in a defined medium (Whittingham, 1971). To this culture medium, different amounts of RTF inhibitor II were added; this inhibitor fraction was chosen for experiment because it contained the highest inhibitor activity (88·7 inh. mU/ml; 1 inhibitor

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Table 1. The effect of inhibitor from ram rete testis fluid on fertilization *in vitro* in mice

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Approximate sperm concentration (sperm./ml × 10^{-6})</th>
<th>Concentration of inhibitor (μg protein/10^6 sperm.)</th>
<th>Inhibitor activity (inh. mU/ml)</th>
<th>Inhibitor activity (inh. mU/10^6 sperm.)</th>
<th>Fertilized ova/total no. of ova (percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-700</td>
<td>2.5</td>
<td>4.0</td>
<td>1.6</td>
<td>5.5</td>
<td>24/33 (73) / 29/58 (50)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>4.0</td>
<td>5.8</td>
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<td>19.4</td>
<td>41/53 (77) / 23/55 (42)</td>
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<td>23.8</td>
<td>38.1</td>
<td>26/40 (65) / 2/43 (5)</td>
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<tr>
<td></td>
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<td>23.4</td>
<td>79.4</td>
<td></td>
<td>23/33 (70) / 1/27 (4)</td>
</tr>
</tbody>
</table>

inh. mU—see text.
milliunit is the amount of inhibitor which reduces the hydrolysis of N-benzoyl-
DL-arginine-p-nitroanilide (BAPA) by trypsin by 1 nmol/min at +37°C. Its
protein concentration was 26 μg/ml. The controls contained an equivalent
volume of 0.9% NaCl; in other experiments, other non-inhibitory fractions
from the CM-cellulose chromatography were shown to have no effect on
fertilization.

When the concentration of the inhibitor was similar (23.8 inh. mU/ml) to
that in RTF (24.1 to 27.2 inh. mU/ml), fertilization was effectively blocked
(Table 1). The results also suggest that fertilization was inhibited more effec-
tively when the number of spermatozoa used was smaller and the inhibitor
concentration was kept at the same level, i.e. when the amount of the inhibitor
per spermatozoon was higher. At lower inhibitor concentration (one sixth of
that occurring in the RTF), a slight but distinct reduction in fertilization rate
was also noted.

Our results confirm the earlier findings of Stambaugh et al. (1969) on the
effect of trypsin inhibitors on fertilization in vitro. These authors, however, used
trypsin inhibitors derived from soybean and ovomucoid, and a high concentra-
tion (0.5 to 1.0 mg/ml) of these inhibitors was needed before fertilization was
prevented. Zaneveld, Polakoski, Robertson & Williams (1971) and Zaneveld,
Robertson, Kessler & Williams (1971), using an in-vivo technique, found that
fertilization was effectively reduced by inhibitors purified from rabbit seminal
plasma or from seminal vesicles at low concentrations, about the same as those
used by us, 1.0 to 2.5 μg/10³ spermatozoa. Schumacher, Swartwout & Zuspan
(1971) failed to block fertilization with polyvalent trypsin-kallikrein inhibitor
(Trasylol®) or with soybean trypsin inhibitor when incubated with ejaculated
spermatozoa or given systemically into rabbits. Their negative result could be
partly explained by our finding that inhibitors purified from the RTF are more
effective against acrosin than is trypsin-kallikrein inhibitor (Trasylol®). This
may be true also in the case of inhibitors occurring in semen or in the secretion
of the seminal vesicles.

It is clear, therefore, that the inhibitor in the RTF is effective in blocking
fertilization, and the testis may be the source of the decapacitation factor in
epididymal seminal plasma. It is now necessary to determine why these
testicular inhibitors are less tightly bound to the spermatozoa than the inhibitors
in ejaculated seminal plasma.

We are grateful to the Underwood Fund for a grant to enable J.S. to come to
England to do this work.

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ANALYSIS OF THE FIRST CLEAVAGE DIVISION
TO DETERMINE THE SEX-RATIO AND INCIDENCE
OF CHROMOSOME ANOMALIES AT
CONCEPTION IN THE MOUSE

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(Received 7th September 1972)

Summary. Fertilized mouse eggs were examined between 27 and 34 hr
after superovulation and mating. Out of 1334 eggs examined, 432 were
already at the two-cell stage. Of the one-cell eggs, 135 were at metaphase
of the first cleavage division with 40 clearly distinguishable chromo-
somes, and it was possible to sex 123 of these preparations. Sixty-two
were found to be male and sixty-one female, corresponding to a sex-ratio
at conception of approximately 1:1. A moderate incidence of triploidy
(4-1%) and aneuploidy (8-3%) was also observed at this stage and one
example of hyperdiploidy, an embryo with 41 chromosomes (mouse
2n = 40). A method for distinguishing between all the autosomes and
the sex chromosomes at the first cleavage metaphase is described.

INTRODUCTION

Since the publication of a description of an air-drying technique for producing
chromosome preparations from mouse eggs (Tarkowski, 1966), there has been
a renewed interest in the value of the primary sex-ratio. Possible effects on the
sex-ratio of external factors such as seasonal variation or population density
can only be studied when the primary sex-ratio of a species is established. The
criteria which Stich & Hsu (1960) applied to somatic cells of the mouse to
identify male and female cells were utilized by Vickers (1967) to estimate
the sex-ratio from air-dried preparations of mouse blastocysts. This was the
earliest developmental stage at which a direct estimation of the sex-ratio had
been reported. Chromosomal preparations from later embryos (Ford &
Woollam, 1963) allowed an estimate of the sex-ratio later in gestation (Vickers,
1969a). Indirect approaches to this problem such as the estimate of the sex-
ratio from mouse litters with no apparent prenatal mortality (MacDowell &
Lord, 1925) are open to criticism mainly on the grounds that these litters may
not be representative of the whole population, and that it would be very
difficult to exclude the possibility of preimplantation loss.

Recent analysis of the first cleavage division (McGaughey & Chang, 1969,
1971; Donahue, 1972a, b; Kaufman, 1973a, b) has demonstrated the accessi-
bility of this stage for study but it has not been possible to identify the individual
chromosomes of the first cleavage division in the mouse with the precision and detail possible for the chromosomes of adult mouse tissues (Francke & Nesbitt, 1971; Miller & co-authors, 1971; Buckland, Evans & Sumner, 1971; Schnedl, 1971).

The first cleavage metaphase has the advantage over subsequent mitoses that at this stage there is a greater variation in size between the longest and shortest chromosomes than is apparent in later divisions. This allows sexing to be carried out by the criteria of Stich & Hsu (1960).

The present investigation was undertaken to determine the sex-ratio in the mouse at conception and to make observations on the incidence of chromosomal anomalies at the first cleavage division. A specific staining procedure was devised which enabled a distinction to be drawn between all the autosomes present at this metaphase; results confirm the validity of sexing cells by the method of Stich & Hsu (1960).

**MATERIALS AND METHODS**

Fifty, female CFLP strain mice (Carworth, Europe), from 8 to 10 weeks old, were induced to superovulate with 10 i.u. PMSG followed at a 48-hr interval by 10 i.u. HCG, and were paired with fertile CFLP males shortly after the HCG injection. Females that had mated were killed between 27 and 34 hr after the HCG injection, and the eggs were flushed from the oviducts.

Air-dried chromosome preparations were made by the method described by Tarkowski (1966) and stained with 2% Giemsa (Giemsa stain R.66, G. T. Gurr) at pH 7·0 for 1 hr. Slides were then dehydrated and mounted with Clearmount (E. Gurr, Ltd). Eggs at the analysable stages of metaphase were examined in detail. All groups where 40 chromosomes could clearly be seen were photographed at ×1250 magnification under oil-immersion and phase-contrast. This usually required three to six photographs to cover the field of the metaphase plate. Composite photographs were then subjected to analysis by two independent observers in order to determine, where possible, the sex of the metaphase group. Karyotyping along the lines described by Stich & Hsu (1960) for somatic cells was possible in the majority of these preparations.

Groups were sexed according to the number of small chromosomes present, whether a pair (female) or three small chromosomes (male). When there were more than three chromosomes in the smallest group, it was necessary to determine whether there was an odd (male) or even (female) number present. The composite photographs were cut up in the more complex groups to aid pairing of the chromosomes. Pairing was based on size alone, as the staining technique did not allow a more accurate identification of autosomes.

Various pretreatment procedures were investigated which enable the observer to distinguish between autosomes in adult mouse mitoses. Banding was obtained by all these means but was very difficult to analyse.

The method finally adopted was as follows: slides of air-dried chromosome preparations were fixed in ethanol and acetic acid (3:1) and treated within a few hours of fixation with a saturated aqueous solution of barium hydroxide for 10 min. Following the hydroxide treatment, slides were incubated for 1 hr
at 60°C in ‘2×SSC’ (0.3 M-sodium chloride plus 0.03 M-trisodium citrate, Buckland et al., 1971) and were then rinsed thoroughly in distilled water and stained with 2% Giemsa for 20 min. The slides were then rinsed briefly in distilled water, thoroughly air-dried, dehydrated with xylene, and mounted with Clearmount. The most reliable results were obtained when a freshly prepared, unfiltered, aqueous solution of barium hydroxide was used.

This pretreatment technique produced consistent banding patterns characteristic for each chromosome. In control preparations treated with ‘2×SSC’ but not with barium hydroxide, chromosomes stained inconsistently, a few showing fine banding while the majority stained uniformly with Giemsa.

RESULTS

A total of 193 cells was obtained at metaphase of the first cleavage mitosis (see Table 1); 168 of these were diploid; eight cells were triploid; five had 60 chromosomes, and four of these appeared to be XXX in constitution, while the fifth could not be assessed. The sixth group had 58 apparently normal chromosomes with two very small chromosomes, while the remaining two groups had 58 chromosomes. Sixteen cells were hypodiploid, eight of these metaphases having

<table>
<thead>
<tr>
<th>Table 1. Analysis of metaphase groups from first cleavage mitosis in mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of metaphases</td>
</tr>
<tr>
<td>Diploid groups with 40 chromosomes</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>With minimal or no overlapping</td>
</tr>
<tr>
<td>With moderate/considerable overlapping</td>
</tr>
<tr>
<td>Diploid groups with less than 40 chromosomes</td>
</tr>
<tr>
<td>(8×39, 4×38, 4 less than 37)</td>
</tr>
<tr>
<td>Triploid groups</td>
</tr>
<tr>
<td>Diploid with 41 chromosomes</td>
</tr>
</tbody>
</table>

In 113 groups, there was agreement by two independent observers without consultation; in ten groups, there was agreement after reassessment and consultation by both observers.

* Of these 135 diploid metaphases, sexing was possible in 123 cases.

39 chromosomes. While this finding may be a guide to the incidence of aneuploidy, chromosome losses can be due to technical mishaps, and these groups were not examined in detail. One hyperdiploid metaphase was observed with 41 chromosomes.

Of the 168 diploid groups containing 40 chromosomes, 135 had minimal or no overlapping of chromosomes and were examined by two independent observers. In 123 of these metaphases, a definite decision was made as to sex. In 113 of these groups, agreement was reached by the two independent observers without consultation while in ten groups, agreement was reached after consultation and reassessment by both observers. There was some doubt
regarding sexing of the remaining twelve metaphases and these were excluded from the series.

The 123 groups in which a definite decision was made as to sex were then analysed according to the time of preparation after HCG, and this information is presented in Table 2. Though the number of preparations for each time interval is small, the ratio did not differ from 1:1 at any stage.

In order to present the facts clearly, it is necessary to compare a normal diploid metaphase group at the 'chromatid' stage of the first cleavage mitosis (Pl. 1, Fig. 1) with a parthenogenetically induced haploid group (Pl. 1, Fig. 2) at the same stage of metaphase. In the parthenogenetically activated egg, no Y chromosome is present. Both these metaphase groups are stained with Giemsa directly after air-drying and without any form of pretreatment. Plate 2, Fig. 3 illustrates a haploid metaphase group at the 'chromatid' stage of the first cleavage mitosis stained with Giemsa after the specific pretreatment procedure described above. Characteristic banding and the slight chromosomal contraction produced is apparent. The group illustrated in Pl. 2, Fig. 3 has been arranged as a karyotype (Pl. 3, Fig. 4) and the chromosomes numbered according to the Standard Karyotype of the mouse (Committee on Standardized Genetic Nomenclature for Mice, 1972). Plate 3, Fig. 5 shows a normal diploid group at early metaphase before chromatid separation stained with Giemsa after the specific pretreatment procedure. This is the karyotype of a normal male mouse, 40 XY, and illustrates the characteristic appearance of the Y chromosome.

A further series of twenty well-spread first cleavage metaphases were examined under phase contrast (unstained) and a definite decision made as to sex in all cases. These preparations were stained with Giemsa after barium hydroxide and '2×SSC' pretreatment and were re-examined. In all cases where the group had been sexed as male, the Y chromosome was morphologically distinguishable from the smallest pair of autosomes. In some cases, it was impossible to distinguish the X chromosome from one or more of the four longest pairs of autosomes because of the similarity of its banding pattern.

Table 2. Numbers of male and female metaphase groups in mice observed at various times after HCG

<table>
<thead>
<tr>
<th>Time after HCG (hr)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>28½</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>29½</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>29½</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>30½</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>32</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>33</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>34</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>61</td>
</tr>
</tbody>
</table>
Fig. 1. First cleavage division, diploid metaphase group at the ‘chromatid’ stage of a mouse. No pretreatment before Giemsa staining. This is a female cell as two small chromosomes are present (arrowed).

Fig. 2. First cleavage division, haploid metaphase group at the ‘chromatid’ stage of a mouse oocyte. No pretreatment before Giemsa staining.

(Facing p. 70)
Fig. 3. First cleavage division, haploid metaphase group at the 'chromatid' stage of a mouse oocyte. Barium hydroxide and "2 x SSC" pretreatment before Giemsa staining, showing chromosome banding.
Fig. 4. Karyotype of haploid metaphase group from mouse oocyte illustrated in Pl. 2, Fig. 3 with chromatids aligned. The chromosomes are numbered according to the Standard Karyotype of the mouse (Committee on Standardized Genetic Nomenclature for Mice, 1972).

Fig. 5. First cleavage division, diploid metaphase group from a mouse oocyte. Barium hydroxide and '2×SSC' pretreatment before Giemsa staining. Karyotype of male mouse, 40 XY.
DISCUSSION

The present investigation has revealed direct evidence on the sex-ratio at conception for the first time in mammals, showing it to be approximately 1:1 in the mouse. The earliest previous series of mouse embryos to have been satisfactorily sexed were at the blastocyst stage (Vickers, 1967, 1969a, b) where the ratio of male to female was found to be 1:1 in mice of the PDE strain, and a similar ratio was observed in a delayed fertilization group. These results, together with those now reported, suggest there is no sex-selective mortality operating at any stage of gestation from fertilization onwards.

Two methods are available to karyotype the first cleavage mitosis. Because of the considerable difference in length between the chromosomes, the method described by Stich & Hsu (1960) for somatic tissues is equally applicable to this division, and is possibly easier to evaluate than at later stages. Groups were sexed by this method according to the number of small chromosomes present, whether a pair (female) or three small chromosomes (male). When there were more than three chromosomes in the smallest group, it was necessary to determine whether there was an odd (male) or even (female) number present. This method is open to the criticism that the Y chromosome cannot be distinguished morphologically with certainty in many groups where the three smallest chromosomes are present, and that the X chromosomes cannot be distinguished at all by this means.

The staining procedure described in this paper was developed to counter these arguments and to distinguish the homologous pairs of autosomes from the sex chromosomes. To distinguish the male karyotype with certainty given a group of 40 chromosomes, it is sufficient to demonstrate 19 paired chromosomes with an additional unpaired large and small chromosome. For the purpose of sexing groups, it does not matter whether the autosomes have a similar banding pattern to adult-type chromosomes or not.

The banding pattern observed in first cleavage metaphases closely resembles the ideogram of the mouse mitotic chromosome complement illustrated in the Standard Karyotype of the mouse (Committee on Standardized Genetic Nomenclature for Mice, 1972). At the first cleavage metaphase, the pattern is more likely to represent the ‘true’ picture of events, as fewer artefacts of chromosomal contraction have been introduced (Sasaki, 1961).

Mitotic figures were sexed by the criteria of Stich & Hsu (1960) under phase contrast (unstained), then checked by the more specific staining method, and a very high concordance obtained. As this was the case, the previous series of metaphases (Table 1) which had been sexed by the same method were considered a valid assessment of the sex-ratio at the first cleavage division.

ACKNOWLEDGMENTS

I would like to thank Dr R. L. Gardner and Dr P. Barlow for advice and discussion, Mrs J. Corrigan for independently analysing the metaphase groups, Miss J. Baff for photographic assistance, and Professor C. R. Austin for his interest and criticism of the manuscript. The work was supported by a grant from the Ford Foundation. The author is a recipient of an M.R.C. Junior Research Fellowship.
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The effect of osmolarity on mouse parthenogenesis

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From the Physiological Laboratory, University of Cambridge

SUMMARY

Eggs from (C57Bl x A2G)F1 mice were activated by treatment with hyaluronidase, which removed the follicle cells, and cultured in vitro. Observations were made 6–8 h after hyaluronidase treatment to determine the frequency of activation and the types of parthenogenones induced. Cumulus-free eggs resulting from hyaluronidase treatment were incubated for 2½ h in culture media of various osmolarities. The frequency of activation was found to be dependent on the postovulatory age of oocytes, while the types of parthenogenones induced were dependent on the osmolarity of the in vitro culture medium and their postovulatory age.

Culture in low osmolar medium suppressed the extrusion of the second polar body (2PB). This decreased the incidence of haploid eggs with a single pronucleus and 2PB and immediately cleaved eggs from 97.5% to 42.3% of the activated population. Where 2PB extrusion had been suppressed, 97.4% of parthenogenones contained two haploid pronuclei. Very few were observed with a single and presumably diploid pronucleus.

Serial observations from 11 to 18 h after hyaluronidase treatment were made on populations of activated eggs as they entered the first cleavage mitosis after 2½ h incubation in medium either of normal (0.287 osmol) or low (0.168 osmol) osmolarity. A delay in the time of entry into the first cleavage mitosis similar to the duration of incubation in low osmolar medium was observed.

Further, eggs were incubated in control and low osmolar culture media containing uniformly labelled [U-14C]amino acid mixture to examine the extent of protein synthesis in recently activated eggs subjected to these culture conditions. An hypothesis is presented to explain the effect of incubation in low osmolar culture medium in delaying the first cleavage mitosis.

INTRODUCTION

The recent publications by Tarkowski, Witkowska & Nowicka (1970) and Graham (1970) on experimental parthenogenesis in the mouse have provided a considerable stimulus to workers in the field of mammalian developmental biology. More recently Tarkowski (1971), Graham (1971, 1972), Mintz & Gearhart (1973) and Kaufman (1973c) have proposed various hypotheses in an attempt to explain why mouse parthenogenones fail to develop beyond the early post-implantation period. At the present time it is not clear whether this is due to asynchrony between the parthenogenone and the uterus at the time of implantation (possibly resulting from the slower cleavage rate of these embryos compared to fertilized eggs), failure resulting from the absence of a cytoplasmic component normally provided by the fertilizing spermatozoon, the exposure and subsequent

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2 Author’s address: Physiological Laboratory, Cambridge CB2 3EG, U.K.
expression of recessive lethal genes (see review by Tarkowski, 1971), an incom¬plete zona reaction in these embryos (Mintz & Gearhart, 1973) or possibly a gene dosage effect resulting from X-chromosome imbalance (Lyon, 1973, personal communication).

The investigation of any of these hypotheses requires a simple experimental approach capable of providing a large number of parthenogenones of various types. Thus a high activation frequency and yield specificity are essential. It was with these criteria in mind that the present investigation was undertaken. Kaufman (1973a) showed that the postovulatory age of oocytes was a critical factor in determining when the activating stimulus was applied. The use of hyaluronidase medium, being a simplification of Graham’s method of activation (Graham, 1970), consistently provided an activation frequency of between 70 and 80% when eggs at the appropriate stage were stimulated. Approximately 98% of activated eggs were of the haploid type with a single pronucleus and second polar body when eggs from females killed between 16 and 20 h after HCG were stimulated, though maximal yields were obtained on stimulating eggs from females killed between 18 and 20 h after HCG. A similar frequency of activation was obtained when eggs from females killed approximately 25 h after HCG were stimulated, though the incidence of the various types of parthenogenones differed markedly, with a 62% incidence of haploid eggs of the immediate cleavage type.

Graham (1971, 1972) has demonstrated that decreasing the osmolarity of the culture medium increases the frequency of suppression of second polar body formation. This experimental approach potentially provides a means of inducing large numbers of diploid parthenogenones only rarely obtained by the use of isotonic culture conditions.

The demonstration that activated eggs with a single pronucleus (without a second polar body) were diploid (Graham, 1972) and the more recent demonstration that eggs with two pronuclei (without a second polar body) were also potentially diploid (Kaufman, 1973c) provided a stimulus for the present investigation into the effect of osmolarity on activated eggs. The present work complements the study of Brinster (1965) on the effect of osmolarity on the in vitro development of fertilized mouse embryos, confirming that mouse oocytes are able to withstand a very wide range in the osmolarity of the environment for limited periods of time without apparent detrimental effects.

MATERIALS AND METHODS

(a) Activation of eggs. Female (C57Bl × A2G)F1 mice 8–10 weeks old were superovulated with 10 i.u. pregnant mares’ serum gonadotrophin (PMSG) followed at a 48 h interval by 10 i.u. of human chorionic gonadotrophin (HCG), and killed between 14 and 25½ h after the HCG injection (ovulation occurs approximately 12 h after the HCG injection). Oocytes were liberated from the ampullae into 0-5 ml of a modified Krebs-Ringer bicarbonate culture medium
Osmolarity and mouse parthenogenesis

containing 4 mg/ml bovine serum albumin (Whittingham, 1971) and 100 i.u./ml hyaluronidase (Koch-Light, ovine testes) contained in an embryological watch-glass, and incubated at 37 °C in 5% CO₂ in air. After 15 min the eggs were isolated from this medium and transferred to another watch-glass containing 0.5 ml of hyaluronidase-free medium under 2 ml of light liquid paraffin. Atretic and fragmented eggs were discarded, presumably ovulated in response to the PMSG injection (Fowler & Edwards, 1957). After approximately 6–8 h the eggs were examined under the 50× magnification of a Wild dissecting microscope to determine the overall activation frequency and types of parthenogenones induced.

(b) Experiments to assess the response of eggs to handling. Female mice were ovulated as described in section (a) and killed at various times after HCG. Oocytes were liberated into culture medium (0.287 osmol) and incubated at 37 °C in 5% CO₂ in air for approximately 6 h. Eggs were then isolated from this medium and transferred to hyaluronidase medium for 5–10 min. This effectively removed the adherent cumulus cells, and allowed the activation frequency and types of parthenogenones induced in response to the stimuli associated with handling to be immediately assessed.

(c) Experiments to determine the effect of osmolarity. In these experiments standard culture medium (see section (a)) was diluted as follows: 1 vol. of water was added to 4 vols. of medium, referred to as 4/5 medium (b), or 2 vols. of water added to 3 vols. of medium, and referred to as 3/5 medium. The measured osmolarities of these media were as follows: undiluted culture medium, 0.287 osmol; 4/5 medium, 0.227 osmol; and 3/5 medium, 0.168 osmol.

Females were killed at various times after HCG and their eggs liberated from the ampullae directly into culture medium containing 100 i.u./ml hyaluronidase as described above. After 10–15 min eggs were isolated and transferred to either (a) undiluted culture medium, (b) 0.227 osmolar or (c) 0.168 osmolar medium. After 21/2 h eggs from all groups were transferred to undiluted culture medium and examined approximately 6 h later to determine the overall activation frequency and types of parthenogenones induced.

Further groups of eggs from females killed between 20 and 21 h after HCG were incubated in hypertonic culture media for 21/2 h prior to transfer to undiluted medium. The increased tonicity was achieved by the addition of solid sodium chloride to the medium. The osmolarities of these hypertonic media were 0.336 and 0.402 osmol.

(d) Experiments to assess the time of onset of the first cleavage mitosis. A detailed description of the method employed to assess the time of onset of the first cleavage mitosis in activated eggs with a single pronucleus and second polar body after incubation in undiluted culture medium has been reported elsewhere (Kaufman, 1973c). A second method was employed with activated eggs which had been incubated in 0.168 osmolar medium.

At approximately 8 h after activation, eggs with a single pronucleus and second polar body and eggs with two pronuclei (without a second polar body) were
isolated from the culture dishes. Eggs of each type were then separated into batches of 10 eggs in 30–50 μl drops of medium under light liquid paraffin in 15 × 60 mm diameter disposable plastic Petri dishes (Falcon) to facilitate further observation (see Kaufman, 1973b). These dishes were returned to the incubator for further culture. At regular intervals from 13 to 18 h after activation the dishes were removed from the incubator and their contents examined under the 50 × magnification of a Wild dissecting microscope. Eggs where the pronuclear outline had disappeared were removed from the culture drops and examined by the air-drying technique (Tarkowski, 1966) to confirm their haploid or diploid status. Disappearance of the pronuclear outline(s) was used as a criterion for entry of the egg into the first cleavage mitosis.

(e) [U-14C]amino acid mixture incorporation into proteins. (C57Bl × A2G)F1 female mice were superovulated and killed 20–21 h after HCG. The general format for this investigation is outlined diagrammatically in Fig. 1. Eggs from three females were treated for 15 min with hyaluronidase medium, the cumulus-free eggs pooled, and assigned to the various treatment groups. Eggs in groups 1–4 were incubated in 0·168 osmolar medium for the first 2½ h of culture, then

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**Fig. 1.** Format of experiment to assess [U-14C]amino acid mixture incorporation into proteins by activated eggs after incubation in low and normal osmolarity culture media. Duration of culture in labelled medium. H, Preincubation period in hyaluronidase medium. A, First period of 1 h 7 min after hyaluronidase treatment. B, Second period of 1 h 7 min after hyaluronidase treatment. C, Second period of 2½ h incubation. D, 2 h period of culture prior to selection of activated eggs for analysis.
transferred to 0.287 osmolar medium, whereas eggs in groups 5–8 were cultured throughout in 0.287 osmolar medium. In groups 1 and 5 eggs were incubated for the first 1 h 7 min in medium containing uniformly labelled [U-14C]amino acid mixture (10 μCi/ml) obtained from the Radiochemical Centre, Amersham (specific activity 57 mCi/m-atom carbon). Eggs in groups 2 and 6 were cultured in labelled medium for the second period of 1 h 7 min of culture. Groups 3 and 7, where eggs were incubated for 2½ h in labelled medium, acted as controls for their respective groups. Eggs in groups 4 and 8 were transferred after 2½ h of culture in unlabelled medium to 0.287 osmolar medium containing the label for 2½ h incubation in this medium. All eggs were washed carefully in unlabelled medium of the appropriate osmolarity after each transfer. After approximately 7 h the contents of all dishes were examined with the 50 x magnification of a dissecting microscope.

All activated eggs were isolated and washed in unlabelled medium at the end of the experiment. Activated eggs were squashed between two discs of glass-fibre paper (Whatman GF/A 2.5 cm diameter). The subsequent procedure for treating these discs was as described by Monesi & Salì (1967), with minor modifications. Discs were taken through several changes of cold 10% trichloracetic acid to precipitate the proteins and remove the acid-soluble non-incorporated label. After the final wash in ether, the discs were air-dried and placed in counting vials with 5 ml scintillation fluid (6 g PPO/l of toluene). Samples were counted in a Tracerlab coru/matic 200 liquid scintillation spectrometer. Counts were corrected for background and quenching.

RESULTS

(i) Factors affecting the types of parthenogenones induced

(a) The effect of postovulatory age of oocytes

Groups 1–4 of Table 1 summarize the effect of postovulatory age on the types of parthenogenones induced in eggs isolated from females killed 14, 16–20, 21 and 25–25½ h after HCG, treated with hyaluronidase prior to in vitro culture in standard (0-287 osmolar) medium (Kaufman, 1973a), while groups 5 and 6 demonstrate the effect of handling alone on eggs isolated at 16 and 18–19½ h after HCG.

Groups 1, 5 and 6 (Table 1) represent the 'background' level of activation resulting from minimal handling of these eggs. The present data showed that this was only an effective activating stimulus to aged eggs, being totally ineffective with recently ovulated material. The stimuli involved here were probably both mechanical and due to transient fluctuations in temperature during the handling period. It should be emphasized, however, that a pulse of hyaluronidase of 10–15 min seemed to be capable of evoking a maximal response in susceptible groups of eggs in the present series, consistently giving an activation frequency of between 70 and 80%. In most experiments when activated eggs were allowed
Table 1. *The effect of postovulatory age and handling on the activation frequency and types of parthenogenones induced*

<table>
<thead>
<tr>
<th>Group</th>
<th>HCG + hours</th>
<th>Activating stimulus</th>
<th>Total no. of eggs</th>
<th>Activated eggs (%)</th>
<th>Overall activation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 pronucleus + 2PB</td>
<td>Immediate cleavage</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>Handling + hyaluronidase</td>
<td>172</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>16-20</td>
<td>Handling + hyaluronidase</td>
<td>465</td>
<td>257 (98.5)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>Handling + hyaluronidase</td>
<td>600</td>
<td>398 (92.6)</td>
<td>20 (4.7)</td>
</tr>
<tr>
<td>4</td>
<td>25-25.5</td>
<td>Handling + hyaluronidase</td>
<td>382</td>
<td>96 (33.3)</td>
<td>179 (62.2)</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>Handling</td>
<td>126</td>
<td>33 (100.0)</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>18-19.5</td>
<td>Handling</td>
<td>229</td>
<td>100 (92.6)</td>
<td>2 (1.9)</td>
</tr>
</tbody>
</table>

* Lower value due to low overall activation frequency in '16 h' group (27.0%, see Kaufman, 1973a).
Table 2. The effect of 2½ hours incubation in culture media of various osmolarities on the types of parthenogenones induced in eggs from females killed 20–21, 15½ and 25½ hours after HCG.

All eggs were preincubated in hyaluronidase medium, and observations made 6–8 h later.

<table>
<thead>
<tr>
<th>Group</th>
<th>HCG + hours</th>
<th>Measured osmolarity of medium (osmol)</th>
<th>Total no. of eggs</th>
<th>Activated eggs (%)</th>
<th>Overall activation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 pronucleus + 2PB</td>
<td>Immediate cleavage</td>
</tr>
<tr>
<td>1</td>
<td>20–21</td>
<td>0.168</td>
<td>1633</td>
<td>366 (29.8)</td>
<td>154 (12.5)</td>
</tr>
<tr>
<td>2</td>
<td>20–21</td>
<td>0.227</td>
<td>107</td>
<td>68 (86.1)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>3</td>
<td>20–21</td>
<td>0.287</td>
<td>737</td>
<td>510 (93.6)</td>
<td>21 (3.9)</td>
</tr>
<tr>
<td>4</td>
<td>20–21</td>
<td>(control)</td>
<td>375</td>
<td>263 (83.5)</td>
<td>19 (6.0)</td>
</tr>
<tr>
<td>5</td>
<td>20–21</td>
<td>0.346</td>
<td>147</td>
<td>94 (97.9)</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>15–25</td>
<td>0.168</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>15–25</td>
<td>0.227</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>25½</td>
<td>0.168</td>
<td>43‡</td>
<td>7 (21.9)</td>
<td>9 (28.1)</td>
</tr>
</tbody>
</table>

* Cleavage rate at 24 h 82.9 %.
† Cleavage rate at 24 h 18.8 %.
‡ A further 46 eggs fragmented.
to remain in culture for a further 18 h almost 100% cleaved to the 2-cell stage (or to the 4-cell stage in the immediate cleavage class).

The significantly higher incidence of immediate cleavage eggs in the 25-25f h group (Table 1, group 4) compared to the incidence in groups 1, 2 and 3 (Table 1) has been discussed elsewhere (Kaufman, 1973a).

(b) The effect of osmolarity

Groups 1–5 of Table 2 demonstrate the effect of incubating cumulus-free eggs from females killed 20–21 h after HCG in media of various osmolarities (range 0.168–0.402 osmol) for 2½ h directly after hyaluronidase treatment. All eggs were transferred to standard culture medium (0.287 osmol) for a further 4–6 h before observations were made on the activation frequency and types of parthenogenones present. Groups 6 and 7 of Table 2 represent the effect of 2½ h incubation in 0.168 and 0.227 osmolar media on eggs from females killed 15½ h after HCG, while group 8 demonstrates the effect of 0.168 osmolar medium on eggs isolated 25½ h after HCG.

Because of the considerable differences which result when eggs are incubated for 2½ h in hypo- and hypertonic media, these categories will be considered separately.

(1) Hypotonic media. The closeness of the overall activation frequencies, being 75.2% in the 0.168 osmolar and 73.8% in the 0.227 osmolar groups (Table 2, groups 1 and 2 respectively) and 74.0% in the control series would seem to confirm the hypothesis that hyaluronidase treatment and handling act as activating stimuli, and that the effect of incubation in hypotonic media during the period when eggs are completing their second meiotic division is to modify the pathway of development taken by these oocytes.

Reference to groups 6 and 7 of Table 2 demonstrate that the activation frequency of eggs isolated from females 15½ h after HCG and added to either 0.168 or 0.227 osmolar medium is very low, suggesting that low osmolarity per se is not an activating stimulus. It is interesting that the two eggs which were activated (see group 6, Table 2) contained a single pronucleus but no second polar body. This class of parthenogenone has been observed by Graham (1971) in his experiments on the effect of osmolarity, though in the present series of experiments this class of parthenogenone was only rarely encountered.

An important feature illustrated in Table 2 is the increase in the incidence of oocytes with two pronuclei from 2.6% in the control series to 56.2% in the 0.168 osmolar group. This increase in response is significant (P < 0.001, χ² = 453.3). In addition there is an increase in the incidence of activated eggs undergoing immediate cleavage from 3.9% to 12.5%, with a corresponding decrease in the incidence of eggs with a single pronucleus and second polar body from 93.6% to 29.8%.

The response of eggs added to 0.227 osmolar medium was intermediate between that observed in the control group and the 0.168 osmolar group.
A small number of eggs were observed in the 0.168 osmolar group with a single pronucleus (presumed diploid) without a second polar body, and this represented 1.5% of the total number of activated eggs in this group. No eggs of this type were observed in the control series.

The results of experiments when eggs isolated from females killed 25.5 h after HCG were cultured for 2 1/4 h in 0.168 osmolar medium are presented in Table 2 (group 8). If only the apparently morphologically normal eggs are considered, the overall activation frequency of 74.4% is very similar to the 74.0% of the control series. The incidence of the various types of parthenogenones closely resembles the response to 0.168 osmolar medium observed in the 20-21 h group, with a high incidence of eggs with two pronuclei rather than of the immediate cleavage type as observed in the control series (group 4, Table 1). The incidence of fragmentation in these eggs was very high, being 51.7% of the total eggs subjected to this treatment. No eggs were observed with a single (presumably diploid) pronucleus resulting from suppression of the second polar body in this time group.

(2) Hypertonic media. The results from experiments where eggs from females killed 20-21 h after HCG were added to either 0.336 or 0.402 osmolar media are presented in Table 2 (groups 4 and 5 respectively). The eggs cultured in 0.336 osmolar medium appeared morphologically normal at 2 1/2 and 6 h after hyaluronidase treatment, and 84.0% were activated. However, eggs which have been cultured in 0.402 osmolar medium all appeared to have an increased perivitelline space after 2 1/4 h, probably due to a decrease in the vitelline volume. At 6 h an overall activation frequency of 65.3% was recorded, but the majority of pronuclei were smaller than usual, and in some of the eggs, especially in the non-activated class, the vitelline outline was irregular. Further observations were made on both these groups of eggs at 24 h after activation to determine how many had progressed to the 2-cell stage (or 4-cell stage in the case of the immediate cleavage class). This data is presented in a footnote to Table 2. It will be apparent from this and previous data (see comment at the end of Results section (a)) that of all the media tested (from 0.168 to 0.402 osmol), the only one which definitely impaired development to the first cleavage division was the 0.402 osmolar medium. Significantly fewer eggs cleaved by 24 h after activation when incubated for 2 1/4 h in 0.402 as compared to 0.336 osmolar culture medium ($P < 0.001$, $\chi^2_{(0)} = 138.69$).

(ii) The effect of low osmolarity on the time of onset of the first cleavage mitosis in activated eggs

Serial observations have been reported elsewhere (Kaufman, 1973c) which enabled the 50% point for activated eggs with a single pronucleus and second polar body (haploid) entering the first cleavage mitosis to be calculated. This
point was achieved approximately 12.4 h after activation when eggs remained in 0.287 osmolar medium during the total culture period. In this analysis 308 eggs were examined at various times between 11 and 15.5 h after their addition to hyaluronidase medium.

Serial observations from 13 to 18 h after activation were made on parthenogenones with a single pronucleus and second polar body (haploid) and eggs with 2 pronuclei without a second polar body (diploid) when activated eggs from females killed 20–21 h after HCG were incubated for 2.5 h in 0.168 osmolar medium. These results are presented in graphical form in Fig. 2, and all regression lines have been drawn by eye.

The time of entry into mitosis of eggs with a single pronucleus and second polar body represents the combined results from observations made on 128 haploid eggs, while serial observations on 226 eggs with two pronuclei provided the information for the second line.

The difference between the 50% points for entry into mitosis in these two groups is approximately 20–30 min. Thus haploid and diploid parthenogenones induced under identical conditions enter mitosis at about the same time after activation.

The difference between the 50% points for entry into mitosis in parthenogenones with a single pronucleus and second polar body between those cultured wholly in 0.287 osmolar medium and those incubated for 2.5 h in 0.168 osmolar medium...
Table 3. Results of labelling experiments to determine the \([U-^{14}C]amino acid mixture incorporation into proteins\)

<table>
<thead>
<tr>
<th>Duration of incubation in labelled medium</th>
<th>Low osmolarity medium for 2(\frac{1}{2}) h after activation</th>
<th>Normal osmolarity medium for 2(\frac{1}{2}) h after activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Groups cpm/egg (mean ± s.E.)</td>
<td>Total no. of eggs examined</td>
</tr>
<tr>
<td>1 h 7 min</td>
<td>1 46.0 ± 3.9</td>
<td>66 5 82.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>2 60.2 ± 1.5</td>
<td>62 6 88.5 ± 2.9</td>
</tr>
<tr>
<td>2(\frac{1}{2}) h</td>
<td>3 95.0 ± 5.8</td>
<td>70 7 147.1 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>4 158.2 ± 16.8</td>
<td>56 8 169.3 ± 2.7</td>
</tr>
</tbody>
</table>

medium is approximately 2\(\frac{1}{2}\) h. This difference in time is very close to the period spent in the low osmolar medium by the group of eggs which were delayed in entering mitosis.

(iii) \([U-^{14}C]amino acid mixture incorporation into proteins\)

The combined results from three experiments are presented in Table 3. Groups 5–8 represent controls for groups 1–4, while groups 3 and 7 act as controls for groups 1 and 2 (combined) and 5 and 6 (combined) respectively. The most interesting feature of this table is the reduced level of incorporation observed in groups 1–3 compared to groups 5–7. When these values are examined in more detail, the level of incorporation during the first hour of incubation in low osmolarity medium is just over one half that occurring in group 5. An increased uptake is observed in the second hour of incubation in low osmolarity medium. This is significantly above the level observed in group 1, but still significantly lower than that in the control (group 6). There is probably no significant difference between the levels of incorporation observed in groups 4 and 8.

DISCUSSION

The results presented in this paper are a detailed analysis of the effect of osmolarity on mouse eggs obtained at various times after the HCG injection of superovulation. Cumulus-free eggs were incubated in media within the range 0.168–0.402 osmol for a period of 2\(\frac{1}{2}\) h directly after activation induced by pre-incubation in hyaluronidase medium. The results presented in Table 1 confirm and extend previous findings that the frequency of activation was dependent on the postovulatory age of oocytes when stimulated (Kaufman, 1973a). When eggs were incubated in media of different osmolarities directly after activation (see Table 2) the proportionate incidence of the various types of parthenogenones induced was considerably altered, though the overall activation frequency was not markedly affected. This either suggests that osmolarity per se is not an
activating stimulus, or that maximal rates of activation may already have occurred in response to the hyaluronidase treatment. Experiments with other strains of mice may be required to clarify this issue.

The response which was most marked and is indicated by the greatest difference in the proportionate incidence of the various types of parthenogenones encountered, was observed when eggs were incubated in 0.168 osmolar culture medium directly after activation. As an alternative to diluting all the components of the medium, lowering of the osmolarity may also be achieved by reducing the level of sodium in the medium (while maintaining the Na/K ratio). The effectiveness of this experimental approach remains to be tested.

The earliest time when second polar body extrusion was commonly observed in (C57Bl × A3G)F1 eggs was approximately 2.5 h after activation, so that incubation for this period in media of different osmolarities allows the investigator to test their effect on populations of eggs which would normally be completing meiosis II.

Graham (1971, 1972) has previously reported that decreasing the osmolarity of culture medium increased the frequency of second polar body suppression, and this observation is confirmed by the present findings. The major difference between the results presented in this series and those reported by Graham relate to the widely differing proportions of parthenogenones encountered with a single pronucleus in the absence of a second polar body (presumably diploids). These were only found in very low numbers in the present series, and several hypotheses may be forwarded to explain this variation. Culture conditions and techniques were not strictly comparable to those used by Graham, while there may be considerable strain variation in response to a similar activating stimulus. In the extensive series reported here, 56% of all eggs activated 20-21 h after HCG and incubated in 0.168 osmolar medium developed two pronuclei in the absence of a second polar body (see Table 2, group 1). These embryos are potentially diploid (Kaufman, 1973c).

The largest class of parthenogenones encountered after incubation in low osmolar medium (as described in this paper) will give rise to heterozygous diploid embryos (assuming crossing over has occurred during meiosis). It is likely that further analysis of these diploid embryos may provide more favourable subject material for the analysis of later development than haploid or homozygous diploid embryos.

The observation that parthenogenones were delayed on entering the first cleavage mitosis after incubation in low osmolar medium stimulated the investigation of the underlying mechanism. The results of the labelling experiments presented here demonstrate that the quantitative assessment of protein synthesis is one aspect of the biochemical events which occur during incubation in low osmolarity medium which can be effectively monitored. Incubating activated eggs in low osmolar medium reduced the overall level of uniformly labelled [U-14C]amino acid mixture incorporation into proteins to about half the level
observed in eggs cultured throughout under control conditions. Statistically significantly higher counts were recorded in the second hour of incubation in the labelled low osmolar medium compared to the first, suggesting that a partial accommodation to the low osmolarity medium may take place. Slightly lower counts were recorded in eggs previously cultured for 2 h in 0.168 osmolar medium compared to control eggs cultured throughout in 0.287 osmolar medium. The significantly lower rate of protein synthesis in eggs cultured for the first 2 h in low osmolar medium may be due to changes in cell membrane permeability which result in a reduced uptake of amino acid precursors, possibly due to the low sodium concentration in the medium (Schultz & Curran, 1970). Alternatively, incubation in low osmolarity medium may result in a shift in the protein species synthesized. Qualitative analysis of protein from variously treated eggs will be required to distinguish between these two possibilities.

The activation of eggs in vitro has the advantage over the in vivo approach (Tarkowski et al. 1970) in that much larger numbers of eggs can be handled by the investigator at any one time, so that synchronous populations can easily be made available for developmental or timing studies. Various classes of parthenogenones have been transferred to the oviducts of pseudopregnant recipients within 6 to 9 h of in vitro activation (Kaufman & Gardner, 1974). A high proportion of the embryos isolated on day 4 appeared to be morphologically normal morulae or blastocysts, or evoked a decidual response when the uteri of recipients were examined on day 6 or 7 of pseudopregnancy. Thus aged eggs isolated 20–21 h after HCG and induced to develop parthenogenetically by in vitro activation are fully capable of developing at least to the blastocyst stage on transfer to pseudopregnant recipients. The other great advantage of the in vitro approach is its relative ease and reliability, and this may lead in the near future to the establishment of haploid and diploid parthenogenetic cell lines which could be made available for genetic and biochemical research.

The labelling experiments reported here were carried out in collaboration with M. A. H. S. The remaining data have been presented by M. H. K. in partial fulfilment for the degree of Doctor of Philosophy of the University of Cambridge. M. H. K. is a recipient of an MRC Junior Research Fellowship and M. A. H. S. is in receipt of an MRC scholarship. The work was supported by a grant to Professor C. R. Austin from the Ford Foundation.

REFERENCES


(Received 23 August 1973, revised 30 September 1973)
Diploid and haploid mouse parthenogenetic development following \textit{in vitro} activation and embryo transfer

By M. H. KAUFMAN$^1$ AND R. L. GARDNER$^2$

From the Physiological Laboratory, Cambridge, U.K.

\textbf{SUMMARY}

Parthenogenetic mouse embryos were selected following \textit{in vitro} activation, and transferred to the oviducts of pseudopregnant recipients. Decidua was evoked by 50–56\% of diploid parthenogenones compared to 35–1\% of haploid embryos with a single pronucleus, 37.5\% of immediate cleavage eggs and 77\% of fertilized eggs (controls). On day 4, 58.7\% of diploid parthenogenones were morphologically normal morulae or blastocysts; over 90\% of these ‘normal’ embryos evoked decidua when retransferred to recipients compared to 8.9\% of abnormal embryos flushed from the ‘transfer’ sides, suggesting that only ‘normal’ embryos could evoke decidua. Potentially diploid parthenogenones remained diploid on chromosomal examination on day 4.

\textbf{INTRODUCTION}

This paper describes a technique which permits a high rate of development to the late preimplantation and early postimplantation stages of selected types of mouse parthenogenones induced by \textit{in vitro} activation (Graham, 1970, 1971, 1972; Kaufman, 1973$^a$, $^b$; Kaufman & Surani, 1974). The advantage of the \textit{in vitro} approach is that it combines a very high rate of activation (Kaufman, 1973$^a$) with the ability to regulate the proportion of the various types of parthenogenones induced. Thus by altering two major variables, the postovulatory age of oocytes at the time of activation (Kaufman, 1973$^a$), and the osmolality of the culture medium during the period immediately after activation (Graham, 1972; Kaufman & Surani, 1974), it is possible to obtain largely haploid or diploid parthenogenones. Eggs activated \textit{in vitro} and incubated throughout the induction period in medium of normal osmolarity will give rise to mainly haploid parthenogenetic embryos. Approximately 98\% of the parthenogenones induced by the activation of eggs from females killed 19–20 h after the HCG injection of superovulation possess a single haploid pronucleus and second polar body (2 p.b.). Activation under similar conditions of oocytes isolated from females killed approximately 25 h after HCG results

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in almost 60% of activated eggs undergoing immediate cleavage. Under both circumstances between 70 and 80% of oocytes are usually activated. Very few diploid parthenogenones are obtained.

Suppression of second polar body extrusion occurs in 56% of the activated population when eggs are incubated in low osmolarity medium (3 volumes medium, 2 volumes distilled water) during the 2 h period directly after hyaluronidase treatment when they would normally be completing their second meiotic division. The frequency of activation is unaffected by this treatment. In a recent series of experiments (Kaufman & Surani, 1974) almost 99% of eggs which retained their second polar body developed two pronuclei. A common first cleavage metaphase spindle develops, which gives rise to a potentially heterozygous diploid embryo (Kaufman, 1973b).

Pronuclear development is usually apparent within 6 h of hyaluronidase treatment, so that selection of embryos for oviduct transfer (Tarkowski, 1959) can be made at this stage.

MATERIALS AND METHODS

Recipients

Recipients for oviduct and uterine transfer were selected by vaginal inspection (Champlin, Dorr & Gates, 1973) at about 10 p.m., and those in oestrus were placed with vasectomized males. At 8 to 10 a.m. the following morning females were checked for evidence of mating, and oviduct transfers were carried out either in the morning or on the afternoon of finding the vaginal plug, that is, on day 1 of pseudopregnancy. The uterine transfers were carried out in the early afternoon of day 3.

Control series of transfers

Separate control series were carried out by each author when early pronucleate fertilized eggs from (C57Bl x A2G)F1 females mated to F1 males were transferred to recipients at about midday on day 1 of pseudopregnancy.

Diploid parthenogenetic transfers

Eggs from (C57Bl x A2G)F1 females killed approximately 20 h after HCG were incubated for 21/2 h in low osmolarity medium directly after hyaluronidase treatment (Kaufman & Surani, 1974). Eggs were either activated at 2 a.m. and transferred to recipients between 9 a.m. and 12 noon (bilateral series), or activated at 9-9.30 a.m. and transferred between 3 and 6 p.m. on day 1 of pseudopregnancy (unilateral series). In these two series potentially diploid parthenogenones with two pronuclei were transferred to recipients within 6-9 h of activation.
In addition to diploid parthenogenones, haploid eggs with a single pronucleus and second polar body and immediate cleavage eggs were also obtained following low osmolarity treatment of eggs, as described in the previous section. These two classes of eggs were transferred separately to recipients within 6–9 h of activation.

Format of experiment

Three series of experiments were performed. In series I, fertilized eggs from (C57B1 × A2G)F₁ females mated to F₁ males were transferred unilaterally or bilaterally to the oviducts of female mice on day 1 of pseudopregnancy. The recipients were examined for uterine implantation sites on day 6 or day 7. In series II, diploid parthenogenones from F₁ females were similarly transferred to the oviducts of another group of recipients. Some of the recipients were examined for uterine implantation sites on day 6 or day 7, while the uterine horns of the remainder were flushed at midday on day 4. The embryos recovered from these females were examined and divided into the following three categories: morphologically normal blastocysts, morphologically normal morulae, and abnormal or degenerate eggs. Some of the blastocysts and morulae were retransferred to the uteri of recipients on day 3 of pseudopregnancy and the remainder were made into air-dried preparations for chromosome analysis. Some of the abnormal and degenerate eggs were also retransferred to the uteri of day 3 recipients, and the uteri examined for implantation sites on day 6 or day 7. In series III, two classes of haploid parthenogenetic eggs were transferred to the oviduct on day 1 of pseudopregnancy and the uteri examined for implantation sites on day 6 or day 7.

RESULTS

Series I. Control transfers

In both the unilateral and bilateral series of transfers 77% of transferred eggs had evoked decidua when the uteri of recipients were examined on day 6 or 7 of pseudopregnancy. These results are presented in Table 1.

Series II. Diploid parthenogenones

In the bilateral transfer series 50%, and in the unilateral series 56% of diploid parthenogenones evoked a decidual response when recipients were killed and their uteri inspected on day 6 or 7 of pseudopregnancy (see Table 1).

In series ‘a’ of the control and diploid parthenogenetic transfers, 5 or 6 eggs were usually transferred bilaterally to each oviduct, while in series ‘b’ a similar number of eggs were always transferred unilaterally (Table 1). No decidua were found in the uterine horns on the non-transferred sides in series ‘b’ when these were inspected on day 6 or 7.
Table 1. Transfers to the oviduct of female recipients on day 1 of pseudopregnancy, analysed on day 6 or day 7

<table>
<thead>
<tr>
<th>Group</th>
<th>Unilateral or bilateral</th>
<th>Total number of eggs transferred</th>
<th>Total eggs transferred to females with implants</th>
<th>Total no. of recipients (oviducts)</th>
<th>Recipients with implants (uteri)</th>
<th>Total no. of decidua</th>
<th>% of embryos implanting, in females with implants</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Fertilized eggs (controls)</td>
<td>(a) Bilateral</td>
<td>35</td>
<td>35</td>
<td>7</td>
<td>7</td>
<td>27</td>
<td>77.1</td>
</tr>
<tr>
<td></td>
<td>(b) Unilateral</td>
<td>67</td>
<td>57</td>
<td>12</td>
<td>10</td>
<td>44</td>
<td>77.2</td>
</tr>
<tr>
<td>II. Diploid parthenogenones</td>
<td>(a) Bilateral</td>
<td>55</td>
<td>40</td>
<td>11</td>
<td>9</td>
<td>20</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>(b) Unilateral</td>
<td>89</td>
<td>89</td>
<td>16</td>
<td>16</td>
<td>50</td>
<td>56.2</td>
</tr>
<tr>
<td>III. Haploid parthenogenones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) Single pronucleus + 2 p.B. class</td>
<td>Bilateral</td>
<td>167</td>
<td>94</td>
<td>18</td>
<td>13</td>
<td>33</td>
<td>35.1</td>
</tr>
<tr>
<td>(ii) Immediate cleavage class</td>
<td>Unilateral</td>
<td>71</td>
<td>56</td>
<td>10</td>
<td>8</td>
<td>21</td>
<td>37.5</td>
</tr>
</tbody>
</table>

Table 2. Diploid parthenogenetic embryos transferred on day 1 of pseudopregnancy, analysed on day 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Unilateral or bilateral transfers</th>
<th>Total no. eggs transferred</th>
<th>Total eggs transferred to females with embryos</th>
<th>Total no. of recipients</th>
<th>Recipients from which embryos recovered</th>
<th>Total no. of embryos recovered</th>
<th>% of embryos recovered from females with embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid parthenogenones</td>
<td>Unilateral</td>
<td>308</td>
<td>288</td>
<td>31</td>
<td>29</td>
<td>100</td>
<td>69</td>
</tr>
</tbody>
</table>
The number of morulae and blastocysts recovered when the uterine horns of other recipients were flushed with culture medium at approximately midday on day 4 are presented in Table 2. Twenty-nine out of 31 uterine horns yielded morulae and/or blastocysts. In addition to morphologically normal morulae and blastocysts (Fig. 1), grossly abnormal or degenerate eggs were often isolated. It was not possible to determine the source of these abnormal eggs in the 'transfer' sides. Degenerate eggs could either result from transferred parthenogenones which failed to develop, or eggs ovulated by the recipient. As activated eggs were all transferred unilaterally in this series (9 or 10 ova/horn), contralateral horns were also flushed to determine the morphology of their contents. Without exception only eggs undergoing degeneration were found to be present. The 29 recipients from which late preimplantation embryos were recovered yielded 100 morulae and 69 blastocysts out of the 288 activated eggs transferred at the one-cell stage. If all of the eggs transferred were recovered
Table 3. Implantation rate on day 6 or 7 of embryos flushed from recipients on day 4 and retransferred to the uteri of other recipients on day 3

<table>
<thead>
<tr>
<th>Groups</th>
<th>Unilateral or bilateral transfers</th>
<th>Total no. of eggs transferred</th>
<th>Total eggs transferred in females with decidua</th>
<th>Total no. of recipients</th>
<th>Recipients with decidua</th>
<th>Total no. of decidua</th>
<th>% of embryos implanting, in females with decidua</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid parthenogenones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) Morulae</td>
<td>Unilateral</td>
<td>18</td>
<td>12</td>
<td>3</td>
<td>2</td>
<td>10</td>
<td>83.3%</td>
</tr>
<tr>
<td>(ii) Blastocysts</td>
<td>Unilateral</td>
<td>30</td>
<td>30</td>
<td>6</td>
<td>6</td>
<td>29</td>
<td>96.7%</td>
</tr>
<tr>
<td>(iii) Abnormal and fragmented eggs</td>
<td>Unilateral</td>
<td>45</td>
<td>30</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>13.3*</td>
</tr>
</tbody>
</table>

* Overall value for six recipients, 8.9 %.
from the uterine horns of the recipients, this represents a minimum of 58.7% normal development. As most uteri were flushed at about midday, the majority of morulae recovered would probably have reached the blastocyst stage by the evening of day 4. Some of these parthenogenetic morulae and blastocysts were retransferred to the uterine horns of recipients on the 3rd day of pseudopregnancy. Recipients were killed on day 6 or 7 of pseudopregnancy and the number of decidual sites determined. These results are presented in Table 3. Twenty-nine out of 30 blastocysts evoked a decidual response and all six recipients had decidua, whereas 10/12 morulae evoked a decidual response in two recipients, the third having no decidua. Blastocysts generally evoked a larger decidual reaction than morulae. Histological examination is being carried out to assess the morphology of these post-implantation conceptuses.

In a parallel series, abnormal and fragmented eggs isolated from ‘transfer’ sides on the 4th day of pseudopregnancy were retransferred unilaterally to the uteri of recipients on the 3rd day of pseudopregnancy (seven or eight ova/horn). Recipients were killed on day 6 or 7, and the number of decidual sites determined. These results are presented in Table 3. Two out of six recipients had no decidua, while the remaining 4 females each had a single decidual site. These decidua were all very small compared to those obtained when diploid parthenogenetic blastocysts were retransferred under similar experimental conditions. It is possible that a few morphologically abnormal morulae may have been transferred in this series and evoked a response similar to that previously observed when diploid parthenogenetic morulae were retransferred to recipients. The four decidual swellings were not at the sites of transfer, which suggests that they were not traumatic in origin.

Sixty-three of the diploid parthenogenetic morulae and blastocysts flushed from recipients on day 4 were examined by the air-drying technique (Tarkowski, 1966) and at least one suitably spread metaphase was examined from each embryo. A total of 75 diploid metaphase spreads were scored. One early morula with 12 blastomere nuclei had 2 haploid metaphases present. This may have resulted from the transfer of an egg which underwent ‘delayed immediate cleavage’ (Graham, 1971, 1972). This abnormal behaviour might also explain the low cell number.

**Series III. Haploid parthenogenones**

A low implantation rate was observed when haploid eggs with a single pronucleus and second polar body and immediate cleavage eggs were transferred to recipients (see Table 1), possibly because of the greater degree of asynchrony between embryos and recipients at the time of implantation (Kaufman, 1973b). A decidual response was evoked on day 6 of pseudopregnancy by 35.1% of haploid eggs with a single pronucleus and 37.5% of immediate cleavage eggs.
DISCUSSION

The similarity between the proportion of morphologically normal embryos recovered from the ‘transfer’ sides (58.7%, 169/288), and the proportion of such embryos which were able to evoke a decidual reaction when recipients were examined on day 6 or 7 of pseudopregnancy (54.3%, 70/129), suggests that only ‘normal’ embryos were responsible for the observed decidua sites. The high decidual response obtained by retransferring parthenogenetic morulae and blastocysts to the uteri of recipients (92.9%, 39/42), compared to the low response obtained when abnormal embryos and fragmented eggs were transferred (8.9%, 4/45), would seem to confirm this hypothesis. However, it remains possible that some of the decidua observed on days 6 and 7 of pseudopregnancy were caused by degenerating eggs of donor origin.

The main advantage of the technique described here over in vivo activation (Tarkowski, Witkowska & Nowicka, 1970) is that it allows the investigator to select the type or types of parthenogenones he wishes to examine during the pre- or early post-implantation period. The advantages and disadvantages of the various methods of activation have been discussed in a recent review by Tarkowski (1971). However, the method outlined in this paper allows the incidence of cytokinetic failure leading to mosaicism in individual classes of parthenogenones, and their respective rates of cleavage, to be examined in detail. This should also greatly increase the possibility of developing selected parthenogenetic cell lines suitable for genetical research.

We wish to thank Professor C. R. Austin and Miss Janet Rossant. The work was supported by the Medical Research Council and the Ford Foundation.

REFERENCES


(Received 18 September 1973, revised 30 October 1973)
Bare-patches, a new sex-linked gene in the mouse, associated with a high production of XO females

II. Investigations into the nature and mechanism of the XO production

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(Received 21 March 1974)

SUMMARY

The high XO production in Bpa + mouse stocks appears to be due, not to Bpa itself, but to a closely linked factor for high XO production (Fxo) which may be a gene or a chromosomal aberration. The change of Bpa Fxo to Bpa non-Fxo occurs with a frequency of about 6%; the reciprocal change from Bpa Fxo to Fxo non-Bpa has not been found. There is some evidence that Fxo might involve a structural alteration such as an inversion or a deletion. The X-chromosome loss is due to non-disjunction which occurs in about one-third of the oocytes, probably mainly at meiosis I. Frequencies of other chromosomal abnormalities found in the stocks are given.

1. INTRODUCTION

Bare-patches (Bpa), a male-lethal, sex-linked gene in the mouse, was found to be associated with a high production of XO offspring (Phillips, Hawker & Moseley, 1973). The genotype associated with this high XO production has been called Fxo for ease of discussion. Numerous questions remained to be answered: (a) on the nature of Fxo, such as whether the effect is due to Bpa itself or to some closely linked factor and whether, if a linked factor is involved, it is in fact a ‘normal gene’ or some chromosomal rearrangement; (b) at what stage in development the X chromosome is lost and by what mechanism.

This paper describes some attempts we have made to answer these questions.

2. MATERIALS AND METHODS

(i) Materials

Three stocks of Bpa were maintained:

1) Bpa + ♀ crossed to (C3H × 101)F₁ ♂ at each generation; the presence of XO animals was tested for by corneal mitotic preparations (Fredga, 1964) of the wild-type females.

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(2) Some $Bpa$ females from (1) were crossed for one generation to $(JU \times C3H)$ and $(C3H \times JU)F_1 \delta$; that is, males carrying different alleles of the $X$-chromosome controlling element $(Xce)$ (Cattanach & Isaacson, 1967), which causes non-random $X$-chromosome activity (Cattanach & Williams, 1972).

(3) For ease of analysis for the presence or absence of $Fx0$ and to collect data on the effect of $Fx0$ on sex-linkage values, $Bpa$ females from (1) were crossed with males carrying the sex-linked genes Tabby ($Ta$) or Blotchy ($Blo$), a different type of male being used for alternate generations. The offspring were classified three times a week until the presence or absence of $Bpa +$ and $XO$ types were determined.

Females from these stocks were divided into two classes: A-line, where their parents, at least, had been shown to be $XO$-producing, and D-line, where they and/or their parents had been proved non-$XO$ producing.

(ii) Methods used in the investigation into the nature of $Fx0$

(1) Most of the data came directly from the normal breeding stocks described above, in which we were able to establish $Fx0$-free lines and from which we were able to obtain information on the linkage of $Bpa$ with other sex-linked genes in the presence and absence of $Fx0$. But, to assess the possibility of obtaining the reciprocal non-$Bpa Fx0$ mice, two further groups of matings were set up. (a) Wild-type females from A-line matings in stock (1) were tested for $XX$ or $XO$ by counting mitotic cells from cultures of ear skin. Known $XX$ females were then crossed to $TaY$ and, if possible, at least 16 female offspring classified for $XO$. (b) Wild-type females from A- and D-line matings of stock (2) were crossed to $TaY$ and those which produced any $TaO$ offspring were themselves scored for $XX$ or $XO$ by the ear culture method referred to above. Proven $XO$ mice were discarded whilst those shown to be $XX$ were allowed to continue breeding to assess the percentage of $TaO$ offspring produced. Finally, those females who produced no $TaO$ amongst 16–20 classified female offspring were killed and their $XX$ genotype confirmed by counts of corneal mitotic metaphases (Fredga, 1964). The offspring from all the matings were classified during the first week for $Bpa +$ to eliminate the possibility that any female was a normal overlap for $Bpa$.

(2) To assess whether the presence or absence of $Fx0$ affected the percentage of pre- and post-implantation death, A- and D-line $Bpa +$ and wild-type sibs from stocks (1) and (2) were mated to $(C3H \times 101)F_1 \delta$; the age of the females varied from 3½ to 6 months. Pregnant females were dissected at about 14 days, corpora lutea were counted and the implants classified. The wild-type A-line females were classified as $XX$ or $XO$ at the time of dissection by corneal mitotic metaphases.

(iii) Methods used in the investigations into the mechanism of X-chromosome loss

(1) Oocytes from A- and D-line $Bpa +$ females from all three stocks were isolated from the ovaries by follicular puncture and cultured in vitro to diakinesis/metaphase I, at which stage chromosome preparations were made by the air-drying method (Henderson & Edwards, 1968).
(2) Control females (C3H × 101)F₁, A-line Bpa + females and D-line Bpa + females from stocks (1) and (2) were super-ovulated with 5 i.u. PMSG followed after a 48 h interval by 5 i.u. HCG. Oocytes were isolated from the ampullae and activated as described by Kaufman (1973a, b) and Kaufman & Surani (1974) at 20–21 h after the HCG injection. The parthenogenones formed (Figs. 1, 2) were scored at 1st cleavage for abnormal chromosome number. The experiment was coded so that the genotype of the female was not known at the time of scoring.

(3) Finally, the oocytes from six stock (2) females, themselves proven to carry Fxo by previous mating, were cultured in low osmolar medium directly after activation to increase the number of eggs in which second polar body suppression had occurred (Kaufman & Surani, 1974), thus allowing the two products of anaphase of meiosis II to be examined (Pathway B, Figs. 1 and 2). In this way non-disjunction at meiosis I can be distinguished from that at meiosis II.
Fig. 2. Diagram showing the products of ovulation and activation from (i) cell with non-disjunction at meiosis I, (ii) cell with non-disjunction at meiosis II, (iii) cell with non-disjunction at meiosis I and II. Pathways A, B and C as in Fig. 1.
3. RESULTS

(i) Investigations into the nature of Fxo

Fig. 3 shows in diagrammatic form, the establishment of definite non-XO producing lines (D-lines) from known XO-producing matings (A-lines) in stock (2), and Table 1 gives data from all three stocks. In at least 5, and possibly 6, of the 91 matings (86 A-line matings + the 5 original D matings) considered in Table 1, the female had lost the XO producing factor: also in 7 of the A matings the number of offspring raised was too few to be sure of their classification. Thus the frequency of the conversion of Bpa Fxo to Bpa non-Fxo ranges from 5/91 to 6/84 or 5.6% to 7.1%.

Fig. 3. Diagram showing the ancestry of stock (2). ●, A-lines; ○, D-lines; ⊙, incompletely tested; ©, mice from these matings used as A line in haploid 1st cleavage metaphases (group D, Table 4) later proved to be from new crossover or D-lines. Numbers = XO/(XO + XX).

Overall there were five XO offspring out of 480 classified in the D-lines (Table 1) giving a value of 1.04% for spontaneous XO production as against an average value of 36% for the A-lines. This difference is highly significant. Whether the chromosome missing was of maternal or paternal origin was in most cases not identifiable: matings of stock (3) (Table 5), however, have shown that in A-lines it is the maternal X that is lost giving OXP. Information on the frequency of production of XO offspring from wild-type known XX females from both A- and D-lines was obtained from crosses put up to search for the reciprocal Fxo non-Bpa chromosome (see below). The data are summarized in Table 2. Overall a frequency of 7/1068 or 0.6% was found, not significantly different from that obtained for the D-line Bpa+ females. During routine scanning of corneal mitotic preparations, 1 out of 327 wild-type stock-2 females was found to carry a metacentric chromosome (Plate 1A; Table 3): she was the offspring of an A-line mother.

The search for the reciprocal Fxo non-Bpa chromosome has not so far been
Examples of unexpected abnormal cells found during these experiments. (A) Mitotic corneal preparation of 38 chromosomes of which 1 was a metacentric (assumed XO). (B) Mitotic corneal preparation of 40 chromosomes of which 1 was metacentric (XXX?). (C) Haploid parthenogenone with 21 chromosomes including 1 ring. (D) Haploid parthenogenone with 20 chromosomes including 1 ring. (E) Diploid parthenogenone with 40 chromosomes including a possible metacentric.
successful. Sixteen wild-type females from stock (1) A-lines were tested by method (a) (see Methods (ii) 1), but only 13 of them produced at least the required 16 classifiable female offspring. One female had one $X^M0$ out of 36 daughters classified; no other XO mice were produced in a total of 330 female offspring (Table 2).

**Table 1. Number of XO animals found and the number of non-Bpa females tested (N) in all stocks from time of establishment of line D1**

<table>
<thead>
<tr>
<th>Line</th>
<th>$XO$</th>
<th>$N$</th>
<th>No. matings</th>
<th>$XO$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1*</td>
<td>47</td>
<td>152</td>
<td>18</td>
<td>30-9</td>
</tr>
<tr>
<td>A2</td>
<td>119</td>
<td>317</td>
<td>37</td>
<td>37-5</td>
</tr>
<tr>
<td>A3</td>
<td>38</td>
<td>96</td>
<td>12</td>
<td>39-6</td>
</tr>
<tr>
<td>A4</td>
<td>18</td>
<td>41</td>
<td>6</td>
<td>43-9</td>
</tr>
<tr>
<td>A5</td>
<td>25</td>
<td>83</td>
<td>13</td>
<td>31-3</td>
</tr>
<tr>
<td>Totals</td>
<td>247</td>
<td>689</td>
<td>86</td>
<td>35-85</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Line</th>
<th>$XO$</th>
<th>$N$</th>
<th>No. matings</th>
<th>$XO$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2†</td>
<td>2</td>
<td>147</td>
<td>12</td>
<td>1-1</td>
</tr>
<tr>
<td>D3†</td>
<td>0</td>
<td>24</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>D5†</td>
<td>0</td>
<td>22</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>D6†</td>
<td>1</td>
<td>109</td>
<td>15</td>
<td>0-9</td>
</tr>
<tr>
<td>Totals</td>
<td>5</td>
<td>480</td>
<td>53</td>
<td>1-04</td>
</tr>
</tbody>
</table>

* Includes another possible D-line.
† $D2$ arose from $A1$ in generation 1; $D5$ from $A2$, generation 2; $D3$ and $D6$ from $A5$, generations 4 and 5 respectively.

**Table 2. Summary of data on the background (i.e. not due to Fxo) frequency of XO mice in these stocks**

<table>
<thead>
<tr>
<th>Genotype of ♀ parent</th>
<th>Line</th>
<th>Stock</th>
<th>$X^M0$</th>
<th>$OX^p$</th>
<th>Total XO</th>
<th>Total ♀ classified</th>
<th>% $XO$*</th>
</tr>
</thead>
<tbody>
<tr>
<td>++</td>
<td>A</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>330</td>
<td>0-30</td>
</tr>
<tr>
<td>++</td>
<td>A</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>446</td>
<td>0-99</td>
</tr>
<tr>
<td>++</td>
<td>D</td>
<td>2, 3</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>322</td>
<td>0-62</td>
</tr>
<tr>
<td>Bpa +†</td>
<td>D</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>480</td>
<td>1-04</td>
</tr>
</tbody>
</table>

* Frequency not corrected for fact that only half the losses of maternal $X$-chromosomes are detectable.
† Data from Table 1; $X^M0$ and $OX^p$ not distinguishable.

This frequency of 1/330 or 0-3% is within that expected for the background spontaneous level. Russell (1968) reports frequencies for $X^M0$ varying from 0-1 to 1-1% (with 0-51% in (101 × C3H)F₁ females (Russell & Montgomery, 1966)). In one or two cases, the females were mated to (C3H × 101)F₁ males instead of $TaY$ and their female offspring scanned by corneal mitotic preparations; in one of these a youngster was found with 40 chromosomes one of which was a metacentric (Plate 1B; Table 3). A further 41 A-line females, together with 21 D-line females
for comparison, from stock (2) were tested by method (b). Of the 41 A-line females, 13 were proved XO and 28 XX, but six of the 28 failed to produce the required 16 young, leaving 22 reasonably tested females, who produced two OXP and two X^M0 offspring out of 446 classified (0.9%) (Table 2). The four XO mice were all from different matings in the ratios 1/20, 1/29, 1/26 and 1/20 respectively. The wild-type D-line females produced one OXP and one X^M0 out of 322 offspring classified or 0.6%, in good agreement with the value found for the wild-type A-line females (Table 2). The frequency of OXP is much higher than the < 0.02% reported by Russell (1961) but, on the other hand, Léonard & Schröder (1968), using C3H females, found a spontaneous value of 0.13% for OXP and 0% for X^M0 in 1508 animals tested. It therefore seems certain that out of the 35 known XX wild-type A-line females from known XO producing matings, none has proved to carry Fxo. Using the method of Carter (1951) so that incompletely tested females (i.e. those which raised less than 16 young) are also included and taking a figure of 9/25 (36%) for the expected XO offspring, the number of females fully tested can be taken as (1 - (16/25)^n), where n is the number of offspring raised by each female. The equivalent-tested for the 16 females tested by method (a) and 28 females tested by method (b) totalled 41.65, as compared with 35 if incompletely tested females are excluded. Thus the observed incidence of Fxo non-Bpa is 0.41.65 and the upper fiducial limit at the 5% level is 9%, not significantly different from the 5.6-7.1% crossover value found for Bpa Fxo to Bpa non-Fxo.

(b) Dissections of pregnant females

The results of dissection of pregnant females from A- and D-lines are presented in Table 4. Both lines show a high level of prenatal death but the values for the D-line are about 7-8% lower, opening up the possibility that Fxo itself is causing some death; on the other hand, the D-line data are from various sources and are very heterogeneous.

(c) Linkage tests

The results from crosses of A- and D-line Bpa+ females on to various sex-linked stocks (stock 3) are presented in Table 5. Here there is a statistically significant

Table 3. Frequency of chromosomal abnormalities other than XO

<table>
<thead>
<tr>
<th>Nature of abnormality</th>
<th>No. abnormal</th>
<th>Total tested</th>
<th>Abnormal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X^M X^P Y</td>
<td>1</td>
<td>1141</td>
<td>0.09</td>
</tr>
<tr>
<td>?? heterozygous for a metacentric*</td>
<td>2</td>
<td>915</td>
<td>0.22</td>
</tr>
<tr>
<td>Parthenogenones containing a ring chromosome</td>
<td>2</td>
<td>228 haploid</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 diploid</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* In addition, one diploid parthenogenone from a known Fxo female (Table 7, group E, and Plate 1E) may have included a metacentric chromosome.
Table 4. Dissection of pregnant females (a) Bpa + ♀ and (b) proven XX sibs

(The females were opened at 13–15 days gestation.)

<table>
<thead>
<tr>
<th>Source</th>
<th>Type and no. of ♀♀</th>
<th>Corpora lutea (CL)</th>
<th>Live (LE)</th>
<th>Dead</th>
<th>Moles</th>
<th>Pre-I*</th>
<th>Post-I*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Line A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stock (1)†</td>
<td>(a) 20</td>
<td>185</td>
<td>60</td>
<td>1</td>
<td>62</td>
<td>11.5</td>
<td>41.8</td>
<td>48.5</td>
</tr>
<tr>
<td></td>
<td>(b) 19</td>
<td>205</td>
<td>129</td>
<td>3</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stock (2)</td>
<td>(a) 8</td>
<td>80</td>
<td>30</td>
<td>1</td>
<td>19</td>
<td>11.5</td>
<td>37.1</td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td>(b) 9</td>
<td>92</td>
<td>62</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>(a) 28</td>
<td>265</td>
<td>90</td>
<td>2</td>
<td>81</td>
<td>11.47</td>
<td>40.35</td>
<td>47.19</td>
</tr>
<tr>
<td></td>
<td>(b) 28</td>
<td>297</td>
<td>191</td>
<td>3</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Line D</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stock (1)†</td>
<td>(a) 12</td>
<td>98</td>
<td>49</td>
<td>4</td>
<td>26</td>
<td>(-5.27)</td>
<td>22.5</td>
<td>18.4</td>
</tr>
<tr>
<td></td>
<td>(b) 11</td>
<td>111</td>
<td>68</td>
<td>2</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stock (2)</td>
<td>(a) 14</td>
<td>144</td>
<td>52</td>
<td>0</td>
<td>51</td>
<td>12.38</td>
<td>45.05</td>
<td>51.85</td>
</tr>
<tr>
<td></td>
<td>(b) 17</td>
<td>196</td>
<td>147</td>
<td>3</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crossovers</td>
<td>(A → D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(a) 8</td>
<td>82</td>
<td>38</td>
<td>1</td>
<td>14</td>
<td>14.9</td>
<td>19.78</td>
<td>31.72</td>
</tr>
<tr>
<td></td>
<td>(b) 20</td>
<td>221</td>
<td>150</td>
<td>0</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>(a) 34</td>
<td>324</td>
<td>139</td>
<td>5</td>
<td>91</td>
<td>7.54</td>
<td>32.87</td>
<td>37.94</td>
</tr>
<tr>
<td></td>
<td>(b) 48</td>
<td>528</td>
<td>365</td>
<td>5</td>
<td>43</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Pre-I, pre-implantation death; Post-I, post-implantation death.
† Includes data from Phillips, Hawker & Moseley (1973).
difference between the crosses of the two lines. In crosses between Bpa and Ta in the presence of Fxo, 0.97% crossover has been seen amongst the male offspring (where classification is unequivocal), as opposed to 8.75% in its absence. Again in the Bpa, Blo crosses, more crossing-over has occurred in the absence of Fxo. Tests are also in progress with Gs, spf and Hq.

Table 5. Linkage of Bpa with Ta and Blo in the presence (line A) or absence (line D) of Fxo

<table>
<thead>
<tr>
<th>Offspring</th>
<th>Cross 1</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>D</td>
<td>Cross 2</td>
</tr>
<tr>
<td>Bpa Ta/ + Blo and</td>
<td>51</td>
<td>42</td>
<td>48</td>
</tr>
<tr>
<td>Bpa +/+ Blo</td>
<td></td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Ta/ +/+ Blo</td>
<td>101</td>
<td>72</td>
<td>76</td>
</tr>
<tr>
<td>+/+ +/+ Blo</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>BloO</td>
<td>51</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>TaY</td>
<td>102</td>
<td>73</td>
<td>47</td>
</tr>
<tr>
<td>+Y</td>
<td>1</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Linkage value</td>
<td>± 0.97</td>
<td>± 8.75%</td>
<td>± 6.00%</td>
</tr>
<tr>
<td>(using 3 data only) %</td>
<td>± 3.2</td>
<td>± 7.4</td>
<td>± 6.6</td>
</tr>
</tbody>
</table>

Standard error between estimates (%)*

<table>
<thead>
<tr>
<th>s.e. of difference</th>
<th>3.3</th>
</tr>
</thead>
</table>

* Standard error for the difference between two estimates, a and b, of a recombination value $= \sqrt{(SE_a)^2 + (SE_b)^2}$. Twice this value is then compared with the difference between the two estimates.

(ii) Investigation into the stage at which the X-chromosome is lost and evidence as to what mechanism is involved

(1) Diakinesis/metaphase I preparations were examined from A-line females. Sixty-three scorable cells were examined, all of which appeared to consist of normal configurations of 20 tetrads. One of the females had oocytes in the dilated ampullar region, and these were also examined. One metaphase II preparation had 21 chromosomes.

(2) Results from the haploid first cleavage metaphase analyses of activated oocytes (Figs. 1 and 2, Pathway A) are presented in Table 6. A low but definite incidence of non-disjunction was observed in the (C3H×101)F1 stock (group A, Table 6 and a significantly higher incidence in the A-line Bpa+ females (group B, Table 6, $X^2 = 15.48, P < 0.001$); the latter females tended to be older (3 to nearly 6 months) than the control females (1 1/2–4 months) but there was no indication of a correlation between age and the distribution of abnormalities in either group. A ring chromosome was observed in two metaphases, one with 21 chromosomes and the other with 20 chromosomes (not included as an abnormal cell in the calculations) (Plate 1 C, D; Table 3).

D-line females (group C, Table 6) showed a similar frequency of abnormal cells
to the control (C3H × 101)F₁ animals. Five other females (group D, Table 6) from their ancestry included as A-line females, also appeared to behave as D-line; their classification as non-Fxo was later confirmed by breeding data (Fig. 3). Although there is significant heterogeneity within this crossover group (group D, Table 6, last column), there is no heterogeneity between groups when the three controls, A, C and D are added ($\chi^2 = 0.663, P > 0.7$).

<table>
<thead>
<tr>
<th>Group</th>
<th>Nos. and type of female</th>
<th>Chromosome complement of metaphases</th>
<th>Abnormal groups (%)</th>
<th>Heterogeneity between $\varnothing$ within groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10 (C3H × 101)F₁</td>
<td>18 19 20 21 22 23</td>
<td>7.56 9 13.2</td>
<td>0.1</td>
</tr>
<tr>
<td>B</td>
<td>10 Bpa + A-line†</td>
<td>17 5 39§ 9§</td>
<td>27.78 9 19.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>C</td>
<td>10 Bpa + D-line</td>
<td>3 94 2</td>
<td>5.05 8 9.9</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>D</td>
<td>5 Bpa + crossovers</td>
<td>1 46 2</td>
<td>6.12 4 14.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>E</td>
<td>6 Bpa + proved to carry Fxo</td>
<td>6 13 6 1</td>
<td>48.00</td>
<td>Data from all 6 lumped</td>
</tr>
</tbody>
</table>

* Degrees of freedom.
† Crossover of 1 or 2 $\varnothing$ to Fxo cannot be excluded.
§ Oocyte not included in calculations.
§ A ring chromosome present in one group of each (the cell with 20 chromosomes not included as abnormal in calculations).

Some of the D-line females had their oocytes activated and induced to develop as immediate cleavage embryos (haploids) or as diploid parthenogenones (Pathways B and C, Figs. 1 and 2). One example at late prometaphase had one pronuclear group with 21 and a second group with 19 chromosomes. This results from non-disjunction at meiosis II.

(3) The oocytes from the six females, previously tested to confirm XO production, were also induced to develop as diploid parthenogenones. In fact 26 cells were haploid having already formed the polar body, and are included in Table 6 (group E). One of these cells contained 23 chromosomes which could indicate non-disjunction at metaphase I and II (see Fig. 2(iii)). Of those cells in which diploidy was induced, four oocytes were at late metaphase of 1st cleavage where the 2 pronuclear contents had already fused, giving 3 cells with 40 (either 20+20 or 19+21) and 1 cell of 42 (21+21). The latter implies non-disjunction at meiotic metaphase I (Fig. 2(i)) or possibly 23+19 indicating non-disjunction at I and II, see Fig. 2(iii). Only eight oocytes were of the immediate cleavage class, where the haploid metaphase from each blastomere could be analysed; six consisted of 20, 20, one of 19, 21 and one of 21, 21. Therefore non-disjunction had occurred at meiotic metaphase II and I respectively. No 19, 19 groups were observed but one anomalous cell occurred with 19, 20 groups, and, also from a known Fxo female
Nature and mechanism of XO production in Bpa mice

(Table 6E), another diploid cell with 40 chromosomes one of which was much longer than normal (Plate 1E). The abnormally long chromosome had a slightly paler central spot and may have been a metacentric or might possibly be the product of unequal crossing-over.

The data on the frequency of production of chromosomal abnormalities, other than the X-chromosome loss caused by Fxo, are listed in Tables 2 and 3.

4. DISCUSSION

Four possible mechanisms were considered to account for the observed high frequency of XO offspring.

(1) That the maternal environment of Bpa + mothers was such that the paternal X-chromosome was lost during early cleavage. This has been excluded by crosses of Bpa + with other sex-linked genes. These have shown that it is the maternal X that is lost (Table 5 and tables 3 and 4 of Phillips, Hawker & Moseley, 1973).

(2) That the XX and XO stem lines developed in the ovaries of these mice, so that the germ line was a mosaic and resulted in oocytes being ovulated with 19 or with 20 chromosomes (Fig. 1(ii)). This was excluded by the results of the diakinesis/metaphase I preparations all of which showed the normal complement of 20 pairs of chromosomes (tetrads).

(3) That the Bpa chromosome was lost in (a) meiosis: this originally seemed the most likely explanation since XXX and XXY types were not seen (but see discussion under (4) below). Otherwise only one cell suggested chromosome loss: this cell, one of the potential diploid parthenogenones where both haploid metaphases could be analysed, consisted of one group of 19 and one of 20 chromosomes (as mentioned at end of Results section). (b) Cleavage stages: this, in the absence of selection for one cell type, would be expected to lead to mosaicism. No definite evidence for this was found except for one wild-type female, offspring of an A-line Bpa + mouse, 10% of whose corneal cells showed a count of 40 and 90% of 39. Thus a low frequency of chromosome loss both at meiosis and in the early cleavage stages cannot be excluded.

Fig. 4. Diagram showing the expected products of non-disjunction at meiosis I and II in Bpa + oocytes.
(4) That non-disjunction of the X-chromosome occurs during meiosis. Non-disjunction should lead to both XXX and XXY as well as XO, but despite the lack of evidence for these two types in the breeding data (see also Phillips et al. 1973), the cytological investigations have demonstrated that oocytes from A-line Bpa+ females do undergo significantly more non-disjunction than control mice (Table 6) and thus this is the main mechanism involved in the X-chromosome loss. Therefore, possible reasons for the absence of XXX and XXY must be considered.

Table 7. Segregation from A- and D-line matings in Table 1
(including only litters where all wild-type classified for XX/XO)
(Figure in parentheses = % of total in that class.)

<table>
<thead>
<tr>
<th>Source</th>
<th>Bpa+</th>
<th>XX</th>
<th>XO</th>
<th>+Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-line stocks (1) and (3)</td>
<td>89</td>
<td>148</td>
<td>76</td>
<td>147</td>
<td>460</td>
</tr>
<tr>
<td>A-line stocks (2) and (3)</td>
<td>69</td>
<td>90</td>
<td>57</td>
<td>111</td>
<td>327</td>
</tr>
<tr>
<td>Total</td>
<td>158</td>
<td>238</td>
<td>133</td>
<td>258</td>
<td>786</td>
</tr>
<tr>
<td>D-line stocks (1) and (3)</td>
<td>154</td>
<td>296</td>
<td>3</td>
<td>317</td>
<td>770</td>
</tr>
<tr>
<td>D-line stocks (2) and (3)</td>
<td>114</td>
<td>187</td>
<td>2</td>
<td>197</td>
<td>500</td>
</tr>
<tr>
<td>Total</td>
<td>268</td>
<td>483</td>
<td>5</td>
<td>541</td>
<td>1270</td>
</tr>
</tbody>
</table>

If non-disjunction were occurring in Bpa oocytes at meiosis II, the XXX and XXY classes would be of the constitution BpaBpa+ and BpaBpa Y (Fig. 4). As all Bpa Y die prenatally (Phillips et al. 1973) the BpaBpa Y would obviously also do so and as 40–50% of Bpa + also appear to die (Table 7) it would not be surprising if BpaBpa + were lethal as well. If, on the other hand, non-disjunction occurred at meiosis I, XXX and XXY would be of the constitution Bpa + + and Bpa + Y and there appears to be no a priori reason why these should die.

Evidence from the oocytes induced to develop as diploid parthenogenones is not conclusive. Only one abnormal oocyte in which both products of anaphase of meiosis II could be examined was available from the control females; this showed non-disjunction at meiosis II (Results section (ii) 2). Therefore non-disjunction is occurring at meiosis II in the controls. From the known Fxo females (Results section (ii) 3) four oocytes gave information; one of 42 chromosomes and one of 21, 21 chromosomes indicated non-disjunction at meiosis I; one of 19, 21 was the result of non-disjunction at meiosis II and the haploid group of 23 (Table 6) indicated non-disjunction at both meiosis I and II. Therefore either Fxo is inducing non-disjunction at meiosis I and II or it acts only at meiosis I. Evidence from the breeding data tends to support the latter hypothesis.

The expectations from Bpa+ crossed to + Y in the D-lines are:

\[ \frac{1}{4} v Bpa+ : \frac{1}{4} + + : \frac{1}{4} Bpa Y : \frac{1}{4} + Y, \]

where \( v \) = the viability of Bpa+. This viability is approximately 60% (Table 7) and the expected live-born births are therefore:

\[ \frac{3}{20} Bpa+ : \frac{1}{4} + + : \frac{1}{4} + Y, \]
with a total of 13/20th live and therefore 7/20th or 35.0% dead. The frequency of prenatal death (Table 4) is in good agreement with this.

In the A-lines the expectations in the various classes are complicated by the contribution from that proportion of oocytes which undergoes non-disjunction. If this proportion is taken as \( p \) then the live-born expectations from normally dividing cells become:

\[
\frac{1}{4} v (1 - p) \text{Bpa} + \frac{1}{4} (1 - p) + \frac{1}{4} (1 - p) + Y,
\]

the products of non-disjunction at meiosis I (Fig. 4):

\[
\frac{1}{4} p \text{XXX} : \frac{1}{4} p \text{XXY} : \frac{1}{4} p \text{OY},
\]

and the products of non-disjunction at meiosis II (Fig. 4):

\[
\frac{1}{8} p \text{XXX} : \frac{1}{8} p \text{XXY} : \frac{1}{8} p \text{OXY} : \frac{1}{4} p \text{XX} : \frac{1}{4} p \text{XY},
\]

assuming that non-disjunction only occurs in the Bpa or Fxo carrying oocyte.

Table 8. The percentage live-born young and total death expected* following non-disjunction at meiosis (M) I or II compared with the observed frequencies

<table>
<thead>
<tr>
<th>Offspring (%)</th>
<th>A-lines</th>
<th>D-lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bpa+</td>
<td>+ +</td>
</tr>
<tr>
<td>Non-disjunction M I</td>
<td>19.4</td>
<td>32.3</td>
</tr>
<tr>
<td>Non-disjunction M II</td>
<td>15.7</td>
<td>38.9</td>
</tr>
<tr>
<td>Observed†</td>
<td>20.0</td>
<td>30.2</td>
</tr>
<tr>
<td>Non-disjunction M I</td>
<td>22.6</td>
<td>37.7</td>
</tr>
<tr>
<td>Non-disjunction M II</td>
<td>22.0</td>
<td>38.5</td>
</tr>
<tr>
<td>Observed†</td>
<td>21.10</td>
<td>38.03</td>
</tr>
</tbody>
</table>

* Expected where the viability of \( \text{Bpa}+ = 60\% \) and the proportion of \( \text{Bpa} \)-carrying oocytes undergoing non-disjunction = 0.33 (groups B and E, Table 4) for A-lines and 0.05 (groups C and D, Table 4) for D-lines.

† Data from stock (i), Tables 4 and 7.

The value of \( p \) observed in activated oocytes from A-line females (Table 6B, E) averaged 33.3% and this figure was used to solve the expectations above, from which were then calculated the percentage expected live-born in each class assuming that the XXX, XXY and OY genotypes are lethal. The results are given in Table 8, together with the observed percentages obtained from Tables 4 and 7. From this table it is obvious that the expectations from meiosis I non-disjunction fit the data more closely than those from meiosis II. The more obvious explanation for the lack of the XXX and XXY types (death of \( \text{BpaBpaX} \) and \( \text{BpaBpaY} \)) does not seem to be valid. Whether \( X^M X^M X \) and \( X^M X^M Y \) are lethal or are eliminated in most cases by cell selection after fertilization is not known: nor is it known whether this lack is due to some property of \( \text{Fxo} \) or is general for the mouse; \( X^M X^M Y \) definitely seems to be viable in man (Edwards, 1971). There is some
evidence that Fxo might be a structural alteration rather than a straightforward gene (see below) and this may contribute to the elimination of these multiple types. The investigation into the nature of Fxo has shown that it can be separated from Bpa but the non-Bpa Fxo crossover type has not been identified. This may be chance or may indicate some interaction so that Fxo only expresses itself in the presence of Bpa.

The finding of significantly less crossing-over between Bpa and Ta and between Bpa and Blo in the presence of Fxo (Table 5), together with a possible increase in prenatal death (Table 4), suggests that Fxo might be a structural alteration. A number of such cases of crossover-suppression have been reported for the mouse, for instance with sex-linked translocations and tobacco mouse translocations (Cattanach, 1966; Cattanach & Moseley, 1973; Lyon & Newport, 1973), with inversions (Roderick & Hawes, 1970) and with a presumed deletion (Wallace, 1972). No evidence of a translocation has been seen cytologically in 63 meiotic metaphases from A-line females but this is not conclusive as some translocations may have as low as 8% abnormal configurations (Ford et al. 1969). It is not possible to distinguish between an inversion and a deletion as a cause of crossover-suppression on the present evidence; all that can be said is that no obvious abnormalities were visible in meiotic preparations. On the other hand the abnormally long chromosome in a diploid A-line cell (see Results (ii) 3 and Plate 1E) and the two-ring chromosomes found in A-line parthenogenones (Tables 3, 6; Plate 1C, D) may be connected with Fxo. No ring chromosomes were found in 172 (C3H × 101)F1 controls nor in approximately 1000 haploid first cleavage stages from (C57Bl × A2G)F1♀ (M. H. Kaufman, unpublished). The presence of some structural alteration as an explanation of the phenomenon seems more likely than that two point-mutations occurred so close together in one animal (see Phillips et al. 1973). Obviously much more information is needed before the whole story is elucidated.

R.S.P. wishes to thank Mrs Susan Hawker and Mrs Jayne Watson for technical assistance.

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Nature and mechanism of XO production in Bpa mice


GENETIC CONTROL OF HAPLOID PARTHENOGENETIC DEVELOPMENT IN MAMMALIAN EMBRYOS

By

MATTHEW H. KAUFMAN, ELIEZER HUBERMAN and LEO SACHS

Genetic control of haploid parthenogenetic development in mammalian embryos

The establishment of haploid cell lines in mammals would be of value for studies on developmental biology, genetics and carcinogenesis. It should be possible to produce such lines by culturing cells from haploid parthenogenetic embryos. Only a limited degree of haploid parthenogenetic development has been obtained with mammals1,4, possibly as a result of the effects of deleterious recessive genes in haploid cells. During the process of selection necessary to produce an inbred strain of animals, there may be a reduction in the number of deleterious genes. Haploid embryos from random-bred animals that have not been subjected to the selection that occurs in inbreeding may, therefore, have a lower development potential than haploid embryos from an inbred strain. To test this possibility, we have compared the development of haploid embryos from one inbred strain of golden hamsters with random-bred animals and have found better development of haploid embryos in the inbred animals.

We used an inbred strain (LSH/SS) and random-bred golden hamsters, because embryonic hamster cells can be readily transformed into cell lines by various viral, chemical and physical agents4,6 and their chromosomes can be easily identified. Parthenogenetic development has been induced in the mouse by electrical stimulation of the oviduct after superovulation with gonadotrophins3,4. This technique also induces a high frequency of parthenogenetic development in hamsters.

The frequency of haploids and diploids was determined in parthenogenetically developing eggs at the single cell pro-nuclear stage, 10-12 h after electrical stimulation, and in

<table>
<thead>
<tr>
<th>Time after electrical stimulation (h)</th>
<th>Random-bred or inbred animals</th>
<th>Total no. of eggs examined</th>
<th>No. of embryos Two-cell</th>
<th>% Activated eggs or embryos per animal*</th>
<th>Embryos with or without pronuclei</th>
<th>% Non-activated eggs or embryos per animal*</th>
<th>Haploid-diploid ratio$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-12</td>
<td>Random</td>
<td>5</td>
<td>96</td>
<td>0</td>
<td>0</td>
<td>57.7 ± 10.2</td>
<td>3.2:1</td>
</tr>
<tr>
<td>10-12</td>
<td>Inbred</td>
<td>5</td>
<td>189</td>
<td>0</td>
<td>0</td>
<td>59.6 ± 9.0</td>
<td>1.8:1</td>
</tr>
<tr>
<td>72-74</td>
<td>Random</td>
<td>26</td>
<td>725</td>
<td>107</td>
<td>65</td>
<td>25.5 ± 4.5</td>
<td>0.2:1</td>
</tr>
<tr>
<td>72-74</td>
<td>Inbred</td>
<td>11</td>
<td>349</td>
<td>81</td>
<td>47</td>
<td>33.5 ± 4.4</td>
<td>1.7:1</td>
</tr>
</tbody>
</table>

* Females (7-12 week old) from random-bred and the LSH/SS inbred strain of golden hamster, were superovulated by intraperitoneal injections of 25 IU of pregnant mare's serum gonadotrophin followed 48 h later by 20 IU of human chorionic gonadotrophin (HCG). Females were anaesthetised with an intraperitoneal injection of 0.3-0.4 ml of a 24 mg ml$^{-1}$ solution of Nembutal in physiological saline 19.5-21 h after the HCG injection.

**Eggs were activated in situ by electric shocks (50 V), as described for mouse eggs7.8. The tips of two steel needles, which served as electrodes, were applied along the length of the ampullar region, and 20-25 shocks applied in rapid succession. The animals were killed either 10-12 h or 72-74 h after treatment. Their oviducts were removed and flushed with phosphate-buffered saline to collect the eggs or embryos. Control groups without electrical stimulation were examined after superovulation and after Nembutal anaesthesia. About 20% of these eggs from the controls developed pronuclei, but no two-cell embryos were observed. When females were stimulated 15-16 h after HCG, only about 13% of the eggs isolated 19.5-21 h later were either two-cell or more advanced embryos. Higher rates of activation were obtained when the oviducts were electrically stimulated 19.5-21 h after HCG, so that this time was used in the experiments. Pronuclear eggs were cultured at 37 °C in NCTC 135 medium9 with 10% foetal calf serum, under light paraffin oil in plastic Petri dishes in an incubator with 5% CO$_2$ in air. The chromosomes of eggs were examined using an air-drying technique9 in the first cleavage mitosis. As with mice2,12 all eggs which originally had a single pronucleus had a single haploid set of chromosomes, whereas those which originally had two pronuclei had a diploid first cleavage metaphase. Cleavage embryos (8-9) beyond the two-cell stage recovered at 72-74 h after electrical stimulation, were examined after culturing for 2-3 h at 37 °C in phosphate-buffered saline containing 4 μg ml$^{-1}$ colcemid. The chromosome ploidy and cell number of these embryos was then determined. The golden hamster has a normal diploid number of 44 chromosomes10. Some 70% of the embryos with mitotic figures had two or more metaphase plates.†

† Eggs were only classified as activated if they had developed a pronucleus or had cleaved to the two-cell or later stage. The percentages of activated and non-activated eggs or embryos per animal are given as the mean ± s.e.

‡ In the 72-74 h groups there were two classes of eggs. Those which progressed to the first cleavage mitosis but failed to cleave to the two-cell stage, and those which were not activated. These two classes could not be distinguished. In all the experimental groups there were 30-40% degenerated eggs.

§ In the 10-12 h groups, the ratios were determined both on the number of pronuclei present, one or two, and on subsequent chromosome counts. The ratios in the 72-74 h groups were determined by chromosome counts on embryos that were more advanced than the two-cell stage. In the 72-74 h groups, the ploidy could not be determined in 30% of embryos from the random-bred and 37% of the inbred animals, because there were no dividing cells.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Number of cells in embryos that had developed beyond the two-cell stage at 72-74 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Random-bred or inbred animals</td>
</tr>
<tr>
<td>Random</td>
<td>4</td>
</tr>
<tr>
<td>Inbred</td>
<td>17</td>
</tr>
</tbody>
</table>

* Mean ± s.e.

Numbers in parentheses are numbers of embryos.
embryos with more than two cells at 72-74 h after stimulation (Fig. 1). About 60% of the eggs were activated in both the inbred and random-bred hamsters at 10-12 h as determined by the presence of one or two pronuclei. At 72-74 h, about 30% of the embryos were either at the two-cell or a more advanced stage. At both times there were 30-40% degenerated eggs. Since the proportion of degenerated eggs was not higher at 72-74 than at 10-12 h, this suggests that 30% of the activated eggs found at 10-12 h after stimulation failed to develop to the two-cell stage in both groups of animals and were indistinguishable from non-activated eggs. Although in previous studies with golden hamsters parthenogenetic development did not occur beyond the two-cell stage, we observed some well developed blastocysts with about 20 cells.

The ratios of haploids to diploids in the random-bred and inbred animals were 3.2:1 and 1.8:1 in the eggs at 10-12 h, and 0.2:1 and 1.7:1 in the embryos at 72-74 h, respectively (Table 1). The haploid to diploid ratio of eggs and embryos was, therefore, similar in inbred hamsters, with more haploids than diploids. In the random-bred animals, the ratio of haploid to diploid eggs was similar to that found in the inbred hamsters, but there was a much lower ratio of haploid to diploid embryos. The results indicate that in the random-bred animals, there was a block in more than 90% of the haploid eggs, so that they did not develop beyond the two-cell stage, but not in the inbred strain of hamsters (nor was there a block in two inbred strains of mice). The average number of cells in embryos which had developed beyond the two-cell stage at 72-74 h, was about the same in both haploids and diploids (Table 2).

These results indicate that the development of haploid parthenogenetic embryos can be genetically controlled. The better developmental potential of haploid embryos from the inbred compared to the random-bred animals presumably results from a reduction in the number of deleterious genes occurring during the process of selection associated with inbreeding.

M. H. K. is a Royal Society—Israel Academy of Sciences Fellow.

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Received January 20, 1975.

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Parthenogenetic activation of mouse oocytes following avertin anaesthesia

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SUMMARY

Avertin anaesthesia induced mouse eggs to become activated parthenogenetically. An increasing incidence of activation was observed when females were anaesthetized 6.5, 9 and 13 h after ovulation, reaching a maximum of 45.8%. In a spontaneously ovulating group approximately 12.5% of all the eggs ovulated, or 27.3% of all the eggs activated evoked a decidual response, and the presence of implanting embryos was confirmed histologically. These findings demonstrate a new and simple method of inducing post-implantation parthenogenetic development in the mouse, and stress the necessity of taking into account the possible consequences of anaesthesia in the early post-ovulatory period.

INTRODUCTION

The induction and examination of mammalian parthenogenones could provide a valuable tool in the experimental analysis of fertilization and early development. This led me to pursue the chance observation that parthenogenetic development was initiated when mice were anaesthetized with avertin, a preparation of tribromoethanol dissolved in amylene hydrate, commonly employed as a general anaesthetic in animal practice.

It was already known that ether anaesthesia could initiate parthenogenetic development (Braden & Austin, 1954a), while heat shock (Braden & Austin, 1954b) and electric stimulation of the oviduct (Tarkowski, Witkowska & Nowicka, 1970; Witkowska, 1973a) could activate mouse eggs in vivo. Treatment of eggs in vitro with hyaluronidase (Graham, 1970; Kaufman, 1973), filtrates of sperm suspensions (Kaufman, 1973), and heat shock (Komar, 1973), are also capable of inducing parthenogenetic activation.

MATERIALS AND METHODS

Eight- to twelve-week-old (C57BI x A₂G)F₁ hybrid female mice were kept under controlled lighting conditions (dark period from 7 p.m. to 5 a.m.), and anaesthetized with an intraperitoneal injection of avertin at various times after spontaneous or induced ovulation. The standard dose of anaesthetic used was

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0·02 ml/g body weight of a freshly prepared 1:80 solution of avertin dissolved in 0·9 % saline.

Spontaneously ovulating females were mated to proven sterile vasectomized males, and anaesthetized at various times throughout the day on which the vaginal plug was observed (day 0 of pseudopregnancy). A second group of females was superovulated with 5 or 10 i.u. pregnant mares' serum gonadotrophin (PMSG), followed 48 h later by 5 or 10 i.u. of human chorionic gonadotrophin (HCG). A third group was mated with vasectomized males shortly after the HCG injection.

In the spontaneously ovulating females the time of ovulation was assumed to be approximately the middle of the dark period (Braden, 1957). The number of eggs ovulated spontaneously was 4·00 ± 0·27 (mean ± s.E.) per oviduct (Table 1). Ovulation occurred approximately 12 h after the HCG injection in the gonadotrophin-induced females.

Spontaneously ovulating females, previously mated to vasectomized males, were anaesthetized 4, 6·5, 9 or 13 h after midnight (the mid-dark point of the mouse photoperiod), while superovulated females were anaesthetized 20 or 25 h after HCG. Saline-injected controls and females mated but not anaesthetized were also examined.

Females were either killed 20–24 h after anaesthesia (group A) when their oviduct contents were examined (day 1), or on the morning of day 3 (group B) when their oviduct and uterine contents were examined, or on days 5, 6 or 7 (group C) to determine the number of decidua present.

RESULTS

In the spontaneously ovulating mice, mating to vasectomized males did not induce activation (Table 1, group 1), nor was activation observed in the females anaesthetized at 4 a.m. (group 2). An increasing incidence of activation was observed when females were anaesthetized 6·5 (group 3), 9 (groups 4 and 5) and 13 h after midnight (group 6), reaching a maximum of 45·8 % (group 6). Increasing the dose of anaesthetic (group 5) did not affect the activation frequency, but increased the incidence of degenerated eggs observed 20–24 h later. Nine out of 11 eggs in the 13-h group were at the 3- or 4-cell stage at the time of examination, suggesting that they underwent immediate cleavage. This is consistent with previous observations on the activation of eggs isolated approximately 13 h after ovulation (Kaufman, 1973).

A low rate of activation was observed at HCG + 25 h (group 8), though a high proportion of the eggs were found to be fragmenting. Reducing the dose of avertin by 50 % (group 9) failed to induce activation. Control females injected with saline at this time showed no evidence of activation (group 10).

Five spontaneously ovulating females of the 13-h group were killed in the morning of day 3 (group B). Two females had only non-activated eggs present
Table 1. Reaction of mouse eggs 20–24 h after avertin anaesthesia

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time of anaesthesia/treatment</th>
<th>No. of oviducts</th>
<th>Fragmenting eggs</th>
<th>Degenerated eggs</th>
<th>Non-activated eggs</th>
<th>Activated eggs</th>
<th>Total eggs</th>
<th>% Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2-cell</td>
<td>3-cell</td>
<td>4-cell</td>
</tr>
<tr>
<td>I. Spontaneous ovulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Mated only (control)</td>
<td>6</td>
<td>---</td>
<td>---</td>
<td>29</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>Midnight* + 4 h</td>
<td>4</td>
<td>---</td>
<td>---</td>
<td>16</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>Midnight + 6.5 h</td>
<td>4</td>
<td>---</td>
<td>---</td>
<td>13</td>
<td>1</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4</td>
<td>Midnight + 9 h</td>
<td>21</td>
<td>5</td>
<td>5</td>
<td>58</td>
<td>13</td>
<td>---</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>Midnight + 9 h (1.5 x standard dose)</td>
<td>8</td>
<td>---</td>
<td>19</td>
<td>3</td>
<td>5</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>6</td>
<td>Midnight + 13 h</td>
<td>6</td>
<td>2</td>
<td>---</td>
<td>11</td>
<td>2</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>II. Induced ovulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>HCG + 20 h</td>
<td>8</td>
<td>29</td>
<td>40</td>
<td>50</td>
<td>44</td>
<td>---</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>HCG + 25 h (standard dose of avertin)</td>
<td>8</td>
<td>70</td>
<td>8</td>
<td>53</td>
<td>6</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>HCG + 25 h (0.5 x standard dose)</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>52</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>10</td>
<td>HCG + 25 h (0.4 ml saline, control)</td>
<td>4</td>
<td>9</td>
<td>1</td>
<td>44</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

* Midnight was the mid-dark point of the mouse photoperiod.
in their oviducts, while a total of 22 eggs were isolated from the remaining three females. Two eggs had degenerated, seven were non-activated, one was at the 2-cell stage, and twelve were at various cleavage stages between the 8-cell and 32-cell stages. The twelve cleavage embryos were examined by air-drying (Tarkowski, 1966), and haploid metaphase plates were observed in three embryos.

Eighteen females anaesthetized 13 h after midnight, and five at 20 h after HCG, were killed on days 5, 6 or 7. In the 13-h group, eight out of eighteen females had no implantation sites, while the remaining ten females had a total of 18 sites. Thus, in the females with implants, approximately 12.5% of all the eggs ovulated, or 27.3% of all the eggs activated, survived at least until the moment of implantation. This is similar to the implantation rate observed

Fig. 1. Six-day-old parthenogenetic embryo at the early egg-cylinder stage, from a spontaneously ovulating female anaesthetized with avertin at approximately 13 h after ovulation. Scale bar represents 300 μm.
when immediate-cleavage embryos activated in vitro were transferred at the pronuclear stage to pseudopregnant recipients (Kaufman & Gardner, 1974). In the induced group anaesthetized 20 h after HCG, two out of five females had no implants, and a total of six implants was observed in the remaining three females. Some of the decidual sites have been examined histologically, and have confirmed the presence of implanting embryos (Fig. 1).

**DISCUSSION**

The present findings confirm that mouse oocytes show an increased tendency to develop parthenogenetically following ageing in the oviduct. Anaesthetics may, by their overall pharmacological action (Krantz & Carr, 1961; Goodman & Gilman, 1970), produce local conditions within the oviduct which stimulate mouse eggs to develop parthenogenetically, though the stimulus involved remains unclear.

These findings stress the necessity of taking into account the possible consequences of anaesthesia, when operative procedures are to be carried out in the early post-ovulatory period. Probably few, if any, of these parthenogenones are capable of prolonged post-implantation viability (Witkowska, 1973b).

It is unlikely that avertin is the only anaesthetic agent which can induce mouse eggs to develop parthenogenetically beyond implantation. While ether anaesthesia was capable of initiating parthenogenetic development (Braden & Austin, 1954a), no activation was observed when eggs were isolated from control oviducts of mice anaesthetized with nembutal (Tarkowski et al. 1970; Witkowska, 1973a), though anaesthesia probably took place during the period when eggs would have been refractory to stimulation by any anaesthetic agent.

While several of the techniques so far described produce limited post-implantation development, oocyte activation by avertin anaesthesia is probably the simplest method of inducing mouse parthenogenesis.

I thank Professor P. F. Kraicer for his criticism of an early draft of this manuscript. The author is a Royal Society–Israel Academy of Sciences programme fellow.

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(Received 15 October 1974)
THE EXPERIMENTAL INDUCTION OF PARTHENOGENESIS IN THE MOUSE

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The aim of this article is to review the latest developments in the field of mouse parthenogenesis. Some of the recent technical advances will be considered which now make it possible to increase the incidence of selected classes of haploid and diploid parthenogenones. The various stimuli which are capable of initiating mouse parthenogenetic development are discussed, as well as the relevant experimental data which might suggest the possible underlying mechanisms in each case. Current ideas on the factors governing the fate of mouse parthenogenones, and a range of problems which may be investigated using activated oocytes, will be discussed.

Most of the reports which have appeared since Tarkowski's (1971) recent review are concerned with advances in methodology in areas already known to induce mouse parthenogenetic development. Thus further reports on in-vitro activation employing hyaluronidase may be found in Graham (1972), Kaufman (1973a, c, d), Biczysko, Solter, Graham & Koprowski (1974), Graham & Deussen (1974), Kaufman & Gardner (1974), Kaufman & Surani (1974), Phillips & Kaufman (1974) and Solter et al. (1974). Studies on the effect of heat shock in vitro have been reported by Komar (1973). Detailed observations relating to earlier work by Tarkowski, Witkowska & Nowicka (1970) on in-vivo activation by electrical shock stimulation of the oviduct have recently been published by Witkowska (1973a, b). Mintz & Gearhart (1973) have compared the properties of the zona pellucidae of fertilised and parthenogenetic embryos also using electrical shock treatment. To this list must be added ether anaesthesia (Braden & Austin, 1954a), and heat shock to the oviducts (Braden & Austin, 1954b), which are both capable of initiating a limited degree of parthenogenetic development in vivo.

Avertin anaesthesia (Kaufman, unpublished data) is also capable of inducing parthenogenetic activation. The embryos produced are capable of achieving a limited degree of post-implantation development similar to those resulting from electrical stimulation of the oviduct (Tarkowski et [25])
al., 1970; Witkowska, 1973b) following the transfer of eggs activated in vitro to pseudopregnant recipients (Kaufman & Gardner, 1974) and the spontaneous activation of ovulated ova in LT/ChReSv mice (Stevens & Varnum, 1974). All these reports confirm that a wide range of stimuli are capable of initiating mouse parthenogenetic development.

The recent observation that spontaneous parthenogenetic development may commonly be observed in the ovaries or, more rarely, in the oviducts or uteri of LT/ChReSv and related strains of mice (Stevens & Varnum, 1974) is especially important in relation to the very high incidence of ovarian teratomas reported in these mice.

The main advances in this field over the past few years have come in defining some of the factors which can increase the proportion of eggs activated, and control the pathways of development taken by parthenogenones following activation in vitro. Thus by altering the postovulatory age of oocytes at the time of activation (Kaufman, 1973a), and the osmolality of the medium during the first 2–3 hour period of culture when activated eggs would be completing meiosis II (Graham, 1972; Kaufman & Surani, 1974), selected types of parthenogenones can be preferentially obtained.

Very little information is available on the underlying mechanisms involved when mammalian eggs are activated to develop parthenogenetically either in vivo or in vitro, and it is not yet possible to relate the diverse range of stimuli which are capable of activating eggs to the early normal events associated with fertilisation.

Activation may be carried out either in vivo or in vitro. The advantages and disadvantages of these two approaches have been discussed by Tarkowski (1971). In order to simplify the discussion on the possible underlying mechanisms involved in these two approaches, each will be dealt with separately.

IN-VITRO ACTIVATION

Hyaluronidase treatment

Kaufman (1973a) noted that sperm-free filtrates can activate aged mouse eggs. However, fertilisation in vitro occurred only when freshly ovulated eggs were used (Kaufman, 1973d). This suggested that certain changes were occurring in the zona within 6–8 hours of ovulation which prevented spermatozoa from penetrating aged eggs in vitro. The activating factor in
sperm suspensions was probably released from the acrosome region. This factor was capable of inducing a type of 'zona reaction' which blocked sperm entry. *In vivo*, in contrast to the situation *in vitro*, approximately 70% of eggs are penetrated up to 27 hours after HCG (human chorionic gonadotrophin), though 80 hours later less than 30% of these ova appeared to be cleaving normally (Marston & Chang, 1964).

Graham (1970, 1971, 1972) demonstrated that a proportion of mouse eggs treated with hyaluronidase 24-9 hours after HCG became activated. More detailed observations by Kaufman (1973a) demonstrated that an increasing proportion of activated eggs were obtained at 16, 18 and 20-1 hours after HCG. The activation frequency in populations of eggs stimulated approximately 25 hours after HCG is similar to that observed at 20-1 hours, although different classes of parthenogenetic embryos are obtained. This is especially marked between 16-20 and 25 hours after HCG, while an intermediate response is observed at 21 hours.

It is not clear whether the zona of a freshly ovulated egg is permeable to hyaluronidase, or whether a change occurs in response to ageing. In addition, very little is known about the properties of the egg's vitelline membrane. Measurements of the electrical changes in the surface of mouse eggs during maturation, or following fertilisation, or during pre-implantation development (Cross, Cross & Brinster, 1973), have demonstrated a pattern of events which is similar to that observed in other species. This suggests that similar events are initiated at fertilisation in all species (Epel, 1972). One interesting observation was that there was no significant change in the membrane potential of unfertilised eggs when this was determined at 13-15 hours and 20-2 hours after HCG. This is of importance since membrane potential changes are one of the first events which can be demonstrated after fertilisation, followed slightly later by the cortical reaction (Epel, 1972).

The effect of pronase

A further demonstration of the effect of ageing was observed in a recent series of experiments on the effect of removing the zona pellucida with pronase (Kaufman, 1973d). Freshly ovulated (HCG +14.5-15.0 hours) and more aged eggs (HCG +21.5-22.0 hours) were treated for 10-15 minutes with 0.25% pronase in phosphate buffered saline. Approximately half of the eggs had been pretreated for 10-15 minutes with medium containing hyaluronidase, and eggs were examined 6-8 hours later. The results of this series of experiments are presented in Table 1. This
demonstrates that almost all of the eggs in the two aged groups treated with pronase became activated, whereas no activation was observed in the two treatment groups where freshly ovulated eggs were involved. It seems likely that pronase causes certain changes in the oocyte plasma membrane, in addition to its proteolytic effect on the zona pellucida.

The unexpectedly high rate of activation observed in the aged eggs (see Table 1) suggests that the cell surface change induced by pronase is related to the induction of activation in these eggs. As freshly ovulated

<table>
<thead>
<tr>
<th>HCG + hours</th>
<th>Treatment</th>
<th>1 pronucleus</th>
<th>+2nd polar body</th>
<th>2 pronuclei</th>
<th>Immediate cleavage</th>
<th>Overall activation frequency* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.5-15.0</td>
<td>Pronase</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/70 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pronase + hyaluronidase</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/47 (0)</td>
<td></td>
</tr>
<tr>
<td>21.5-22.0</td>
<td>Pronase</td>
<td>66</td>
<td>0</td>
<td>6</td>
<td>72/73 (98.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pronase + hyaluronidase</td>
<td>41</td>
<td>2</td>
<td>4</td>
<td>47/48 (97.9)</td>
<td></td>
</tr>
</tbody>
</table>

* Observations made 4–5 hours after treatment.

eggs treated with pronase do not respond in this way, it seems likely that the surface change which takes place acts as a trigger mechanism. The membrane changes induced by the activating spermatozoon are presumably dissimilar, in that the triggering of the specific changes associated with fertilisation can occur in both freshly ovulated and relatively aged oocytes.

Some recent information has been provided by Pienkowski & Koprowski (1974). They have shown that a similar amount of concanavalin A (Con A) binds to the surface of fertilised and unfertilised eggs. They found that fertilised eggs in culture agglutinate in a concentration of 10 μg/ml of Con A, while unfertilised eggs require 2000 μg/ml before they will agglutinate. Exposure of unfertilised eggs to pronase for 10 minutes changed their surface properties in such a way that they then agglutinated at the lower concentration of Con A.
Komar (1973) has recently reported on the effect of heating mouse oviducts containing ova to temperatures between 43.0 and 45.5°C for periods of time varying from 5–10 minutes. Females were either superovulated and their oviducts excised between 14.5 and 17.5 hours after HCG, or ovulated spontaneously, and mated with vasectomised males. In the spontaneously ovulating group, dissections were carried out between 08.00 and 11.00 hours on the day a vaginal plug was detected. The response observed depended on both the temperature and the duration of treatment. At all temperatures tested, haploid eggs predominated over diploids. Of the heat-treated eggs 15.4% developed to the morula or blastocyst stage after 4 days in organ culture, compared with 57.4% of fertilised eggs.

As neither the lighting schedule nor the normal time of spontaneous ovulation in the strains examined was reported, it is impossible to be certain whether heat shock applied in vitro is effective in activating recently ovulated eggs (within 2–3 hours of ovulation). While the number of eggs undergoing different classes of reaction have been tabulated, there is no mention of whether these eggs originated from induced or spontaneously ovulating mice. There is also no information as to whether they were isolated from 'A' strain or Swiss Albino females. The absence of this information considerably reduces the value of the data. Had these various groups been tabulated separately, it might have been possible to establish whether heat is the primary stimulating agent in electric shock treatment in vivo.

FACTORS WHICH MODIFY THE INITIAL PATHWAYS OF DEVELOPMENT OF PARTHENOGENONES

The spontaneous central migration of the second meiotic spindle in response to postovulatory ageing of the oocyte

One of the earliest histological changes which may be observed in ageing oocytes is the rotation and migration of the second meiotic spindle from the periphery to the centre of the egg (Szollosi, 1971).

As the developmental pathway taken by activated eggs depends on the location of the spindle at the time of activation, eggs in which the spindle is peripheral will normally extrude a second polar body. Those in which
the spindle is centrally located will either undergo immediate cleavage or, if cytokinesis does not occur, develop two haploid, or a single, diploid pronucleus. Indirect evidence of spindle migration was first detected about 17 hours after HCG (approximately 5 hours after ovulation). In Fig. 1 the proportion of eggs in which the spindle was assumed to be centrally located at the time of activation has been plotted against the time after HCG when eggs were isolated and activated in vitro. These data indicate that the spindle would be centrally located in almost all ova by approximately 27 hours after HCG. The very low incidence of eggs undergoing immediate cleavage or with two pronuclei observed up to 21 hours after HCG, suggests that hyaluronidase treatment does not accelerate spindle migration.
PARTHENOGENESIS IN THE MOUSE

The effect of culture in hypotonic medium

When eggs were cultured in hypotonic medium during the first few hours after hyaluronidase treatment, retention of the second polar body and the formation of two pronuclei occurred in a high proportion of the activated eggs (Kaufman & Surani, 1974). No increase in activation frequency was observed following culture in hypotonic medium compared with untreated controls. These eggs are potentially diploid, as the two pronuclear chromosome sets unite on the first cleavage spindle (Kaufman, 1973c). While second polar body retention had been observed following culture in hypotonic medium, Graham (1972) recorded a high incidence of activated eggs with a single (presumed diploid) pronucleus, and a much smaller proportion with two pronuclei (see also Graham & Deussen, 1974). Similarly, Graham & Deussen (1974) observed that culture in hypotonic medium following hyaluronidase treatment increased the number of activated eggs in some mouse strains. Whether these differences are due to strain variation in the mice employed, or differences in experimental procedure, is not clear.

The time of entry into the first cleavage mitosis of 50% of activated eggs cultured in hypotonic medium was 15 hours after hyaluronidase treatment (Kaufman & Surani, 1974). Pronucleus formation occurred at about the normal time, that is, within 3–4 hours of activation, though the entry of these eggs into the first cleavage mitosis was delayed by approximately the duration of culture in hypotonic medium. Amino acid incorporation is significantly reduced during the culture period in hypotonic medium, and this may be related to a reduction in carrying capacity, due to the low Na⁺ in the medium (Schultz & Curran, 1970).

Retention of the second polar body is probably due to a direct inhibitory effect on the oocyte plasma membrane and microfilament system. Thus culture in hypotonic medium superficially resembles the effect of cytochalasin B on cells (Defendi & Stoker, 1973) and early cleavage embryos (Snow, 1973). As a result of failure of extrusion of the second polar body, both products of anaphase II are retained within the egg vitellus. However, the spindle apparatus remains functionally intact. From their earliest appearance at prometaphase until the onset of metaphase of the first cleavage mitosis, the two pronuclear chromosome sets always appear to be synchronous in their degree of condensation (Kaufman, 1973c). This contrasts with the situation in fertilised eggs, where chromosome condensation in the male and female pronuclei is asynchronous until the two groups unite on the spindle equator (McGaughey & Chang, 1971; Donahue, 1972a; Kaufman, 1973b). Graham (1971) originally stated that eggs with
two pronuclei underwent a process of delayed immediate cleavage, which invariably produced haploid embryos, and that diploid embryos were exclusively formed from haploid eggs by doubling of the haploid set of chromosomes. The occurrence of haploid–diploid mosaics is evidence that regulation to diploidy certainly does occur in some blastomeres (Tarkowski et al., 1970; Witkowska, 1973a). The majority of diploid embryos probably result from suppression of second polar body extrusion, rather than suppression of the first cleavage division. Delayed immediate cleavage was not observed by Kaufman (1973c) following serial observations on large numbers of eggs with two pronuclei, or by Witowska (1973a).

**IN-VIVO ACTIVATION**

*Electrical stimulation of the oviduct*

The underlying mechanisms which induce eggs to undergo parthenogenetic activation *in vivo* are more difficult to examine. At least two distinct classes of stimuli may be differentiated – those which are capable of activating recently ovulated eggs, and those which are only capable of activating aged eggs. Electrical stimulation of the oviduct (Tarkowski et al., 1970) belongs to the first class of stimuli. This type of stimulation may also induce aged eggs to develop parthenogenetically, but research on this topic has not been reported to date. Most of the other stimuli which activate eggs *in vivo* belong to the second class, and these include ether anaesthesia (Braden & Austin, 1954a), heat shock to the oviduct (Braden & Austin, 1954b) and avertin anaesthesia (Kaufman, unpublished data).

All of these stimuli must alter, either directly or indirectly, the biochemical environment within the oviduct. Electrical stimulation may induce activation by a local heating effect or by causing a change in the ionic composition of the oviduct fluid, or by a direct effect on the oocyte's membrane potential.

Gwatkin, Williams, Hartmann & Kniazuk (1973) demonstrated that electrical stimulation of hamster oocytes *in vitro* caused the cortical granules to rupture and release a trypsin-like protease into the perivitelline space. Presumably activation was not induced, as these authors did not comment on polar body extrusion or nucleus formation in these eggs. Cortical granule breakdown may occur when freshly ovulated mouse eggs are activated in this way, when the cortical granules are still peripherally
located. The demonstration of a partial zona reaction by Mintz & Gearhart (1973) suggests that either their stimulation was suboptimal, or that a proportion of the cortical granules had already spontaneously migrated from the periphery of the eggs in response to postovulatory ageing. The normal mouse zona reaction may be a quantitative response which is only induced when the products of all the cortical granules are released.

One of the first changes which takes place following sperm penetration in normal fertilisation is the zona reaction (Braden, Austin & David, 1954). This is probably caused by the release of a trypsin-like protease from the cortical granules into the perivitelline space. This substance interacts with the zona pellucida to cause a change in its physical properties, rendering it impermeable to spermatozoa (Austin & Braden, 1956; Barros & Yanagimachi, 1971; Gwatkin et al., 1973). It has long been recognised that a change occurs in the zona pellucida following fertilisation, which renders it resistant to proteolytic enzymes (Smithberg, 1953; Chang & Hunt, 1956; Mintz, 1962). Mintz & Gearhart (1973) demonstrated that parthenogenetic embryos induced by electrical stimulation of the oviduct showed a response in the time taken to dissolve the zona with pronase intermediate between that seen in unfertilised and fertilised eggs. It remains to be seen whether this ‘incomplete’ zona reaction plays any role in the death of these embryos. Migration of the cortical granules from the periphery of the unfertilised egg occurs spontaneously in response to ageing in the oviduct following ovulation (Szollosi, 1971), so that delayed mating would, in any case, induce an abnormal response. This does not, however, completely preclude embryo viability (see Marston & Chang, 1964), and would be analogous to the situation in parthenogenones induced in vitro, where postovulatory ageing is an important factor in the induction of high rates of activation.

The effect of avertin anaesthesia

Of more general interest has been the recent demonstration (Kaufman, unpublished data) that avertin anaesthesia is capable of inducing a relatively high rate of parthenogenetic activation and development. An activation frequency of 46 % was observed in a spontaneously ovulating population of (C57Bl × A2G)F1 females anaesthetised 13 hours after the ‘mid dark point’ of their photoperiod. Ovulation was assumed to have occurred at about this time (see Braden, 1957). Females were killed 20–4 hours after anaesthesia, and the number of cleavage embryos recorded. A high proportion of the embryos were at the 3- or 4-cell stage at the time of examination, suggesting that they underwent immediate cleavage. This
is consistent with previous observations on the activation of eggs isolated approximately 13 hours after ovulation (Kaufman, 1973a, HCG + 25-hour group). When 18 spontaneously ovulating mice anaesthetised 13 hours after ovulation were killed on days 5, 6 or 7 (day of anaesthesia referred to as day 0), the overall mean number of implants per horn was 0.50 (18/36), or 0.90 (18/20) if only the females with implants are considered. Approximately 12.5% of all the eggs ovulated, or 27.3% of all the eggs activated, survived at least until the moment of implantation.

The pattern of activation following avertin anaesthesia was similar to that observed after hyaluronidase treatment of oocytes in vitro, in that an increasing incidence of activation was observed when females were anaesthetised at 4, 6.5, 9 or 13 hours after the mid dark point. Neither the injection of saline nor the stimulus of mating to vasectomised males induced activation.

A similar implantation rate was observed in superovulated females anaesthetised 20 hours after HCG, where the initial activation frequency was 29.6%. However, nearly all of the activated eggs developed a single pronucleus and extruded a second polar body. When examined on day 7, out of 5 females 2 had no implants, and a total of 6 implants was observed in the remaining 3 females.

It is interesting that the implantation rates observed in this series are similar to those observed when eggs activated in vitro were transferred at the pronuclear stage to pseudopregnant recipients (Kaufman & Gardner, 1974). Twenty-nine per cent of all immediate cleavage embryos transferred evoked decidual reactions, or 37.5% if only the females with implants are considered. Corresponding figures for haploid embryos with a single pronucleus and second polar body were 19.8% and 35.1%, respectively.

Braden & Austin (1954a) noted that 17 out of 98 mouse eggs became activated following ether anaesthesia carried out 10–14 hours after ovulation, though development was only observed to the 2- or 4-cell stage. It is not clear whether all anaesthetics given at the appropriate period after ovulation would induce parthenogenetic development. No activation was observed in eggs isolated from control oviducts of mice anaesthetised with nembutal (Tarkowski et al., 1970; Witkowska, 1973a), though anaesthesia may have been performed during the period when these eggs would have been refractory to stimulation by any anaesthetic agent.
THE ULTRASTRUCTURE OF PARTHENOGENONES

Recently several ultrastructural analyses of parthenogenones induced by hyaluronidase treatment of oocytes in vitro have been carried out (Biczysko et al., 1974; Solter et al., 1974), and it is now possible to compare the morphological changes occurring in these embryos with those occurring during meiotic maturation (Calarco, Donahue & Szollosi, 1972), in unfertilised (Zamboni, 1970) and fertilised 1-cell eggs (Hillman & Tasca, 1969; Zamboni, 1971, 1972; Zamboni, Chakraborty & Smith, 1972), and early cleavage embryos.

In their ultrastructural study Solter et al. (1974) examined 1-cell and cleaving embryos which had been activated in vitro, and demonstrated numerous differences between the parthenogenones and fertilised eggs at similar stages of development. No cortical reaction was observed in these parthenogenones, and numerous cortical granules and vacuolated mitochondria were usually found near the cell membrane. Primary nucleoli persisted during mitosis, cytokinesis was generally irregular, and fragments of cytoplasm were commonly seen between dividing cells. This analysis suggested that numerous disturbances affecting various cell systems were occurring during early cleavage of the parthenogenones. Apart from the abnormal cortical reaction and the persistence of primary nucleoli, these authors suggested that RNA and protein synthesis were probably also disturbed.

Considering how morphologically grossly abnormal all these parthenogenones appeared to be, it is difficult to see how such embryos could survive beyond implantation. One explanation might be that the parthenogenones which were examined would not, in any case, have survived beyond a few cleavage divisions. Thus, most of the observations may have been carried out on potentially inviable embryos, as only approximately 5–10 % of eggs activated in vitro are capable of development to the blastocyst stage in culture (Graham, 1971). A more favourable approach might be the examination of parthenogenetic embryos following oviduct transfer (Kaufman & Gardner, 1974), where 37–60 % of embryos, depending on their ploidy, survive beyond implantation.

THE DEVELOPMENT OF PARTHENOGENONES

Measurement of the duration of the first cleavage mitosis in haploid and diploid parthenogenones and fertilised eggs (Kaufman, 1973b, c, d) demonstrated that the overall duration was related to the ploidy, rather
than to whether eggs were of parthenogenetic origin or not. Thus the
duration of the first cleavage mitosis in fertilised eggs and diploid partheno-
genones was about 120 minutes, compared with about 160 minutes in
haploid embryos. Later, Kaufman & Surani (1974) demonstrated that both
classes of parthenogenones entered the first cleavage mitosis at approxi-
mately the same time after activation. Further observations will be required
to determine whether subsequent mitoses and intermitotic intervals in
haploid and diploid parthenogenones follow a similar pattern. If this were
the case, by the time the blastocyst stage is reached immediate cleavage
embryos would be expected to be one cleavage division ahead of haploids
derived from eggs in which the second polar body was extruded.

Immediate cleavage embryos may reach the blastocyst stage earlier if
both classes cleave at the same rate, and cell number is the only factor
involved in cavitation. This may not prove to be the case, and early-
cavitating blastocysts of immediate cleavage origin may prove to have
twice as many blastomeres as haploids originating from eggs in which
extrusion of the second polar body occurs. This would be consistent with
previous findings (Tarkowski & Wroblewska, 1967), which demonstrated
that blastocoel formation was initiated when blastomeres of the appropriate
age were present. Cavitation occurred despite the fact that some embryos
contained only a third or a quarter the normal number of cells.

These observations on fertilised eggs (Tarkowski & Wroblewska, 1967)
are not strictly correlated with parthenogenetic development. Witkowska
(1973a) found that many parthenogenones did not cavitate despite the
accumulation of very large numbers of cells. This analysis also suggested
that the transformation of morulae into blastocysts was not connected with
ploidy, as haploid, haploid–diploid mosaic and diploid blastocysts con-
tained on the average more cells than morulae. The mitotic activity of
most of these parthenogenones was only slightly less than in fertilised
embryos, when measured in numbers of cell cycles. The number of
nuclei present in parthenogenones recovered on the 5th day was correlated
with the degree of ploidy; haploids had the greatest number of cells, di-
ploids the smallest, and mosaics were intermediate. The relationship be-
tween ploidy and cell number had previously been predicted by Beatty &
Fischberg (1951) and confirmed by Edwards (1958), though with less
convincing data.

Detailed observations were also made on the progress of these embryos
(Witkowska, 1973a), which showed that the development of eggs from
spontaneously ovulating pseudopregnant females was superior to that of
eggs from superovulated females. No comparable analyses have been per-
formed on the pre-implantation stages of development of parthenogenones
induced by any other technique, apart from the studies on the time of entry into the first cleavage division and the overall duration of the first cleavage mitosis referred to above. In a subsequent paper on the post-implantation development of parthenogenones induced by electrical stimulation (Witkowska, 1973b) over 150 implantation sites were examined from the 5th to the 10th day of pseudopregnancy. The number of living embryos recovered decreased steadily with every day, while the most advanced embryos recovered were two living embryos found on the 9th and 10th day. This provides strong evidence that development may occasionally proceed beyond this stage.

SPONTANEOUS PARTHENOGENETIC DEVELOPMENT

Stevens & Varnum (1974) have recently reported that LT/ChReSv (referred to as LT) mice show a very high incidence of ovarian teratomas, and they present evidence which suggests that these probably originate from oocytes which develop parthenogenetically within the ovary. A smaller incidence of implantation sites evoked by oocytes which activated spontaneously following ovulation was also noted. The spontaneous parthenogenones which were implanted died between days 5 and 7 at approximately the same developmental stage as most experimentally induced parthenogenones.

The early stages of development of ovarian embryos were observed in all mice over 18 days of age, while in older mice, some parthenogenones reached stages comparable to normal 6-7 day embryos, after which stage they became disorganised. As teratomas were only observed in mice old enough to have developed corpora lutea, these authors hypothesised that a hormonal factor might be a possible aetiologic agent. Impressive as the circumstantial evidence appears to be, that these teratomas arise from the spontaneous activation of ovarian oocytes, inconvertible proof of their parthenogenetic origin would only be provided by the demonstration of haploid metaphases in cell lines derived from these tumours. At the present time, it is impossible to exclude the possibility that normal ovarian tissue in the region of degenerating parthenogenetic embryos may be induced to develop into teratomas, because teratomas in these mice always develop near to parthenogenetic islands of cells.

The fact that the parthenogenones which develop from ovulated eggs implant in virgin females, suggests that their hormonal status is in some way disturbed. Thus, in addition to spontaneous activation, these females
also seem to be capable of becoming spontaneously pseudopregnant. This may be the mechanism by which spontaneously activated blastocysts are allowed to implant.

THE USE OF CHROMOSOME ANALYSIS OF THE FIRST CLEAVAGE MITOSIS OF PARTHENOGENONES

The normal morphology of the first cleavage mitosis in haploid parthenogenones has recently been described (Kaufman, 1973c). This has allowed a comparison to be made between the appearance of the chromosomes at this stage, and at metaphase II, in the parallel events in fertilised eggs (McGaughhey & Chang, 1971; Donahue, 1972a, b; Kaufman, 1973b), and in the subsequent pre-implantation cleavage mitoses (Tarkowski, 1966). This study (Kaufman, 1973c) clearly demonstrated the greater morphological simplicity of the chromosomes at the first cleavage metaphase compared to metaphase II, and to the chromosomes of cleavage embryos. It is frequently very difficult to analyse metaphase II chromosome groups, even to the extent of determining the exact number of dyads present.

The chromosomes observed at the first cleavage metaphase of the haploid parthenogenone represent half of those participating in the second meiotic cleavage. Both products of anaphase II may be examined if oocytes are induced to develop as immediate cleavage embryos, or as potentially diploid eggs with two haploid pronuclei. In both these types of parthenogenones the two pronuclei enter prometaphase approximately synchronously, so that air-dried preparations of these eggs shortly after their pronuclear outlines have disappeared will allow the two chromosome groups to be examined. This type of analysis would be particularly useful for detecting gross morphological damage to chromosomes induced during oogenesis by chemical agents or X-irradiation, as chromosomal aberrations would be easier to assess at the first cleavage metaphase than at metaphase II (Rohrborn & Hansmann, 1971). When the second cleavage division in fertilised eggs is used for this type of analysis (Rohrborn, Kuhn, Hansmann & Thon, 1971) other difficulties arise, due to the increase in asynchrony between blastomere nuclei and the additional presence of a male chromosome set.

Oocytes from ‘Bpa’ mice (Phillips, Hawker & Moseley, 1973) and related stocks were examined (Phillips & Kaufman, 1974) to determine the mechanism underlying the high production of XO female offspring. Oocytes were induced to develop parthenogenetically, and both products
of anaphase II examined as outlined above. X-chromosome loss was found to be due to non-disjunction, which occurred in about one-third of the oocytes from 'Bpa' females, mainly at meiosis I, though evidence of non-disjunction at meiosis II was also observed. This type of analysis has allowed meiotic events occurring within the ovary to be examined in detail for the first time.

A further important use of this approach could be the examination of oocytes, matured in vitro, to determine whether these show any evidence of chromosome imbalance which could explain why so few are capable of fertilisation in vitro (Cross & Brinster, 1970). Similarly, the chromosome constitution of oocytes ovulated by aged female mice should be re-examined to determine whether the reduced chiasma frequency observed with increasing maternal age (Henderson & Edwards, 1968) manifests itself by this stage. This would confirm or refute the observations of Jagiello & Polani (reported in Fowler & Edwards, 1973, p. 69, as personal communication) who stated that all mouse oocytes examined at metaphase II were diploid (see also Fowler & Edwards, 1973, for discussion).

Very little information is available which could help to establish why mouse parthenogenones do not survive beyond the early post-implantation period. Various theories have been proposed to account for their premature death (Graham, 1971, 1974; Tarkowski, 1971; Mintz & Gearhart, 1973). In these parthenogenones gene expression might be disordered (possibly because of an altered nuclear–cytoplasmic ratio), or their extensive genetic homozygosity may expose recessive lethal genes. Alternatively a compound from the male gamete may be necessary for normal development to take place. This component may be cytoplasmic or nuclear, and may function in normal X-inactivation (Brown & Chandra, 1973).

The observation that their slower rate of development compared to normal embryos would result in asynchrony between the embryo and the uterus, does not seem to be a major factor in causing their premature death. Embryos isolated at the morula and blastocyst stage which were transferred to recipients at an earlier stage of pseudopregnancy implanted, but an increased survival rate was not obtained (Witkowska, 1973b; Kaufman & Gardner, 1974).

The decidual response induced by these embryos appears to be normal, which suggests that their trophoblastic component at least is functioning normally at the time of implantation. Thus one explanation which could account for the early death of these embryos, is the failure of normal
development and functional capacity of their inner cell mass or embryonic component. A balance between these two entities is essential for normal embryonic growth to take place (Gardner, 1971; Gardner & Johnson, 1972; Gardner, Papaioannou & Barton, 1973), while direct contact with the uterine mucosa is probably necessary for inner cell mass development (Tarkowski, 1962).

The microsurgical transfer of parthenogenetic inner cell masses to trophoblast vesicles derived from normal blastocysts, and the reciprocal transfer of inner cell masses from normal blastocysts to trophoblast vesicles from parthenogenetic embryos, would give invaluable information on the developmental potential of both these components. The transfer of these reconstituted chimaeric blastocysts to suitable recipients may be the only means of obtaining advanced parthenogenetic embryonic growth. Chromosome and enzyme markers would serve to differentiate between the two components of the chimaera. The transfer of chimaeric embryos formed by the fusion of parthenogenetic to fertilised morulae to pseudo-pregnant recipients (Graham, 1970) gave inconclusive results, as no evidence of colour marker genes known to be present in the parthenogenones were expressed in the live-born young.

In addition to the ultrastructural anomalies found by Solter et al. (1974), evidence of abnormal development may be observed in some embryos during early cleavage. A small proportion of embryos develop binucleate blastomeres following electrical stimulation of the oviduct (Tarkowski, Witkowska & Nowicka, 1970). This presumably accounts for the haploid-diploid mosaic blastocysts observed after this treatment. Similar mosaic embryos may also occur following other types of activation, but comparable detailed analyses have not been reported. It is also unclear whether the various classes of haploid embryos are equally susceptible to this form of anomalous development. Transfer of selected types of embryos at the pronuclear stage, followed by detailed serial cytological examination during early cleavage, and chromosome analysis at the blastocyst stage, would provide this information. However, the possibility remains that the cytoplasmic damage which results in failure of cytokinesis in some blastomeres may be due to the type of stimulation employed, rather than to a particular susceptibility of one class of embryo compared to another. To date, no examples of diploid-tetraploid mosaics have been reported. It is also equally unclear whether the 'incomplete' zona reaction observed by Mintz & Gearhart (1973) plays any significant role in the post-implantation death of these embryos.

It is curious that post-implantation embryos obtained by electrical stimulation of the oviduct, avertin anaesthesia, in-vitro activation and
embryo transfer at the pronuclear stage, or those resulting from spontaneous activation, all appear to die shortly after implantation. The egg-cylinder stage is quite commonly seen, but only very rarely are more advanced embryos observed.

A great deal more information will be required on the normal pre- and post-implantation stages of development of parthenogenones before any meaningful comparisons can be made with the events occurring in normal embryos.

The different types of stimuli which have been employed to activate mouse eggs in vivo and in vitro, and the possible underlying mechanisms involved in each case have been considered. A correspondingly much smaller proportion of this article has been concerned with the application of these techniques to the understanding of early development. It is hoped that within the next few years further improvements in technique will overcome the present limitations of growing post-implantation parthenogenetic embryos.

I would like to thank Dr C. F. Graham for drawing my attention to certain papers 'in press', and Dr E. Huberman for his helpful criticism of the manuscript. The author is a recipient of a Royal Society–Israel Academy of Sciences Programme Fellowship.

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The early development of haploid and aneuploid parthenogenetic embryos

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The early development of haploid and aneuploid parthenogenetic embryos

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SUMMARY

The early development of parthenogenetically activated oocytes has been studied in C57BL x CBA-T6T6 (F1T6) translocation heterozygote mice and C57BL x CBA-LAC (F1LAC) mice. All F1T6 oocytes had either a quadrivalent or a univalent-trivalent configuration at meiosis I; no such chromosome configurations were observed in the F1LAC oocytes. At ovulation 36.5% of the F1T6 oocytes had 19 or 21 chromosomes, whereas 97% of the F1LAC had the normal haploid chromosome number of 20. After parthenogenetic activation, chromosome counts at metaphase of the first cleavage mitosis were made of the eggs with a single pronucleus following extrusion of the second polar body. These activated eggs had similar frequencies of 19, 20 and 21 chromosomes as had the oocytes at ovulation. The activated 1-cell eggs were transferred to the oviducts of pseudopregnant recipients and the embryos recovered 3 days later. At this stage of development, most of the F1T6 embryos with 19 chromosomes were no longer found, but the frequency of 21-chromosome embryos was similar to the frequency of 21-chromosome oocytes and activated eggs. There was a similar mean number of cells in the embryos with 20 and 21 chromosomes.

The results indicate that nearly all the embryos with 19 chromosomes failed to develop, probably beyond the 2-cell stage, whereas oocytes with 21 chromosomes had a similar development to oocytes with 20 chromosomes up to the morula stage.

INTRODUCTION

Spontaneous abortions and cases of congenital malformations in man can be associated with chromosome abnormalities (Carr, 1971; Fowler & Edwards, 1973), so that it is important to determine the developmental potential of aneuploid embryos. An abnormal chromosome constitution in the embryo can be produced by meiotic non-disjunction during gametogenesis, and in normal diploid animals there is generally an extremely low incidence of this non-disjunction (Beatty, 1970; Rohrborn, 1972; Hulten & Lindsten, 1973; Fowler & Edwards, 1973). For experiments on aneuploid embryos, it is therefore necessary to use a system which produces a higher level of chromosome abnormality. This situation occurs in mice which are translocation heterozygotes (Lyon & Meredith, 1966; Searle, Ford & Beechey, 1971; Eicher & Green, 1972).

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The T6 translocation involves a reciprocal exchange between segments of chromosomes 14 and 15 (Miller et al. 1971; Nesbitt & Francke, 1971; Eicher & Green, 1972). During meiosis in T6/+ mice, the small T6 marker chromosome (T6M) and large translocation products associate with their two normal partners either in a quadrivalent or univalent–trivalent configuration, the univalent being the T6M chromosome (Eicher & Green, 1972; Forejt, 1974). T6/+ heterozygote mice therefore ovulate both haploid and aneuploid oocytes, as a result of non-disjunction at meiosis I (Eicher & Green, 1972).

The present experiments were undertaken to determine the effect of aneuploidy on the development of parthenogenetic mouse embryos. Oocytes ovulated by T6/+ F₁ mice, which were expected to have a high frequency of aneuploidy, were compared with oocytes from non-translocation-bearing F₁ mice, which were expected to have a low incidence of aneuploidy. Only the parthenogenetically activated eggs which developed a single pronucleus following extrusion of the second polar body, and were potentially haploid (Kaufman, 1973a; Graham & Deussen, 1974), were examined. The development of these embryos was followed during the pre-implantation period. Chromosome analyses were made at the first cleavage mitosis, and on the 4th day following transfer of the activated eggs at the pronuclear stage to the oviducts of pseudo-pregnant recipients. The early developmental potential of hypo- and hyper-haploid oocytes was compared with oocytes possessing the normal haploid chromosome complement.

**MATERIALS AND METHODS**

(a) Activation of eggs. Eggs were isolated from the ampullar region of the oviduct of 8- to 12-week-old (C57BL × CBA-T6T6)F₁ and (C57BL × CBA-LAC)F₁ female mice (hereafter referred to as F₁T6 and F₁LAC respectively) at 25–26 h after the human chorionic gonadotrophin (HCG) injection for superovulation. The activation technique was similar to that described elsewhere (Kaufman, 1973a, b; Kaufman & Surani, 1974), the only modification being that all culture was carried out under light paraffin oil in 60 × 15 mm plastic Petri dishes (Falcon plastics, No. 3002) instead of in embryological watch-glasses. Five to six hours after activation the eggs were examined to determine the overall activation frequency and types of parthenogenones induced.

Eggs which developed a single pronucleus following extrusion of the second polar body were either retained in culture or isolated at this stage and transferred to the oviducts of pseudopregnant recipients. In the experiments where activated eggs were allowed to remain in standard embryo culture medium (Whittingham, 1971) for a further 6 h, embryos were transferred to fresh medium containing 0-5–1 μg/ml colchicine or colcemid and then examined by air-drying (Tarkowski, 1966) 10–12 h later. By this time, nearly all the embryos had entered the first cleavage mitosis. The preparations were stained with 3%
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lactic acetic orcein or 4% Giemsa. The number of chromosomes present in each metaphase group was recorded.

(b) Observations on the chromosome complement of oocytes at ovulation. F₁T6 and F₁LAC females were superovulated and their eggs isolated at approximately 14–16 h after HCG. The eggs were released directly into medium containing hyaluronidase to remove adherent cumulus cells, and transferred after 5–10 min to 1% sodium citrate solution before air-drying (Tarkowski, 1966). A maximum of three oocytes were carefully placed on each microslide.

(c) Oviduct transfer and the recovery of embryos from recipients on the 4th day of pseudopregnancy. Eight- to twelve-week-old spontaneously ovulating F₁LAC and F₁T6 females were examined by vaginal inspection (Champlin, Dorr & Gates, 1973) at approximately 8 p.m. and those in oestrus were mated to proven sterile vasectomized males. Females with vaginal plugs the following morning were later used as recipients. The day on which a vaginal plug was found was considered the first day of pseudopregnancy. All mice were kept under similar lighting conditions, where the period of darkness lasted from 3 p.m. to 8 a.m.

Activated eggs were transferred to the right oviduct (Kaufman & Gardner, 1974), generally in batches of 10–12, between 3 and 5 p.m. on the afternoon of the first day of pseudopregnancy. All the mice were anaesthetized with 0.02 ml/g body weight of a freshly prepared 1:80 solution of avertin dissolved in 0.9% saline.

Recipients were killed on the 4th day of pseudopregnancy and both uterine horns flushed with phosphate-buffered medium (Whittingham & Wales, 1969). In ten females, the oviducts were also flushed with medium but no eggs were recovered. All cleaving embryos more advanced than the 2-cell stage were transferred to standard embryo-culture medium containing 0.5-1 μg/ml colchicine or colcemid, and incubated in this medium for 4-6 h. Embryos were then examined by air-drying, and stained as described in section (a). The number of blastomere nuclei present was recorded, as was the chromosome number of the cells in mitosis.

RESULTS

(a) The chromosome complement of oocytes at ovulation and of embryos at the first cleavage metaphase

Analysis of diakinesis/metaphase I chromosomes from oocytes isolated at the germinal vesicle stage and cultured for 4 h in standard embryo-culture medium, has shown that all F₁T6 oocytes had either a quadrivalent or a univalent–trivalent configuration (Fig. 1A, B). No such chromosome configurations were observed in the F₁LAC oocytes. The F₁T6 showed a much higher frequency of aneuploid oocytes (37.8%) than the F₁LAC oocytes (3.4%). The majority of the aneuploid oocytes in the F₁T6 had 19 or 21 chromosomes with about equal frequency, and the one aneuploid out of 29 F₁LAC oocytes examined had
Table 1. *The chromosome complement of oocytes at ovulation; and of parthenogenetic embryos at the first cleaving metaphase and on the 4th day of development* *

<table>
<thead>
<tr>
<th>F₁ hybrid</th>
<th>Stage</th>
<th>Total no. of oocytes or embryos examined</th>
<th>Chromosome number</th>
<th>Aneuploid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>F₁T6</td>
<td>1. Metaphase II (recently ovulated oocytes)</td>
<td>74</td>
<td>15</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>2. First cleavage metaphase</td>
<td>110</td>
<td>12</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>3. Embryos recovered on the 4th day</td>
<td>74</td>
<td>1</td>
<td>59</td>
</tr>
<tr>
<td>F₁LAC</td>
<td>1. Metaphase II (recently ovulated oocytes)</td>
<td>29</td>
<td>1</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>2. First cleavage metaphase</td>
<td>61</td>
<td>2</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>3. Embryos recovered on the 4th day</td>
<td>57</td>
<td>—</td>
<td>50</td>
</tr>
</tbody>
</table>

* Embryos with 40 or 42 chromosomes are also included in these data.
† The T6 marker chromosome was present in the metaphases of 6 of these 7 embryos, and these embryos must therefore have originated from the F₁T6 recipients.
‡ The aneuploid embryos originating from the F₁T6 recipients are not included in this calculation.

Table 2. *The pathways of development of parthenogenetic embryos at 5–6 h after in vitro activation*

<table>
<thead>
<tr>
<th>F₁ hybrid</th>
<th>Total no. of eggs examined</th>
<th>1 pronucleus + 2PB*</th>
<th>Activated eggs (%): immediate cleavage</th>
<th>Nil 2PB*</th>
<th>Overall activation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 pronucleus</td>
<td>2 pronuclei</td>
<td></td>
</tr>
<tr>
<td>F₁T6</td>
<td>1284</td>
<td>863 (92.8)†</td>
<td>35 (3.8)</td>
<td>1 (0.1)</td>
<td>31 (3.3)</td>
</tr>
<tr>
<td>F₁LAC</td>
<td>623</td>
<td>428 (93.7)</td>
<td>20 (4.4)</td>
<td>1 (0.2)</td>
<td>8 (1.8)</td>
</tr>
</tbody>
</table>

* Second polar body.
† The percentage of the total eggs activated is given in parentheses.
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19 chromosomes (Table 1). There was also a higher incidence of aneuploids in F1T6 (26.4%) compared to F1LAC (3.3%) eggs at metaphase of the first cleavage mitosis. As at ovulation, the majority of aneuploids at the first cleavage metaphase had 19 or 21 chromosomes in the F1T6, and the two aneuploids in the F1LAC both had 19 chromosomes (Table 1).

About 93% of the F1T6 and F1LAC activated oocytes developed a single pronucleus following extrusions of the second polar body (Table 2).

(b) The chromosome complement and cell number of embryos on the 4th day of development

A total of 511 activated 1-cell F1T6 eggs were transferred to 44 (34 F1LAC and 10 F1T6) recipients, and 412 cleaving embryos were recovered from the uterine horns on the side of transfer on the 4th day of pseudopregnancy. Two hundred and seventy-six embryos were more advanced than the 2-cell stage, but 16 of these were lost during the air-drying procedure. One hundred and thirty-six of the 260 embryos had cells in mitosis and unequivocal chromosome counts could be made in 74 of these embryos. Thirteen of these 74 embryos had 21 chromosomes, one embryo had 42 chromosomes, and one 4-cell embryo had a single metaphase with 19 chromosomes (Table 1). The T6M chromosome was absent in the embryo with 19 chromosomes, but present in all of the embryos with 21 chromosomes (Fig. 1C). One of the embryos with 21 chromosomes also contained a large metacentric chromosome.

A total of 284 activated 1-cell F1LAC eggs was transferred to 24 F1T6 recipients, and 276 cleaving embryos were recovered from the uterine horns on the side of transfer. One hundred and ninety-one embryos were more advanced than the 2-cell stage, but 17 of these were lost during the air-drying procedure. Seventy-eight of the 174 embryos had cells in mitosis and unequivocal chromosome counts could be made in 57 of these embryos. Seven of these 57 embryos had 21 chromosomes. The T6M chromosome was absent in one of these embryos with 21 chromosomes (Fig. 1D). As the six embryos with 21 chromosomes in which the T6M chromosome was present were all isolated from F1T6 recipients, these could only have originated from activation of the recipients' ova.

Of the F1T6 embryos recovered on the 4th day 10.3% were 'haploid/diploid' mosaics. Thirteen embryos were 20/40 mosaics and one embryo was a 21/42 mosaic (Table 3). These mosaics have been classified under the groups with 20 or 21 chromosomes in the data in Table 1, as have the 'diploid' embryos with 40 or 42 chromosomes. The classification was based on chromosome counts of a mean of between three and four cells in mitosis per embryo. Of the F1T6 embryos 8.1% had 40 and 1.4% had 42 chromosomes. All of the F1LAC 'diploids' had 40 chromosomes.

The mean cell number of eleven F1T6 embryos with 21 chromosomes recovered from the transfer side and a further five similar embryos recovered from the control series was 11.7, while the mean cell number of 49 F1T6
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embryos with 20 chromosomes was 12.0. The mean cell number of all the embryos which were more advanced than the 2-cell stage was similar in the F1T6 and F1LAC series (Table 3).

(c) The chromosome complement and cell number of eggs and embryos recovered from the control uterine horns of recipients on the 4th day of pseudopregnancy

As avertin anaesthesia may induce parthenogenetic development (Kaufman, 1975), the control uterine horns also were studied in recipients on the 4th day of pseudopregnancy. A total of 36 and 47 cleaving embryos was recovered from the control uterine horns of 29 F1T6 and 25 F1LAC recipients, respectively. Thirty of the F1T6 and 35 of the F1LAC embryos were more advanced than the 2-cell stage. The mean number of cleaving embryos recovered from the F1T6 and F1LAC control uterine horns was therefore 1.2 and 1.9 per horn respectively.

Fifteen of the F1T6 and 24 of the F1LAC embryos had cells in mitosis. All five of the F1T6 embryos in which unequivocal counts could be made had 20 chromosomes. Thirteen of the F1LAC embryos in which unequivocal counts could be made had 20 chromosomes, while two embryos had an aneuploid chromosome complement. One of these aneuploid embryos had 21 chromosomes (the T6M was not present), while the other had 41 chromosomes, one of which was a large metacentric chromosome (Fig. 1E). The mean cell number of the F1T6 and F1LAC embryos which were more advanced than the 2-cell stage was 10.2 and 13.4, respectively.

In 9 out of 34 F1T6 recipients and 8 out of 34 F1LAC recipients in which more cleaving embryos were recovered from the uterine horns than activated 1-cell eggs were transferred, the mean number of extra cleaving embryos was 1.3 and 1.5 per horn respectively.

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**Figure 1**

Air-dried chromosome preparations. A–D stained with Giemsa, E stained with lactic acetic orcein. The bar on each photograph represents 5 μm.

A. F1T6 (T6 translocation heterozygote) oocyte at diakinesis-metaphase I, with 18 bivalents and a chain quadrivalent (arrowed).

B. F1T6 oocyte at diakinesis-metaphase I, with 18 bivalents, a univalent (the T6 marker chromosome, T6M, short arrow), and a trivalent (long arrow).

C. Metaphase plate with 21 chromosomes including the T6M (arrowed), from an 18-cell F1T6 parthenogenetic embryo.

D. Metaphase plate with 21 chromosomes from a 15-cell F1LAC parthenogenetic embryo.

E. Metaphase plate with 41 chromosomes including a large metacentric chromosome (arrowed), from a 5-cell F1LAC parthenogenetic embryo.
Table 3. The chromosome complement and cell number of parthenogenetic embryos recovered on the 4th day of development

(i) Chromosome complement of embryos which were more advanced than the 2-cell stage

<table>
<thead>
<tr>
<th>F&lt;sub&gt;1&lt;/sub&gt; hybrid</th>
<th>Total no. of embryos examined</th>
<th>'Haploid'*</th>
<th>'Haploid/diploid'** mosaiics</th>
<th>'Diploid'**</th>
<th>Total no. of embryos with cells in mitosis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>F&lt;sub&gt;1&lt;/sub&gt;T6</td>
<td>260</td>
<td>106</td>
<td>14</td>
<td>16</td>
<td>136</td>
</tr>
<tr>
<td>F&lt;sub&gt;1&lt;/sub&gt;LAC</td>
<td>174</td>
<td>66</td>
<td>5</td>
<td>7</td>
<td>78</td>
</tr>
</tbody>
</table>

(ii) Cell number of embryos recovered on the 4th day of development

<table>
<thead>
<tr>
<th>F&lt;sub&gt;1&lt;/sub&gt; hybrid</th>
<th>Total no. of embryos examined</th>
<th>Cell number</th>
<th>Mean cell number of embryos with more than 2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>F&lt;sub&gt;1&lt;/sub&gt;T6</td>
<td>396</td>
<td>2</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3-4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9-16</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>17-32</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>33-64</td>
<td></td>
</tr>
<tr>
<td>F&lt;sub&gt;1&lt;/sub&gt;LAC</td>
<td>259</td>
<td>85</td>
<td>7.8</td>
</tr>
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<td>59</td>
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<td></td>
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<td>69</td>
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<td>37</td>
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<tr>
<td></td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* 'Haploid' includes 19-21 chromosomes, and 'diploid' includes 40-42 chromosomes.

† These embryos were scored as 'haploid', 'haploid/diploid' mosaiics or 'diploids' without necessarily making an unequivocal count of the chromosome number.
DISCUSSION

We have compared the early developmental potential of haploid and aneuploid oocytes isolated from genetically similar normal and translocation-bearing mice. Oocytes, activated eggs and embryos from the translocation-bearing F3T6 mice had a much higher frequency of aneuploidy than those from the non-translocation-bearing F3LAC mice. Eggs with 20 and 21 chromosomes had a similar development, at least up to the morula stage. The mean cell number of the embryos with 20 and 21 chromosomes was similar, while the proportion of 21-chromosome embryos was about that expected from the incidence of oocytes ovulated with 21 chromosomes. The presence of this additional chromosome, therefore, did not hinder the early haploid embryonic development. Eggs with 20 chromosomes which have a balanced genetic constitution can contain either the normal chromosomes 14 and 15, or the small (T6M) and large translocation products. Eggs with 21 chromosomes must contain three out of four of these normal and translocation products, following non-disjunction at meiosis I. All the embryos with 21 chromosomes from the F3T6 series contained the T6M, in contrast to the frequency of 50% expected if the distribution were random. The two embryos with 21 and 41 chromosomes (one of the 41 chromosomes was a large metacentric), which did not include a T6M chromosome, both came from the F3LAC series, and may have been disomic for chromosomes other than numbers 14 or 15.

There were many oocytes and activated eggs with 19 chromosomes in the F3T6. The observation of only a single 4-cell embryo with 19 chromosomes out of 129 analysable embryos recovered from both the transfer and control sides in the F3T6 and F3LAC series, suggests that this condition is almost certainly inviable very early in development. Most embryos with 19 chromosomes probably fail to develop beyond the 2-cell stage. Contraction of the chromosomes as a result of the colchicine or colcemid treatment prevented identification of the T6M chromosome in the first cleavage metaphase, and the single 4-cell embryo with 19 chromosomes did not contain a T6M chromosome. Fewer cleaving embryos were recovered in the F3T6 than the F3LAC series (80.6% and 97.2%, respectively, of the embryos transferred). When the number of embryos recovered from the control uterine horns is taken into account, the proportion of transferred embryos recovered was probably nearer 66% in the F3T6 and 87% in the F3LAC series, respectively. The reduction of the 19-chromosome population, from approximately one-fifth of the activated eggs on day 1 to only one embryo recovered on the 4th day in the F3T6 series, could account for the reduced recovery rate of cleaving embryos in the F3T6 series. Haploid F3T6 parthenogenetic embryos may also die very early in development because they contain an unbalanced genome following chiasma formation at meiosis I (Searle et al. 1971).

Fertilization of oocytes with 21 chromosomes can lead to the production of
viable offspring with 41 chromosomes which are T6M trisomics. In contrast to the high rate of early development of the parthenogenetic embryos with 21 chromosomes, the 9-day-old embryonic (Evans & Meredith, reported as personal communication in Cattanach, 1967), and post-natal incidence of mice with 41 chromosomes produced after fertilization was 2–7% (Cattanach, 1967; Eicher & Green, 1972). These results suggest that most embryos with the additional T6M chromosome die later.

All the embryos transferred, and most of those originating from activation of the recipients’ own ova, were initially haploid. Thus the high incidence of embryos with 20 or 21 chromosomes recovered on the 4th day, rather than haploid/diploid mosaics or diploid embryos, suggests that the process of cell division is normal in most of the haploids.

Data from the F1LAC series confirm the observation of Graham & Deussen (1974), that culture of oocytes in medium of normal osmolarity directly after activation in vitro does not induce non-disjunction at meiosis II. The very low incidence of aneuploidy observed in recently ovulated oocytes from 8- to 12-week-old F1LAC mice also supports previous observations that the occurrence of non-disjunction at meiosis I is rare in oocytes from young non-translocation-bearing mice (Rohrborn, 1972, and following discussion; Uchida & Lee, 1974). However, even in non-translocation-bearing animals there can be more aneuploidy as a result of non-disjunction at meiosis I in old females (Henderson & Edwards, 1968; Gosden, 1973; Yamamoto, Shimada, Endo & Watanabe, 1973).

The further study of aneuploid embryos should be able to determine which other genetic deficiencies or duplications influence embryonic viability. The present experimental approach should therefore prove useful in further elucidating the genetic basis of normal and abnormal embryonic development.

M.H.K. is a recipient of an MRC Travelling Fellowship.

REFERENCES

Haploid and aneuploid parthenogenetic development


(Received 10 April 1975, revised 2 July 1975)
Complete preimplantation development in culture of parthenogenetic mouse embryos

By MATTHEW H. KAUFMAN1 and LEO SACHS2

From the Department of Genetics, Weizmann Institute of Science, Rehovot, Israel

SUMMARY

The present experiments were undertaken to determine whether, in parthenogenesis, heterozygous embryos develop better than homozygous embryos. Such experiments may provide an approach to elucidating whether fertilized embryos develop better than parthenogenetic ones because of heterozygosity, or if the sperm provides another contribution necessary for complete embryonic development. The parthenogenetic embryos studied included uniform haploids after extrusion of the second polar body, mosaic haploids in which each blastomere contained a genetically different haploid nucleus, and heterozygous diploid mouse embryos. Eggs were activated and cultured in a chemically defined medium. About three times as many mosaic haploid or heterozygous diploid eggs developed beyond the 4-cell stage after 98–100 h and to the blastocyst stage after 120 h in culture, than uniform haploid eggs. This indicates that the development of parthenogenetic embryos is probably under genetic control and that there was a better development of the heterozygous embryos. Mosaic haploid embryos showed the same high frequency of development as heterozygous diploids. The results therefore indicate that heterozygosity provided a developmental advantage even when distributed between two genetically different clones of cells in the same embryo.

INTRODUCTION

Fertilization, in addition to introducing the sperm, results in heterozygosity. This raises the question whether the better development of fertilized compared to parthenogenetic embryos is due only to heterozygosity, or whether the sperm provides another contribution necessary for complete embryonic development (Tarkowski, 1971; Graham, 1974). One approach to answering this question is to study whether, in parthenogenesis, heterozygous embryos have a better development than homozygous embryos.

The mouse was chosen for the present study because its eggs can be efficiently activated in vitro (Graham, 1970; Kaufman, 1973a, b) and conditions for the culture of preimplantation fertilized embryos have been established (Whitten, 1956; Brinster, 1963; Biggers, Whittingham & Donahue, 1967; Whittingham & Biggers, 1967; Whitten & Biggers, 1968). Parthenogenetic eggs of various

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types, with different genetic constitutions, may be obtained by controlling the post-ovulatory age of oocytes at activation and the culture conditions during the first few hours after activation (Graham, 1972; Kaufman, 1973a; Graham & Deussen, 1974; Kaufman & Surani, 1974). As a result of crossing-over, both products of meiosis I are genetically dissimilar and three classes of parthenogenetic embryos can be obtained, depending on whether the second polar body is expelled or not (Tarkowski, 1971; Graham, 1974). Under normal circumstances one set of chromosomes is discarded with the second polar body, leaving the egg with a single haploid set of chromosomes (uniform haploid). The embryos which result from this type of development would be expected to be homozygous at all genetic loci. When, however, second polar body extrusion is inhibited, the egg may either contain one heterozygous diploid or two genetically different haploid pronuclei (Fig. 1). The third class of parthenogenetic development has been termed 'immediate cleavage' (Braden & Austin, 1954). In this case the egg divides into two equally sized blastomeres instead of forming a second polar body, so that each blastomere contains a genetically different pronucleus. When eggs undergo 'immediate cleavage' or 'delayed cleavage' (Fig. 1; the 'delayed immediate cleavage' of Graham, 1971) both blastomeres give rise to separate clones of cells within the embryo. Since these clones are genetically dissimilar these embryos will be referred to as 'mosaic haploids'.

We have examined in the present experiments the preimplantation development of uniform haploid, mosaic haploid and heterozygous diploid parthenogenetic embryos. Eggs which developed a single diploid pronucleus (Fig. 1) were not observed. The development of genetically different parthenogenetic embryos was also compared with the development of fertilized embryos. Oocytes were obtained from (C57BL × CBA)F₁ hybrid females as these had previously been successfully employed (Graham, 1970; Kaufman & Sachs, 1975) in in vitro activation studies. All eggs were cultured in a simple chemically defined medium (Whittingham, 1971).

MATERIALS AND METHODS

(a) Parthenogenetic activation of eggs

Eggs were isolated from the ampullar region of the oviduct of 8- to 12-week-old (C57BL × CBA-LAC)F₁ female mice (hereafter referred to as F₁LAC) at 21–21.5 h after human chorionic gonadotrophin (HCG) injection for superovulation. Oocytes were liberated from the ampullae into a modified Krebs–Ringer bicarbonate culture medium containing 4 mg/ml bovine serum albumin (Whittingham, 1971) and 100 i.u./ml hyaluronidase (Koch-Light, ovine testes) between 08.30 and 09.30 h, and incubated at 37 °C in 5 % CO₂ in air. After 10–15 min the eggs were isolated from this medium, transferred to hyaluronidase-free medium and culture continued for a further 4–5 h. All culture
was carried out under light paraffin oil in 60 x 15 mm plastic Petri dishes (Falcon plastics No. 3002). Eggs were examined under the 50 x magnification of a Wild dissecting microscope to determine the overall activation frequency and types of parthenogenetic eggs induced.

Activated eggs of three types were observed at this time, namely those which developed a single pronucleus following extrusion of the second polar body, eggs which developed two pronuclei in the absence of second polar body extrusion, and eggs which underwent immediate cleavage. Activated eggs of each type were isolated and transferred, generally in batches of 10 or 15, to 30-50 μl drops of culture medium under oil in separate Petri dishes. The activated eggs were retained in culture for a further 94-96 h. Eggs and embryos were then classified according to whether they had fragmented or degenerated, or were at the 2-, 3- or 4-cell stage, or were more advanced in development. Only embryos which were more advanced than the 4-cell stage were examined by air-drying (Tarkowski, 1966). In a few experiments embryos were cultured in medium containing 0.5 μg/ml Colcemid for approximately 2 h prior to air-drying, to accumulate an increased number of cells in mitosis.
A maximum of three embryos were carefully placed on each microslide, and preparations were stained with 4% Giemsa. The air-drying technique allowed the number of blastomere nuclei present to be recorded, and the ploidy of the cells in mitosis to be determined.

Parthenogenetic embryos were also cultured for a total of 120 h to determine the proportion which were capable of developing to the blastocyst stage.

(b) Recovery of fertilized 1-cell eggs

Eight- to twelve-week-old spontaneously ovulating F₁LAC females were examined by vaginal inspection (Champlin, Dorr & Gates, 1973) at approximately 20.00 h, and those in oestrus were mated to fertile F₁LAC males. A further group of 8- to 12-week-old F₁LAC females were treated with an intraperitoneal injection of 5 i.u. pregnant mares' serum gonadotrophin followed 48 hours later by 5 i.u. of HCG. The gonadotrophins were injected at 12.00 h, and females were placed with fertile males shortly after the HCG injection. Both groups of females were checked at approximately 08.00 h the following morning for evidence of mating. Those with vaginal plugs were killed 1–2 h later and the fertilized eggs released into medium containing hyaluronidase. After 10–15 min the cumulus-free eggs were transferred to hyaluronidase-free medium, and retained in culture for a further 98–100 h. Eggs and embryos were then classified according to their stage of development and those more advanced than the 4-cell stage were examined by air-drying. The preparations were stained and analysed as described in section (a).

RESULTS

(a) Pathways of development of parthenogenetic eggs
4–5 h after in vitro activation

As cytoplasmic cleavage took place about 6–8 h after activation in most of those eggs with two pronuclei that subsequently underwent delayed cleavage, all eggs were examined 4–5 h after hyaluronidase treatment. This allowed the immediate-cleavage eggs to be distinguished from the eggs that developed two pronuclei (see Fig. 1). The overall activation frequency in the present series of experiments was 70% (Table 1), and the incidence of eggs which fragmented was 0.5%.

(b) Development beyond the 4-cell stage of parthenogenetically activated and fertilized 1-cell eggs after 98–100 h in culture

Twenty-one per cent of the total 1-cell parthenogenetically activated eggs with a single pronucleus that were retained in culture for 98–100 h progressed beyond the 4-cell stage, compared to 66% of the 1-cell eggs with two pronuclei, and 65% of the eggs that underwent immediate cleavage. In the two fertilized series, 96% and 72% of the 1-cell eggs isolated from the spontaneously
Parthenogenetic development in culture

Ovulating and gonadotrophin-induced females, respectively, developed beyond the 4-cell stage during a similar period in culture (Table 2). Seventy-four per cent and 67% of the spontaneously ovulating and gonadotrophin-induced embryos, respectively, that developed beyond the 4-cell stage had become blastocysts, while the remaining embryos were all morulae.

Table 1. The pathways of development of parthenogenetic eggs at 4–5 h after in vitro activation

<table>
<thead>
<tr>
<th>Activated eggs</th>
<th>Overall activation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of eggs examined</td>
<td>1 pronucleus + 2nd polar body</td>
</tr>
<tr>
<td>2756</td>
<td>1725 (88.9)*</td>
</tr>
</tbody>
</table>

* The percentage of the total number of eggs activated is given in parentheses.

(c) The cell number and ploidy of embryos which developed beyond the 4-cell stage after 98–100 h in culture

The cell numbers of the parthenogenetic and fertilized embryos which developed beyond the 4-cell stage after 98–100 h in culture are presented in Table 2. The mean cell number of the single pronuclear parthenogenetic embryos was significantly lower than that of the 2-pronuclear and immediate-cleavage embryos, while the mean cell numbers of the two groups of fertilized embryos were approximately double those of the 2-pronuclear and immediate-cleavage embryos. As the initiation of development in the fertilized eggs probably took place 5–8 h before activation was induced in the parthenogenetic eggs, this would only partially account for the greater mean cell numbers of the fertilized embryos. This suggests that the cleavage rate of the fertilized eggs was on average faster than that of the parthenogenetic embryos when all groups were cultured under similar conditions.

The ploidy of the embryos with cells in mitosis at the time of analysis is presented in Table 3. The mitoses were scored as 'haploid', 'diploid' or 'tetraploid' without necessarily making an unequivocal count of the chromosome number, though accurate counts could be made in a high proportion of the cells examined. Seventy-two per cent of the single pronuclear eggs had only haploid mitoses present, having the expected ploidy for this type of embryo. Sixteen per cent and 76%, respectively, of the eggs which developed two pronuclei appeared to be either uniform haploids or uniform diploids. This suggests that a higher proportion developed as diploids than underwent 'delayed cleavage' (Fig. 1). Eighty-one per cent of the immediate cleavage embryos had only haploid mitoses present, having the expected ploidy for this type of embryo. All (11/16) of the immediate cleavage embryos in which unequivocal counts could be made had either 20 or 40 chromosomes.
Table 2. *The cell number of parthenogenetic and fertilized embryos which developed beyond the 4-cell stage after 98–100 h in culture*

<table>
<thead>
<tr>
<th>Parthenogenetic or fertilized group</th>
<th>Type</th>
<th>Total number of activated eggs cultured</th>
<th>Total number of embryos which developed beyond the 4-cell stage</th>
<th>Cell number</th>
<th>Mean cell number of embryos with more than 4 cells ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parthenogenetic</td>
<td>One pronucleus + second polar body</td>
<td>1053</td>
<td>220</td>
<td>93 85 35 6 1</td>
<td>12.3 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Two pronuclei without second polar body</td>
<td>65</td>
<td>43</td>
<td>7 12 17 6 1</td>
<td>21.9 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Immediate cleavage</td>
<td>71</td>
<td>46</td>
<td>15 15 9 6 1</td>
<td>17.9 ± 2.3</td>
</tr>
<tr>
<td>Fertilized</td>
<td>Spontaneous ovulation</td>
<td>68</td>
<td>65</td>
<td>2 8 12 25 18</td>
<td>48.2 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>Gonadotrophin-induced ovulation</td>
<td>58</td>
<td>42</td>
<td>2 4 13 19 4</td>
<td>38.3 ± 3.4</td>
</tr>
</tbody>
</table>
Table 3. The ploidy of parthenogenetic and fertilized embryos which developed beyond the 4-cell stage after 98–100 h in culture

<table>
<thead>
<tr>
<th>Parthenogenetic or fertilized group</th>
<th>Type</th>
<th>Total number of embryos examined</th>
<th>Total number of embryos with cells in mitosis*</th>
<th>Ploidy of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Haploid</td>
</tr>
<tr>
<td>Parthenogenetic</td>
<td>One pronucleus + second polar body</td>
<td>220</td>
<td>87 (39.6)†</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Two pronuclei without second polar body</td>
<td>43</td>
<td>25 (58.1)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Immediate cleavage</td>
<td>46</td>
<td>16 (34.8)</td>
<td>13</td>
</tr>
<tr>
<td>Fertilized</td>
<td>Spontaneous ovulation</td>
<td>65</td>
<td>49 (75.4)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Gonadotrophin-induced ovulation</td>
<td>42</td>
<td>28 (66.7)</td>
<td>—</td>
</tr>
</tbody>
</table>

* The ploidy of these embryos was scored without necessarily making an unequivocal count of the chromosome number.
† The percentage of the total number of embryos examined is given in parentheses.
In the two fertilized series 92% and 93% of the spontaneously ovulating and gonadotrophin-induced groups, respectively, had only diploid mitoses present. One embryo in the gonadotrophin-induced group was a diploid-tetraploid mosaic, while the remaining five embryos in which the ploidy could be determined had only tetraploid mitoses present.

Table 4. Development of parthenogenetic eggs to the blastocyst stage after 120 h in culture

<table>
<thead>
<tr>
<th>Type</th>
<th>Total number of activated eggs cultured</th>
<th>Total number of eggs which developed to the blastocyst stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>One pronucleus + second polar body</td>
<td>188</td>
<td>40</td>
</tr>
<tr>
<td>Two pronuclei without second polar body</td>
<td>33</td>
<td>19</td>
</tr>
<tr>
<td>Immediate cleavage</td>
<td>29</td>
<td>16</td>
</tr>
</tbody>
</table>

(d) Development of parthenogenetic eggs to the blastocyst stage after 120 h in culture

When the 2-pronuclear and immediate cleavage eggs were retained in culture for 120 h, 58% and 55% of these eggs, respectively, developed to the blastocyst stage compared to 21% blastocysts from the eggs with one pronucleus (Table 4).

DISCUSSION

In the present experiments, we have examined the preimplantation development in culture of three types of parthenogenetic embryos with different genetic constitutions, namely uniform haploids, mosaic haploids and heterozygous diploid embryos. These three out of the four possible types of pronuclear eggs (Fig. 1) were found 4–5 h after activation according to whether second polar body extrusion occurred or not, whether the egg underwent immediate cleavage, and depending on whether the egg contained one or two pronuclei. The development of these three types of parthenogenetic eggs was compared with fertilized eggs.

The results show that about three times as many parthenogenetic eggs developed beyond the 4-cell stage in the eggs with two haploid pronuclei or after immediate cleavage, compared with eggs that developed a single haploid pronucleus following extrusion of the second polar body. Similar results were obtained when eggs were examined after 98–100 h in culture, or when the rate of development to the blastocyst stage was determined after 120 h in culture.
In order to substantiate the hypothesis that preimplantation parthenogenetic development is under genetic control, other possible explanations for the poorer development of the uniform haploids must be considered and excluded. Three obvious differences exist between the uniform haploids and the other two classes of embryos. Only in the uniform haploids is a certain volume of cytoplasm lost following second polar body extrusion. The actual volume of cytoplasm involved is extremely small, and similar to that lost following fertilization. Here, second polar body extrusion is both a normal and necessary process with no apparent detrimental effect on the resulting embryo. Thus the loss of this volume of cytoplasm in the uniform haploids is unlikely to account for the poorer development of these embryos. It is more difficult to evaluate the importance of the different nuclear–cytoplasmic ratios of the uniform haploid compared to the immediate cleavage and 2-pronuclear embryos. It has generally been assumed that the ratio of 2N:1 observed in fertilized embryos must be optimal, and that other ratios are in some way sub-optimal. Thus the ratios observed in the 2-pronuclear and immediate cleavage eggs of 2N:1 and N:0.5, respectively, might be optimal, whereas the ratio of N:1 observed in the uniform haploids might be sub-optimal. Evidence which tends to suggest that this general hypothesis may be an oversimplification has recently been published by Modlinski (1975). This author demonstrated that the preimplantation development potential of fertilized mouse eggs from which one of the two pronuclei had been withdrawn depended on whether the remaining pronucleus originated from the male or female gamete. In this case the resulting nuclear–cytoplasmic ratios were similar, but the development potential was clearly not so. These findings must shed some doubt on the importance which should be assigned to the concept of an optimal nuclear–cytoplasmic ratio controlling early embryonic development, and on this explanation for the present findings.

These three classes of parthenogenetic embryos are also genetically dissimilar. The eggs with two haploid pronuclei developed either as mosaic haploids or heterozygous diploids, the immediate cleavage eggs as mosaic haploids, and the eggs with a single haploid pronucleus as uniform haploids. The mosaic haploid embryos contain two genetically different clones of cells. Since these mosaic embryos showed the same high frequency of development as heterozygous diploids, this presumably indicates that heterozygosity in an embryo, even when distributed between two clones of cells, provides a developmental advantage. Products produced by the two clones of cells may diffuse from one to the other, so as to compensate for missing products or balance the effect of deleterious genes. It remains to be seen whether the heterozygous diploids have a developmental advantage over mosaic haploids beyond the preimplantation period. The higher implantation rate of the heterozygous diploids over the homozygous haploids and mosaic haploids (Kaufman & Gardner, 1974) suggests that this cooperation may not be as efficient at later
stages of development. The fact that 21% of the uniform haploids were able to achieve a comparable stage of development to the mosaic haploids, suggests that the actual number of deleterious factors that might inhibit preimplantation development are relatively few in inbred strains (Kaufman, Huberman & Sachs, 1975).

All of the immediate cleavage embryos in which unequivocal counts could be made had either 20 or 40 chromosomes. This suggests that normal segregation of chromosomes occurred at the second meiotic division when embryos of this type were induced to develop under the present culture conditions. Previously Graham & Deussen (1974) had demonstrated that over 60% of immediate cleavage embryos had abnormal karyotypes. The present findings suggest that these abnormal karyotypes may have resulted from differences in the culture conditions after activation.

No homozygous diploid embryos were obtained with certainty in the present experiments. Diploid eggs of this type would show complete genetic homozygosity at all loci, and thus may have no developmental advantage over uniform haploid embryos. It should be possible to produce homozygous diploid embryos from uniform haploid eggs by inhibition of their first cleavage division with, for example, Cytochalasin B (Snow, 1973), and it would be interesting to compare their development with uniform haploid and heterozygous diploid embryos.

A higher proportion of the fertilized embryos developed beyond the 4-cell stage after 98–100 h in culture, and their cleavage rate was on average faster than that of all three types of parthenogenetic embryos. Whether their better development was due to a greater degree of heterozygosity in the fertilized compared to the parthenogenetic embryos, or was due to the presence of some other factor contributed by the sperm remains to be determined. If a factor from the sperm is necessary for advanced embryonic development, the production of chimaeric embryos by the aggregation of one parthenogenetic with one fertilized embryo (see Graham, 1970) may be the only means of obtaining complete parthenogenetic development. If, however, the degree of heterozygosity is the critical factor, more advanced development could possibly be obtained by aggregating two or more parthenogenetic embryos from different genetic backgrounds. A parallel can be drawn to the situation in tetraparental mice (Tarkowski, 1961; Mintz, 1962), especially where these have been used for the survival of embryos with lethal genes (Mintz, 1962; Eicher & Hoppe, 1973).

F₁ hybrid mice were used in the present experiments because it had previously been shown that a high proportion of their fertilized 1-cell eggs developed to the blastocyst stage in simple chemically defined medium (Whitten & Biggers, 1968). One-cell eggs from inbred and random-bred animals have so far generally failed to develop beyond the 2-cell stage in culture (Biggers, Whitten & Whittingham, 1971). However, if an inbred strain rather than an F₁ hybrid
Parthenogenetic development in culture

could be found whose preimplantation fertilized and parthenogenetic eggs developed completely in vitro, this might provide additional information on the genetics of early development without the possible complicating factor of using $F_1$ hybrids.

The present results demonstrate that in vitro activated eggs may be routinely cultured from the 1-cell stage to the blastocyst in chemically defined medium, and that the development of parthenogenetic eggs is probably under genetic control. This supplements the previous findings on the genetic control of haploid parthenogenetic development, which suggested that haploid embryos had a greater developmental potential if they were derived from inbred rather than from random-bred animals (Kaufman, Huberman & Sachs, 1975).

M.H.K. is a recipient of an MRC Travelling Fellowship.

REFERENCES


(Received 26 August 1975)
The incidence of chromosomally unbalanced gametes in T(14; 15)6 Ca heterozygote mice

BY M. H. KAUFMAN

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(Received 5 January 1976)

SUMMARY

When T6/+ female mice were mated to non-translocation-bearing males, the relative viability of the embryos at 13.5-14.5 days gestation was about 39%. About 36% of the oocytes ovulated by T6/+ females were aneuploid, as a result of non-disjunction at meiosis, the majority having either 19 or 21 chromosomes. However, aneuploidy only accounts for a proportion of the embryonic loss in T6/+ x +/+ matings, as many of the embryos with 41 chromosomes survive postnatally. The present findings indicate that approximately 50% of the oocytes ovulated with the normal haploid number of chromosomes (n = 20) were genetically unbalanced as a result of adjacent segregation, and that a high proportion of the resultant embryos die in the early postimplantation period. In the present study non-translocation-bearing mice which were genotypically similar to the T6/+ females acted as controls.

1. INTRODUCTION

The present experiments were undertaken to investigate the genetic constitution and development potential of the eggs ovulated by female mice which were heterozygotes for the T6 translocation. This translocation involves a reciprocal exchange between segments of chromosomes 14 and 15 (Miller et al. 1971; Nesbitt & Francke, 1971; Eicher & Green, 1972). During meiosis in T6/+ mice, the small T6 marker chromosome (T6M) and large translocation products associate with their two normal partners either in a quadrivalent or univalent-trivalent configuration, the univalent being the T6M chromosome (Eicher & Green, 1972; Forejt, 1974). T6/+ heterozygote mice ovulate both haploid and aneuploid oocytes as a result of non-disjunction at meiosis I (Eicher & Green, 1972; Kaufman & Sachs, 1975). The majority of the aneuploid eggs ovulated contain 19 or 21 chromosomes with about an equal frequency.

Carter, Lyon & Phillips (1955) had previously noted that the relative viability of the post-implantation embryos in T6/+ females mated to non-translocation-bearing males was about 36%, while Cattanach (1967) showed that fertilization of the oocytes with 21 chromosomes could lead to the production of viable offspring with 41 chromosomes. The post-natal incidence of mice with 41 chromosomes was 2-7% (Cattanach, 1967; Eicher & Green, 1972). Viable offspring with 39 chromosomes, but excluding the XO condition, were not reported in these series. In a more
recent study, Eicher (1973) reported that translocation trisomic offspring were only produced in crosses involving T6/+ females, never T6/+ males, when these were mated to non-translocation-bearing males and females, respectively. In this series, the incidence of translocation trisomic offspring in the T6/+ $2\times +/++$ crosses was about 16%.

The preimplantation development potential of parthenogenetically activated haploid, hypo- and hyperhaploid oocytes from T6/+ females with 20, 19 and 21 chromosomes, respectively, have recently been examined (Kaufman & Sachs, 1975). These authors found that activated 1-cell eggs, at the first cleavage mitosis, had frequencies of 19, 20 and 21 chromosomes similar to oocytes at ovulation. On the third day of development, few embryos with 19 chromosomes remained, but the frequency of 21-chromosome embryos was similar to the frequency of 21-chromosome oocytes and activated 1-cell eggs. Thus the presence of an additional chromosome did not appear to hinder preimplantation haploid embryonic development. In this series, the incidence of aneuploidy in the oocytes ovulated by the T6/+ females was about 38%. All the embryos with 21 chromosomes from the T6/+ series contained the T6M chromosome, in contrast to the expected frequency of 50% if the distribution were random.

The present experiments were carried out to determine the extent of the embryonic loss, and the stage of pregnancy when embryonic death occurs, when T6/+ females are mated to non-translocation-bearing males. The dominant lethal assay was used to determine the stage and extent of the embryonic loss in these matings. Earlier work on translocations in the mouse had suggested that the majority of the genetically unbalanced embryos would die in the pre- or early post-implantation period (Snell, Bodemann & Hollander, 1934; Snell, 1946; Carter et al. 1955). Non-translocation-bearing female mice with a similar genetic background acted as controls for these studies. The main function of the controls was to provide information of the level of embryonic loss occurring in the T6/+ females, due to factors unrelated to the T6 translocation.

2. MATERIALS AND METHODS

All the mice used in the present experiments were obtained from the main breeding colony of the Weizmann Institute of Science, Rehovot, Israel. CBA-T6T6 and CBA/Lac male mice were mated to C57BL females. The resultant F1 hybrid mice are hereafter referred to as F1T6 and F1LAC, respectively. The F1T6 mice were heterozygotes for the T6 translation (T6/+), whereas the F1LAC mice did not carry the T6 translocation (+/+).

10- to 12-week old F1T6 and F1LAC females were given 5 i.u. of pregnant mares' serum gonadotrophin followed 48 hours later by 5 i.u. of human chorionic gonadotrophin (HCG). Females were killed either 8–10 or 14–16 hours after the HCG injection. In the 8–10 hour group the ovaries were removed and pre-ovulatory oocytes obtained by follicular puncture. The chromosome constitution of these oocytes was then determined by air-drying (Tarkowski, 1966). The presence or
Unbalanced gametes in T6 heterozygote mice

absence of a quadrivalent or univalent-trivalent configuration, and the stage of meiotic maturation were determined, namely diakinesis, diakinesis-metaphase I, or metaphase I. Eggs were isolated from the ampullar region of the oviducts of females killed 14-16 hours after HCG. Eggs at metaphase II were examined by air drying, and the number of chromosomes in each egg determined. All preparations were stained with 4% Giemsa following the air-drying procedure, and a maximum of three eggs placed on each microslide. Further groups of 10- to 12-week old F₁ hybrid females of both types were mated to fertile F₁LAC males. Females were checked each morning and those with vaginal plugs were isolated and a maximum of three females caged together. The day on which a plug was observed has been termed the first day of pregnancy. Females were killed at approximately midday on either day 13 or 14 of gestation. The contents of their uterine horns were examined, and the number of resorptions and dead and live embryos noted. The ovaries were then examined to determine the number of corpora lutea present.

Reciprocal skin grafts were performed between groups of CBA-T6T6 and CBA/Lac males as a gross means of assessing their genetic similarity. No rejection was observed in any of the recipients when the sites were examined at 100-120 days after the grafting procedure. This suggests that these two strains were probably either identical, or at least very similar at all of the major and minor loci.

3. RESULTS

(a) The chromosome complement of oocytes during meiotic maturation

Follicular oocytes from F₁T6 females isolated between 8 and 10 hours after the HCG injection for superovulation were analysed by air-drying. Classification of oocytes was made on morphological criteria alone, so that chromosome preparations were divided into diakinesis, diakinesis-metaphase I, or metaphase I groups.

Table 1. Chromosome configurations observed in oocytes at meiosis I

<table>
<thead>
<tr>
<th>F₁ hybrid</th>
<th>Stage of meiosis</th>
<th>Total examined</th>
<th>Quadri-valent</th>
<th>Univalent</th>
<th>Trivalent</th>
<th>Bivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>present</td>
<td>present</td>
<td>only</td>
<td></td>
</tr>
<tr>
<td>F₁T6</td>
<td>Diakinesis</td>
<td>16</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diakinesis-metaphase I</td>
<td>19</td>
<td>16</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Metaphase I</td>
<td>42</td>
<td>31</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>F₁LAC</td>
<td>Diakinesis, diakinesis-metaphase I, metaphase I</td>
<td>46</td>
<td>0</td>
<td>0</td>
<td>46</td>
<td></td>
</tr>
</tbody>
</table>

This classification was therefore independent of the time after HCG when oocytes were isolated. All preparations contained either a single quadrivalent or a univalent-trivalent configuration. The incidence of univalent-trivalent configurations appeared to increase as meiotic maturation progressed. Thus in only 6-3% of diakinesis preparations was a univalent-trivalent configuration found, whereas 26-2% of metaphase I preparations were of this type (Table 1). If a number of
oocytes at a more advanced stage of meiotic maturation had been obtained, a higher proportion of univalents might possibly have been observed. A few preparations of this type were examined, but found to be technically very difficult to analyse.

In many of the chain quadrivalents in which the T6M was terminal, its association appeared to be very loose. This was especially marked at metaphase I. Thus in many of these preparations the T6M chromosome seemed only to be linked by a single chromatid to the sub-terminal member of the chain. A possible sequence of events in the formation of univalents from chain quadrivalents in T6/+ females is illustrated (Fig. 1). Only bivalent configurations were observed in 46 diakinesis-metaphase I preparations isolated under similar conditions from the control F1LAC females.

Fig. 1. Possible sequence of events in the formation of univalents from chain quadrivalents during meiotic maturation in T6/+ oocytes.

(b) The chromosome complement of F1T6 and F1LAC oocytes at ovulation

The chromosome complement of recently ovulated oocytes from F1T6 translocation-bearing and F1LAC non-translocation-bearing mice is presented in Table 2. 37.8% of the oocytes ovulated by F1T6 females had an aneuploid chromosome constitution. The majority of these aneuploid oocytes had 19 or 21 chromosomes with about an equal frequency. The one aneuploid oocyte in the F1LAC series had 19 chromosomes (Table 2). These results have been summarized elsewhere (Kaufman & Sachs, 1975).

(c) An assessment of embryonic loss when T6/+ and control females were mated to non-translocation-bearing males

All females in which a vaginal plug was observed were considered to have mated, and were killed either on day 13-5 or 14-5 of gestation (day of vaginal plug = day 1 of pregnancy). In both series all mated females were found to be pregnant when
dissections were carried out. In the experimental series 17 T6/+ females and in the control series 14 F1,LAC females were mated to non-translocation-bearing males. The total corpora lutea counts, numbers of resorptions, dead and live embryos for both series are presented in Table 3. The proportionate incidence of preimplantation and early and later postimplantation embryonic deaths (Table 3, section b) have been obtained from the figures presented in Table 3, section (a). In contrast to the very low incidence of early postimplantation embryonic loss in the control series (1.6%), 46.8% of the total zygotes produced in the F1,T6 series died at this stage of gestation. The proportion of live embryos observed on days 13.5–14.5 of gestation was 38.7% and 93.6% of the total zygotes produced in the F1,T6 and control series, respectively. The estimated preimplantation embryonic losses were 10.4% and 4.8% in the F1,T6 and control series, respectively.

4. DISCUSSION

In order to be able to assess what proportions of eggs ovulated by T6/+ mice have the normal haploid number of chromosomes (n = 20), but are genetically unbalanced, two distinct approaches may be employed. Embryos at various stages of development may be karyotyped using giemsa or fluorescent banding techniques. This was the approach taken by Oshimura & Takagi (1975), but has the
disadvantage that isolation of embryos at the egg cylinder stage, in the very early post-implantation period when maximum embryonic losses would be expected, is technically very difficult. A second approach based on the dominant lethal assay was employed in the present study. This approach gives information of the incidence and developmental potential of unbalanced gametes, but is unable to distinguish between gametes with different genotypes. In this respect, the two approaches are complementary. In extrapolating from the results obtained in the present experiments, where translocation-bearing T6/+ and genotypically similar but non-translocation-bearing +/- control mice were used, certain assumptions have to be made. It has generally been accepted that in mice the corpora lutea count represents the total number of ova ovulated, there being an extremely low incidence of polyovular follicles in this species. Second, that all the eggs ovulated by non-induced females have an equal chance of becoming fertilized, even those with an abnormal genetic constitution. Third, that the F1T6 and F1LAC mice used in the present experiments were genotypically identical the only difference being that the F1T6 were heterozygotes for the T6 translation (T6/+). No rejection was observed when skin grafts were made between the two male parental strains used to produce the F1T6 and F1LAC mice, namely CBA-T6T6 and CBA/Lac. While the experimental and control females may not have been identical at all genetic loci, the graft data suggests that the differences between them were probably very small. Fourth, that nearly all the zygotes with 39, and over one half of those with 41 chromosomes would die at some stage during embryonic development. One estimate of viability in embryos with 41 chromosomes was that reported by Evans & Meredith (cited in Cattanach, 1967) who noted that approximately 6% of the total 9-day embryonic population in their series was of this type. This proportion was similar to the post-natal incidence observed by Cattanach (1967). In a more recent study Oshimura & Takagi (1975) noted that approximately 13% of the viable embryos examined on days 16.5-18.5 of gestation had 41 chromosomes. The postnatal incidence of translocation trisomics in Eicher’s series (Eicher, 1973) was about 16%.

When in the present series of experiments, the uterine contents and ovaries of pregnant T6/+ and +/- females previously mated to non-translocation-bearing males (+/+) were examined on days 13.5-14.5 of pregnancy, certain obvious differences between these two groups were observed. The incidence of preimplantation and early and later postimplantation embryonic losses were higher in the T6/+ series than in the +/- control females. The 4.8% preimplantation embryonic mortality observed in the control series could either have been due to the failure of oocytes to become fertilized, or to the loss of zygotes at any stage between fertilization and implantation. The embryonic loss in this group must have been due to factors unrelated to the T6 condition. The comparable preimplantation loss in the T6/+ series was 10.4%. The preimplantation embryonic loss in the T6/+ was presumably made up of two components, the first due to factors unrelated to the T6 condition, and the second resulting directly from the T6 state. By extrapolation from the control data it seems likely that the second component might account for
approximately 5–6% of the total embryonic loss in the T6/+ females. This is considerably lower than might have been expected if all zygotes with 39 chromosomes die prior to implantation. This therefore suggests that, at least in the present series, a high proportion of genetically unbalanced fertilized embryos, which may have deletions or duplications of genetic material, were capable of development beyond implantation. It is not clear whether these embryos were only capable of evoking a decidual response or of developing for a limited period beyond implantation. If all oocytes with 19 or 21 chromosomes were capable of fertilization, they would account for approximately 36% of the initial zygote population. Assuming all embryos with 39 chromosomes were inviable as some stage during the prenatal period, this alone would account for approximately 18% of the expected total embryonic loss observed in the T6/+ females. In the Evans & Meredith series (cited in Cattanach, 1967) and that of Cattanach (1967), about 50% of the embryos with 41 chromosomes died prenatally. More recently Oshimura & Takagi (1975) observed that 10–15% of oocytes from T6/+ mice had either 19 or 21 chromosomes. Karyotypes carried out on day 6.5 of gestation demonstrated that a high proportion of the embryos with 41 chromosomes were still viable, in contrast to the very low incidence of embryos with 39 chromosomes. The 13% incidence of viable embryos with 41 chromosomes present on days 16.5–18.5 of gestation reported by these authors would seem to suggest that in their series very few embryos of this type died during the prenatal period. It is obviously impossible to extrapolate from the present findings what proportion of embryos with 41 chromosomes were viable at the time of dissection as karyotyping was not carried out.

The expected embryonic mortality in the T6/+ females due to aneuploidy would be about 23–35%. This estimate accounts for the incidental preimplantation losses unrelated to the T6 state, and assumes that all embryos with 39 chromosomes were inviable, and that 40–100% of embryos with 41 chromosomes were viable. As the overall embryonic viability in the T6/+ series was about 39%, the observed embryonic loss was nearer to 61% of the estimated original zygote population. If one assumes that about 50% of the embryos with 40 chromosomes or, by extension almost 50% of the oocytes ovulated with 20 chromosomes were genetically unbalanced this figure fits well with expectation. This is consistent with previous observations where it has been shown that genetically unbalanced embryos are more likely to die in the early postimplantation period than at other times prenatally (Snell, 1946).

These data support the findings of Oshimura & Takagi (1975), and suggest that the incidence of adjacent segregation in oocytes which undergo normal disjunction at meiosis I in the T6/+ females employed in the present study was about 50%. Further, that the incidence of non-disjunction was not less than 36% in these mice. It should also be noted that the overall viability in T6/+ × +/+ ♂ matings has been remarkably consistent, ranging from 36–39% in three series (Carter et al. 1955; Oshimura & Takagi, 1975; present series), despite the fact that the genetic backgrounds of the non-translocation-bearing strains have varied widely.
It is unclear whether the increase in the incidence of univalent-trivalent configurations which appeared to be related to meiotic maturation in the T6/+/ oocytes, may also be observed in male translocation heterozygotes. A detailed re-evaluation of the preparations examined by E. P. Evans (cited in Eicher & Green, 1972) and Oshimura & Takagi (1975) of meiotic configurations from T6/+ males, where the incidence of quadrivalents was 63% and 54% and univalent-trivalents was 37% and 46% respectively, might help to clarify this point. In both these series, the univalent was always the T6M chromosome. The present findings on the configurations at first meiosis may be relevant to the difference in fertility observed between male and female translocation-bearing mice (Lyon & Meredith, 1966), and to the production of translocation trisomies by female but not male translocation carriers (Eicher, 1973).

I wish to thank Dr Mary Lyon and Professor Moshe Feldman for their advice and discussion, Mr N. Sharaby for carrying out the skin grafting described in the text, and Professor Leo Sachs, the Genetics Department, Weizmann Institute of Science, Rehovot, Israel, for providing me with laboratory facilities. The author was a recipient of an MRC Travelling Fellowship.

REFERENCES


Deleterious effect of an anaesthetic on cultured mammalian embryos

The teratogenic effect of certain tranquillisers on human and animal pregnancies has been known for many years 1-9. Anaesthetics and other central nervous system depressants may also be teratogenic when given during early pregnancy, possibly because of the inhibitory action of these agents on mitosis 10. Anaesthetics can also activate unfertilised mouse eggs in vitro, inducing them to develop parthenogenetically 11,12. Chronic exposure to low levels of anaesthetics may result in a significant increase in spontaneous abortions and the birth of children with congenital abnormalities 13,14. Experiments with rodents 15 which seemed to demonstrate that chronic exposure to anaesthetics could be teratogenic in early pregnancy have proved difficult to repeat 16, so that doubt has now been cast on the validity of these earlier results. Attention has also been drawn to the possible risk to the human foetus of surgery carried out during pregnancy 17. In the experiments reported here we have demonstrated a direct, deleterious effect on early postimplantation rat embryos in culture of a single exposure to different doses of an anaesthetic.

Headfold-stage rat embryos of the CFHB strain were explanted during the afternoon of day 10 of gestation (day of finding sperm in the vagina=day 1 of pregnancy), and cultured in vitro for 24 or 48 h in heat-inactivated (56°C, 30 min) homologous IC serum 18, prepared from blood withdrawn from the aorta of rats anaesthetised with ether. During heat inactivation the bottle containing the serum was opened twice to drive off as much ether as possible. Serum from the same batch was used for both control and experimental culture groups, with Avertin (Winthrop) added to the experimental groups. Except where shown in Table 1, the medium was equilibrated with a gas mixture containing 5% O2, 5% CO2 and 90% N2 for the first 24 h and 5% CO2 in air for the rest of the culture period. These conditions give optimum development in vitro for embryos explanted at the headfold stage (D. A. T. New, personal communication). Since the purpose of the cultures was to test the teratogenic effect of a specific anaesthetic agent, the pregnant females were not killed by etherisation but by cervical dislocation. Where embryos from more than one female were used in one experiment, these were divided equally between control and experimental groups.

In preliminary studies the embryos were cultured in watchglasses or 60-ml roller bottles by New's technique 19. With this equipment the gas phase is considerably greater in volume than the volume of the medium. In this instance no teratogenic effect was obtained even with twice the standard anaesthetic dose of Avertin. In subsequent cultures, sealed embryological watchglasses or 12x75 mm plastic test tubes (Falcon, 2003) containing 3 ml and 5 ml of medium, respectively, were used, such that the volume occupied by the gas phase was less than 0.1 ml and 0.5 ml, respectively.

The standard dose of Avertin used to anaesthetise intact rats was used, where 1 ml of culture medium was assumed to be equivalent to 1 g body weight of an intact rat. The standard dose of Avertin used was 0.02 ml of a freshly prepared 1.2% solution of Avertin dissolved in 0.9% saline per ml of serum (or g body weight of an intact rat), equivalent to approximately 240 p.p.m. (by volume). Further groups of embryos were cultured for 24 h in serum containing twice, four times or six times the standard dose of Avertin. Some of these embryos were examined histologically, while others were transferred with the medium to a 60-ml roller bottle containing 2.4 ml of fresh anaesthetic-free serum and cultured for a further 24 h. At the end of culture, at 24 h or 48 h, embryos were examined under a dissecting microscope and an assessment of development was made using purely morphological criteria, such as the presence or absence of a beating heart, size of the neural tube, the adoption of the foetal position (turning), somite number and the yolk sac diameter. Embryos which were not examined histologically were dissected free from embryonic membranes and their protein content was determined microspectrophotometrically 20.

When embryos were cultured with a large gas phase, there was no difference between controls and those cultured in once or twice the standard anaesthetic dose of Avertin for periods ranging from 10 min to 24 h (the results of this and subsequent cultures are presented in Table 1). When the volume of the gas phase was reduced, embryos grown for 24 h in twice the standard anaesthetic dose clearly developed less well (somites indistinguishable, less embryos with a heartbeat) than controls, and four and six times the standard anaesthetic dose had an even greater effect. When the culture period was extended to 48 h and the protein content of the embryos was estimated, there was a significant difference (P<0.0025) between control embryos and those cultured in twice the standard dose of Avertin.

The reduction in volume of the gas phase also resulted
in a significant difference (P<0.0025) in protein content between control embryos and those grown in the standard dose of Avertin. As in twice the standard dose, all embryos cultured for 48 h developed a heartbeat and closure of the neural folds was unaffected. The frequency of embryos which had 'turned', however, was decreased in the standard dose and twice the standard dose of Avertin compared with the controls. Differentiation, assessed by somite number, was unaffected by the standard dose, but was greatly retarded in twice the standard dose.

Histological examination of control embryos and those cultured in twice the standard dose of Avertin confirmed the detrimental effect of the anaesthetic on differentiation. In the experimental embryos the somites were poorly differentiated and fewer in number (Fig. 1a) compared with control embryos (Fig. 1b). As well as this effect on the paraxial mesoderm the heart seemed to be affected (Fig. 1c). In four out of seven embryos cultured in twice the standard dose of Avertin, cell debris was present in the amniotic cavity (Fig. 1d). This was also found when embryos were cultured in four and six times the standard dose. In the latter group the embryonic material consisted almost entirely of dead or dying cells. Debris was not found in the amniotic cavity of control embryos.

These experiments have demonstrated that the anaesthetic Avertin can be teratogenic in the rat, and that the degree of embryotoxicity is dose dependent. When a range of parameters of development was examined, rat embryos which had been cultured in serum containing Avertin were retarded compared with controls. It is not clear whether the type of growth retardation which was observed in apparently normally differentiating embryos as a result of treatment with standard dose of Avertin could be corrected by compensatory growth later in gestation.

The main advantage of the model used here over, for example, cell lines in tissue culture as a teratological test system, is that the use of whole embryos more closely resembles the situation in vivo. There is, however, the important difference that all the observations reported here result from the direct effect of known doses of Avertin on the embryo, independent of secondary maternal factors such as placental permeability. Further, the anaesthetic is metabolised in vivo, and the metabolic products may be more or less teratogenic than the original agent.

In view of these positive results, the factors which remain to be examined include the establishment of the minimal teratogenic dose and exposure duration of a range of anaesthetics. Experiments should also be carried out with embryos of different gestational ages, and different species. It would also be interesting to compare the sensitiveness of our experimental model with other models now in use for teratological testing.

It seems likely that anaesthetics other than Avertin may be teratogenic in species other than the rat. It would therefore be of clinical importance to determine whether any of the anaesthetic agents commonly used in human practice have a similar embryotoxic action when administered during early pregnancy.

We thank Dr D. A. T. New for advice and discussion, and Mrs S. M. Jackson for technical assistance. This work was supported by a grant from the Ford Foundation. C.E.S. is supported by an MRC grant to Dr G. M. Morris.

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Received January 13; accepted March 3, 1976.
Effect of anaesthesia on the outcome of pregnancy in female mice

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It has long been known that certain anaesthetics may impair cell division, presumably by an effect on spindle and associated protein (Andersen, 1966; Fink, 1971). What is not known is whether the germ cells of adult female mammals, in the "resting" or dictyate stage of meiotic prophase, are susceptible to these agents. Oocytes are particularly vulnerable cells, as mutagens may either impair their fertilizability or reduce the developmental potential of the resultant zygotes. These aspects of the possible mutagenic effect of anaesthetics may be monitored by assessing fetal loss during the pre- and postimplantation stages of pregnancy. The type of analysis used in the present study provides a quantitative means of assessing the influence of anaesthesia before conception on the subsequent fertility of females. It also provides a means of comparing the overall incidence of embryonic loss in the experimental groups with that occurring in unanaesthetized control females.

Ten- to twelve-week-old randomly bred CFLP mice (Anglia Laboratories) were allocated to a control group of 48 females (Group 1), or to an experimental group of 48 (Group 2) or 72 (Group 3) females. The females in Groups 2 and 3 were anaesthetized at approximately 12.00 h (designated Day 1) with a single i.p. injection of either 1·5 times (Group 2) or twice (Group 3) the standard dose of tribromoethanol (Avertin: Winthrop) normally used to anaesthetize an intact mouse. The standard dose of a freshly prepared 1·2% solution of Avertin dissolved in 0·9% NaCl is usually 0·02 ml Avertin/g body weight, and was not used in this study because a longer period of anaesthesia than the normal 10–20 min was required. Group 3 contained more animals because preliminary experiments had shown that this dose was close to the LD<sub>50</sub> for this anaesthetic agent. All the females recovered from the anaesthetic within 1·0–1·5 h (Group 2) or 2·0–3·0 h (Group 3). Thirty-five deaths occurred in Group 3 within 10 days of the anaesthesia, but most were in the first 72 h. All females that survived the anaesthetic were subsequently paired on Day 21 with proven fertile CFLP males and checked each morning for 6 days for evidence of mating. Females with vaginal plugs were isolated and a maximum of three females caged together, while those that had not mated by Day 27 were discarded. The day on which a plug was observed was termed Day 1 post coitum (p.c.). All females were killed at approximately 12.00 h on Day 14 p.c.; the contents of the uterine horns were examined, and the numbers of resorbing, dead and live embryos were noted. The ovaries were then examined to determine the number of corpora lutea present.

The results are shown in Table 1, and are expressed in terms of early postimplantation deaths (resorptions) and later postimplantation deaths (dead embryos), while the difference between corpora lutea counts and total implantations gave a measure of the preimplantation losses. The total embryonic losses were significantly higher in Group 3 than in Group 1 ($\chi^2 = 13·14, P < 0·005$), but the increase in Group 2 was not significantly different from the control value ($\chi^2 = 2·19$). The embryonic loss was most marked in the preimplantation period, being 13% for Group 2 ($\chi^2 = 4·64, P < 0·05$) and 17% for Group 3 ($\chi^2 = 11·47, P < 0·005$) compared with the level of 8% in Group 1. A slight, but not significant, increase in resorption was observed in the Group 3 animals ($\chi^2 = 2·06$).

These experiments demonstrate that in mice a single period of anaesthesia with Avertin between 3 and 4 weeks before the onset of pregnancy is capable of adversely influencing the subsequent fertility of the female. However, the similar corpora lutea counts observed in the 3 groups of animals suggest that this treatment does not influence the number of eggs ovulated. The preimplantation embryonic loss in the anaesthetic-treated females may be caused by eggs which failed to become fertilized, or eggs which became fertilized but failed to implant.
It is not clear whether the increase in embryonic losses observed in Groups 2 and 3 resulted from a mutagenic effect of the anaesthetic on the germ cells or not. This is especially likely to be the case if fertilization occurred but the embryos died before implantation. Further studies will obviously be required to investigate this possibility. The present results provide no clues to the underlying mechanism of action of anaesthetics on female germ cells. Preliminary studies (unpublished) indicate that anaesthetics may interfere with normal chromosome segregation in a small proportion of oocytes, leading to an increase in the incidence of non-disjunction. Further observations will be required to investigate this phenomenon and the biochemical and ultrastructural changes induced by anaesthetics on germ cell organization and function. For instance, anaesthetics may affect genetic or extragenetic processes in the cell, which may result in impaired cytoplasmic organization or in an alteration of the surface properties. However, the mechanism by which normal mitosis is disrupted would not necessarily be the same as that which might affect cells in meiotic prophase. For example, the genome of oocytes in the adult ovary may be altered so that subsequent development of these cells is impaired when meiosis is resumed just before ovulation. Acute exposure to high levels or chronic exposure to low levels of anaesthetics during spermatogenesis may affect the genome of the fertilizing spermatozoon, and could explain why the unexposed wives of male anaesthetists and operating room personnel have an increased risk of bearing children with congenital abnormalities (American Society of Anesthesiologists, 1974). It would also be of interest in the present context to compare the incidence of congenital abnormalities in the offspring of female mice anaesthetized before or during pregnancy and of unanaesthetized controls. A direct dose-dependent deleterious effect of Avertin on early postimplantation rat embryos in culture has already been observed (Kaufman & Steele, 1976).

I thank Dr Mary Lyon and Dr Azim Surani for advice and discussion. This work was supported by a grant from the Ford Foundation.

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*Received 12 July 1976*
Normal postimplantation development of mouse parthenogenetic embryos to the forelimb bud stage

Pre- and postimplantation development of parthenogenetic embryos is of interest in analyses of the role of the male genome in early mammalian development, differentiation, and in haploid and diploid gene expression in embryogenesis. Diploid and haploid mouse parthenogenones evidently possess the capacity to form teratomas and give rise to differentiated tissues\textsuperscript{10} although normal organogenesis has not been observed to progress beyond the early somite stage in \textit{in utero}. Birth of live parthenogenetic rabbits has been reported\textsuperscript{11}, and Beatty\textsuperscript{12} has estimated that 1 in 200 embryos induced under similar conditions can be expected to reach term. There is, however, some doubt about the validity of this early work since subsequent attempts to repeat it have failed to obtain development beyond the blastocyst stage\textsuperscript{11,12}. Mouse parthenogenones develop to the egg cylinder stage but only one 8-somite embryo has so far been obtained\textsuperscript{12-14}. We have examined the postimplantation development of diploid mouse parthenogenones and report that a high proportion progressed to somite embryos when blastocysts were transferred to pseudopregnant recipients. Recipients were ovarioctomised following embryo transfer and their pregnant state maintained with exogenous hormones. Two apparently normal forelimb-bud stage embryos with a beating heart and yolk sac circulation were obtained. Other embryos isolated from the same recipient showed disorganised development, and a twin conceptus was obtained from a further recipient.

Cumulus masses from the oviducts of 8-12-week old (C57BL x CBA)F\textsubscript{1} hybrid mice were released at about 18 h after the HCG injection for superovulation into modified Krebs-Ringer bicarbonate embryo culture medium\textsuperscript{11} containing 4 mg ml\textsuperscript{-1} bovine serum albumin but lacking calcium and magnesium salts (M.A.H.S. and M.H.K., in preparation). After 5-6 h adherent cumulus cells were removed with hyaluronidase, and the eggs examined to determine the activation frequency and types of parthenogenones induced (Table 1). Activated eggs were separated into their different types, transferred to normal embryo culture medium\textsuperscript{15} and retained in culture for a further 90 h. By this time 91 out of 178 of the presumptive diploid\textsuperscript{16,17} 2-pronuclear embryos had developed to the expanded blastocyst stage. Blastocysts were transferred to a single uterine horn of d 3 pseudopregnant recipients previously mated to proven sterile vasectomised males (day of finding vaginal plug=d 1 of pseudopregnancy). The recipients were bilaterally ovarioctomised immediately after transfer of blastocysts and thereafter maintained on exogenous steroid hormones. After an initial hormone-free period of 2 d, recipients were maintained for 4 d on 1 mg progesterone daily per animal, during which time blastocysts presumably enter into quiescence.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Types of parthenogenones induced in HCG + 18 h group in medium lacking Ca\textsuperscript{2+} and Mg\textsuperscript{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of eggs examined</td>
<td>Activated eggs (%)</td>
</tr>
<tr>
<td>731</td>
<td>1 pronucleus + 2PB*</td>
</tr>
<tr>
<td></td>
<td>58 (11.8)</td>
</tr>
</tbody>
</table>

*Second polar body.

Implantation was initiated by injecting 20 ng oestradiol along with progesterone for 3 d, and thereafter pregnancy maintained with 8 ng oestradiol and 1.6 mg progesterone daily per female.

Implantation began about 24 h after the initial injection of oestradiol, which is equivalent to about d 5 of pregnancy, and recipients were killed at intervals between the 6th and 11th day of "pregnancy". About 80% of the transferred blastocysts implanted (Table 2), and all implantation sites up to d 9 were immediately fixed in Bouins solution and examined histologically. After d 8 of "pregnancy", embryos were dissected from the uterus and examined under a

![Fig. 1](https://example.com/fig1.png)

Fig. 1 a, Parthenogenetic mouse embryo with about 25 somites photographed within its amnion, unfixed preparation. b, Near sagittal section of embryo in a showing large number of well formed somites (arrowed). Embryo fixed in Bouins about 4 h after isolation from the uterus. Scale bar represents 0.2 mm; 1 and 2, first and second branchial bars; O, otic vesicle; Ht, heart; Rh, rhombencephalon with 4th ventricle; A, amnion; Y, yolk sac; V, 4th ventricle.
Dissecting microscope. The two most advanced embryos obtained were at the forelimb-bud stage on the 11th d of “pregnancy”, both had about 25 somites and were apparently healthy with beating hearts and a good yolk sac circulation at the time of isolation from the uterus. These embryos were later fixed for histology (Fig. 1a and b).

Several factors may account for the improved results reported here compared with the only other comprehensive study on postimplantation mouse parthenogenetic development.\(^1\)\(^2\) The mode of activation of eggs in Ca\(^{++}\)/Mg\(^{++}\)-free medium has not been used before and may be less traumatic than electrical stimulation of eggs in vitro\(^3\) or hyaluronidase activation in vitro\(^4\)\(^5\)\(^6\). The choice of the F\(_1\) hybrid where one-cell eggs develop to the blastocyst stage in vitro\(^7\)\(^8\)\(^9\) is unusual since embryos from other strains do not normally progress beyond the 2-cell stage in vitro. The use of ovariotomised recipients may permit a better synchrony between blastocysts and the uterus before implantation commences. Blastocysts in this instance were allowed to enter into quiescence before implantation was stimulated. An increase in cell number occurs as embryos enter into quiescence\(^10\) and would proportionately double the number of cells in blastocysts before implantation.

Parthenogenetic blastocysts are similar to normal fertilised blastocysts when examined ultrastructurally\(^11\). Their cells can also differentiate into a variety of cell types in ectopic sites\(^12\)\(^13\). In the present study 35% of implanted embryos developed into normal egg cylinders, and 25% developed to somite-stage embryos.

The present study indicates that non-fertilised parthenogenetic eggs are capable of apparently normal development to a very advanced state in the absence of any genetic or extragenetic contribution from sperm. Since a greater number of embryos develop normally after fertilisation, the role of sperm is probably to prevent imbalance in the genetic constitution of the zygote. In the present study 60–70% of the embryos which successfully implanted died shortly thereafter, leaving about 25% to develop normally to somite embryos. In some embryos anomalous development may manifest itself in disorganisation of the embryo such that individual cells remain viable but are unable to form into tissues and organs. Survival of parthenogenomes to term will depend on the extent of genetic imbalance in individual embryos which may affect development during critical stages of organogenesis.

Parthenogenetic embryos will be valuable to determine the developmental potential of genetically dissimilar embryos. The role of sperm in the activation of unfertilised eggs can be studied by morphological, genetic and biochemical comparison between fertilised and different types of parthenogenetic embryos. It may be possible to establish haploid and diploid cell lines from unfertilised parthenogenomes. This would facilitate biochemical studies on cell surface constituents and their role in histotypic contact and cellular interactions. The disorganisation of some postimplantation parthenogenomes probably reflects abnormalities in their cell surface properties which may be analysed by comparisons with the cells of normal embryos.

We thank Mrs Andrea Burling for technical assistance and Mr D. Thurlbourn for caring for the mice. This work was supported by a grant from the Ford Foundation.

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Received September 16; accepted November 15, 1976.

EFFECT OF ANAESTHETIC AGENTS ON EGGS AND EMBRYOS

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In this paper the findings of recent in vivo and in vitro experiments designed to investigate the effects of anaesthetics on mammalian eggs and embryos will be considered in detail, as will the biochemical and ultrastructural observations which may shed some light on their possible mode of action during early development.

A great deal of work is being carried out at the present time to investigate how local and general anaesthetics influence the cell surface and plasma membrane, and intracellular cytoplasmic processes. The rapid advances taking place in this area have almost exclusively been aimed at investigating the biochemical and molecular interrelationship between the external environment of the cell, the structure of the plasma membrane, and the microtubules and microfilaments of the cellular cytoskeletal system. While this information may not appear to shed much light on the possible mechanisms of anaesthesia, it provides certain clues which may help to explain recent clinical observations that the reproductive efficiency of operating theatre personnel who are chronically exposed to low levels of anaesthetic gases is impaired.

There is now a considerable body of circumstantial evidence which seems to confirm the notion that anaesthetics, both in clinical and subclinical dosage, may be teratogenic during certain stages of pregnancy. The evidence in favour of this hypothesis has been building up over the last 10 years. Epidemiological studies which have been carried out on operating theatre personnel (e.g. Vaisman, 1967; Askrog & Harvald, 1970; Lencz & Nemes, 1970; Cohen et al., 1971; Knill-Jones et al., 1972; Corbett et al., 1974; American Society of Anesthesiologists, 1974; Knill-Jones et al., 1975) have recently been briefly reviewed by Smithells (1976), who comes to the general conclusion that 'there are good reasons for believing that there are teratogenic forces at work in operating theatres'. Up to now no definite cause-effect relationship has been established between exposure to trace levels of waste anaesthetic gases and the increased incidence of spontaneous abortions and congenital abnormalities found both in females who work within the operating theatre environment and in the unexposed wives of males who work in operating theatres.
Measurements of the concentration of different anaesthetic gases in operating room air, and specifically within the inhalational zone of the anaesthetist, have been made by various workers (eg. Linde & Bruce, 1969; Corbett & Bell, 1971; Corbett, 1972), and range from barely detectable to very high levels (peak concentration of nitrous oxide of 9700 ppm, Corbett & Bell, 1971).

In addition to the effect of trace anaesthetic concentrations, attention has also been drawn to the possible risk to the human foetus of surgery carried out during pregnancy (Shnider & Webster, 1965). This topic has been discussed in detail by Smith (1968) who considered experimental and clinical evidence for the possible teratogenicity of anaesthetic agents, premedicants and supplemental drugs (eg. narcotics, hypotensive and vasopressor agents, antihistamines and the muscle relaxant drugs). While most of his review is directed towards anaesthesia at later stages of pregnancy than considered in the present discussion, he has directed attention to some of the marked alterations which occur in maternal physiology during anaesthesia (eg. hypoxia, electrolyte disturbances, hypercapnia and increased oxygenation) that may in themselves be foetotoxic either when acting alone or synergistically with one another or with the anaesthetic agent. However, as Wilson (1954) has pointed out, not all alterations in maternal physiology during pregnancy, no matter how severe they may be, are capable of affecting the subsequent offspring, though this may, of course, depend on the duration of the stress and the stage of development of the foetus at the time of exposure.

The results of a large number of teratological studies in which pregnant animals have been subjected to various subanaesthetic and anaesthetic concentrations of different anaesthetics are summarised, and attention drawn to the salient features. The effect of anaesthesia on female germ cells at the 'resting' or dictyate stage of meiosis is also considered in detail, as recent indirect evidence suggests that anaesthesia carried out before conception may, under certain circumstances, affect the outcome of pregnancy.

THE OPERATING THEATRE ENVIRONMENT - CLINICAL STUDIES

In one of the earliest studies concerned with occupational disease in anaesthetists, Vaisman (1967) reported an unusually high incidence of headache, nausea and fatigability, and that 18 out of 31 pregnancies studied ended in spontaneous abortion. A smaller survey of Hungarian anaesthetists of both sexes (Lenicz & Nemes, 1970, cited in Corbett, 1972) revealed that only 10 out of 31 deliveries were normal; of the remaining 21 pregnancies, 10 ended in spontaneous abortion, 9 in 'pathologic' pregnancies and 2 in premature delivery.

Cohen et al. (1971) reported a 29.7% incidence of spontaneous abortions (10 out of 36 pregnancies) in 67 operating room nurses compared to a control
incidence of 8.6% (3 out of 34 pregnancies) in 92 general duty nurses with no history of exposure to an operating room environment. These authors also compared the incidence of spontaneous abortions in 50 female anaesthetists (38.7%, 14 out of 37 pregnancies) and 81 female physicians (10.3%, 6 out of 58 pregnancies). The differences between the exposed and unexposed groups in both series were highly significant (p<0.045 and p<0.0035, respectively).

Knill-Jones et al. (1972) obtained obstetric information from 563 female anaesthetists and 828 women doctor control subjects. The anaesthetists were divided into two groups depending on whether they had or had not worked during the first and second trimesters of pregnancy. When the ratio of abortion/total pregnancies was examined, the ratio was found to be significantly greater in the "working" anaesthetists (18.2%) compared with the control group (14.7%). The ratio in the anaesthetists "not at work" was 13.7%. The incidence of congenital abnormalities was significantly greater (6.9%) where the mother had worked than when she had not worked (2.9%, p<0.02) but not significantly different from the control frequency (4.9%). Involuntary infertility was twice as frequent among anaesthetists (12%) as in the control group.

In a recent study by Corbett et al. (1974) information on the incidence of birth defects was obtained from 621 nurse anaesthetists. 4.7% births were recorded in pregnancies during which the mother had worked, and 261 pregnancies in which the mother had not. No significant difference was seen in the incidence of major malformations. However, there appeared to be a significant difference in the incidence of minor defects, 16.4% in the working nurse anaesthetists and 5.7% in those that had not worked during pregnancy. The majority of these malformations consisted of skin defects and inguinal hernias.

In the recent national study in the U.S.A. (American Society of Anesthesiologists, 1974) 49,583 exposed operating room personnel were compared with 23,911 unexposed control individuals. The results indicated that the exposed group of females had an increased risk of spontaneous abortion (17.0 ± 0.9% to 19.5 ± 0.9%) compared to unexposed controls (14.4 ± 1.4% to 15.7 ± 3.3%). The incidence of congenital abnormalities was also higher in the exposed group (9.6%) compared to the controls (5.9%, p 0.01). The exposed group of females also had a greater risk of developing cancer (1.3 to 2 fold risk), and hepatic disease (1.3 to 2.2 fold risk), and renal disease (1.2 to 1.4 fold). No increased risk of cancer or renal disease was observed in male anaesthetists, but a similar chance of developing hepatic disease was reported. No increased risk of spontaneous abortions was observed in the unexposed wives of male operating room personnel, but an increased risk of bearing children with congenital abnormalities was noted in the unexposed wives of male anaesthetists (p=0.04). The types of abnormalities reported involved most of the major organ systems.
In the latest study of this type, Knill-Jones et al. (1975) surveyed 5507 male doctors (of whom 26% were anaesthetists, 9% surgeons) in the U.K. The incidence of spontaneous abortion in pregnancies involving the exposed group was 11.1%, compared to 10.9% in their control series. However, maternal exposure alone was associated with a frequency of 15.9% compared to 10.9% where neither parent was exposed \( p < 0.01 \). A slight increase in the incidence of minor abnormalities was observed in association with male exposure (4.5%) compared to controls where neither parent was exposed (3.2%, \( p < 0.01 \)), largely accounted for by an increase in the reporting of minor congenital abnormalities by the exposed group.

THE EFFECT OF ANAESTHETICS ON GAMETES

No experimental information is available on the possible effects of anaesthetics on gametogenesis in males or females, though indirect evidence from the recent Danish report (Askrog & Harvald, 1970), the American Society of Anesthesiologists national study (American Society of Anesthesiologists, 1974) and Knill-Jones et al. (1975) that spermatogenesis might be affected by chronic exposure to subclinical levels of anaesthetics will be discussed later. Assessment of any effect on gametogenesis in the female is complicated by the fact that most of oogenesis occurs prenatally. In this section some recent observations on the effects of anaesthesia in rodents will be outlined, and an attempt made to discuss their possible significance.

The current requirements of various government agencies for reproductive and teratological testing of new drugs have recently been discussed by Berry and Barlow (1976). At least three reports of laboratory tests on the effects of anaesthetics on fertility and reproductive function have recently appeared in the literature (Bruce, 1973; Kennedy et al., 1976; Kaufman, 1976). Other studies of this kind have undoubtedly been carried out by interested drug companies, but their results are not generally available. The few reports of in vivo and in vitro teratological studies on the effect of anaesthesia on pregnant animals and early postimplantation embryos which are available are discussed later.

The effect of anaesthetics on ovarian oocytes at the 'resting' or dictyate stage of meiosis

While it has long been known that anaesthetics and other CNS depressants may impair cell division (for reviews see Andersen, 1966; Fink, 1971), few attempts have been made to establish whether the germ cells of adult female
mammals, in the 'resting' or dictyate stage of meiotic prophase, are susceptible to these agents. In a recent study (Kaufman, 1976) these aspects of the possible effect of anaesthetics were monitored by assessing the influence of anaesthesia carried out 21-27 days before conception on the subsequent fertility of females. The incidence of pre- and post-implantation embryonic loss in the experimental groups was compared with that occurring in unaesthetised control females (Group 1). As the effect of prolonged anaesthesia was being tested, females were anaesthetised with a single i.p. injection of either 1.5 times (Group 2) or twice (Group 3) the standard dose of tribromoethanol (Avertin:Winthrop) normally used to anaesthetise an intact mouse. The standard dose of a freshly prepared 1.2% solution of Avertin dissolved in 0.9% NaCl is usually 0.02 ml Avertin/gm body weight. As anaesthesia induced by the standard dose of Avertin usually only lasts for 10-20 minutes its effect was not tested. The twice standard Avertin dose was close to the LD$_{50}$ for this agent in this series when the body temperature during anaesthesia was not maintained by external warmth. In subsequent studies in which a similar dose of Avertin was given, and the body temperature maintained during anaesthesia, the mortality was in the region of 25-30%. The total embryonic losses were significantly higher in Group 3 (p<0.005) than in Group 1, but the increase in Group 2 was not significantly different from the control value. The embryonic loss was most marked in the preimplantation period, being 13% for Group 2 (p<0.05) and 17% for Group 3 (p<0.005) compared with the level of 8% in Group 1. The similar corpora lutea counts which were observed in the 3 groups of animals, suggests that this treatment did not influence the number of eggs ovulated.

The increased embryonic losses reported by Kaufman (1976) seems to be at variance with the findings in other studies in which the authors concluded that halothane given before conception produced no adverse effect on female fertility in mice (Bruce, 1973) and rats (Kennedy et al., 1976). However, because of the different experimental approaches employed in these 3 studies comparison of results is almost impossible. In the study by Bruce (1973), for example, a very low subanaesthetic dose of halothane (16 ppm) was given to mice for 7 hr/day, 5 days/week for 6 weeks prior to mating, while Kennedy et al. (1976) exposed rats for 1 hour daily either 1-5, 6-10 or 11-15 days before mating to an anaesthetic concentration of halothane (1.3%-1.43%) in air. Obvious differences between these various studies are the use of an inhalational anaesthetic (halothane) versus an agent given i.p. (Avertin), the use of the rat as an experimental animal by Kennedy et al. (1976), and the different type and degree of anaesthesia achieved by these different anaesthetics. Thus the level of narcosis obtained using an inhalational agent from a constant flow gassing apparatus is likely to be much more uniform
than that achieved following an i.p. injection of any anaesthetic agent. Certainly in the Avertin studies where relatively high doses of this agent were given i.p., profound narcosis very rapidly ensued. It was difficult to assess the level of anaesthesia achieved, but recovery usually occurred within 1.0 - 1.5 h with the 1.5 times standard dose or 2.0-3.0 h with the twice standard dose group when body temperature was not maintained by external warmth. It can only be assumed that the high circulating levels of Avertin which were initially achieved following the i.p. injection of this agent had an adverse effect on a proportion of the oocytes contained within the ovary. In addition, it seems unlikely that a significant effect on fertility would have been observed by Kaufman had lower doses of Avertin been used.

Several explanations may be proposed to account for the recent experimental findings of Kaufman (1976) and the increased incidence of involuntary infertility and spontaneous abortions seen in operating room personnel. One explanation for the observed increase in embryonic loss might be that chronic exposure to trace levels or acute exposure to high levels of anaesthetics may be mutagenic either during spermatogenesis or during the dictyate stage of meiotic prophase. This might manifest itself in an increase in the incidence of dominant lethality. Long-term genetic studies in mice have recently been initiated to test this possibility (M.F. Lyon, personal communication), but the results will not be available for some time. In addition to this possible mechanism of action, in which it is presumed that damage might be induced at the molecular level in the genetic material of the germ cells, anaesthetics may induce the production of gametes with an abnormal chromosome constitution. Various mechanisms could be envisaged which might produce gametes of this type. For example, simple numerical anomalies may be produced as a result of non-disjunction at meiosis I or II, or more subtle structural anomalies such as balanced or unbalanced translocations, segment inversions etc. as a result of more extensive chromosome damage.

The technical and organizational problems involved in carrying out cytogenetic analysis of sufficient human abortus material from this source makes it unlikely that a direct assessment would be feasible on a large enough scale to produce a meaningful result. Large numbers would have to be examined because of the high incidence of chromosome anomalies which are normally present in spontaneous abortions (Carr, 1965, 1971) where no aetiological factors are normally found. In any case, critical material from the pre- and post-implantation period is likely to be lost before pregnancy is diagnosed and would therefore not be available for analysis.
Experimental attempts to investigate the possibility that pre- and early post-implantation embryonic losses may, at least in part, be due to numerical anomalies have recently been made by Kaufman (unpublished). Oocytes at the germinal vesicle stage were isolated from the ovaries of randomly-bred CFLP mice (Anglia Laboratories) anaesthetised with 1.5 times or twice the standard dose of Avertin 2 weeks before, and from unanaesthetised controls. Groups of between 30 and 50 eggs/treatment were incubated in vitro for 4 or 13 hours under standard embryo culture conditions (Diggers et al., 1971) in small drops of modified Krebs-Ringer bicarbonate medium (Whittingham, 1971) containing 4 mg/ml bovine serum albumin. No difference was observed in the proportion of eggs which underwent germinal vesicle breakdown in the 3 groups looked at after 4 hours, or had completed maturation to metaphase II (13 hour groups). However, analysis of the chromosomes of all maturing eggs (method of Tarkowski, 1966) indicated that there was one interesting difference between the morphology of the chromosomes in the experimental and control groups which was probably accentuated by the lengthy (about 20 mins) hypotonic citrate pre-treatment of eggs prior to fixation. Between 30% and 40% of preparations in the experimental groups showed evidence of abnormal chromatid separation (Figure 1a). This feature was not observed in controls (Figure 1b). Attempts to demonstrate a similar effect in eggs isolated at 15-16 hr after the HCG injection for superovulation pretreated for only 10 minutes in hypotonic citrate were not successful.

Because of the technical difficulties involved in accurately estimating the number of chromosomes present in metaphase II preparations a parallel study in which the eggs were activated to take them through to the first cleavage mitosis was undertaken. (C57BL x CBA)F₁ eggs were isolated at about 20 h after HCG and induced to develop parthenogenetically by hyaluronidase treatment in vitro (Kaufman, 1973a). In these preliminary experiments females had previously been anaesthetised with the standard dose of Avertin at intervals between 25 hr before and 2 hr after the HCG injection for superovulation. The chromosome constitution of the activated eggs was determined at the first cleavage mitosis (Kaufman, 1973b). The activation frequency was in the region of 70-80%, and accurate chromosome counts could be made in a very high proportion of preparations. The results of this study are presented in Table 1, and indicate that Avertin anaesthesia carried out during the oestrus cycle leading to ovulation may induce non-disjunction in a small proportion of eggs. Further studies will be required to determine whether this plays any role in the embryonic losses observed when mice are anaesthetised 3 or more weeks before conception. It would in addition be interesting to examine the chromosome constitution of fertilized eggs at the first cleavage mitosis and
Figure 1a (upper): Chromosome complement of mouse oocyte at prometaphase II. Oocyte isolated from the ovary at germinal vesicle stage and cultured in vitro for 13 hours. Some sister chromatids have disjoined (arrows). The female had been anaesthetised with 1.5 times the standard dose of Avertin two weeks before. Giemsa stain.

Figure 1b (lower): Chromosome complement of control oocyte at metaphase II. All chromatid pairs joined at their centromeres. Giemsa stain.
at later preimplantation stages of embryonic development. It is unfortunate that only gross numerical anomalies can be ascertained by this method of analysis, and that most structural anomalies cannot be detected by this means.

<table>
<thead>
<tr>
<th>Group Number</th>
<th>Time of anaesthetic HCG - hours</th>
<th>No. of females</th>
<th>Total No. eggs analysed</th>
<th>Chromosome no. 18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>Percent aneuploid eggs</th>
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<tr>
<td>1</td>
<td>Nil (control)</td>
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If anaesthesia does, in fact, increase the incidence of meiotic nondisjunction, then it is likely that it is the interrelationship between the centromere and the meiotic spindle apparatus on which attention should be focused. Very little is known about the ultrastructure of the centromere (or kinetochore), though slightly more is known about its functional activity as a result of recent studies by Telzer et al. (1975). These workers have shown that the centromeres of isolated chromosomes of Hela cells are the only regions on these chromosomes which can act as microtubule assembly sites, and have suggested from this evidence that centromeres are also likely to possess this capacity in vivo.

Many studies have been made on the mitotic spindle apparatus (for review of early work see Mazia, 1961), and numerous models proposed to explain its functioning during mitosis (e.g. McIntosh et al., 1969). The latest models describe an equilibrium between the mitotic spindle and its subunits and the cross-bridges which interconnect spindle microtubules. It has been postulated that these intertubule links are active mechanochemical units capable of sliding adjacent tubules over one another. Allison and Nunn (1968) have hypothesised that microtubular protein units are held together by low energy bonds which are easily and reversibly broken, and have suggested that in the presence of general anaesthetics, for example, depolymerisation of these tubules can occur.
This has recently been confirmed by Hinkley and Telser (1974), who demonstrated that halothane caused microfilament breakdown in cultured mouse neuroblastoma cells, and by Hanchke et al. (1974) and Nicolson et al. (1976) using local anaesthetics such as lidocaine and tetracaine.

It has been known for many years that anaesthetic agents are capable of interfering with cell division and that metaphase arrest occurs when plant and animal cells are exposed to anaesthetics at concentrations usually within the clinical range (for reviews see Andersen, 1966; Fink, 1971). The inhibitory action on cell division which may be induced by these agents is similar in appearance to the well-defined arrest of mitosis induced by colchicine (c-mitosis, Levan, 1938). It seems likely that anaesthetics in appropriate concentrations would have a similar inhibitory effect on meiotic spindle activity. What is less clear is the effect that these agents may have on 'resting' or dictyate stage ovarian oocytes when the chromosomes are decondensed and the spindle present in a disassembled state. Exposure to trace levels of anaesthetics during the dictyate stage of meiotic prophase may eventually interfere with the normal subunit assembly mechanism which is activated during meiotic maturation. The possibility also exists that exposure to these agents may also produce non-specific chemically-induced changes in the genetic material of oocytes via mutation, the production of major gene defects or chromosome aberrations, or damage to the centromeric region of these chromosomes with the possibility of chromosome imbalance in the resultant products of division. These suggestions go some way towards explaining both the clinical and experimental observations, but should only be considered as a very tentative working hypothesis at this stage.

Delayed ovulation induced by barbiturates

Everett and Sawyer (1950) demonstrated that a range of barbiturates were able to suppress the mid-cycle surge of LH in the rat, delaying ovulation by 24 hours. To produce this effect moderate or profound anaesthesia was usually required, and the sodium salts of the following agents were found to be effective, amytal, barbital, dial, phenobarbital and nembutal.

The chromosome constitution and developmental potential of the oocytes released from delayed follicles (often termed "overripe follicles") have been examined by Butcher and Fugo (1967, see also Butcher, 1975, for summary of recent results). These workers reported a decreased implantation rate when nembutal-treated females were mated to fertile males, and an increased incidence of chromosome anomalies in foetuses at mid-gestation (4.6%) compared to the control level of 1.5% (Butcher & Fugo, 1967). Recent ultrastructural studies on oocytes from delayed follicles by Peluso and Butcher (cited by
Butcher, 1975) demonstrated a range of cytoplasmic changes which included a reduction in the normal number of cortical granules, and changes in mitochondrial morphology. The chromosome constitutions of preimplantation rat embryos isolated from pentobarbital-treated females have also been studied by Mikamo and Hamaguchi (1975). Little effect on meiotic divisions and fertilization was observed, but the incidence of chromosomally mosaic blastocysts indicated that non-disjunction or anaphase lagging must have occurred during the early cleavage divisions.

These recent findings by Butcher (1975) and Mikamo and Hamaguchi (1975) suggest that most of the effects on embryonic development previously reported by these workers was probably due to barbiturate-induced changes in the pre-ovulatory oocyte. These studies in the rat may well explain the recent observations by Kaufman (1976, and unpublished) who examined the chromosome constitution and developmental potential of oocytes from Avertin-treated mice (see earlier).

Effect of anaesthetics on ovulated unfertilised eggs

Rodent eggs may be induced to develop parthenogenetically in vivo when intact animals are anaesthetised shortly after the occurrence of ovulation. Thus rat eggs have been activated parthenogenetically with ether (Thibault, 1949; Austin & Braden, 1954), chloroform, ethyl chloride, ethyl alcohol, paraldehyde, nitrous oxide and i.p. Nembutal (Austin & Braden, 1954), and mouse eggs with ether (Braden & Austin, 1954) and i.p. Avertin (Kaufman, 1975). Apart from the report by Kaufman (1975), where postimplantation parthenogenetic development was obtained when pseudopregnant mice were anaesthetised with Avertin, previous workers had only reported the occasional development of these eggs as far as the 2 or 4 cell stage. The incidence of activation and types of parthenogenones induced is probably related to the post-ovulatory age of eggs at the time of anaesthesia. In the mouse, for example, a similar pattern of response had previously been demonstrated when eggs were isolated at various times after ovulation and activated in vitro with hyaluronidase (Kaufman, 1973a). The underlying mechanisms whereby anaesthetics may induce unfertilized rodent eggs to develop parthenogenetically was initially thought to be due to the production of tissue anoxia (Austin & Braden, 1954). It now seems more likely that anaesthetics initiate a chain of events at the cell membrane which result in the release of sequestered intracellular calcium ions. The hypothesis that similar events may be initiated in eggs of all species, and that this is the 'universal activating mechanism', has recently been proposed by Steinhardt et al. (1974). These authors came to this conclusion as a result of studies on oocyte activation in several species including mammals using the divalent
ionophore A23187. Similar events are also thought to occur at fertilization (Steinhardt & Epel, 1974; Steinhardt et al., 1974).

Extracellular Ca^{2+} ions are also important for second polar body extrusion in mouse parthenogenetic eggs (Surani & Kaufman, unpublished). The presence of an optimal level of extracellular calcium within the ampulla of the mammalian oviduct may facilitate an increase in the intracellular pool of free Ca^{2+} ions within the oocyte during sperm penetration. An optimal ionic concentration may also be a prerequisite for second polar body extrusion following fertilization, being the normal means of reconstituting the diploid state. Cytochalasin B, which disrupts microfilaments, also prevents second polar body extrusion (Balakier & Tarkowski, 1976) probably by displacement of the second metaphase spindle from the periphery of the egg (Johnson et al., 1975). This is relevant in the present context because anaesthetics may competitively affect Ca^{2+}-sensitive functions necessary for microtubule maintenance (Wilson et al., 1970) and might even increase intracellular Ca^{2+} concentrations to levels sufficient to induce microtubule depolymerization (Kirschner & Williams, 1974) and microfilament breakdown (Hinkley & Telser, 1974; Nicolson et al., 1976). Mg^{2+} is required for the polymerization of tubulin, and high levels of Ca^{2+} strongly inhibit this process (Weisenberg, 1972). Intracellular levels of these and other ions could therefore regulate cyclic changes in the formation and breakdown of the cellular cytoskeletal system which are essential in cell division. All the evidence suggests that maintenance of the normal interrelationship between divalent cations and the cellular cytoskeletal system is of fundamental importance, especially in early development, and that this delicate balance may be affected by anaesthetics. Thus even subclinical anaesthetic levels in certain mammalian species may be capable of influencing both meiotic and mitotic chromosome segregation, polar body extrusion, and cytokinesis.

Influence of anaesthetics on spermatogenesis

In three clinical studies (Askrog & Harvald, 1970; American Society of Anesthesiologists, 1974; Knill-Jones et al., 1975) observations have been made on the outcome of pregnancy in unexposed wives of male anaesthetists. The two more recent reports are not in accord with the earlier report of Askrog & Harvald (1970) in which an increase in the incidence of spontaneous abortion and premature delivery had been observed. These findings may, however, have resulted from other factors such as, for example, the effect of maternal age on spontaneous abortion, since they compared obstetric histories before and after starting employment in anaesthetics. A slight increase in the incidence of congenital abnormalities was observed in the American Society of Anesthesiologists (1974) study and by Knill-Jones et al. (1975). Because of
the small numbers involved and the high incidence of minor anomalies, caution must be exercised in interpreting these findings. At the most these studies indicate that chronic exposure to trace levels of anaesthetics may have an adverse influence on spermatogenesis, but until further information becomes available the case remains unproven.

Two recent attempts have been made to investigate this problem using rodents (Bruce, 1973; Kennedy et al., 1976). In one study (Bruce, 1973) male mice were exposed to trace levels of halothane 7 hr/day, 5 days/week for 6 weeks prior to mating, while Kennedy et al. (1976) exposed male rats for 1 hr daily to anaesthetic concentrations of this agent for 1-5, 6-10 and 11-15 days prior to mating. Both groups reported negative findings, with no increase in the incidence of pregnancy loss when unexposed females were mated to exposed males. Unfortunately, both studies fall short of the present requirements of various government agencies for testing the effect of drugs on male fertility (see Berry & Barlow, 1976) who recommend that treatment in the male should be carried out at least 60-80 days before mating. Further studies, possibly involving the use of other species (see Jackson, 1970; Baker, 1972), will obviously be required to clarify this issue.

TERATOLOGICAL STUDIES

A number of experiments have been carried out to investigate the effect of halothane, both in subanaesthetic and anaesthetic concentrations and for various exposure durations, on pregnant rats (Basford & Fink, 1968; Katz & Clayton, 1973; Chang et al., 1974; Kennedy et al., 1976; Lansdown et al., 1976), mice (Bruce & Koepke, 1969; Bruce, 1973) and rabbits (Kennedy et al., 1976). Similar studies to test the effect of nitrous oxide on pregnant rats (Farbrook et al., 1965; Fink et al., 1967; Shepard & Fink, 1968; Corbett et al., 1973) and anaesthetic concentrations of sodium pentobarbital on pregnant mice (Setala & Nyyssonen, 1964) have also been reported. In two further studies, the effect of nitrous oxide/halothane mixtures were tested in pregnant rats (Wittman et al., 1974) and hamsters (Bussard et al., 1974).

In these studies three different situations have been tested, namely chronic exposure to subanaesthetic concentrations of these agents, ranging from trace levels up to levels associated with drowsiness and inability to feed, and the effect of a single or repeated exposure to concentrations which induce narcosis. This latter class of experiments where animals have been repeatedly exposed to anaesthetic concentrations of these agents, in some cases for many hours each day, is not particularly close to the clinical situation. In other reports, where subanaesthetic and anaesthetic concentrations have been given, very high levels of skeletal anomalies (e.g., Basford & Fink, 1968) and foetal losses (e.g., Katz & Clayton, 1973) have been observed in controls, suggesting
that factors other than anaesthetics alone may have contributed to the high incidence of embryonic mortality and morbidity in these studies. In attempting to summarize these findings and present a balanced assessment of their possible clinical significance, these various considerations have been taken into account. Very little information is available on the effect of anaesthetics during the preimplantation period, and only one study has been carried out to investigate the direct effect of an anaesthetic agent on early postimplantation embryos in culture (Kaufman & Steele, 1976).

The specific days of pregnancy on which animals were exposed to treatment, referred to in the following pages, is that found in the original papers. In most cases the authors have referred to the day of finding sperm in the vagina or noted the presence of a vaginal plug as Day 1 of pregnancy. A few authors refer to this as Day 0 of pregnancy. The particular system used is, unfortunately not always clearly stated. In addition, it has long been known that the reaction of the embryo to specific compounds varies not only from species to species but also within a given species, between each strain and even between individuals of the same strain (for review, see Tuchmann-Duplessis, 1969). The importance of these genetic differences in sensitivity to different agents and the rationale for choosing a particular experimental animal in teratological studies has recently been discussed by various authors (eg. Axelrod, 1970; Berry & Barlow, 1976). Thus cortisone, for example, is a potent teratogenic drug in the mouse and rabbit but does not produce malformations in the rat; the same dose of this drug produces cleft palates in 17% of strain C57BL/6 Jax mice and in 100% of strain A/Jax (Fraser et al., 1954). The use of different strains of experimental animals may partly account for apparently conflicting findings reported by various workers and reviewed here, where, for example, increased foetal loss and the presence of malformations was observed in some studies but not in others. If further information is required on these various points, reference should be made to the original articles.

The effect of halothane

In a recent study by Iamsdow et al. (1976) no teratogenic or foetotoxic effect was observed at autopsy on day 22 when rats were exposed to subanaesthetic levels of halothane ranging from 50-3200 ppm on days 8-12 and 1600 ppm on days 1-21 of pregnancy. No increase was found in foetal death and resorption rates, growth retardation or in the frequency of skeletal anomalies.

No increase in the incidence of embryonic losses or gross congenital anomalies were reported by Chung et al. (1974) when rats were exposed to a concentration of 10 ppm halothane throughout pregnancy. However, the progeny
showed histological changes in the tissue of the central nervous system (vacuoles, some neuronal necrosis, etc.) which these workers hypothesised might later be associated with behavioural changes and learning defects. The testing of offspring of exposed females by studies on behavioural and functional development has recently been incorporated into the new British guide-lines for studies on reproduction (Committee on Safety of Medicines, 1974), and the principles and practice of behavioural teratology testing are discussed in detail by Barlow and Sullivan (1975).

In a recent study by Kennedy et al. (1976) rats were exposed to anaesthetic concentrations of halothane (1.35-1.4%) on days 1-5, 6-10 and 11-15 of pregnancy. No increase was observed in the incidence of skeletal or gross congenital anomalies. These workers also exposed rabbits to anaesthetic concentrations of halothane (2.15-2.3%) on days 6-9, 10-13 and 15-18 of pregnancy with similar negative results when these animals were autopsied on day 29.

In contrast to these generally negative findings, Basford and Fink (1968) reported an increased incidence of embryonic loss and skeletal anomalies in their experimental group when rats were exposed to anaesthetic doses of halothane (0.8%, which induced light sleep within 1 hour) for 12 hr/day on either day 6, 7, 8, 9 or 10 of pregnancy or daily during this period. These workers also reported a diurnal variation in response with a higher incidence of resorptions when anaesthetics were administered during the day than at night.

The findings of Katz and Clayton (1973) that low concentrations of halothane were both teratogenic and foetotoxic are particularly difficult to interpret as very high rates of foetal loss were observed in their control series. In a slightly more complex study, Bruce (1973) exposed various strains of mice to subanaesthetic concentrations of halothane (about 16 ppm) 7 hr/day, 5 days/week for 6 weeks before conception, and daily throughout pregnancy. No adverse effect on the pregnancy outcome was observed in the treatment group compared to the controls. However, as the results from three strains of mice were pooled, and corpora lutea counts were not given, it is difficult to draw any useful conclusions from this study. In a previous study by Bruce and Koepke (1969) very few mice mated when exposed daily to 0.1% halothane in air, the reason for this was not established. An increase in deaths occurred in this group of mice which was neither enhanced nor antagonised by any effects of radiation (1.44 R/hour gamma radiation).

The effect of nitrous oxide

Various groups of workers have examined the effect of nitrous oxide on pregnancy in the rat. In the majority of these studies, animals were exposed to quite high concentrations of this agent either 8 or 24 hr/day for up to
nine successive days during the postimplantation period, test systems far removed from the clinical situation. Only in the study by Corbett et al. (1973), where groups of rats were exposed to 100 ppm and 1000 ppm for 8 hr/day on days 10-13, 14-19 or 10-19 of pregnancy, were subanaesthetic doses given. These workers also exposed rats for 24 hr/day from day 8-13 to levels of 15000 ppm. At this higher dose, an increase in the incidence of foetal death was observed. At the lower doses tested, a diurnal effect was observed in that a significant increase in foetal loss occurred when the anaesthetic was given from 6 a.m. to 2 p.m., but not when animals were exposed from 2 p.m. to 10 p.m. This is similar to the diurnal effect reported by Basford and Fink (1968) noted in the previous section.

In the study by Fink et al. (1967) rats were exposed for 24 hr/day to gas mixtures containing 45-50% nitrous oxide for either 2, 4 or 6 days from day 8 of pregnancy. In the report by Shepard and Fink (1968), further details of this earlier study are given, and additional findings on the effect of exposure of rats to 70% nitrous oxide either 24 hr/day from days 5-11 or for a single exposure period lasting 24 hr on days 5, 6, 7, 8, 9, 10 or 11. Similar findings are reported in both these studies, with an increased incidence of skeletal (100% of foetuses examined in the experimental series) and visceral anomalies, and reduced birth weight in surviving foetuses. A selective loss of male foetuses was thought to have occurred in the early postimplantation period with a concomitant alteration in the sex-ratio at birth. Shepard and Fink (1968) reported that a maximum effect on the incidence of skeletal anomalies occurred when rats were anaesthetised on day 9 of pregnancy. However, a significant increase in skeletal anomalies was found in unanaesthetised females starved for 24 hr on day 7, and others exposed for 48 hr to 10% oxygen on days 8 and 9. A moderate incidence of skeletal anomalies was also found in the control series. The early report by Parbrook et al. (1965) in which rats were exposed to 60% nitrous oxide during early pregnancy is difficult to evaluate due to the almost complete absence of details of their treatment schedules.

Exposure to mixtures of halothane and nitrous oxide

In two studies, experimental animals have been exposed to mixtures of halothane and nitrous oxide. Wittman et al. (1974, cited in Kennedy et al., 1976) exposed rats for 12 hr/day on days 6 and 10 of pregnancy to a gas mixture containing 0.3% halothane in nitrous oxide/oxygen, and reported a 34% incidence of foetal loss compared to a control rate of 1%. The control level was not increased by exposure to nitrous oxide alone. In the second study of this type, Bussard et al. (1974) exposed hamsters for 3 hr/day on days 9, 10 and 11 of pregnancy to anaesthetic concentrations of nitrous oxide/halothane.
At autopsy on day 15 (of a 16 day pregnancy) an increase in foetal loss (resorptions) was observed, surviving foetuses were reduced in weight and length compared to controls, with a maximum response on day 11. No effect on sex-ratio was found.

Exposure to sodium pentobarbital

In two series of experiments, Setala and Nyssonen (1964) exposed mice on days 1-4 and 1-15 of pregnancy to narcotic levels of sodium pentobarbital (i.p. injection of 1.6 mgm daily), and reported reduced fertility from 80-90% in controls to about 50% in the experimental series, and a high incidence of a wide variety of congenital abnormalities. Very few experimental details are to be found in this report.

In these last sections the reactions of the foetus to drugs given to the mother have been outlined. While several techniques have been described which could be modified to test the direct action of anaesthetics on the foetus, either in vivo or in vitro, in only one study has the direct effect of an anaesthetic agent on the isolated foetus in culture been described.

The effect of Avertin on rat embryos in culture

Kaufman and Steele (1976) using the technique of postimplantation embryo culture described by New (1971) and New et al. (1973) examined the effect on headfold stage rat embryos of a single exposure to different levels of Avertin. The 'standard' dose of Avertin used was similar to the dose normally given to anaesthetise a rat for a short period, assuming that 1 ml of culture medium be equivalent to 1 gm body weight of an intact rat. Groups of embryos were cultured for 24 hr in 1x, 2x, 4x and 6x the standard dose of Avertin. Some embryos were examined histologically at this stage, while others were cultured for a further 24 hr. At the end of culture, embryonic development was assessed, and the protein content of embryos which were not examined histologically was determined. After 48 hr in culture there was a significant difference (p<0.0025) in the protein content between controls and embryos cultured in 1x and 2x Avertin. Development, assessed by somite number and other morphological criteria, was unaffected by 1x Avertin, but was greatly retarded in 2x Avertin (Figure 2a, b, c). In over half the embryos in this group which were examined histologically cell debris was present in the amniotic cavity (Figure 2d). This study therefore demonstrated that Avertin had a direct, dose-dependent teratogenic effect on the early postimplantation rat embryo. By slightly modifying this technique, it should be possible to determine the minimal teratogenic dose and exposure duration of a range of anaesthetics on embryos of different gestational ages and different species.

This test system in which isolated foetuses are exposed for prolonged periods to anaesthetics in vitro is justified on several grounds. It provides
information on the direct effect of known doses of the anaesthetic on the embryo independent of secondary maternal factors such as placental permeability, and the presence of metabolic products which may be more or less teratogenic than the original agent. Further, the organs of the foetus may be unable to detoxify and excrete many of the anaesthetic substances which he may receive from the mother, and thus be subject to these agents for a much longer period of time than the adult animal with normally functioning detoxification mechanisms (Smith, 1968).

The overall trend in these studies seems to indicate that anaesthetics can act as a positive teratogenic stimulus. In many of these studies, pregnant animals have been chronically exposed to very high levels of anaesthetics, particularly during the early postimplantation period. The few studies in which animals have been exposed to trace levels of halothane throughout pregnancy report a less convincing effect on foetal loss and the appearance of gross congenital anomalies in the offspring. However, at least one study (Chang et al., 1974) indicates that subtle changes in the histological appearance of the central nervous system may be induced under these conditions. In contrast to these findings, a frank teratogenic and foetotoxic effect has been reported by Katz and Clayton (1973), but the validity of these results has been questioned because of the high rate of foetal loss present in their control series. When higher doses of halothane were used, the reports are equally conflicting, varying from the totally negative findings of Kennedy et al. (1976) in the rat and rabbit, to the high incidence of foetal losses and skeletal anomalies, reported by Basford and Fink (1968), in the rat. In the majority of studies in which experimental animals were exposed to high levels of nitrous oxide both teratogenicity and foetotoxicity have been reported, though these studies may be criticised on the grounds that they are far removed from the clinical situation. In the single study where rats were exposed to trace levels of nitrous oxide (Corbett et al., 1973) during the latter half of the organogenetic period, a diurnal response was observed. No effect on pregnancy occurred when rats were anaesthetised from 2 – 10 p.m., but a significant effect on foetal mortality occurred when animals were

Figure 2 (opposite): (a) top left. Near sagittal section of conceptus cultured for 24 h in twice the standard dose of Avertin. Two poorly formed somites can be seen (arrowed). (b) top right. Sagittal section of control conceptus cultured for 24 h. Note the larger number of well formed somites (arrowed) compared with (a). (c and d) bottom left and right. Frontal sections of conceptuses cultured for 24 h in twice the standard dose of Avertin. The infused heart primordia (Ht) on either side of the head is shown in (c), and the cellular debris (arrowed) found in the amniotic cavity of experimental embryos is shown in (d). The scale is the same for all figures: bar represents 0.1 mm; P, foregut; H, head; Ht, heart.
exposed from 6 a.m. - 2 p.m. This difference in response is difficult to explain except in terms of variations in growth of rat foetuses at different times throughout the day.

In the experimental models which most closely approximate to the clinical situation, where operating room personnel are chronically exposed to trace levels of anaesthetics, the overwhelming trend seems to be in the direction of a negative or at most a marginally positive teratogenic effect. However, a much more consistently positive teratogenic effect is observed when animals are subjected to a single acute or chronic exposure to high levels of an anaesthetic agent. Both situations are not strictly comparable to the conditions prevailing in operating theatres, as in most instances a mixture of different anaesthetic gases are likely to be present in varied concentrations depending on the distance from the anaesthetic apparatus and the technique of anaesthesia being used at any given time.

MECHANISMS OF TERATOGENESIS

In order to understand some of the possible mechanisms of action of anaesthetics on the conceptus, it is necessary in the first instance to briefly consider the sites at which anaesthetics may act. Most of the studies which have been carried out to investigate the mode of action of anaesthetics at the cellular level indicate that nearly all aspects of cell structure and function are sensitive to these agents (Allison, 1971; Geddes, 1971), for example, anaesthetics block electron transport at various sites along the respiratory chain (Snodgrass & Piris, 1966), impair normal functioning of many enzyme systems within mitochondria (Conney, 1967), and impair liver microsomal activity which is intimately concerned with drug metabolism (Geddes, 1971). Exposure to anaesthetics or their degradation products may induce structural changes in lipo-protein complexes in mitochondrial membranes which may in turn alter the activity of enzymes responsible for the coupling of phosphorylation to electron transport, and lead to depression of cellular respiratory activity (Weinbach & Garbus, 1969). Glucose consumption in cultured cells is generally increased in the presence of anaesthetics, with a resultant rise in lactate levels. Both these changes are associated with depression of cell multiplication (Pink & Kenny, 1966). The effects of these agents on the cell membrane have been discussed earlier in relation to their possible effect on the microtubules and microfilaments of the cellular cytoskeletal system (for recent review, see Nicolson, 1976). It seems likely that chronic exposure to these agents may modify maternal metabolism and that this may affect the conceptus either as a result of the production of toxic metabolites, or by a failure to produce some basic cell constituent (Tuchmann-Duplessis, 1969; Beck, 1976).
Theoretically anaesthetics act on the mother, the foetus, and the foeto-maternal barrier. The factors which govern whether the embryo will be affected by a particular drug are complex. The means of administration of anaesthetics, whether inhalational, intravenous or intraperitoneal, and their initial and residual plasma and tissue levels may also be critical. To an extent the susceptibility of the foetus may also depend on the general condition of the mother, her dietary, nutritional and hormonal state at the time of drug administration. Thus, for example, the changes which occur in the hormonal state of the mother during pregnancy may serve to increase the risk to the foetus at these times. Exposure during early pregnancy may result in genetic damage to the embryo, or, secondary to maternal factors, prevent implantation or cause early postimplantation mortality, possibly by inducing changes in the concentration of certain ions or other essential constituents of the oviduct or uterine luminal fluid. Most drugs cross the placental system to some extent, often reaching the embryo without being modified, and would then be in a position to affect the embryo directly. This is thought to be one of the major routes of drug action. In many instances there may be very little maternal toxicity associated with considerable embryotoxicity. Changes in response to certain drugs during pregnancy in rodents, for example, may depend on the development of the chorio-allantoic placenta (Wilson et al., 1963; Beck & Lloyd, 1966).

Essentially teratogens may act at different structural levels within the developing embryo. Thus disturbances of protein and nucleic acid synthesis, and other metabolic pathways may result when these agents act at the molecular level. Teratogens may induce defects of intra- and extra-cellular differentiation with resultant disturbances of cell multiplication. Cell movement and cell contacts may be affected, and this may lead to interference with normal induction processes. Interference at all these levels may, by direct or indirect means, lead to structural and/or functional defects of organogenesis (Menkes et al., 1970; Poswillo, 1976). The type and extent of the damage incurred depends to a large extent on the exposure duration. Exposure during early development may lead to the death of the embryo, whereas exposure at later stages may lead to the production of major or minor malformations, functional anomalies or retarded growth of the foetus. It is well known that differences in response to teratogenic stimuli are observed among individuals of the same strain. This would seem to be the case with operating theatre staff who are exposed to trace levels of anaesthetics, where only a small but significant proportion of those at risk appear to be affected in this way. No overall picture has, as yet, emerged to support the hypothesis that anaesthetics are particularly specific in their teratogenic action. Death usually occurs either during the preimplantation period, or early in the postimplantation period, or foetuses survive and are born with a range of predominantly minor congenital
malformations. Detailed analysis of abortus material from this source may eventually demonstrate a common factor which leads to embryonic death at this stage of gestation.

**CONCLUSIONS**

In this review, I have attempted to put into its proper perspective the small amount of experimental work which has been carried out on ovarian oocytes and preimplantation stages of mammalian embryonic development, as I believe that it is this area rather than in the postimplantation period where more effort should be concentrated in the future. This obviously applies to both in vivo and in vitro studies, where attempts should be made to establish minimal teratogenic levels of the commonly used anaesthetic agents. Most of the clinical studies indicate that the commonest types of reproductive problems encountered in operating room personnel are associated with the significant increase in the incidence of involuntary infertility and spontaneous abortion compared to unexposed controls. Additional problems encountered include premature delivery and the birth of congenitally malformed children. That the majority of the problems encountered by operating room personnel fall into the first group indicates that the pathology, in the case of females, is either already present within the oocyte by the time ovulation occurs, or is induced shortly after conception, possibly during the preimplantation period. In a small proportion of males exposed to trace levels of anaesthetics, adverse changes probably occur during spermatogenesis.

It has generally been considered that teratogenic changes may be induced in response to chronic exposure to trace levels of anaesthetics. However, as indicated by some of the experimental studies reviewed here, a single acute exposure to anaesthetic levels of these agents may also produce similar clinical symptoms if exposure occurs during the critical periods of gametogenesis, embryogenesis or organogenesis. Until minimal teratogenic doses are established for these developmental stages, careful attention should be taken to see that the smallest possible number of people are exposed to any unnecessary risk (for recommendations, see American Society of Anesthesiologists, 1974; Department of Health and Social Security, Circular HC(76)38, 1976). If it can be convincingly demonstrated that the installation of efficient scavenging devices in all operating theatres significantly reduces the incidence of pregnancy loss and the birth of congenitally malformed children in operating theatre staff, then this is surely a small price to pay.

While there still appears to be little human data on the effect of surgery carried out during pregnancy, Smith's (1968) suggestion that, when applicable, 'spinal' (intrathecal) block rather than general anaesthesia should be carried out in early pregnancy seems, at least in theory, to be a reasonable one.
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BRIEF NOTES

Influence of Extracellular Ca\(^{2+}\) and Mg\(^{2+}\) Ions on the Second Meiotic Division of Mouse Oocytes: Relevance to Obtaining Haploid and Diploid Parthenogenetic Embryos

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Received August 30, 1976; accepted in revised form April 1, 1977

The types of mouse parthenogenones obtained in a medium modified with respect to Ca\(^{2+}\) and/or Mg\(^{2+}\) ions were investigated in "spontaneously" activating eggs after culturing cumulus masses in vitro for 5 hr. The second meiotic division was affected in eggs cultured in medium lacking Ca\(^{2+}\) and Mg\(^{2+}\) or Ca\(^{2+}\) alone, resulting in suppression of second polar body extrusion in a high proportion of cases, giving rise to two pronuclear eggs or eggs that underwent immediate cleavage. Extrusion of the second polar body occurred normally when the cumulus mass was cultured in complete medium and, in a high proportion of eggs, when Mg\(^{2+}\) alone was lacking in the medium. The results are discussed with reference to the second meiotic division. The method provides an efficient way for obtaining a large number of different types of parthenogenetic embryos.

INTRODUCTION

Unfertilized mammalian eggs are stimulated to undergo further development after fertilization or parthenogenesis after applying a variety of stimuli in vitro and in vivo (1). Parthenogenetic activation of mammalian eggs in vitro occurs both after treatment of unfertilized eggs with enzymes such as hyaluronidase (2, 3) and in response to ionophorous antibiotics such as A23187 (4). Activation, especially of older eggs, can occur "spontaneously," presumably in response to mechanical stimulation when cumulus masses are released into a medium in vitro (5).

Several different types of parthenogenetic eggs can be identified after activation of unfertilized eggs resulting from aberrations of the second meiotic division (Fig. 1). In unfertilized eggs, two genetically dissimilar sets of chromosomes are present which are the products of the first meiotic division. Under normal circumstances after fertilization, and in some eggs undergoing parthenogenetic development, one set of chromosomes is discarded with the second polar body at the second meiotic division. The parthenogenetic eggs in this case retain one haploid pronucleus. The extrusion of the second polar body at the second meiotic division is affected in eggs developing parthenogenetically, if the culture medium is altered. For example, second polar body extrusion is largely inhibited when eggs are cultured in low osmolar medium (5, 6, 7), giving rise to diploid parthenogenones with two haploid pronuclei. If the second polar body fails to develop, some eggs may undergo immediate cleavage (8) with the formation of two equal-sized blastomeres each containing a haploid pronucleus, both of which make cellular and genetic contributions to the developing embryo. A fourth type of parthenogenetic egg is less frequently observed. Here, second polar body extrusion is inhibited, and a single diploid pronucleus forms (see Fig. 1).

In these studies, we wished to investigate how alterations in the culture me-
bicarbonate medium containing 4 mg/ml of bovine serum albumin (9). This group of
eggs is referred to as the control group. In
the experimental groups, the cumulus
masses were placed in medium which
lacked both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} ions or only one
of the two ions. The culture drops in Fal¬
con petri dishes (No. 3002) were covered
with light liquid paraffin oil and were
placed in a continuous flow incubator at
37°C in an atmosphere of 5% CO\textsubscript{2} in air for
5 hr. The eggs were released from the cu¬
mulus masses at the end of the incubation
period by enzyme treatment for 5 min in
medium containing 100 IU/ml of hyaluron¬
idase to facilitate examination of eggs, so
that both the activation frequency and the
types of parthenogenones induced could be
determined. The eggs were examined un¬
der a Wild M.5 dissecting microscope at
50× magnification. Pronucleus formation
in the egg was taken as an indication that
the eggs had been activated. Four different
kinds of parthenogenones were obtained
(Fig. 1).

**RESULTS**

As observed previously, the activation
frequency increased with postovulatory
aging of the oocyte (3), so that higher rates
were observed in groups isolated from fe¬
males at 20 as compared to 16 hr after
HCG (Table 1). The 18-hr group showed an
intermediate response. The activation fre¬
cuency in the medium lacking both Ca\textsuperscript{2+}
and Mg\textsuperscript{2+} ions was similar to that observed
in the control group and in the medium
lacking Ca\textsuperscript{2+} alone. A higher activation
frequency was, however, observed in the
medium lacking Mg\textsuperscript{2+} alone. Modification
of the culture medium in which activation
of the eggs occurred caused a marked al¬
teration in the types of parthenogenones
obtained. In the control group, the major¬
ity of the activated eggs developed a single
haploid pronucleus following extrusion
of the second polar body. This was especially
marked in the 18 hr group. However, in
eggs which were activated in medium
lacking Ca\textsuperscript{2+} and Mg\textsuperscript{2+} or Ca\textsuperscript{2+} alone, only
a very small proportion of the activated
eggs were of this type, there being a con¬
comitant increase in the number of eggs
which failed to extrude a polar body (i.e.,
those which developed either two haploid
pronuclei or a single diploid pronucleus) or
which underwent immediate cleavage. In
the experimental group in which Ca\textsuperscript{2+} was
present in the medium, but Mg\textsuperscript{2+} was ab¬
sent, an intermediate response was ob¬
served. In this group, 41% of the activated
eggs developed a single pronucleus follow¬

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<th>Medium</th>
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<td>Complete</td>
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<td>136</td>
<td>6 (75.0)</td>
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<tr>
<td></td>
<td>2</td>
<td>18</td>
<td>142</td>
<td>57 (90.5)</td>
<td>4 (6.3)</td>
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<tr>
<td></td>
<td>3</td>
<td>20</td>
<td>265</td>
<td>88 (57.1)</td>
<td>15 (9.7)</td>
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<tr>
<td>Without</td>
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<td>16</td>
<td>54</td>
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<td>4 (57.1)</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>5</td>
<td>18</td>
<td>167</td>
<td>2 (2.2)</td>
<td>34 (37.4)</td>
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<tr>
<td>and Mg\textsuperscript{2+}</td>
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<td>20</td>
<td>44</td>
<td>1 (3.4)</td>
<td>2 (2.9)</td>
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<tr>
<td>Without</td>
<td>7</td>
<td>18-19</td>
<td>159</td>
<td>5 (6.5)</td>
<td>29 (37.7)</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>8</td>
<td>18-19</td>
<td>152</td>
<td>62 (40.8)</td>
<td>36 (23.7)</td>
</tr>
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</table>
Fig. 1. Classification of parthenogenones following activation of unfertilized mouse eggs (15). Normal meiotic division gives rise to polar body extrusion, leaving one haploid pronucleus. When second polar body extrusion is suppressed, eggs either undergo immediate cleavage or develop two haploid or one diploid pronuclei (see text).
ing second polar body extrusion (Fig. 2).

**DISCUSSION**

Our results indicate that the parthenogenetic activation frequency of mouse eggs was not affected when Ca\(^{2+}\) and Mg\(^{2+}\) ions were omitted from the complete medium. Indeed, the activation frequency was substantially greater when Mg\(^{2+}\) alone was omitted from the medium, though the reason for this increase is not known. The second meiotic division, in which extrusion of the second polar body normally occurs, was markedly affected by alterations in the ionic composition of media. Thus, when Ca\(^{2+}\) and Mg\(^{2+}\) or Ca\(^{2+}\) alone was omitted from the culture medium, either the normal meiotic division was suppressed, or eggs underwent immediate cleavage. However, normal meiotic division occurred when cumulus masses were incubated in the complete medium and, in a large proportion of activated eggs, when Mg\(^{2+}\) alone was omitted from the medium.

The manner in which alterations of the extracellular Ca\(^{2+}\) and Mg\(^{2+}\) ions affect the early development of parthenogenetic eggs and the second meiotic division, in particular, is unknown. Decreasing osmolarity causes suppression of the second meiotic division (5, 6, 7), and cytochalasin B, which disrupts microfilaments, produces similar results (10). In addition, cytochalasin B destroys regional differences in the cell-surface concanavalin A receptors, and causes displacement of the second metaphase spindle from the periphery (11). Alterations in the levels of extracellular Mg\(^{2+}\) and Ca\(^{2+}\) ions may have an effect on the cytoskeletal structure and function of the eggs, but more precise determinations of the ionic content of the media and direct observations of cytoskeletal structures and functions are required before a meaningful assessment of the underlying mechanisms can be made. Both Ca\(^{2+}\) (12) and Mg\(^{2+}\) (13) ions are thought to play critical regulatory roles in mitotic division of cells, and decreasing the concentration of Mg\(^{2+}\) ion in the medium causes inhibition of mitotic division (13). The role of these ions in meiotic division requires further investigation.

The method of parthenogenetic activation described here is reliable and provides a means of obtaining large numbers of different types of parthenogenetic embryos by simply altering the ionic composition of the medium. The different types of haploid and diploid parthenogenetic embryos obtained by this method will allow biochemical, genetic, and ultrastructural analyses of the development of the parthenogenones to be initiated. This should facilitate determination of the role of the male gamete in the activation process and in early mam-
malian development and the influence of haploid and diploid gene expression during embryogenesis. In a recent study, we obtained diploid parthenogenones from (C57BL × CBA) F₁ mice by culturing eggs in Ca²⁺ and Mg²⁺-free medium as described here. Following transfer to pseudopregnant recipients, some developed to the forelimb-bud stage (14).

We thank Dr. David Epel for his helpful criticisms of the manuscript and Andrea Burling for technical assistance. The work was supported by a Ford Foundation grant to Professor C. R. Austin.

REFERENCES
Development to term of chimaeras between diploid parthenogenetic and fertilised embryos

Parthenogenetic mouse embryos have the potential for extensive cellular proliferation as well as differentiation into various cell types. But this potential has been realised only when parthenogenetic embryos have been transferred to extrauterine sites, and in spontaneously occurring ovarian teratomas and teratocarcinomas of parthenogenetic origin. The development of mammalian parthenogenetic embryos in utero is restricted, with no conclusive evidence that they can develop to term. Several hypotheses have been proposed to account for their poor development. For example, deleterious recessive genes may affect the viability of their cells, possibly because of their extensive homozygosity, or disorganised growth and limited life span may result from anomalies of cellular interactions. But the extensive cellular proliferation and differentiation of parthenogenetically derived cells which occurs in extrauterine sites is not entirely consistent with these explanations, and indicates that parthenogenones probably have a relatively stable genetic constitution. Indeed, these studies stress the likely importance of cellular environment for cytodifferentiation, provided in this instance by the extrauterine host tissue. There is a precedent for supposing that if the environment is critical for cytodifferentiation, parthenogenetic cells should be able to form chimaeras with cells derived from fertilised embryos. Teratocarcinoma cells and cells carrying known lethal alleles can develop into viable chimaeras when aggregated with cells from normal embryos. Previous attempts to achieve development to term of aggregation chimaeras between parthenogenetic and fertilised embryos were apparently unsuccessful. We have introduced inner cell masses (ICMs) from diploid parthenogenetic embryos into intact fertilised mouse blastocysts, and we report here the development of a chimaera to term.

Mouse oocytes isolated 18 h after injection of human chorionic gonadotrophin for superovulation from (C57BL×CBA) F1 females were activated in vitro in medium lacking Ca2+ and Mg2+ salts, and the diploid parthenogenetic embryos allowed to develop to the blastocyst stage in culture. Of 397 activated eggs 37 (14%) had one pronucleus and the second polar body; 160 (60.6%) had two pronuclei without the second polar body; eight (3%) had one pronucleus without the second polar body, and 59 (22.3%) went into immediate cleavage. The overall activation frequency was 66.5%. The embryos were homozygous for the isozyme of glucose phosphate isomerase (GPI). Gpi-1/2-Gpi-1/2 and carried coat colour and eye colour genes of the C57BL and CBA strains. Of the 160 activated eggs with two pronuclei without the second polar body, 81 (50.6%) reached the blastocyst stage. We obtained 43 ICMs from the blastocysts and were able to transfer 20 to recipient blastocysts. ICMs were isolated by a technique involving exposure of blastocysts to the calcium ionophore A23187. The parthenogenetic blastocysts were exposed to A23187 at the final concentration of 2×10^-6 M for 20-30 min. This caused selective lysis of trophoderm cells, leaving the ICM cells intact (M.A.H.S., D. Torchiana and S.C.B., in preparation). Individual ICMs were introduced microsurgically into blastocysts on the 4th day of pregnancy obtained by matings of Gpi-1/2-Gpi-1/2 albino C57BL/6j mice (Anglia Laboratory Animals Ltd). After 3-4 h culture at 37 °C, blastocysts were transferred to the uterus of d3 pseudopregnant CFLP mice previously mated to proven sterile vasectomised males (the day when vaginal plug was found was d1 of pseudopregnancy). Four blastocysts were transferred to each of the five recipients.

One female examined on d9 of pregnancy contained four implantation sites. Four apparently normal early somite embryos were recovered. The embryos and their respective ectoplacental cones were separately typed for GPI. All four embryos were chimaeric, whereas the ectoplacental cones only contained the Gpi-1/2 isozyme band. Two embryos probably contained 40-50% contribution from parthenogenetic cells, whereas the other two contained between 5 and 30% contribution from parthenogenetic cells. This assessment was made subjectively from the intensity of staining Gpi-1/2 and Gpi-1/2 isozyme bands (Table 2). The other four females were autopsied on d19 of pregnancy. Three of them were not pregnant, having no evidence of implantation sites. One female contained two embryos (Fig. 1a). The smaller embryo was dead, although morphologically an apparently normal male. This embryo showed pigmentation of the sclera and iris and had probably survived up to d15-17 of pregnancy. The larger embryo was alive at autopsy, and initiated spontaneous respiratory movements. Attempts were made to foster the foetus, but it was unfortunately not accepted by the foster mother. Anatomical examination of this embryo showed it to be an apparently normal female. The eyes were dissected out and showed pigmentation of the choroid (Fig. 1b) and iris, as well as the retina. All major organs from the two embryos were typed for GPI and found to be chimaeric. Between 40 and 50% of the cells in the smaller, dead embryo were of partheno-

![Image](https://example.com/image1.png)

**Fig. 1 a, Chimaeric embryos isolated from d19 pregnant female.** The larger embryo was alive at autopsy and was an apparently normal female. The smaller embryo was dead and had probably developed until d15-17 of pregnancy and was an apparently normal male. b, An eye dissected from the larger embryo showing pigmentation of the choroid. The iris and retina were also pigmented.
genetic origin, while in the live embryo the contribution was considerably smaller, being in the region of 10–25% (Table 1).

These results demonstrate that cells from diploid parthenogenones, in addition to their potential for proliferating and differentiating in ectopic sites, can participate with cells from fertilised embryos in the development to term of an apparently normal chimaeric embryo. We tentatively suggest that a large number of such chimaeras may be able to develop to term when the parthenogenetic contribution is probably not more than 20% of the total cell population in the individual tissues and organs. The survival and integration of parthenogenetic cells in such chimaeras is probably largely influenced by the environmental conditions determined by the cells from the fertilised embryo, in contrast to the extensive but haphazard cell growth and differentiation which occurs when parthenogenetic and fertilised embryos are transferred to ectopic sites. The exact nature of this environmental influence remains unclear, but there are several probable ways in which such influences may be mediated. For example, exposed sites at the cell surface and/or secreted molecules may compensate for any deficiencies in parthenogenetic cells, permitting their normal development and differentiation. This type of influence may be involved in the 'rescue' of androgen-resistant germ cells. There is evidence for metabolic cooperation between genetically diverse cell types through permeable junctions which enables metabolically deficient cells to function normally. Similar cellular interactions between parthenogenetic and normal fertilised cells may also ensure cooperation of parthenogenetic cells in normal development.

These results suggest that it should be possible to obtain mature adult chimaeras. This would enable us to examine whether the parthenogenetically derived cells are fully differentiated in these chimaeras, and to examine whether they contribute to the normal functioning of all tissues and organs including the germ cells. This study also demonstrates that the presence of a genetic or extragenetic contribution from the male may not be a prerequisite for the normal development and differentiation of mammalian cells. Experiments are in progress to establish the differentiation potential of both haploid and diploid parthenogenetic cells.

We thank Andrea Burling for technical assistance, Dr R. L. Hamil, Eli Lilly Co for A22187 and Dr Miklos P. Salgo for helpful suggestions. The work was supported by a grant from the Ford Foundation. M.A.H.S. is supported by an MRC project grant.

Note added in proof: One apparently normal chimaeric male which is now 4-weeks-old, has been obtained after further transfers of six operated blastocysts. Similar chimaeras have recently been reported by Stevens et al.

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Table 1 The contribution made by parthenogenetic cells in chimaeras assessed from GPI typing

<table>
<thead>
<tr>
<th>Embryo no.</th>
<th>Day of pregnancy at autopsy</th>
<th>Description of embryos</th>
<th>Embryo (excluding membranes)</th>
<th>Ectoplacental cone</th>
<th>Blood</th>
<th>Liver</th>
<th>Lung</th>
<th>Brain</th>
<th>Heart</th>
<th>Gonad</th>
<th>Kidney</th>
<th>Amnion</th>
<th>Eye</th>
<th>Carcass</th>
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<tbody>
<tr>
<td>1</td>
<td>d9</td>
<td>10-15omite stage</td>
<td>40</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>5</td>
<td>ND</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>&quot;</td>
<td>30</td>
<td>ND</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>5</td>
<td>d19</td>
<td>d15-17, male dead</td>
<td>40</td>
<td>ND</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>6</td>
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<td>25</td>
<td>10</td>
<td>15</td>
<td>10</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

ND, not detected; NE, not examined, tissue unsuitable.

*Values represent subjective assessment from GPI analysis based on the time of appearance of the Gpi-1^+ and Gpi-1^- bands and the relative intensities of the two bands.

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X chromosome inactivation in diploid parthenogenetic mouse embryos

The mechanism by which one X chromosome in normal cells of female mammals becomes inactive while the other, apparently identical, chromosome remains active is not known. In kangaroos the paternally derived X chromosome is preferentially inactivated in most tissues, and in mice and rats similar preferential inactivation of the paternal X chromosome occurs in the cells of the extraembryonic membranes. Paternal X inactivation in kangaroos led Cooper to postulate that passage of the X chromosome through male gametogenesis was an important factor in its inactivation. He and Brown and Chandra suggested that paternal X inactivation was a primitive form and that random X inactivation as seen in adult eutherian mammals had evolved from it. Brown and Chandra further suggested that passage of one chromosome set through male gametogenesis or fertilisation led to chromosomal imprinting. A locus concerned in the control of X chromosome activity was postulated to become inactive when imprinted, and one active copy of this gene could maintain the activity of one X chromosome. In marsupials this gene was postulated to lie on the X chromosome itself, but on an autosome in eutherians. Thus, in eutherians the number of active X chromosomes in any animal should be equal to the number of maternally derived (and therefore non-imprinted) autosome sets. In this context it is interesting to study X inactivation in artificially formed parthenogenetic embryos, in which all chromosomes are of maternal origin. We report here that diploid parthenogenetic mouse embryos, at the late egg cylinder stage, showed a single late replicating chromosome, indicating that X inactivation had occurred normally. Because in the production of these embryos polar body formation had been suppressed, it seems that neither passage of chromosomes through male gametogenesis, nor fertilisation, nor probably the effects of peripheral egg cytoplasm are required for normal X inactivation in the mouse.

In the normal mouse embryo X chromosome inactivation is thought to occur at the late blastocyst stage, and

Fig. 1 a–c. Autoradiographs of cells from diploid parthenogenetic mouse embryos. a, Single unlabelled chromosome in heavily labelled cell. b, Same cell after removal of silver grains. c, Single heavily labelled chromosome in otherwise lightly labelled cell (characteristic late-S replication pattern). d, Apparent X chromatid body in some interphase nuclei. Ilford Nuclear Research emulsion, gel form L4, was used for autoradiography. Slides were exposed for 3 weeks at room temperature, developed in Amido developer, fixed and stained with Giemsa. Silver grains were removed by transferring slides through xylene and graded alcohols to potassium ferricyanide, sodium thiosulphate and distilled water.
a late replicating X chromosome can be recognised at about the 5- to 7-d stage. Thus, studies of DNA replication in parthenogenetic embryos at the late egg cylinder stage should indicate whether normal X inactivation has occurred.

Experimental parthenogenetic mouse embryos have been produced by various means, and Kaufman et al. have achieved development of a high proportion of diploid parthenogenotes to the egg cylinder stage or later. We have now studied DNA replication by 3H-thymidine labelling at the late egg cylinder stage in diploid parthenogenetic embryos produced by their method. Eggs were activated by culturing cumulus masses containing recently ovulated oocytes for 5-6 h in medium lacking calcium and magnesium salts. Then the cumulus cells were removed with hyaluronidase, and the successfully activated presumptive diploid eggs (which developed two pronuclei after suppression of second polar body formation or extrusion) were cultured for a further 90 h in normal embryo culture medium, by which time approximately 60% had reached the blastocyst stage. Blastocysts were then transferred to pseudopregnant recipient females, which had been mated to proven sterile vasectomised males 2.5 d earlier. Recipients were ovariectomised bilaterally at the time of transfer of blastocysts, and pregnancy was maintained by exogenous hormones, so that the time of implantation could be controlled. Implantation began about 24 h after the first of a series of oestradiol injections, which were started 6 d after blastocyst transfer. Females were killed after 4 d of injections when the embryos were expected to be at a stage equivalent to about day 7.5-8.5 of normal development. The embryos were dissected free from the decidua and embryonic membranes, and placed in Eagle's medium, supplemented with foetal calf serum (20%) at 37 °C.

In the mouse, because of its many segments of constitutive heterochromatin, the asynchronously replicating X chromosome is more readily detectable by its failure to take up H-thymidine early in the S period, than by its excess labelling at the end of S. Embryos were allowed to incorporate H-thymidine (final concentration 5 µCi ml⁻¹; specific activity 6 Ci mmol⁻¹) for 30 min at 37 °C, followed immediately by three washings with unsupplemented medium, and a chase of medium containing a 100-fold excess of unlabelled thymidine for 10 h. Colchicine, at a final concentration of 1 µg ml⁻¹, was present during the final 2 h of the 10-h incubation. Air-dried slides were prepared according to a modification of the method of Wroblewska and Dyban and autoradiographs were prepared.

Of six embryos studied four yielded scorable mitoses. Cells with 40 chromosomes were scored for uptake of label, and for presence or absence of a single large unlabelled chromosome, presumed to be an X chromosome. In all four embryos some cells showed a clear asynchronously replicating chromosome (Table 1). In all cells this was a large chromosome, of approximately the expected size of an X chromosome (Fig. 1). In most cases the asynchronous chromosome was unlabelled in an otherwise fully labelled cell (Fig. 1a, b), but in a few cells there was a single heavily labelled chromosome in a lightly labelled set (Fig. 1c). In addition some interphase cells showed an apparent X chromatin body (Fig. 1d).

The proportion of cells showing any label varied somewhat (from 17 to 42%) among the embryos, but the proportion of labelled cells with an asynchronous chromosome showed reasonable consistency, with a mean of 62% (range 56-80%). The failure to detect an asynchronous chromosome in the remaining cells does not of course mean that one is not lacking, because cell cycle times may have been variable, with some cells being mid-S, and therefore unsuitable for showing asynchronous replication, at the time of the 3H-thymidine pulse. The finding of a few cells with a replication pattern characteristic of late S emphasises this point.

Our interpretation is thus that the asynchronously replicating chromosome seen in all four embryos was a late replicating (and therefore inactive) X chromosome. Clearly differentiation of two X chromosomes within a cell can take place in embryonic development without the need for fertilisation or for passage of either chromosome, or indeed any autosome, through male gametogenesis.

A similar conclusion was reached by McCaw and Latt who found late replicating X chromosomes in human ovarian teratomata, which arise by parthenogenetic growth of germ cells, and by Linder and Power on the basis of X-linked gene expression in such teratomata. Our work strengthens the finding by dealing with embryos which were undergoing fairly normal development (up to the relevant stage), rather than teratomata in which the genetic programming might be in some way disturbed. Thus, these results are inconsistent with the expectation from Brown and Chandra's hypothesis that the number of active X chromosomes in any cell should be equal to the number of maternally derived autosomes. In their development of the original hypothesis they reconciled the X inactivation seen in certain human ovarian teratomata and a diploid/triploid mosaic by postulating that chromosomal imprinting occurred not during male gametogenesis but during passage of chromosomes through the egg cytoplasm. Normally this would occur at fertilisation, but in the cases mentioned the effect was produced by re-entry of the polar body. Our embryos were inconsistent even with this, because the parthenogenetic activation was produced in combination with suppression of polar body formation. Further evidence against Chandra and Brown's ideas has come from human triploid foetuses of one of which had two active X chromosomes although only a single autosome set from the mother and another which had a single active X chromosome but two haploid sets from the mother.

Thus we conclude that in eutherian mammals there is no evidence that chromosomal imprinting is required for the initiation of X-chromosome inactivation. This does not, of course, exclude the possibility of imprinting in normal embryogenesis. Preferential inactivation of the paternally derived X chromosome (manifested by late replication as detected by acridine orange fluorescence after BUdR incorporation) has been found in the extraembryonic membranes but not the foetus itself of both the mouse and the rat. Thus it seems probable that in eutherians, as in marsupials, imprinting of the paternally derived X chromosome does occur, but that the cells of the embryo proper escape from imprinting before X inactivation. One might expect therefore that in parthenogenetic embryos X inactivation would be in some way abnormal in the extraembryonic membranes. Further work is necessary to test this point.

M.H.K. thanks Mrs S. C. Barton and Dr M. A. H.
Surani for help. The work was supported by a grant from the Ford Foundation.

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Received 6 October; accepted 16 December 1977.

Chromosome analysis of early postimplantation presumptive haploid parthenogenetic mouse embryos

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SUMMARY

The chromosome constitution of early postimplantation presumptive haploid parthenogenetic mouse embryos was examined. All the embryos isolated were at the egg-cylinder stage and seven contained dividing cells. In two of the apparently healthy embryos only haploid mitoses were seen, whereas in five others an approximately equal proportion of haploid and diploid mitoses was observed. Out of 52 cells in which unequivocal counts could be made, only one contained more than the euploid number of chromosomes (mouse, n = 20). Possible reasons for the poorer development of haploid compared to diploid parthenogenetic embryos are discussed.

INTRODUCTION

The aim of the present study was to investigate whether parthenogenetically activated haploid mouse embryos contain actively dividing haploid cells in the early postimplantation period.

Previous attempts to study the chromosome constitution of presumptive haploid postimplantation embryos have always been unsuccessful, primarily because of technical difficulties associated with their extremely poor development. Tarkowski and his colleagues (Tarkowski, Witkowska & Nowicka, 1970; Witkowska, 1973) obtained reasonable rates of parthenogenetic development to the egg-cylinder stage, but their experimental approach did not allow the original ploidy of these embryos to be determined. Recently a novel experimental approach (Kaufman, Barton & Surani, 1977) has allowed a high proportion of parthenogenetically activated diploid embryos to reach the egg-cylinder stage, and a more modest proportion to develop to the forelimb-bud stage, equivalent to about day 9-5-10-5 of normal (fertilized) development. This experimental approach was used in the present investigation to obtain presumptive haploid egg-cylinder stage embryos.

Embryos which undergo immediate cleavage (Braden & Austin, 1954) were studied. These result when parthenogenetically activated eggs divide into two equal sized blastomeres instead of extruding a second polar body. Each blasto-

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mere therefore contains one of the products of the second meiotic division. These are genetically dissimilar as a result of crossing over events at the first meiotic division, and consequently give rise to two distinct clones of cells within the cleaving embryo. This has accordingly been termed mosaic haploid development (Kaufman & Sachs, 1976). Some of the theoretical reasons which might account for the observed developmental advantage of immediate cleavage embryos over the single pronuclear type, where the nucleus of each cell is genetically identical, have been considered elsewhere (Kaufman & Sachs, 1976).

In a preliminary study immediate cleavage-derived egg cylinders were examined histologically. Many of the cells in mitosis appeared to have far fewer chromosomes than are usually seen in comparable diploid material. However, because of the obvious difficulties involved in attempting to extrapolate cytogenetic data from histological material, air-dried preparations from disaggregated egg cylinders were examined in the present study.

**MATERIALS AND METHODS**

Cumulus masses containing recently ovulated oocytes were isolated from the oviducts of 8- to 12-week-old (C57BL × CBA) F₁ hybrid female mice at 18–19 h after the HCG injection for superovulation, and released into modified Krebs–Ringer bicarbonate embryo culture medium lacking both calcium and magnesium salts (Kaufman et al. 1977; Surani & Kaufman, 1977). About 5–6 h later the cumulus cells were removed with hyaluronidase after a 5–10 min incubation period in standard embryo culture medium (Whittingham, 1971) containing 100 i.u./ml of this enzyme. This treatment facilitated examination of the eggs and enabled the activation frequency and types of parthenogenones induced to be determined.

Only the eggs which underwent immediate cleavage following activation were further studied. Eggs of this type were transferred to standard embryo culture medium and incubated for a further 90 h. A proportion developed to the blastocyst stage, and was subsequently transferred to a single uterine horn of day-3 pseudopregnant recipients previously mated to proven sterile vasectomized males (day of finding vaginal plug = day 1 of pseudopregnancy). Directly after the transfer procedure had been carried out recipients were bilaterally ovariectomized and thereafter maintained on exogenous steroid hormones. After an initial hormone-free period of 2 days recipients were maintained for 2 days on 1 mg progesterone daily, followed by three daily injections of 20 ng oestradiol and 1·6 mg progesterone daily per female.

Recipients were killed on the ninth day after the embryo transfer procedure, after 3 days of oestrogen injections. All uterine horns containing implantation sites were removed and the decidua placed in phosphate buffered saline. Embryos were dissected out under a dissecting microscope using watchmaker's forceps, care being taken to isolate the egg-cylinder (embryonic) region from the
**Analysis of haploid mouse embryos**

### Table 1. The pathways of development of parthenogenetic eggs at 5–6 h after in vitro activation

<table>
<thead>
<tr>
<th>Total number of eggs examined</th>
<th>Activated eggs</th>
<th>Overall activation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 pronucleus + 2nd polar body</td>
<td>2 pronuclei without 2nd polar body</td>
</tr>
<tr>
<td>1375</td>
<td>205 (20.4)*</td>
<td>449 (44.7)</td>
</tr>
</tbody>
</table>

* The percentage of the total number of eggs activated is given in parentheses.

ectoplacental cone region. Only the egg-cylinder region was further examined.

Air-dried preparations of the disaggregated egg cylinders were made by the technique described by Wroblewska & Dyban (1969). With the exception of the initial hypotonic citrate stage, where 30 min treatment was found to be optimal, all other stages were considerably shortened. Thus 2–3 min fixation in 3:1 ethanol-acetic acid mixture, and about 10 min pre-staining in 2% orcein in 50% acetic acid was found to be adequate. Complete disaggregation usually took place after 10–20 min in the 50% lactic-acetic acid solution. The slides were subsequently stained with 2% orcein and the air-dried material examined by phase-contrast microscopy.

**RESULTS**

When eggs were examined after incubation for 5–6 h in medium lacking calcium and magnesium salts, the activation frequency was 73.0%, and 33.4% of the activated eggs were of the immediate cleavage type (Table 1). After a further 90 h incubation period in standard embryo culture medium 112 of the immediate cleavage eggs developed to the blastocyst stage. A total of 96 of these presumptive haploid embryos were transferred to 16 pseudopregnant recipients. Fourteen females contained implants at autopsy, and the implantation rate in the pregnant females was 63.2%. Out of a total of 48 implants ten egg-cylinder stage embryos were isolated. The embryos ranged in size from early to advanced egg cylinders (Fig. 1a), being approximately equivalent to fertilized embryos of between 6.5 and 7.5 days gestation. Seven of the embryos were apparently healthy and contained between 1% and 4% dividing cells (Table 2). Three additional egg cylinders were obviously unhealthy, containing a high proportion of pyknotic nuclei and no cells in division.

The incidence of haploid and diploid metaphases in the healthy egg cylinders varied considerably between embryos. Two embryos had only haploid metaphases, whereas in the remaining five about half of the cells in division were haploid and the rest diploid. A single tetraploid cell was encountered. Out of
Fig. 1. For legend see opposite.
Table 2. The ploidy of seven morphologically normal presumptive haploid egg cylinders

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Stage of development</th>
<th>Total cells examined*</th>
<th>Ploidy of cells in mitosis†</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Haploid</td>
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<tr>
<td>1</td>
<td>Advanced egg cylinder</td>
<td>&gt; 1100</td>
<td>13</td>
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<td>2</td>
<td>Advanced egg cylinder</td>
<td>&gt; 1000</td>
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<td>3</td>
<td>Advanced egg cylinder</td>
<td>&gt; 1000</td>
<td>12</td>
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<td>4</td>
<td>Small egg cylinder</td>
<td>&gt; 400</td>
<td>8</td>
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<td>5</td>
<td>Small egg cylinder</td>
<td>350–400</td>
<td>2</td>
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<tr>
<td>6</td>
<td>Small egg cylinder</td>
<td>250–300</td>
<td>3</td>
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<tr>
<td>7</td>
<td>Small egg cylinder</td>
<td>180–200</td>
<td>3</td>
</tr>
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</table>

* These values provide only an approximate estimate of the total cell population in these embryos as many cells were lost during the disaggregation procedure.
† The ploidy of 35 out of a total of 51 haploid cells and 21 out of a total of 57 diploid cells were scored without being able to make an unequivocal count of the chromosome number.

DISCUSSION

The present study is the first to conclusively demonstrate that presumptive haploid embryos may contain a population of actively dividing haploid cells at the egg-cylinder stage. Further, that in those cells in which unequivocal counts could be made, almost all contained the normal haploid (mouse, *n* = 20) or diploid number of chromosomes. In the absence of more sophisticated techniques of chromosome analysis it is, of course, impossible to know whether segments of chromosomes were lost or were involved in rearrangements as was apparent in the chromosomally diploid teratoma cell lines examined by Iles et al. (1975) which were derived from presumptive haploid embryos.

Fig. 1. (a) Transverse section of presumptive haploid advanced egg-cylinder stage embryo approximately equivalent to fertilized embryo of between 6.5 and 7.5 days gestation. (b) Air-dried preparation of haploid mitosis from disaggregated egg-cylinder stage embryo. (c) Diploid mitosis from disaggregated haploid-diploid mosaic egg-cylinder stage embryo. (d) Tetraploid mitosis (in early anaphase) from disaggregated haploid-diploid-tetraploid mosaic egg-cylinder stage embryo. (e) Haploid metaphase figure (arrowed) from same embryo as shown in Fig. 1(a). (f) Diploid metaphase figure (arrowed) from presumptive diploid parthenogenetic embryo at the egg-cylinder stage.
Previous studies (Kaufman, unpublished) have indicated that the cleavage rate of haploid embryos is often somewhat slower than that of diploid parthenogenones during the second half of the preimplantation period. In addition, recent observations on the number of cells present in the inner cell mass (ICM) of immediate cleavage-derived haploids, heterozygous diploid parthenogenones and fertilized mouse embryos at the expanded blastocyst stage (Kaufman and Surani, unpublished observations) have indicated that the haploids usually contain fewer cells than diploid parthenogenones, and that the ICMs of the diploids generally contain less than 50% of the number normally present in fertilized blastocysts. Ansell & Snow (1975) have suggested that a critical minimal cell number is required in the ICM for normal embryonic development. Probably fewer haploid than diploid parthenogenones would have achieved this even after several cell divisions. Presumably these relatively ICM-deficient presumptive haploid blastocysts would be capable of evoking a decidual response with, on closer inspection, only minimal evidence of embryonic development. These observations might well explain the lower rate of development to the egg-cylinder stage of the presumptive haploids in the present study (15%) compared to 35% achieved by diploid parthenogenones subjected to similar experimental treatment (Kaufman et al. 1977).

The incidence of apparently completely haploid postimplantation embryos observed in this study, though small, suggests that the haploid state is compatible with a considerable degree of cellular differentiation and apparently normal morphogenesis. A more extensive study will obviously be required to clarify whether the presence of diploid cells conveys any form of developmental advantage to the cells which remain haploid, and to determine whether a relationship exists between the incidence of diploidy and the degree of development which may be achieved in haploid–diploid mosaic embryos.

The relative stability of haploid cells from fungi, higher plants and anuran species (Freed & Mezger-Freed, 1970; Gupta & Carlson, 1972) suggests that the problems associated with the production of a stable haploid mammalian cell line (see Graham, 1974) should not be insurmountable. The two approaches which hold out most promise at the present time, via transplantable teratocarcinomas (Iles et al. 1975) and through the production of cell lines from preimplantation (Hogan & Tilly, 1977) and early postimplantation embryos, are both under active investigation. Attempts are also being made to examine more advanced presumptive haploid parthenogenetic mouse embryos.

I thank Mrs S. C. Barton and Mrs A. Burling for technical assistance, and Dr M. A. H. Surani for allowing me to present some of our unpublished observations. This work was supported by a grant from the Ford Foundation.
REFERENCES


(Received 5 September 1977, revised 5 January 1978)
The Experimental Production of Mammalian Parthenogenetic Embryos

MATTHEW H. KAUFMAN

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I. INTRODUCTION

Parthenogenesis has been defined as the production of an embryo, with or without eventual development into an adult, from a female gamete in the absence
of any contribution from a male gamete (modified after Beatty, 1957). Interest in the production of mammalian parthenogenetic embryos was initiated in the 1930's and early 1940's with the experiments of Pincus and his co-workers with rabbit eggs. Further studies on rabbit and rodent eggs were reported by Thibault in the 1940's, by Austin and Braden and also by Chang in the 1950's, and more recently by Graham and Tarkowski in the 1970's. A complete review of the literature in the field of mammalian parthenogenesis will not be given, but extensive background information on the subject has been provided by Beatty (1957, 1967, 1972), Austin (1961), Austin and Walton (1960), Tarkowski, (1971, 1975), Graham (1974), and Kaufman (1975a). A few of the more important recent contributions in this area of research are also indicated in the relevant sections of the text. In this chapter, emphasis is placed largely on the methodology of activation and production of parthenogenetic mouse embryos. The mechanisms of action of the various stimulating agents, and other aspects of early development, are dealt with only briefly. The importance of this material in providing a new approach to investigating problems associated with early mammalian development are indicated, and some future lines of research are suggested.

II. CULTURE METHODS RELEVANT TO IN VITRO PARTHENOGENETIC ACTIVATION

A. Choice of Medium

Krebs-Ringer bicarbonate or Tyrode's solution supplemented with bovine serum albumin, with lactate, pyruvate, and glucose as energy sources have been found adequate for parthenogenetic activation by most workers. The main advantage of this type of medium is that it has a known chemical composition, which probably facilitates biochemical analysis on embryos. These media can also maintain preimplantation development to the blastocyst stage in vitro of certain strains of activated eggs that are not subject to the "2-cell block." The addition of serum and the use of more complex biologic media are not essential and may give a greater variability in results, and may indeed be detrimental in this system.

Protein-free stock solutions of embryo culture medium should be prepared at weekly or at most 2- to 3-weekly intervals. Lactate and pyruvate are essential energy substrates, and pyruvate is labile and undergoes spontaneous decarboxylation in dilute solution. Glucose is also generally added to culture media and may help to improve the developmental potential of the activated embryos, especially for prolonged culture of activated 1-cell eggs to the blastocyst stage, as well as serving as an additional energy substrate. A detailed study of the energy
requirements of preimplantation mouse embryos has been given by Biggers (1971) and Whittingham (1971). A few of the defined embryo culture media that have been successfully used in various in vitro parthenogenetic activation studies are Whittingham’s medium (Whittingham, 1971—favored by Kaufman), modifications of White’s medium (see Graham, 1970), and Whitten’s medium (Whitten, 1971—favored by Graham).

Details of methods for the preparation of culture media are to be found in many sources, but reference should be made to the papers by Whitten and Biggers (1968), Biggers et al. (1971), and Whittingham (1971). The latter two papers also provide information on routine preimplantation embryo culture work, which are equally suitable for parthenogenetic studies. For most purposes, particularly where long-term embryo culture is contemplated, a McIntosh and Fildes pattern anaerobic jar gassed with humidified 5% CO₂ in air, or 5% CO₂, 5% O₂, 90% N₂, and incubated at 37°C, is adequate. When frequent observations on embryos in culture are required, such as in studies on the entry of embryos into the first cleavage division (Kaufman, 1973a,b), a continuous-flow incubation chamber is preferable, but not absolutely essential.

When only a limited amount of technical assistance is available, it is probably safer to use disposable plastic petri dishes, which are specifically recommended for tissue culture work (e.g., Falcon plastic petri dishes, Cat. No. 3002), rather than embryologic watchglasses or glass petri dishes if these items cannot be washed to the high standard which is essential for reliable embryo culture work. Eggs and embryos are cultured in drops of medium under light paraffin oil according to the method originally described by Brinster (1963).

**B. Choice of Animals**

The activation frequency achieved depends as much on the strain of mice employed, whether they are inbred or randomly bred or F₁ hybrids, as on the culture conditions or the type of stimulus applied to eggs in situ. It is also possible to obtain parthenogenetic activation of some mutant strains of mice (e.g., Bpa, see Phillips and Kaufman, 1974). High rates of activation and development may also be achieved when oocytes isolated from females that are heterozygous for particular translocations are activated (e.g., T6/+ , see Kaufman and Sachs, 1975). Eggs isolated from XO mice are also readily activated (M. H. Kaufman, unpublished).

There seems little evidence for impaired preimplantation development in haploid and diploid parthenogenetic embryos that may possess an additional X chromosome or autosome, or a segment of one or other of these chromosomes. Equally, these genetically unbalanced parthenogenetic embryos appear to be capable of development beyond implantation, but generally die shortly thereaf-
Other workers advocate Nembutal recipients. The anesthetic usually activated eggs solution of (Avertin:Winthrop).romoethanol between 5 and oviduct transfers. Activation usually carried are is that is carried out; males and females are checked the next morning for evidence of mating, and oviduct transfers carried out early in the afternoon of finding the vaginal plug, that is on day 1 of pseudopregnancy. Uterine transfers of morulae or blastocysts are usually carried out in the morning or afternoon of day 3 of pseudopregnancy. Activation is normally carried out between 8:00 and 9:00 a.m and pronuclear eggs selected at about 2:00 p.m for immediate transfer to recipients. Batches of between 5 and 10 or more activated eggs, depending on the availability of activated eggs and recipients, are transferred to the oviducts of lightly anesthetized recipients. The anesthetic usually used for this procedure is tribromoethanol (Avertin:Winthrop). The standard dose of a freshly prepared 1.2% solution of Avertin dissolved in 0.9% NaCl is 0.02 ml Avertin/gm body weight. Other workers advocate Nembutal or ether anesthesia. As it is likely that all
anesthetics are capable of inducing a proportion of the recipient’s own eggs to develop parthenogenetically (Kaufman, 1975b), it may be advisable in certain critical experiments to use genetic or biochemical markers for one or other stock of mice (e.g., see Kaufman and Sachs, 1975). It is well to be aware that this factor may complicate, for example, interpretation of implantation rates not only in parthenogenetic studies, but also where fertilized embryos are transferred under similar circumstances. This complication does not arise when embryos are transferred to day 3 recipients, as the recipient’s own eggs are no longer capable of being activated parthenogenetically.

III. ISOLATION OF EGGS FROM SUPEROVULATED AND SPONTANEOUSLY OVULATING FEMALES

A. Superovulation

The main advantage of superovulation is that large synchronous populations of eggs can be obtained at the convenience of the experimenter, and that the time of ovulation can be accurately assessed to within about an hour. However, there are often considerable variations in the response of different strains of mice to superovulation treatment (see Gates, 1971, for discussion). It will almost certainly be necessary to try different hormone regimens when untested stocks are to be employed. The injection of 1-5 or even 10 IU PMSG followed 48 hours later by 1-5 or 10 IU HCG will usually provide large numbers of eggs, and receptive females which will mate with sterile or fertile males if this is required. While one hormone regimen may induce the ovulation of particularly large numbers of eggs, another, usually smaller, dose may synchronize estrus and encourage a higher proportion of the hormonally induced population to mate. Ovulation occurs in most strains between 11 and 12 hours after the HCG injection for superovulation, and the time of the HCG injection provides a very useful baseline for most in vitro and in vivo studies. Some strains of mice respond poorly to exogenous hormone treatment, and, in these cases, eggs should be obtained from spontaneously ovulating females.

Some controversy exists over the supposed inferiority of superovulated compared to spontaneously ovulated eggs (see Elbling, 1973). However, most authorities consider that the general convenience and availability of large numbers of eggs that can usually be obtained following superovulation probably far outweighs the possible detrimental effect of the hormone treatment. The poorer pregnancy outcome in superovulated females is attributed either to “crowding effects” within the uterus, or to hormonal imbalance. For in vitro parthenogenetic studies, however, 1-cell eggs are normally isolated from the ampullary region of the oviduct into embryo culture medium.
B. Spontaneous Ovulation

Spontaneously ovulating mice at a suitable stage of the estrous cycle can be selected by vaginal inspection using the criteria outlined by Champlin et al., (1973). This technique is usually extremely reliable, and normally well over half the females selected on the afternoon or evening of proestrous ovulate that night. While fewer eggs are generally obtained from spontaneously ovulating females, only the very occasional degenerating or fragmented egg is normally found within the cumulus mass. This, however, has to be balanced with the advantages of superovulation outlined above.

When oocytes are isolated from spontaneously ovulating females, it is essential to note the mid-dark point of the lighting schedule in the animal room in which the mice are normally kept, as this gives a reasonable indication of the time of ovulation (Braden, 1957). This, in addition, provides a convenient baseline value for certain timing studies (see Kaufman, 1975b).

IV. REACTION OF EGGS TO PARTHENOGENETIC STIMULATION

Most mammalian eggs are ovulated at metaphase of meiosis II, and these only complete second meiosis if activated by appropriate stimuli. Under normal conditions in vivo, the activation stimulus is provided by the fertilizing spermatozoon, but it seems likely that a series of biochemical events which closely resemble this type of activation can be induced by a wide range of stimuli. Not only is meiosis resumed in this case, but, under optimal conditions, parthenogenetic embryonic development may proceed well beyond implantation, possibly even to the birth of viable offspring.

Following parthenogenetic activation eggs progress along different developmental routes, depending on whether the second polar body had been extruded or not, whether cytokinesis results in the production of blastomeres of equal or unequal volume, and on whether haploid or diploid nuclei are formed at the various meiotic divisions (Beatty, 1957, 1967). The different routes of early development are illustrated in Fig. 1.

At the completion of second meiosis, which usually occurs within about 2-3 hours of activation, second polar body extrusion normally occurs. Under certain experimental conditions, this may be the commonest type of reaction seen. If, instead of extruding the second polar body, the egg divides into two equal blastomeres, this is referred to as "immediate cleavage" (Braden and Austin, 1954a). If cytokinesis does not occur at the completion of second meiosis, either two haploid pronuclei or a single diploid pronucleus develops within about 4-5 hours of activation.
Confusion may arise in certain strains of mice in determining the different types of parthenogenones induced due to the persistence of the first polar body. In some strains, the first polar body disperses or lyses soon after ovulation, whereas in others it may persist for many hours and, thus, may be confused with the second polar body. When the first and second polar bodies are both present, they are usually morphologically quite dissimilar in appearance, the cytoplasm of the second polar body looking the healthier of the two. 

Pronucleus formation usually commences within a few hours of the completion of meiosis II. The incidence of the different types of parthenogenones observed at this time depends on the strain of mouse employed, the postovulatory age of the oocyte at the time of activation, the culture conditions at and during the first few hours following activation, and, to an extent, the type of stimulation employed.

Under normal conditions, the single pronucleus, or two pronuclei that form contain either the exact haploid, or diploid number of chromosomes. Unequal chromosome segregation may occur, for example, as a result of nondisjunction or chromosome lagging. The incidence of these events probably varies from strain to strain, or may result from the experimental conditions at the time of
activation. The oocyte may extrude small cytoplasmic fragments, or one or many subnuclei may be formed in addition to the presence of one or more "pronuclei." Subnuclei usually contain one or several chromosomes, so that when these are present the egg is likely to have an aneuploid karyotype. Most of these anomalous events are probably due to suboptimal conditions at or following activation.

Only in the immediate cleavage and delayed cleavage types of eggs are the two chromosome sets genetically dissimilar as a result of crossing-over events at meiosis I. Here, the two blastomeres give rise to two genetically different clones of cells, and this has accordingly been termed mosaic haploid development (Kaufman and Sachs, 1976). When a single haploid pronucleus forms following second polar body extrusion, a single clone of cells results, and this has been termed uniform haploid development. When a single diploid pronucleus forms, or the contents of two haploid pronuclei amalgamate at the first cleavage metaphase, the resultant embryo will be homozygous at some loci and heterozygous at others. The extent of homozygosity or heterozygosity will be dependent on the chiasma frequency. Uniform diploid parthenogenetic development would only occur in embryos with two haploid or a single diploid pronucleus in the unlikely event that the chiasma frequency was zero. This type of embryo may, however, be induced experimentally when the first cleavage division of uniform haploid embryos is inhibited.

V. EFFECT OF POSTOVULATORY AGING OF THE OOCYTE ON THE ACTIVATION FREQUENCY AND TYPES OF PARTHENOGENONES INDUCED

Detailed observations on the effect of the postovulatory age of the oocyte at the time of activation (usually referred to as hours after spontaneous ovulation, or hours after the HCG injection for superovulation—ovulation occurs about 11–12 hours after HCG) have been presented by Kaufman (1973c). This study demonstrated that the activation rate tended to rise with an increase in the postovulatory age of oocytes. Thus, when oocytes were released from the oviduct at 14 hours after HCG into culture medium containing hyaluronidase and the cumulus-denuded eggs transferred to enzyme-free medium 5–10 minutes later, no eggs were activated. At 16, 18, and 20 hours after HCG, an increasing proportion of oocytes became activated, reaching a maximum of between 75 and 85% at 18–20 hours after HCG (see Fig. 2). The majority of the stimuli which are capable of activating oocytes in vitro show a similar type of response. A similar pattern has also been observed when oocytes are stimulated in situ with Avertin anesthesia (Kaufman, 1975b). However, when eggs are stimulated in situ by an electric shock, or in vitro by heat shock, activation of more recently ovulated eggs (up to and including HCG + 16
2. Experimental Production of Parthenogenetic Embryos

hours) may be achieved, though the incidence of fragmentation, and atresia is often quite high. If eggs are stimulated beyond about 13 hours after ovulation has occurred (beyond HCG + 25 hours), an increased incidence of fragmentation and atresia is to be expected beyond that normally found when eggs within the range HCG + 18–24 are activated. A slight increase in the proportion of apparently healthy pronuclear eggs which fail to progress beyond the first cleavage division may also be observed.

In addition, a different pattern is usually seen in the incidence of the various types of parthenogenones induced when eggs are activated at different times after ovulation. This is probably related to the migration of the second meiotic spindle from the periphery to the center of the egg, which tends to occur with postovulatory aging of the oocyte (see Szollosi, 1971). This central migration probably explains the high incidence of immediate cleavage and the slight but significant increase in the number of eggs observed in which one of two pronuclei form in the absence of second polar body extrusion. Reducing the osmolarity of the culture medium immediately after activation (Graham and Deussen, 1974; Kaufman and Surani, 1974) and activating eggs in medium lacking calcium and/or magnesium ions (Kaufman et al., 1977; Surani and Kaufman, 1977) are other methods of altering the incidence of the various types of parthenogenones induced.
Further observations on the effect of aging on the activation frequency have been reported by Kaufman (1975a), who treated batches of freshly ovulated (HCG + 14.5–15.0 hours) and more aged (C57BL × A2G)F1 eggs (HCG + 21.5–22.0 hours) for 10–15 minutes with 0.15% pronase in phosphate-buffered saline. Half of the eggs had been pretreated with hyaluronidase to remove cumulus cells. In the earlier time group, no activation occurred, whereas in the later time group very high rates of activation were observed (98–99%). A single pronucleus developed following second polar body extrusion in the majority of the activated eggs.

The general observation that oocytes tend to be more readily activated with increased postovulatry aging is probably also related to changes in the metabolism of the egg. The cumulative effect of these aging changes appears to be inversely related to the ease with which eggs may undergo normal monospermic fertilization and is presumably also related to the increased incidence of abnormal fertilization seen with gamete aging (Marston and Chang, 1964; Austin, 1970).

VI. BASIC TECHNIQUES

The basic techniques that have been employed recently for inducing mouse oocytes to develop parthenogenetically may for convenience be divided into two major groups, depending on whether oocytes are (a) isolated from the oviduct and stimulated in vitro, or (b) stimulated in situ within the oviduct of an anesthesitized animal (in vivo techniques).

A. In Vitro Activation

1. Stimulation Induced by Handling Alone When Oocytes Are Released into Standard Embryo Culture Medium

In many strains of mice, the stimuli involved in handling oocytes during their isolation from the oviduct and release into suitable embryo culture medium may be sufficient to induce a high proportion of eggs to initiate parthenogenetic development. The factors involved when oocytes are stimulated by handling alone may include mild mechanical disturbance of the vitelline membrane, or possibly chemical stimulation such as may result from a change in the pH, temperature, or even chemical composition of their microenvironment. The proportion of eggs that may become activated under these circumstances varies considerably and seems largely to depend on the strain involved and on the postovulatory age of the oocyte at the time of activation. This latter factor will not only influence the overall activation frequency, but it will also tend to modify the proportionate incidence of the different types of parthenogenones observed.
A similar type of response is usually observed when activation is induced by handling or following enzyme stimulation, though the activation frequency tends to be higher when oocytes are stimulated in the presence of hyaluronidase. Thus, about 45–65% of oocytes from several strains of mice isolated at about 18–19 hours after HCG became activated in response to handling alone (Kaufman and Surani, 1974; Kaufman et al., 1977; M. H. Kaufman, unpublished), compared to an activation frequency of 70–75% when eggs from similar strains were activated in the presence of hyaluronidase.

2. The Effect of Releasing Oocytes into Medium Lacking Calcium and/or Magnesium Ions

The chemical composition of the culture medium appears to be one of the major factors which can influence not only the activation frequency, but also the incidence of the different types of parthenogenones (Surani and Kaufman, 1977). The type of response obtained when cumulus masses from (C57BL × A2G)F1 hybrid mice isolated at about 18 hours after the HCG injection for superovulation were released into standard embryo culture medium (Whittingham, 1971) lacking Ca\(^{2+}\) and/or Mg\(^{2+}\) is presented in Fig. 3. The eggs were examined about 5 hours after the cumulus masses had been released into the various culture media. To facilitate observation on oocytes at this time, the cumulus masses were incubated for 5–10 minutes in medium containing hyaluronidase to denude the eggs of adherent follicle cells. The activation frequency and the incidence of the various types of parthenogenones could then be assessed.

When eggs were cultured in medium lacking calcium and magnesium, the activation frequency was similar to that observed in the control group (standard medium) and in the medium lacking calcium alone. A considerably higher activation frequency was observed when eggs were cultured in medium lacking magnesium alone, but the explanation for this finding is not immediately apparent. In standard medium and medium lacking Ca\(^{2+}\) and/or Mg\(^{2+}\), the incidence of the various types of parthenogenones obtained varied considerably (see Fig. 3).

In the control group, the majority of the activated eggs developed a single haploid pronucleus following second polar body extrusion, whereas in the Ca\(^{2+}\) and Mg\(^{2+}\)-free medium as well as in the medium lacking Ca\(^{2+}\) alone, only a small proportion of the eggs were of this type. A corresponding increase was observed in the eggs which either failed to extrude a polar body (i.e., those which developed either two haploid or a single diploid pronucleus) or those which underwent immediate cleavage. In the medium in which Ca\(^{2+}\) was present but Mg\(^{2+}\) absent, an intermediate response was obtained.

When (C57BL × CBA)F1 eggs which had been activated in medium lacking Ca\(^{2+}\) and Mg\(^{2+}\) were retained in culture for up to 100 hours in standard medium, a moderate proportion of the haploid and diploid parthenogenones developed to the blastocyst stage. Highest rates of development were observed in the 2-pronuclear type where about 50–60% of the eggs developed to the blastocyst
Fig. 3. (A) Incidence of different types of parthenogenones induced and (B) activation rate when eggs were activated in complete medium, and medium lacking Ca\(^{2+}\) and/or Mg\(^{2+}\). Cumulus masses from (C57BL × A\(_2\)G)F\(_1\) female mice were released into various media at 18 hours after HCG, and observations made 5–6 hours later. Coding for different routes of development following activation same as for Fig. 2. (Data from Surani and Kaufman, 1977.)

stage in vitro (Kaufman et al., 1977; M. H. Kaufman, unpublished). These diploid parthenogenetic blastocysts were transferred to a single uterine horn of day 3 pseudopregnant recipients previously mated to vasectomized males. The recipients were bilaterally ovariectomized immediately after transfer of blastocysts and thereafter maintained on exogenous steroid hormones. After an initial hormone-free period of 2 days, recipients were maintained for 4 days on 1 mg progesterone daily, during which time blastocysts presumably entered into quiescence. Implantation was initiated by injecting 20 ng estradiol along with progesterone for 3 days, and, thereafter, pregnancy was maintained with 8 ng estradiol and 1.6 mg progesterone daily per female.

Implantation commenced about 24 hours after the initial injection of estradiol, which is equivalent to about day 5 of pregnancy, and recipients were killed at intervals between the 6th and 11th day of "pregnancy". About 80% of the transferred blastocysts implanted, 35–40% developed to the egg cylinder stage (see Fig. 4), and 25% to somite embryos. All implantation sites up to day 9 were immediately fixed in Bouin’s solution and examined histologically. After day 8 of "pregnancy," embryos were dissected from the uterus and examined under a dissecting microscope. The two most advanced embryos obtained were at the
Fig. 4. Different stages of early postimplantation development of diploid (2-pronuclear type) parthenogenetic embryos following the transfer of (C57BL × CBA)F₁ blastocysts to the uteri of pseudopregnant recipients. Egg cylinder stages approximately equivalent to days (A) 5, (B) 6, and (C) 7 of gestation. The scale bar represents 0.1 mm and is the same for all three embryos.
Fig. 5. (A) Parthenogenetic mouse embryo with about 25 somites photographed within its amnion, unfixed preparation. (B) Near-sagittal section of embryo in (A) showing large number of well-formed somites (arrowed). Embryo fixed in Bouin's solution about 4 hours after isolation from the uterus. Scale bar represents 0.2 mm. 1 and 2, First and second branchial bars; O, otic vesicle; Ht, heart; Rh, rhombencephalon with fourth ventricle; A, amnion; Y, yolk sac; V, fourth ventricle. (From Kaufman et al., 1977.)
2. Experimental Production of Parthenogenetic Embryos

Fig. 6. Basic technique for obtaining complete development in vitro to the blastocyst stage following activation of eggs in medium lacking Ca\textsuperscript{2+} and Mg\textsuperscript{2+}. Approximate timing of the different stages is indicated.
forelimb-bud stage on the 11th day of "pregnancy"; both had about 25 somites and were apparently healthy with beating hearts and a good yolk sac circulation at the time of isolation from the uterus. These embryos were later fixed for histology (see Fig. 5). Development of presumptive haploid embryos to the advanced egg cylinder stage has also been achieved using a similar experimental approach (Kaufman, 1978). Because of the effectiveness of this activation technique, the various in vitron stages involved are illustrated diagrammatically in Fig. 6.

The activation induced by handling described here is not comparable to, and should not be confused with, spontaneous activation. This phenomenon occurs only infrequently in the mouse but quite commonly in the hamster (Austin, 1956; Yanagimachi and Chang, 1961; Longo, 1974). However, in the hamster spontaneous development has not been observed to progress beyond the 2-cell stage, though studies in which hamster oocytes in situ were stimulated by electric shock treatment demonstrated that parthenogenetic development to the blastocyst stage could be achieved in this species (Kaufman et al., 1975).

3. Hyaluronidase Activation

Graham (1970) demonstrated that a high proportion of mouse oocytes could be activated in vitro if they were incubated in culture medium containing hyaluronidase for a sufficient time to denude the oocytes of adherent cumulus cells. Mouse oocytes were released into simple embryo culture medium kept initially at 5°C, and hyaluronidase crystals were added until a final concentration of about 100 IU/ml of the enzyme was present. After about 5 minutes, the cumulus-free eggs were isolated; washed several times in enzyme-free medium; then cultured at 37°C under 5% CO₂, 5% O₂, 90% N₂ for varying periods of time. A proportion of the activated eggs developed to the morula and blastocyst stage, but, as the parthenogenones had not been segregated into their various types at the pronuclear stage, the developmental potential of the individual types could not be assessed.

The basic technique may be simplified by the prior addition of hyaluronidase to the embryo culture medium to achieve a final concentration of between 100 and 300 IU/ml. Secondly, the period of incubation in hyaluronidase medium may be carried out at 37°C in either the same gas mixture employed by Graham, or under 5% CO₂, 95% air (see Kaufman, 1973c). The concentration of hyaluronidase in the medium and the duration of exposure to this enzyme do not appear to be critical. In various studies (M. H. Kaufman, unpublished observations), exposure for about 10 minutes (range 5–20 minutes) has usually resulted in high rates of activation. Eggs should be washed once or twice in enzyme-free medium before transfer to drops of similar medium under light paraffin oil for long-term culture. When activation is carried out as described above, second polar body extrusion usually occurs within about 2–3 hours, and pronuclear
2. Experimental Production of Parthenogenetic Embryos

formation within about 4–5 hours. Entry of parthenogenones into the first cleavage division occurs at about 12–14 hours after activation. Mitosis in the haploids lasts about 2½ hours compared with about 2 hours in fertilized eggs of the same strain (Kaufman, 1973b). However, it is likely that the exact timing of these events will vary slightly from one strain of mouse to another.

When oocytes from a single source were activated by the two techniques outlined in this section, similar rates of activation were obtained (see Graham and Deussen, 1974).

4. Effect of Culture of Cumulus-Denuded Oocytes in Hypo- and Hypertonic Medium Directly after Hyaluronidase Treatment

The osmolarity of the medium in which eggs are cultured during the first few hours following activation plays an important part in influencing the incidence of the different types of parthenogenones obtained and may also, in some strains, influence the activation frequency. The pattern of activation obtained in one series of experiments when hyaluronidase-treated oocytes were cultured for 2½ hours in media whose measured osmolarity ranged between 0.168 and 0.402 OsM (Kaufman and Surani, 1974) is illustrated in Fig. 7. In this study, hypotonicity was achieved by diluting the culture medium with distilled water (see Graham, 1971, 1972) and hypertonicity by the addition of solid sodium.

Fig. 7. Effect of incubation for 2½ hours in media of different osmolarities directly after hyaluronidase activation on (A) the incidence of the various types of parthenogenones induced, and (B) the activation frequency. Eggs from (C57BL × A2G)F1, female mice were isolated at 20–21 hours after HCG and observations made 6–8 hours later. The total number of eggs examined in each group is given in parentheses, and the cleavage rate at 24 hours in brackets. Coding for different routes of development same as for Fig. 2. (Data from Kaufman and Surani, 1974.)
chloride to the medium. Little difference was observed in the overall activation frequency between the control and the experimental treatment groups. In similar studies by Graham and Deussen (1974), an increase in the activation frequency, compared to control levels, was observed in some strains of mice when eggs were cultured in hypotonic media. It is unclear whether these differences were due to strain variation or differences in experimental technique.

As may be seen from Fig. 7, the most obvious effect of culturing eggs in low osmolar medium (0.168 OsM) was to increase the incidence of 2-pronuclear eggs from about 3% to 56% of the activated population. A moderate increase was also seen in the incidence of eggs undergoing immediate cleavage. An intermediate effect was observed when eggs were cultured in 0.227-OsM medium.

Apart from the slight effect on the incidence of the different types of parthenogenones induced when oocytes were cultured in hypotonic media compared with the control pattern, the main effect appeared to be on development. All of the activated oocytes in the control and low osmolar series developed to the 2-cell stage in culture compared with 83% and 19% of the activated eggs cultured in 0.336- and 0.402-OsM medium, respectively.

Culturing eggs in hypotonic (0.168 OsM) medium does not appear to have a detrimental influence on their pre- or early postimplantation development as 50–56% of the 2-pronuclear type successfully implanted following transfer at the pronuclear stage to the oviducts of suitable recipients (Kaufman and Gardner, 1974). When similar eggs were isolated on day 4, 59% of these diploid parthenogenones appeared to be morphologically normal morulae or blastocysts.

5. The Effect of Heat Shock

Two recent studies have investigated the effect of heat shock on mouse oocytes in vitro. In the first study (Komar, 1973), excised oviducts containing superovulated (HCG + 14.5-17.5 hours), and spontaneously ovulated ova from “A” strain and Swiss Albino females were subjected to a heat shock of 43°-45.5°C lasting for 5–10 minutes. The excised oviducts were placed in a test tube containing 0.5 ml Ringer’s solution, and the tube was immersed in a thermostatically controlled water bath set at various temperatures between 43° and 45.5°C for 5, 7½, or 10 minutes. The oviducts were then transferred to medium and retained in culture for either 5–10 hours to assess the activation rate, or for 4 days to assess their development potential. Highest rates of activation were observed (57.0%) when oviducts were maintained at 44°-44.5°C, although 37.7% of the activated eggs contained subnuclei. Slightly higher rates of activation were observed when eggs were exposed to temperatures ranging from 43°-45.5°C for 5 minutes compared to groups heated for 7½ or 10 minutes. At all temperatures tested, haploid eggs predominated over diploid. After 4 days in organ culture, 15.4% of the heat-treated eggs that were recovered had developed to the morula or blastocyst stage, compared with 57.4% of fertilized eggs cultured under similar condi-
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6. Activation Induced by Ionophore

Steinhardt and Epel (1974) demonstrated that exposure of sea urchin eggs to the divalent ionophore A23187 induced a cortical reaction indistinguishable from that initiated by sperm. This event was associated with elevation of the "fertilization membrane," a respiratory burst, an increase in protein and DNA synthesis, and typical plasma membrane conductance changes. Subsequent studies with sea urchin, *Xenopus,* and hamster oocytes (Steinhardt et al., 1974) demonstrated that the ionophore was capable of inducing activation in all three species.

A 5 mM stock solution of A23187 was prepared in dimethyl sulfoxide and stored in the dark. When the stock solution was diluted for use with oocytes,
continuous stirring was required to ensure good mixing, since the ionophore is insoluble in water and some precipitation tends to occur. Final concentrations of the ionophore are, therefore, maximum estimates.

Hamster eggs were isolated from randomly bred superovulated females at 15-17 hours after HCG. The eggs were freed from cumulus cells by treatment for 15 minutes with 0.1% hyaluronidase (300 IU/ml), then rinsed thoroughly in Ca$^{2+}$- and Mg$^{2+}$-free culture medium in which the albumin was replaced with 0.2% polyvinylpyrrolidone, and the pH adjusted to 7.3. Highest rates of activation were achieved when oocytes were exposed to a 3-$\mu$M concentration of the ionophore in Ca$^{2+}$- and Mg$^{2+}$-free medium throughout the incubation period. Continuous exposure to concentrations above 10 $\mu$M were detrimental to the eggs. Brief exposure to 3- to 10-$\mu$M concentrations of the ionophore (2 minutes), followed by culture in Ca$^{2+}$ and Mg$^{2+}$-free medium was also effective. The majority of eggs showed cortical granule breakdown within 30 minutes, and well-developed pronuclei were formed within 2 hours. However, protein synthesis was not maintained, mitosis was delayed, and cleavage, if it took place, was abnormal (Steinhardt and Epel, 1974; Steinhardt et al., 1974).

To date, no information has been published on the effect of the ionophore on mouse eggs in culture. Pretreatment with hyaluronidase to remove cumulus cells would be less acceptable in this species, where this enzyme is capable of inducing high rates of activation in the absence of the ionophore. However, this would not apply to recently ovulated eggs (HCG + 12-14 hours), which are usually resistant to stimulation by this enzyme.

**B. In Vivo Activation**

Various experimental techniques have been employed which are capable of inducing mouse eggs to develop parthenogenetically *in situ*, within the oviduct of the anesthetized animal. The two activation methods which have so far been most rigorously investigated are (1) electric shock applied to the eggs within the ampullary region of the oviduct of anesthetized animals (Tarkowski *et al.*, 1970), and (2) the use of anesthesia alone, without surgical intervention (Kaufman, 1975b). A third method has also been employed, namely, heat shock to the eggs within the exteriorized oviduct of anesthetized mice (Braden and Austin, 1954a). This approach gave reasonable rates of activation, but little further information is available on this technique beyond the original description. Indirect information is, however, available on its effect on the oocyte from experiments which investigated the effect of heat shock applied to eggs shortly after mating (Braden and Austin, 1954b).

Under certain experimental conditions, the anesthesia necessary to carry out the first and third techniques indicated above may complement the stimuli involved in electric shock or heat shock treatment. Similarly, in electric shock
treatment, local heating of the tissues may play a role in the activation process. These factors complicate interpretation of the findings and assessment of the underlying mechanisms involved.

1. Electric Shock

Tarkowski et al. (1970) were the first to report the successful use of electric shock stimulation to activate mouse eggs in situ. Eggs within the ampullary region of the oviduct were stimulated by a single electric shock produced from an 8-μF capacitor charged through a rectifier from an ac source of 30, 40, or 50 V, with an automatic 'charge-discharge' switch. The oviducts were brought to the exterior through a dorsolateral incision, and the current was passed across the ampullary region using the tips of two steel needles as electrodes. All females were anesthetized with Nembutal (0.01 ml/gm body weight of a 6 mg/ml solution).

Groups of mice from the A, CBA-p, and CBA-T6T6 inbred strains were anesthetized with Nembutal either at 14–16 hours after the HCG injection for superovulation, or between 8:00 AM and 11:00 AM in the case of spontaneously ovulating females. Mice were examined 4–10 hours after electric stimulation to assess the immediate effects of this procedure, and at daily intervals until the 10th day of pregnancy (day of finding vaginal plug = day 1 of pregnancy). In preliminary experiments, operations were performed unilaterally to assess any influence of the anesthetic, but, as no eggs from the control oviducts showed signs of activation, later operations were performed bilaterally. The results of this and a further similar series of experiments have been described in great detail by Witkowska (1973a,b). All of the types of parthenogenones illustrated in Fig. 1 were observed. In addition, in about 8–10% of the activated eggs second polar body extrusion failed to occur, and more than two nuclei were seen (presumed to be subnuclei).

The activation rate in the spontaneously ovulated eggs was about 50%, compared with approximately 40% in the superovulated group, with the majority of the activated eggs developing a single haploid pronucleus following second polar body extrusion. The incidence of degenerated, nonactivated, and activated classes was related to the voltage used, with the highest activation frequency occurring when a 40-V shock was applied. With a stronger current, the number of degenerated eggs increased, while the number of nonactivated eggs decreased. Rates of implantation were relatively high, especially in the spontaneously ovulating A-strain females, where between a quarter and a third of the activated eggs survived until implantation. When results from the three strains of mice and experiments using different voltages are pooled, the implantation rate for the whole series was about 0.7 implantations per horn.

Twelve out of 19 of the implantation sites examined on days 6 and 7 contained egg-cylinder stage embryos, but the mortality increased rapidly when implants
were examined on the 8th through 10th days of pregnancy. On the 9th day, a single healthy egg-cylinder embryo was found out of a total of 81 implants examined, and on the 10th day a single morphologically normal 8-somite embryo was found out of 16 implants examined (data from Table 3, Witkowska, 1973b).

The main advantage of this technique is that, under optimal conditions, moderate rates of postimplantation development, at least to the egg-cylinder stage, may be expected to occur when relatively freshly ovulated eggs are stimulated by an electric shock. Its main limitation is that it does not allow an assessment of the development potential of different types of parthenogenetic embryos to be made, as a mixed population of haploid and diploid embryos are induced.

This technique, with suitable modifications (up to 20 or 25 50-V shocks may be required) was found to be effective in stimulating hamster eggs to develop to the morula and blastocyst stage (Kaufman et al., 1975), though it is likely that local heating of the tissues may have influenced the outcome of these experiments. In a previous study, Gwatkin et al. (1973) demonstrated that the cortical granules in electrically stimulated hamster eggs disappeared rapidly after the stimulus was applied and that the zona reaction was also induced.

Studies on spontaneous and induced parthenogenetic activation suggest that there may be considerable variation in the incidence of cortical granule breakdown under different in vivo and in vitro conditions (spontaneous activation in the hamster, see Austin, 1956; Longo, 1974; cold-shock-induced activation in rabbit eggs, see Flechon and Thibault, 1964; Longo, 1975; hyaluronidase-induced activation in mouse eggs, see Solter et al., 1974), and that it would be inappropriate at this stage to draw general conclusions from the results obtained with one technique or from a single species.

Mintz and Gearhart (1973) demonstrated that parthenogenetic embryos induced by electric stimulation showed a response in the time taken to dissolve the zona pellucida with a dilute pronase solution intermediate between that seen in unfertilized and fertilized eggs. However, the significance of these observations on the development potential of parthenogenones remains to be established.

2. Effect of Anesthesia

Various authors have demonstrated that rodent eggs may be induced to develop parthenogenetically in situ when intact animals are anesthetized after ovulation has occurred. Thus, rat eggs have been activated parthenogenetically with ether (Thibault, 1949; Austin and Braden, 1954), chloroform, ethyl chloride, ethyl alcohol, paraldehyde, nitrous oxide, and intraperitoneal (ip) Nembutal (Austin and Braden, 1954), and mouse eggs with ether (Braden and Austin, 1954a) and ip Avertin (Kaufman, 1975b). Apart from the study with Avertin, where postimplantation development was obtained, previous workers had only reported the occasional development of these eggs as far as the 2- or 4-cell stage. While only the Avertin experiments will be discussed in detail here, it seems
likely that a range of anesthetics given at an appropriate time after ovulation are probably also capable of inducing mouse eggs to develop parthenogenetically beyond implantation.

In a recent study (Kaufman, 1975b), spontaneously ovulating and superovulated (C57BL × A₂G)F₁ hybrid mice were killed about 24 hours after anesthesia with tribromoethanol (Avertin: Winthrop, dose 0.02 ml/gm body weight of a freshly prepared 1.2% solution of Avertin dissolved in 0.9% saline). In both groups of females, an increasing activation frequency was observed when anesthesia was carried out at about 6.5, 9, and 13 hours after ovulation, reaching a maximum of about 46%. No activation was induced when females were anesthetized at about 4 hours after ovulation, 7% were activated at 6.5 hours, and 17% at 9 hours after ovulation. Both the activation rate and the incidence of the various types of parthenogenones induced was related to the postovulatory age of eggs at the time of anesthesia, and a pattern of response was observed similar to that seen when mouse eggs were activated in vitro by handling alone, or following activation with hyaluronidase (Kaufman and Surani, 1974).

In a spontaneously ovulating group of females anesthetized about 13 hours after ovulation, approximately 13% of all the eggs ovulated, or 27% of all the eggs activated, survived until implantation. Preliminary studies had demonstrated that the majority of the eggs activated in this time group were undergoing immediate cleavage, so that it is likely that most of the embryos which implanted were haploid in origin.

3. Effect of Heat Shock

Braden and Austin (1954a) observed that about 50% of spontaneously ovulated mouse eggs had initiated parthenogenetic development when these were examined 4–5 hours after heat shock had been applied to the oviduct in situ. Mice were anesthetized with Nembutal about 8–12 hours after ovulation, a dorsolateral incision made in the body wall, and the ovaries and oviducts brought to the exterior and immersed for 5–10 minutes in a water bath maintained at 44–45°C. Most of the activated eggs developed a single haploid pronucleus following extrusion of the second polar body. A few eggs underwent immediate cleavage, and a single egg developed two pronuclei in the absence of a second polar body. A few 2- and 4-cell eggs were isolated 48–70 hours after heat-shock stimulation.

In a similar study, Braden and Austin (1954b) investigated the effect of hot shock applied to eggs 3 hours after mating. This increased the incidence of polyspermy (dispermy) from 0.3% to 3.8%, and of eggs exhibiting suppression of second polar body formation from 0.5% to 12.4%. This study also confirmed earlier observations of Fischberg and Beatty (1952) that triploidy could result from dispermy or from suppression of second polar body formation (digyny). In Fischberg and Beatty's (1952) series, a single 6-cell haploid egg was isolated which was probably parthenogenetic in origin.
C. Microsurgical Methods for Obtaining Nonparthenogenetic Haploid Embryos

Two promising experimental techniques have recently been described which allow a limited degree of haploid development to be initiated. Both methods require a considerable degree of microsurgical skill and have yet to be fully evaluated. Modlinski (1975) has obtained haploid embryonic development to the blastocyst stage following the microsurgical removal of one pronucleus from fertilized mouse eggs, while Tarkowski and Rossant (1976) have also obtained advanced preimplantation development when fertilized 1-cell eggs were bisected and transferred to the oviducts of suitable recipients following the insertion of these fragments into empty zonae pellucida.

Attempts have also been made (Graham, 1970) to produce chimeras by the aggregation of parthenogenetic and fertilized 8-cell embryos and morulae, and survival to term of definite chimeric individuals by this means has recently been reported by Stevens et al. (1977). When the inner cell masses of diploid parthenogenones were inserted microsurgically into intact fertilized blastocysts that were subsequently transferred to pseudopregnant recipients, the development to term of chimeric embryos with contributions from parthenogenetically-derived and fertilized cells was also successfully achieved (Surani, Barton, and Kaufman, 1977).

D. Parthenogenetically Derived Spontaneous Ovarian Tumors in LT Strain Mice

Another potential source of parthenogenetic cells is the LT strain of mice, which has a high incidence of spontaneous parthenogenetic development of ovarian oocytes to apparently normal egg cylinder or primitive streak stage embryos (Stevens and Varnum, 1974; Stevens, 1975). Subsequent growth of parthenogenones is disorganized, and both teratomas and teratocarcinomas are often found in the ovaries of these mice. Spontaneously activated eggs may also be recovered from the oviduct and uterus in this strain, and were used in the experiments of Stevens et al. (1977) described above.

VII. CONCLUSIONS

With the availability of experimental methods which give high rates of parthenogenetic activation, and, in some cases, development beyond implantation, it seems likely that rapid advances in our understanding of the activation process and the factors which influence early mammalian development can be expected within the next few years. Even the limited advances that have been achieved in
the few months prior to the writing of this chapter have necessitated a radical reevaluation of the importance of certain embryologic and genetic factors which were generally thought to play an essential role in controlling mammalian embryonic development. For example, it is almost certain that we will have to reexamine our ideas as to the precise role of sperm and the importance of X chromosome activity in development (Kaufman et al., 1978).

While a high proportion of mouse parthenogenones undoubtedly die in the early postimplantation period possibly as a result of intrinsic deficiencies within the embryonic genome, the recent demonstration that a few parthenogenetic mouse embryos were capable of surviving at least to the forelimb-bud stage (Kaufman et al., 1977) suggests that improved methods of activation and greater control over the hormonal status of the recipient may allow a higher proportion of embryos to survive beyond implantation, possibly even to term.

While there have been several attempts to obtain parthenogenetic cell lines from the “growths” which develop when parthenogenetic embryos are transferred to ectopic sites (Graham, 1970; Iles et al., 1975), the establishment of a stable haploid mammalian cell line has yet to be achieved. However, the apparently normal morphology of the somite-stage diploid parthenogenetic embryos recently described by Kaufman et al. (1977, and unpublished observations), coupled with the considerable range of cell types identified in the growths described above, and the observations of Van Blerkom and Runner (1976), which suggest that parthenogenetic blastocysts are similar at the ultrastructural level to normal fertilized blastocysts, are all extremely encouraging signs.

Parthenogenetic embryos would be particularly valuable in the analysis of many early developmental processes, as they would allow the complex events which take place at activation, during the pre- and early postimplantation period, and during morphogenesis and organogenesis in haploid and diploid parthenogenones to be compared with similar events in fertilized embryos. The availability in the near future of haploid and diploid mammalian parthenogenetic cell lines will also be of great value for studies in developmental biology, genetics, and carcinogenesis.

It is hoped that the methods described in this chapter for achieving high rates of activation and advanced parthenogenetic development will encourage embryologists, geneticists, and biochemists to avail themselves of this potentially extremely valuable but largely unexploited source of material.

ACKNOWLEDGMENT

The author’s techniques described in this chapter have largely been developed under grants from the Ford Foundation to Professor C. R. Austin.
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2. Experimental Production of Parthenogenetic Embryos


Induction of monozygotic twinning in the mouse

VINCristine sulphate (Oncovin, Lilly) is a drug commonly used in the chemotherapy of acute leukaemias in children, lymphomas and certain other malignant conditions. We describe here experiments investigating the teratogenicity of this drug during the early stages of embryogenesis in the mouse. We report that a low incidence of gross anomalies was observed in fetuses on days 10 and 12 of gestation when pregnant females were treated with a single dose of this agent on the sixth, seventh or eighth day of gestation. However, contrary to expectation, a high proportion of litters from females treated on the seventh and eighth day of gestation contained at least one set of monozygotic twins. In this respect the present study is unique in that an experimental method of inducing a low but significant incidence of identical twinning in a mammal is reported. Furthermore, this work provides direct evidence regarding the comparatively late stage of embryonic development when identical twin formation may be induced in the mouse, as the most advanced stage at which twinning could be induced with vincristine was at the early headfold stage. However, the highest incidence of monozygotic twinning was obtained when pregnant females were treated on the morning of the seventh day of gestation, when embryos would be expected to be at the advanced egg-cylinder stage. It was in this latter group that a single conjoined twin of the janiceps type (cephalothoraco-pagus) was obtained. The possible aetiology of monozygotic and conjoined twin formation is briefly discussed here in the light of these experimental findings.

Female mice, 6–8-week old virgin C57BL (Anglia), were mated with males of the same strain, and isolated on the morning of finding a vaginal plug (designated the first day of pregnancy). Females were injected intraperitoneally with 0.3 mg per kg vincristine sulphate in 0.2 ml normal saline on the morning of the sixth, seventh or eighth day of gestation. Controls were injected with a similar volume of normal saline. Autopsies were carried out on either the tenth or twelfth day of gestation. The urterine contents were examined, and the number and location of resorption sites and normal and abnormal fetuses recorded (Table 1).

In the only anomalous fetuses encountered, the region overlying the fourth ventricle was apparently collapsed, giving this region of the hindbrain an abnormally flattened appearance.

These fetuses were otherwise apparently quite normal and healthy at the time of autopsy. It is not immediately clear whether the neural tissue in the hindbrain region of this comparatively small population of fetuses would have developed into, for example, an encephalocoele which would have been recognisable at a later stage of gestation. It is also unclear whether these fetuses would have survived to term or into the early post-natal period. A higher incidence of fetuses with abnormal heads of this type was observed in the population of fetuses isolated from females treated on the seventh day (6.2%), compared with those isolated from females treated on the sixth (2.6%) and eighth (2.6%) day of gestation. This low incidence of easily recognisable neural tube pathology is of interest in the light of earlier observations of Joneja and Ungh tavonz1, who investigated the effect of an intraperitoneal injection of this agent in mice on the ninth day of pregnancy. In addition to an increase in the incidence of resorptions, these workers observed that out of 77 fetuses examined at term 5.2%, 11.7% and 6.5%, respectively, were either microcephalic, encephalic, or had an encephalocele. This difference may be related to the strain of mice used, or more probably to the later time of administration of this teratogen. Similar central nervous system anomalies have also been reported when hamsters were injected with this agent on the eighth day of gestation7.

Another observation that seemed to be related to the time of exposure of pregnant females to this agent in the present series was the incidence of monozygotic twins. Four sets of twins, one pair of which was conjoined, were observed when females were treated on the seventh day of pregnancy. Of the five females autopsied on the tenth day, three each had one set of twins, and a single pair of conjoined twins (Fig. 1) was isolated from one out of four females autopsied on the twelfth day of gestation. One additional set of twins was obtained from a female treated on the eighth day. At least three of the twins shared a common amniotic cavity. No twins were observed in the fetuses isolated from females treated on the sixth day of gestation. Apart from the conjoined twins all the other twin fetuses seemed to be morphologically normal.

The main interest in the present study lies in the experimental production of monozygotic twinning in the mouse, as the normal incidence in this and most other mammalian species is thought to be extremely low4,5. In fact, polyembryony is extremely rare and only thought to occur regularly in certain species of armadillo6. It is generally thought that monozygotic twins probably result from the fission of a single embryonic axis shortly after the blastocyst stage has been reached, most probably during the early pre-implantation period. It is therefore obvious that the period of embryogenesis that was most susceptible to the twinning stimulus provided by vincristine in the present study was from the advanced egg-cylinder stage to the early headfold stage of development, and it is at this latter stage that mouse embryos have a distinct head process and primitive streak8. Rarely, incomplete separation of these embryonic axes occurs, and results in the formation of conjoined twins. This seems the simplest explanation for the isolated examples of conjoined twins reported in this and in earlier studies in which pregnant hamsters4,9, rabbits10 and rats11 were treated with teratogens, and as spontaneous occurrences in other species.

The remarkably few reports in the literature in which monozygotic and conjoined twins have been recorded as spontaneous events and following treatment of pregnant animals with teratogens seem to indicate that twins of these types were previously encountered only as isolated occurrences, unlike the present study, where they appeared to be a definite response to the vincristine treatment.

The present experimental approach thus allows for the first time detailed examination of the early stages in the development of monozygotic twins, at precise intervals after the twinning stimulus has been given. Although the exact nature of the twinning stimulus evoked by vincristine remains to be established, it should now be possible to investigate monozy-
Table 1  Incidence of resorptions, normal and abnormal fetuses and monozygotic twins in female mice treated with vincristine

<table>
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<tr>
<th>Treatment</th>
<th>Day of injection</th>
<th>Day of autopsy</th>
<th>Total females examined</th>
<th>Implantation sites</th>
<th>Living fetuses</th>
<th>Abnormal fetuses</th>
<th>Abnormal heads</th>
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Female mice were injected with vincristine sulphate or saline (controls) on the sixth, seventh or eighth day of gestation. * An additional two litters contained only resorbed embryos.

gotic twinning in relatively controlled conditions.

We thank J. F. Crane for photographic assistance. The work was supported by a grant from the Ch’ia Tsio Project.

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Received 13 September; accepted 31 October 1978.
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The teratogenic effect of acetaldehyde: implications for the study of the fetal alcohol syndrome

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(Accepted 19 January 1978)

INTRODUCTION

Various authors have suggested that acetaldehyde, the primary metabolite of ethyl alcohol, may be responsible for the toxic, dependency-producing, and teratogenic properties of ethanol (Akabane 1970; Truitt & Walsh, 1971; Rahwan 1974; Mulvihill & Yeager, 1976). Despite the extensive literature on the sympathomimetic properties (Kumar & Sheath, 1962; Walsh, Hollander & Truitt, 1969) and general pharmacology of acetaldehyde (cf. Akabane, 1970), no experimental work has been carried out to study its possible effect on the developing mammalian embryo. In contrast to this paucity of data on the developmental pharmacology of acetaldehyde, many workers have investigated the influence of ethanol on embryonic and fetal development.

Ethanol has been given to various species of laboratory animals by a number of different routes, e.g. by intraperitoneal and intravenous injections, by gavage, in the drinking water or in a liquid diet (Papara-Nicholson & Telford, 1957; Sandor & Amels, 1971; Chernoff, 1977; Tze & Lee, 1975; Kronick, 1976; Rosman & Malone, 1976; Randall, Taylor, Tabakoff & Walker, 1977; Henderson & Shenker, 1977). These procedures have produced developmental and/or growth retardation, an increased incidence of resorptions and perinatal mortality, as well as anomalies of the central nervous system. In addition, cardiovascular, skeletal, digital, and ocular defects have been noted in the offspring of many experimental animals either at term or in the early postnatal period. In only one of the ethanol studies have mid-gestation embryos been examined (Sandor & Amels, 1971). We believe that this last mentioned experimental approach is particularly important, especially when the direct effect of this treatment on the embryo is to be investigated, as early 'teratogenic' effects may be obscured either by compensatory growth during the remainder of gestation, or by embryonic loss prior to term.

In man, a similar association between a high maternal ethanol intake during pregnancy, growth deficiency, and the presence of certain congenital anomalies in the offspring, has recently been recognized. This has now been termed the 'fetal alcohol syndrome' (Jones, Smith, Ulleland & Streissguth, 1973). Some of the features commonly associated with this condition include: intrauterine growth retardation, and ocular, genito-urinary, cardiac, cranio-facial, and joint abnormalities (Hanson, Jones & Smith, 1976; Mulvihill & Yeager, 1976). However, probably the greatest cause for concern relates to the very high incidence of mental retardation and brain dysfunction seen in these infants (Smith, Jones & Hanson, 1976). Many infants with this syndrome have presumably been misdiagnosed, and others diagnosed correctly only after the birth of a more severely affected younger sibling (Barry & O'Nuallain, 1975; Palmer, Ouellette, Warner & Leichtman, 1974). This may be due in part to the absence of specific information on the effects of different doses of ethanol.
consumed during critical developmental periods. It is, therefore, essential that information of this type be obtained to determine at what stage of gestation the fetus is particularly vulnerable to a high maternal ethanol intake, to determine the nature of the teratogenic insult, and the possible aetiological agent or agents involved.

As ethanol is known to be rapidly metabolized in the body, we decided to investigate the possible teratogenic properties of its primary metabolite, acetaldehyde. In order to study the effect of this agent free from the effects of ethanol, pregnant mice were injected intravenously with moderate doses of acetaldehyde during the early post-implantation period when critical morphogenetic events are known to be taking place.

We report that acetaldehyde in the dose range employed in this study had a profound effect on fetal growth and development which was apparent when embryos were examined on day 10 of pregnancy, and that compensatory growth did not appear to take place later in gestation when this agent was no longer being administered. Further, we observed a marked dose-response effect when various parameters of embryonic and fetal growth and development were assessed. Certain anomalies of the central nervous system were seen, the most marked being failure of closure of the neural tube in the cranial region. A few embryos with cardiovascular anomalies were also observed.

In order to achieve a degree of uniformity throughout the present text, conceptuses isolated prior to and including day 11 of pregnancy have been referred to as 'embryos', while those isolated after day 12 have been termed 'fetuses'. The day of finding a vaginal plug has been designated day 1 of pregnancy.

MATERIALS AND METHODS

Animals

Ten to twelve weeks old randomly bred virgin CFLP female mice (Anglia Laboratories) were mated with individually caged, proven fertile (C57BL x CBA)F1 hybrid males. Females were checked each morning for evidence of mating. Plug-positive females were isolated in cages in groups of 2-4 individuals. The experimental animals were housed in temperature (22 ± 2 °C) and light controlled conditions (13 hours light, 11 hours dark – mid-point of the dark phase at 12.30 a.m.). Laboratory feed and water were available ad libitum throughout.

Groups of female mice were caged with males, and most were found to have mated within 7-10 days. Pregnant females were injected intravenously with either 0.1 ml of 0.9% saline/25 g body weight (controls) or a similar volume of saline containing either 2% (v/v) (Group A1.0) or 1% (v/v) (Group A0.5) of acetaldehyde (Fisons, A0080). All injections were made into the tail vein at about 9.00 a.m.

Pregnant females were injected either with saline alone, or saline containing acetaldehyde, in the morning, on days 7, 8 and 9. All females were autopsied on the afternoon of either day 10 or day 19.

The A1.0 dose of acetaldehyde produced brief locomotor ataxia in the majority of the animals. The LD50 for this agent was 5-3% (v/v) of acetaldehyde. The A0.5 dose was calculated to produce blood levels of acetaldehyde slightly above those seen in human chronic alcoholics during intoxication (Majchrowicz & Mendelson, 1970), though obviously in our experiments this elevated circulating level would be present for only a limited period of time.

Pregnant females were killed by cervical dislocation, the uterine horns isolated,
and their contents carefully examined to determine the total number of implantation sites present in each horn and the location of resorption sites. On day 10 individual decidual swellings were placed in physiological saline, and embryos dissected free of their membranes with fine watchmakers’ forceps.

**Examination of embryos and fetuses**

A brief description of the developmental stage of each embryo was recorded – e.g. whether the embryo was at the headfold stage (‘unturned’), or was ‘partially’ or ‘completely turned’ (Theiler, 1972) – and the presence of any gross abnormalities noted. The crown–rump length of the ‘turned’ embryos was measured using a micrometer eyepiece to assess differences in size between controls and experimentally treated embryos, and to determine the degree of shrinkage occurring during preparation of material for scanning electron microscopy. All embryos were washed several times in physiological saline, then allocated to one of 3 groups depending on whether they were to be examined histologically (embryos placed in 50% Bouin’s solution for 24 hours then stored in 70% alcohol), or biochemically to determine their protein content by the Miller (1959) modification of the Lowry technique (embryos were immediately frozen and stored at −20 °C for analysis at a later date). A third group of embryos was fixed, dehydrated, and sputter coated using a modification of the technique described by Eager, Johnson & Thurley (1976) for scanning electron microscopy. The embryos were viewed in a Cambridge S600 scanning electron microscope operated in the secondary mode at 15 kV.

On day 19 the uterine horns were isolated and the presence and location of any resorption sites noted. Fetuses were dissected free of their membranes and the umbilical cord severed close to the anterior abdominal wall. The crown–rump length of all fetuses was determined, and the presence of gross abnormalities recorded. All fetuses were sexed externally, weighed on a Mettler balance, then either transferred to 50% Bouin’s solution for histological examination or stored in 70% alcohol for free-hand razor blade sectioning at a later date.

**Statistical analysis**

Data on maturation parameters and maternal weight gain were analysed with a One way analysis of variance, and data on resorptions by the Chi-squared test. Student’s t-test was used to analyse data on protein content.

Three females had either less than four or more than ten implantation sites in one uterine horn, and were excluded.

**RESULTS**

**Influence of treatment on fertility and maternal weight gain**

A very high proportion of the plug-positive females were found to be pregnant at autopsy. Thus of the control, A0.5 and A1.0 females 7/8, 11/11 and 9/10, respectively, were found to be pregnant at autopsy on day 10; and 5/6, 5/5 and 4/6 on day 19. In the course of the experiment one A0.5 female was found dead in its cage, and was excluded from the analysis. There were no other maternal deaths.

There was no significant difference between the control and experimental groups in the mean initial weights of females on day 1, nor was there any significant difference in the total weight gains between the different groups when pregnant females were examined during the course of their gestation.

When the mean number of implantation sites per female in the control and
Table 1. Incidence of resorptions observed on days 10 and 19 following acetaldehyde and saline injections on days 7, 8 and 9 of gestation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total pregnant females</th>
<th>Total implantation sites</th>
<th>Total embryos</th>
<th>Total resorptions* (%</th>
<th>Total pregnant females</th>
<th>Total implantation sites</th>
<th>Total fetuses</th>
<th>Total resorptions† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control (saline only) (C)</td>
<td>7</td>
<td>71</td>
<td>64</td>
<td>7 (9-8)</td>
<td>5</td>
<td>44</td>
<td>43</td>
<td>1 (2-3)</td>
</tr>
<tr>
<td>2. 1-0% acetaldehyde in saline (A₀-5)</td>
<td>11</td>
<td>104</td>
<td>71</td>
<td>33 (31-7)</td>
<td>5</td>
<td>50</td>
<td>41</td>
<td>9 (18-0)</td>
</tr>
<tr>
<td>3. 2-0% acetaldehyde in saline (A₁-0)</td>
<td>9</td>
<td>95</td>
<td>51</td>
<td>44 (46-3)</td>
<td>4</td>
<td>42</td>
<td>29</td>
<td>13 (31-0)</td>
</tr>
</tbody>
</table>

* C < A₀-5 < A₁-0; χ²C−A₀-5 = 11-35, P < 0-001; χ²C−A₁-0 = 25-25, P < 0-001; χ²A₀-5−A₁-0 = 4-39, P < 0-05 (Chi-squared test).
† C < A₀-5,A₁-0; χ²C−A₀-5 = 4-9, P < 0-05; χ²C−A₁-0 = 9-4, P < 0-01; χ²A₀-5−A₁-0 = 1-29, P < 0-3 (Chi-squared test).
‡ 0-1 ml of 0-9% saline/25 g body weight.

Table 2. Crown–rump lengths of embryos measured on day 10 and fetuses on day 19 following acetaldehyde and saline injections on days 7, 8 and 9 of gestation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total embryos measured</th>
<th>Crown–rump length (mm ± s.e.)*</th>
<th>Total fetuses measured</th>
<th>Crown–rump length (mm ± s.e.)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control (saline only) (C)</td>
<td>50</td>
<td>2-8±0-1</td>
<td>43</td>
<td>23-6±0-3</td>
</tr>
<tr>
<td>2. 1-0% acetaldehyde in saline (A₀-5)</td>
<td>64</td>
<td>2-6±0-04</td>
<td>41</td>
<td>21-2±0-3</td>
</tr>
<tr>
<td>3. 2-0% acetaldehyde in saline (A₁-0)</td>
<td>35</td>
<td>2-2±0-1</td>
<td>29</td>
<td>22-1±0-4</td>
</tr>
</tbody>
</table>

* A₁-0 < A₀-5 < C, F = 23-18, P < 0-001 (One way analysis of variance).
† A₀-5 < A₁-0 < C, F = 16-77, P < 0-001 (One way analysis of variance).

Experimental groups was compared, no significant difference was found on days 10 or 19. However, when the incidence of resorptions in the various groups was compared, a significant difference was observed both on days 10 and 19, with a considerably higher proportion of resorptions in the A₁-0 group than in the controls. The A₀-5 showed an intermediate response, with a significantly higher proportion of resorptions than in the controls, but lower than in the A₁-0 series (Table 1). The decreased number of implants (including resorptions) seen on day 19 as compared with day 10 possibly reflects early post-implantation embryonic losses, as these would probably only be represented on day 19 by small areas of haemosiderin pigmentation located at the original resorption sites.
Table 3. Protein content of embryos determined on day 10 and weight of fetuses on day 19 following acetaldehyde and saline injections on days 7, 8 and 9 of gestation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 10</th>
<th>Day 19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total embryos examined</td>
<td>Protein content of embryos* (μg ± S.E.)</td>
</tr>
<tr>
<td>1. Control (saline only) (C)</td>
<td>37</td>
<td>192±10</td>
</tr>
<tr>
<td>2. 1-0% acetaldehyde in saline (A₀.₅)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3. 2-0% acetaldehyde in saline (A₁₀)</td>
<td>14</td>
<td>135±15</td>
</tr>
</tbody>
</table>

* A₁₀ < C, t = 3-17, P < 0-004 (Student’s t-test).
† A₁₀ < A₀.₅ < C, F = 13-46, P < 0-001 (One way analysis of variance).

Observations on embryonic/fetal development

Observations on day 10

In the control animals 4 out of a total of 64 embryos were either ‘unturned’ (1) or only ‘partially turned’ (3). A similar incidence was observed in the A₀.₅ animals, where 5 out of 71 embryos were of this type (2 ‘unturned’; 3 ‘partially turned’). A significantly higher incidence of developmental retardation was observed in the A₁₀ series where 16 out of 51 embryos were either ‘unturned’ (7) or only ‘partially turned’ (9).

The number of females with at least one developmentally retarded embryo was 3/7, 4/8 and 5/7 for the control, A₀.₅ and A₁₀ animals, respectively. In the control series, one female had one, and two females two, retarded embryos; in the A₀.₅ group three females each had one, and one female two retarded embryos; while in the A₁₀ group, three females had one, five, and seven retarded embryos, respectively, while two additional females each had two retarded embryos.

The lengths of the ‘turned’ embryos in the A₁₀ series were significantly less than the controls, while the A₀.₅ group was intermediate, being significantly shorter than the controls but longer than the A₁₀ embryos (Table 2). Typical control and A₁₀ experimental embryos are shown in Figure 1 to illustrate the degree of developmental retardation produced by this treatment.

A similar trend was seen when the protein content of embryos from the control and experimental groups was assessed, controls having a significantly greater protein content than experimental embryos (Table 3).

Observations on day 19

By day 19 the only effect which was still clearly related to acetaldehyde administration on days 7–9, apart from the incidence of resorptions discussed earlier, was fetal weight, with the A₀.₅ fetuses weighing significantly less than the controls but more than the A₁₀ fetuses (Table 3). This relationship did not hold for crown–rump length, however, as the A₀.₅ fetuses were significantly smaller than the controls and slightly smaller than the A₁₀ fetuses (Table 2).
Incidence of congenital anomalies

The most common morphological abnormalities encountered on day 10 in this study have involved the cranial and caudal extremities of the central nervous system. Most of the anomalies in the cranial region resulted from failure of the anterior neuropore to close at the proper time. In most of the early ‘turned’ embryos in the control series the anterior neuropore appeared to be almost completely closed (Fig. 2A, B). The extent of closure was less marked in a moderate proportion of the experimental series (particularly in the A1.0 group), and in some embryos complete or partial failure of the anterior neuropore to close was observed (Fig. 2C, D). In these embryos a similar degree of retardation of closure in the caudal region of the neural tube (the posterior neuropore) was also commonly seen (Fig. 3A), though this was even more difficult to assess except in extreme cases, as the posterior neuropore normally closes considerably later (by about the 30–34 somite stage, Thieler, 1972). Grossly malformed experimental embryos are illustrated (Figs. 2C, D, 3A) along with similar views of control embryos (Figs. 2A, B, 3B) to show the extent of neural tube closure normally observed at this stage of development. Abnormal embryos were only seen in the experimental series. Three out of a total of 39 ‘turned’ A1.0 embryos were observed in which the mid- and hind-brain regions were abnormally flattened from side to side, giving this part of the head an exaggerated crest-like appearance (Fig. 4A, B). This was only seen in one of 59 control embryos examined at about the same stage of development. None of 69 embryos in the A0.5 group had this appearance.

The only cardiovascular anomalies seen on day 10 were found in the A1.0 group; one embryo had an over-expanded pericardium, and a second showed dextrocardia. Superficial examination of fetuses isolated on day 19 did not reveal any congenital anomalies either in the controls or in the experimental groups.

DISCUSSION

The present results demonstrate that acetaldehyde is both teratogenic and embryo-toxic when administered intravenously to pregnant mice during the early post-implantation period, and that the extent of the effect is dose-dependent. When the offspring of animals injected with acetaldehyde on days 7–9 of gestation were examined on day 10, we observed embryonic growth retardation and developmental delay; compensatory growth was not apparent when fetuses were examined on day 19. A significant increase in embryonic and fetal losses was observed in the acetaldehyde-treated groups as compared to controls both at mid-gestation and at term. Most of the abnormalities observed in this study were of the central nervous system,
Fig. 2. (A, B) Posterolateral views of two early 'turned' control embryos isolated on the afternoon of day 10, to illustrate the normal range of anterior neuropore closure encountered at this stage of development. (C, D) Similar views of two early 'turned' experimental embryos (group) to show partial and complete failure of anterior neuropore closure. Retarded closure of the posterior neuropore was also apparent in these embryos. In the mid-dorsal region of embryo C there is, in addition, an extensive area where the neural tube has failed to close.
Fig. 3. (A) Lateral view of the experimental embryo illustrated in Fig. 2D, to show the extent of the neural tube abnormalities which may be seen in a severely affected embryo. The cranial extremity of the neural tube has almost completely failed to close except for a small bridge of tissue in the region of the mid-brain. The caudal extremity of this embryo is also severely affected, largely due to retardation of closure of the posterior neuropore. (B) Lateral view of control embryo (same embryo as shown in Fig. 2B) to illustrate the normal extent of posterior neuropore closure expected at this stage of development.

and seemed mainly to result from failure of normal closure of the cranial and caudal regions of the neural tube.

The anomalies of the central nervous system reported here are of particular interest as narrowing of the head (Tenbrinck & Buchin, 1975), abnormal EEG (Havilicek & Childaeva, 1976), cortical atrophy (Jones, Smith, Streissguth & Myrianthopoulos, 1974), and mental deficiency (Mulvihill & Yeager, 1976; Smith et al. 1976) have all been seen in human infants diagnosed as suffering from the fetal alcohol syndrome.

In addition to being the first report of the effect of acetaldehyde on mammalian embryonic development, the present findings strongly suggest that acetaldehyde is likely to be the agent responsible for some of the teratogenic effects observed in the fetal alcohol syndrome.

Acetaldehyde appears to reproduce many of the general effects seen when ethanol itself is administered to pregnant laboratory animals, including (a) developmental and/or growth retardation, (b) an increased incidence of resorptions, (c) anomalies of the central nervous system, and (d), more rarely, cardiac anomalies. Similar effects,
Fig. 4. (A, B) Lateral and posterolateral views of a 20-24 somite stage experimental embryo (A1.0 group) showing a wedge-shaped deformity of the mid- and hind-brain region. (C) Posterolateral view of a control embryo at a similar stage of development to show the normal rounded contours of this region.
however, are produced by many teratogens which act on pregnant females during the early post-implantation period. The specific anomalies seen in the present study, while not identical to those observed in the ethanol studies reported elsewhere, involved the central nervous and cardiovascular systems. Specific defects (skeletal and ocular) reported elsewhere may require that treatment be initiated earlier or continued over a longer period. In addition, we cannot exclude the possibility that some of the anomalies reported previously may have been species specific, due to the direct effect of ethanol itself, or resulted from continuous high blood levels of acetaldehyde.

There is no information available on the rate of transfer of acetaldehyde either into or out of the embryonic compartment during the early post-implantation period. Its small molecular weight and lipid solubility strongly suggest a direct effect on embryonic development, though we cannot exclude at the present time a specific maternal or placentotoxic action with secondary effects on the fetus. Observations on placental tissue at different stages of gestation may clarify this issue. Further investigations are in progress to study both the biochemical and anatomical nature, and possible underlying mechanisms, of the acetaldehyde-induced teratogenic effects reported here.

**SUMMARY**

Pregnant mice were injected intravenously with either saline alone (controls) or 1% or 2% acetaldehyde in saline, on days 7, 8 and 9 of gestation. The uterine contents were examined either on day 10 or on day 19.

Acetaldehyde-treated females had more resorptions both at mid-gestation and at term, though no significant difference in maternal weight gain was observed. Experimental embryos examined on day 10 were smaller, and had a higher incidence of developmental delay, as compared to controls. Anomalies of closure of the cranial and caudal regions of the neural tube were the most commonly noted defects. When examined on day 19, acetaldehyde-treated fetuses were significantly smaller and weighed less than controls.

These results suggest a role for acetaldehyde in the production of some of the teratogenic effects of ethanol manifested in the fetal alcohol syndrome.

We wish to thank Dr D. H. M. Woollam for valuable discussion, Mr C. Burton for photographic assistance, and Mr W. Mouel and Mr K. Thurley for their assistance with the scanning electron microscopy. The work was supported by the Ford Foundation, the Peel Medical Research Trust and the Ch’lia Tsio Project (Downing College, Cambridge).

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ON
MAMMALIAN PARTHENOGENETIC DEVELOPMENT

By MH Kaufman
(Cambridge, UK)

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Appearing in Bibliography Reprod., 33:261, Apr. 79.

The term parthenogenesis was first employed by Richard Owen in 1849 to describe 'procreation without the immediate influence of a male'. More recently it has been defined as 'the production of an embryo, with or without eventual development into an adult, from a female gamete in the absence of any contribution from a male gamete' (modified after Beaty, 1957).

This simple definition excludes two closely related phenomena, (a), where the egg is stimulated to complete the second meiotic division and to undergo further development by a spermatozoon, which, however, does not contribute any genetic material to the developing embryo (gynogenesis), and (b), where the egg is stimulated to develop by a spermatozoon, but where the male genome alone takes part in subsequent development (androogenesis). References to recent experimental studies on the production and subsequent development of haploid mammalian embryos following the microsurgical removal of a single pronucleus, and the bisection of one-cell fertilized eggs, have also been excluded on these grounds.

An extensive literature exists, dating back to Pflüger (1863) or possibly even earlier, in which descriptions have been given of parthenogenetic development within the oocytes of most mammalian species, including man. Many of the illustrations presented in these papers show either single-cell eggs with one or more nuclei, early cleavage stages, morulae, and in a few instances apparently normal blastocysts developing in either normal or atretic follicles. It has long been considered that intrafollicular parthenogenetic development of this type within the ovary may in some way give rise to a proportion of ovarian tumours (e.g. teratomas), and recently the parthenogenetic origin of certain human and murine ovarian tumours has been confirmed both biochemically and cytogenetically. While the relevance of this material to gonadal tumour induction is obvious, the purely descriptive accounts in the earlier literature are of more limited value, and have not been included in this bibliography. These accounts require careful evaluation, as in many cases it is almost impossible to determine with certainty whether the examples cited are indeed parthenogenetic in origin or simply represent degenerative changes (e.g. fragmentation) in atretic follicles. However, because of the recent interest in, and experimental use of, the LT strain of mice (in which a high incidence of spontaneous parthenogenetic development occurs within the oocytes of adult females), the relevant references to teratoma formation in LT mice have been included as have recent references to human teratomas in which their parthenogenetic mode of origin has been investigated.

References to spontaneous activation and development of ovulated eggs (particularly in the hamster) have been included in this bibliography because completion of the second meiotic division, and the presence of early cleavage stages within the oviduct, is not an uncommon finding in some species. Indeed, spontaneously activated one-cell eggs, and early cleavage stages from this source have provided useful material for several recent ultrastructural studies. Spontaneous development of ovulated eggs to more advanced pre-implantation stages, and even occasionally to development beyond implantation, so far, however, only appears to occur regularly in LT strain mice.

A high proportion of the literature in the field of experimental parthenogenesis has been primarily concerned with the development of both in vitro and in vivo techniques for achieving high rates of parthenogenetic activation and subsequent development of different classes of haploid and diploid embryos. Because of the considerable difficulty in achieving reasonable rates of development to the blastocyst stage and beyond, relatively few references are available in which the development potential of haploid and diploid parthenogenetic embryos, and chimaeras between parthenogenones and fertilized embryos, have been investigated in detail. For the same reason, very few studies are available on the ultrastructure of these embryos which would allow their morphology to be compared with fertilized embryos at similar stages of development.

Until quite recently no attempts had been made to examine the biochemical and morphological changes associated with the process of activation in parthenogenetically stimulated mammalian ova. Several preliminary reports have recently been published which have attempted to rectify this omission, and studies along these lines should eventually allow a comparison to be made between the initial changes which take place at and shortly after activation in mammalian and non-mammalian species.

It is hoped that this bibliography will serve as an introduction to this rapidly expanding and exciting, but so far relatively unexploited, field of experimental embryology. To facilitate this purpose, review articles and a few more general references to mammalian parthenogenetic development have also been included.


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Relationship Between Steroidogenesis and Oocyte Maturation in Rat Graafian Follicles Cultured \textit{in vitro}

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\textbf{ABSTRACT}

Rat Graafian follicles were dissected from ovaries during the morning of proestrus and incubated in various media. After an 8 h incubation period, ovarian follicles were collected and analyzed, by radioimmunoassay, for progesterone, 17\textalpha-hydroxyprogesterone, androstenedione, estradiol-17\textbeta, and dihydrotestosterone. Following incubation in proestrus (a.m.) serum, preovulatory follicles secreted all these steroids and the output of progesterone, 17\textalpha-hydroxyprogesterone and androstenedione was markedly stimulated following the addition of LH to the media.

Follicle-enclosed oocytes resumed meiosis when incubated in proestrus serum without added LH. However, germinal vesicle breakdown (GVBD) under these conditions was considerably slower than that observed following addition of LH to the medium. "Spontaneous" maturation of oocytes in proestrus serum was associated with increased production of steroids. Oocyte maturation and steroidogenesis both occurred following incubation of ovarian follicles in serum obtained from hypophysectomized rats. Addition of LH to the media markedly accelerated these responses. These results suggest that oocyte maturation and steroidogenesis also occur in cultured ovarian follicles in the absence of gonadotrophic hormones. The relationship between steroidogenesis and/or spontaneous oocyte maturation are discussed.
INTRODUCTION

During the afternoon of proestrus in the rat, high levels of LH are found in the blood (1) and a few hours later several oocytes resume meiosis (2). 8 h after the LH surge, the follicles rupture liberating the oocyte. In order to study the interaction between LH, the follicular cells and the oocyte, several workers have isolated Graafian follicles prior to the LH surge and measured their response to the LH in vitro (3, 4, 5, 6).

Previous work has shown that follicle-enclosed oocytes will only mature in vitro in the presence of LH (3, 4) but that oocytes released from follicles will mature "spontaneously" (7, 8). LH also has a stimulatory effect on follicular steroidogenesis (5, 6, 9, 10) probably through its action on cyclic AMP (11).

The experiments described in this paper were designed to examine the pattern of steroids produced by rat Graafian follicles in vitro and to determine the effect of LH on steroidogenesis and oocyte maturation. The follicles were cultured in proestrus (a.m.) serum in order to mimic as closely as possible their environment prior to the LH surge. In some instances hypophysectomized rat serum was used as a culture medium to eliminate the effect of endogenous gonadotrophins on steroidogenesis and oocyte maturation.

MATERIALS AND METHODS

Culture of Graafian Follicles

Large rat Graafian follicles (>0.8 mm) were isolated during the morning of proestrus and cultured as described previously (12). Briefly, after dissection, the follicles were pooled and 4-6 were transferred to a glass roller bottle containing 1 ml of either proestrus (a.m.) rat serum or hypophysectomized rat serum. The incubation medium was equilibrated with 66% O₂, 34% N₂. The stoppered bottles were rolled continuously on cylindrical rollers at 37°C. After an 8 h incubation, the medium was aspirated and frozen at -20°C for steroid assay at a later date, and the follicles were transferred to a petri dish for oocyte examination. Oocytes were classified into three morphologically distinct groups depending on whether the germinal vesicle was present (GV+) or had broken down (GVBD) or that the germinal vesicle had broken down and a polar body was present (GVBD+PB). Oocytes which had undergone germinal vesicle breakdown were termed mature.

Assay of Steroids

Steroids in the incubation medium were extracted with diethyl ether and fractionated on celite columns (13) and the concentrations
of progesterone (14), 17α-hydroxyprogesterone (15), estradiol-17β (16), androstenedione (17) and dihydrotestosterone (18) were measured by radioimmunoassay.

RESULTS

Follicles Incubated in Proestrus (a.m.) Rat Serum

After an 8 h incubation in proestrus (a.m.) serum, 65% of the follicle-enclosed oocytes had matured in the absence of added LH (Table I). The endogenous levels of LH in proestrus (a.m.) serum of these rats is 47.6 ± 15.0 ng/ml (Readhead and Abrahám, unpublished observations). In cultures with added LH (10 µg oLH), 93% of the oocytes matured, half of the matured oocytes had polar bodies.

At the end of the incubation period, levels of progesterone, 17α-hydroxyprogesterone, estradiol-17β, androstenedione and dihydrotestosterone in the medium had increased (Fig. 1, Schuetz et al., unpublished). The addition of LH to the medium stimulated the follicular output of progesterone, 17α-hydroxyprogesterone and androstenedione, however, it did not stimulate the output of estradiol-17β or dihydrotestosterone.

Follicles Incubated in Hypophysectomized Rat Serum

Follicles incubated in hypophysectomized rat serum show a similar pattern of steroid output as those grown in proestrus serum. The levels of progesterone, 17α-hydroxyprogesterone and androstenedione in the medium increase after the addition of LH (Fig. 2, Schuetz et al., unpublished). The response of estradiol-17β to LH differs in the different media. Follicles cultured in hypophysectomized serum increase their output of estradiol-17β in response to LH whereas those cultured in proestrus serum do not.

After an 8 h incubation, 50% of the follicle-enclosed oocytes mature in response to LH (Table I).

DISCUSSION

Previous work has shown that follicle-enclosed rat oocytes mature in vitro after LH is added to the medium (3). The data presented here confirm this finding and in addition demonstrate for the first time that follicle-enclosed oocytes also mature "spontaneously" in the absence of gonadotrophins. However, germinal vesicle breakdown (GVBD) under these conditions is rather slow (8 h) compared to GVBD in response to LH (2.5 h) (12).
Figure 1. Output of steroids (ng/ml medium) by rat Graafian follicles after an 8 h incubation in proestrus (a.m.) serum, with and without added LH (10 μg/ml).
Figure 2. Output of steroids (ng/ml medium) by rat Graafian follicles after an 8 h incubation in hypophysectomized rat serum, with and without added LH (10 µg/ml).
"Spontaneous" oocyte maturation also occurs in oocytes liberated from their follicles (7, 8). This is believed to be due to the oocytes escaping from an inhibitor of maturation present in the follicle. A polypeptide which will inhibit "spontaneous" oocyte maturation has been found in porcine follicular fluid (19) and in hamster and bovine follicular fluid (20).

The mechanism for the resumption of meiosis by oocytes is not fully understood. In amphibians steroids trigger oocyte maturation (21) and progesterone-induced maturation is under translational control since cyclohexamide inhibits maturation (22, 23, 24). In mammalian oocytes, no direct association between steroidogenesis and oocyte maturation has been established, and various conflicting pieces of evidence regarding the subject have been described. Lieberman et al. (25) showed that inhibitors of steroidogenesis, such as cyanoketone or aminoglutethimide do not block LH-induced maturation in follicle-enclosed rat oocytes in vitro. In contrast to this, pig oocyte maturation in vivo is associated with elevated levels of estrogens in the follicular fluid (26). Rat Graafian follicles have the capacity to produce progesterone, 17α-hydroxyprogesterone, androstenedione, estradiol-17β and dihydrotestosterone in the absence of gonadotrophins, but LH greatly stimulates the production of progesterone, 17α-hydroxyprogesterone and androstenedione. The data presented here shows that oocyte maturation whether "spontaneous" or LH-induced is always associated with increased levels of steroids in the medium. Thus, it is tempting to suggest that steroids play a role in initiating the resumption of meiosis in rat oocytes.

REFERENCES

(4) R. M. Moor. J. Reprod. Fert. 32 (1973) 545.


**TABLE I**

**OOCYTE MATURATION IN FOLLICLES INCUBATED**

**IN PROESTRUS (a.m.) OR HYPOPHYSECTOMIZED RAT SERUM**

<table>
<thead>
<tr>
<th>Incubation Medium</th>
<th>Total No. Oocytes</th>
<th>GV+</th>
<th>GVBD</th>
<th>GVBD + PB</th>
<th>Degenerate or Lost Oocytes</th>
<th>Degenerate % Maturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proestrus Serum</td>
<td>26</td>
<td>8</td>
<td>13</td>
<td>4</td>
<td>1</td>
<td>65</td>
</tr>
<tr>
<td>Proestrus Serum + LH (10 µg/ml)</td>
<td>15</td>
<td>0</td>
<td>7</td>
<td>7</td>
<td>1</td>
<td>93</td>
</tr>
<tr>
<td>Hypophysectomized Serum</td>
<td>34</td>
<td>14</td>
<td>17</td>
<td>0</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Hypophysectomized Serum + LH (10 µg/ml)</td>
<td>35</td>
<td>0</td>
<td>19</td>
<td>15</td>
<td>1</td>
<td>97</td>
</tr>
</tbody>
</table>

(Schuetz, Kaufman, Abraham and Readhead, unpublished).

- **GV+** germinal vesicle present
- **GVBD** germinal vesicle breakdown
- **GVBD + PB** germinal vesicle breakdown and the presence of the 1st polar body
Influence of Copper on the Early Post-Implantation Mouse Embryo: An in vivo and in vitro Study

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Summary. Female mice were injected intravenously with copper sulphate on either the 7th day (early egg cylinder stage of development), the 8th day (late egg cylinder stage), or the 9th day (early somite stage of development), and examined on the 10th day of gestation. Injection on the 7th day was found to be embryo-lethal; when females were injected on the 8th day, the majority of the surviving embryos exhibited anomalies of the neural tube and/or the heart, while injection on the 9th day resulted in a very low incidence of anomalies. The most common malformations seen on the 10th day involved failure of closure of the neural tube in the head region of the embryo, and various types of anomalies of cardiac rotation and shape. When additional females injected on the 8th day were examined on the 12th day, a high proportion of the fetuses examined had developed exencephaly.

A further group of embryos from untreated females were explanted on the 9th day and cultured in vitro in various concentrations of copper sulphate. The lowest levels tested had little obvious effect on neural tube closure. Intermediate doses resulted in retarded and anomalous embryonic development, while the highest levels employed resulted in neural tube and cardiac anomalies similar to those produced in vivo.

The results demonstrate both the direct toxic effect of copper on embryonic development and that the stage of embryonic development at the time of exposure determines both the nature and the extent of the effect.

Key words: Mouse - Copper sulphate - Teratogenicity.

Introduction

Copper salts have been known to be toxic since as early as 1567, when Paracelsus described their deleterious effects in miners and smelters of copper ore (Davenport, 1953). However, only since the recent observation that the addition of
copper to intrauterine contraceptive devices increases their efficiency (Zipper et al., 1969) have the effects of copper on embryonic development been the subject of experimental investigation.

Copper wire was found to prevent blastocyst implantation when it was inserted into the uterine lumen of rats, hamsters, and rabbits prior to implantation (Chang et al., 1970). When the copper wire was inserted after implantation, an increased incidence of resorptions was observed; however, surviving embryos were morphologically normal (Chang and Tatum, 1973).

In vitro studies have shown copper to be blastocyst-lethal at concentrations above 2.5 \times 10^{-5} \text{ M} in the mouse (Brinster and Cross, 1972; Naeslund, 1972) and to be lethal to 15- and 30-somite rat embryos at concentrations higher than 5 \times 10^{-4} \text{ M}, although lower levels had no observable effect on development (Webb and Coppola, 1976).

Unlike the above studies which demonstrated embryo-lethal, but not teratogenic effects of copper, Ferm and Hanlon (1974) have shown that copper salts administered to pregnant hamsters on day 8 of gestation resulted in various types of developmental anomalies in surviving offspring, many of which involved midline closure defects of the neural tube and the anterior thoracic and abdominal walls.

The present study was undertaken to investigate the effect of copper on early post-implantation development in the mouse. In vitro studies were also carried out to investigate the direct effect of this agent on embryonic development during the early somite period.

**Materials and Methods**

Virgin 6- to 8-week-old CFLP (Anglia Labs.) female mice were mated with males of the same strain, and isolated on the morning of finding a vaginal plug (designated the first day of pregnancy). Food and water were available to the animals ad lib., and the colony was maintained under controlled conditions of humidity, temperature, and lighting (dark phase from 7 p.m. to 5 a.m.).

**A. In vitro Study**

On the morning of either the 7th (embryos at the egg cylinder stage of development), 8th (advanced egg cylinder to primitive streak stage), or 9th (early somite stage), or on the evening of the 7th day (designated 7^{1/2} of pregnancy, females were injected intravenously via the tail vein with 0.1 ml of 0.06 M copper sulphate solution containing 0.80 mg copper per ml in sterile saline, or 0.1 ml of saline alone (placebo control). At approximately six min after injection, this dose produced a level of 1.5 \mu g copper per ml serum (control level 0.8 \mu g/ml serum). Determinations were carried out according to the method described by Walshe (1963).

On the 10th day, females were killed by cervical dislocation and the uterine contents examined. The total number of implants was recorded, as was the number of resorption sites. All embryos were dissected free of their membranes into phosphate buffered saline (PBS, pH 7.3). The crown-rump length of all embryos that had adopted the fetal position ('turned') was recorded, as was the presence of any gross defect. Embryos were then randomly allocated into two groups for subsequent protein analysis by the Miller (1959) modification of the Lowry technique, or for examination by scanning electron microscopy (SEM) as described in section D. An additional two females injected with copper sulphate on the 8th day were autopsied on the 12th day and the uterine contents examined as described above.
Copper and Post-Implantation Embryonic Development

B. In vitro Study

On the morning of the 9th day of pregnancy, embryos with an intact yolk sac and ectoplacental cone were explanted into 30-ml roller bottles (New et al., 1973) containing 2 ml of heat-inactivated, immediately-centrifuged rat serum prepared as described by Steele and New (1974) to which 50 μg per ml streptomycin had been added. The medium was gassed for 10–15 min at 12-h intervals throughout the culture period with 5% CO₂ in air.

Copper sulphate solutions prepared in 0.1 ml of distilled water were added to the various serum samples, so that the final concentrations of copper sulphate were: \(5 \times 10^{-6} \) M (0.32 μg copper/ml, the X group), \(2.5 \times 10^{-3} \) M (1.60 μg copper/ml, the 5X group), and \(5 \times 10^{-3} \) M (3.2 μg copper/ml, the 10X group), respectively; 0.1 ml of distilled water was added to the control serum (group C). Three or four 2–4 somite embryos were randomly allocated to each roller bottle.

At the end of the 36-h culture period an assessment of embryonic development was made by using purely morphological criteria, and the presence of any gross anomalies noted. The crown-rump length was measured and ‘turned’ embryos were then separated into two groups either for subsequent protein analysis or for examination by SEM.

C. Combined in vivo and in vitro Study

Other pregnant females were injected with copper sulphate solution on the 8th day, and embryos isolated 24 h later, on the morning of the 9th day. Embryos were explanted into control serum and cultured for 36 h. Analysis of embryonic development was the same as described in section B. The experimental treatments described in sections A, B and C are indicated in Tables 1 and 2.

D. Electron Microscopy

Embryos were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 h, transferred to 0.1 M sodium cacodylate buffer containing 10% sucrose, and stored at 4° C. For SEM, embryos were dehydrated, critical-point dried, then sputter coated using a gold target. The material was then viewed in a Cambridge Stereoscan S600 microscope.

Results

In vitro Studies

Autopsies on the 10th Day. The incidence of resorptions and the presence of anomalies in surviving offspring were closely related to the time of injection of copper sulphate solution (Table 1). When administered on the morning of the 7th day, no viable embryos were observed on the early afternoon of the 10th day, the uterine horns containing only resorption sites. When females were injected on the evening of the 7th day, four contained only resorptions, while the remaining two females contained only ‘unturned’ embryos. Administration on the morning of the 8th day resulted in a four to five fold increase in the incidence of resorptions compared to controls. Surviving litters were either ‘unturned’ or had a high incidence of gross anomalies. Treatment on the morning of the 9th day also resulted in an approximately five-fold increase in the incidence of resorptions. Besides a slight reduction in the crown-rump length of experimental embryos compared to controls, a lower incidence of anomalies was observed in this group (5.1%) compared to animals exposed to copper sulphate on the 8th day (87.1%).
Table 1. Observations on day 10 following injection of copper sulphate on either day 7, 8, or 9 of gestation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Groups</th>
<th>Injection day</th>
<th>Total females</th>
<th>Total implants</th>
<th>Total resorptions</th>
<th>Total dead embryos</th>
<th>Total abnormal</th>
<th>Mean crown-rump length (±SE)a</th>
<th>Mean protein content µg/embryo (±SE)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper sulphate</td>
<td>1</td>
<td>7</td>
<td>7</td>
<td>74</td>
<td>74</td>
<td>2</td>
<td>42</td>
<td>1.9 ± 0.02</td>
<td>112 ± 5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8</td>
<td>12</td>
<td>153</td>
<td>18</td>
<td>1</td>
<td>81/92</td>
<td>2.3 ± 0.02</td>
<td>130 ± 4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9</td>
<td>6</td>
<td>78</td>
<td>11</td>
<td>2</td>
<td>0</td>
<td>2.3 ± 0.02</td>
<td>137 ± 2</td>
</tr>
<tr>
<td>Saline (controls)</td>
<td>4</td>
<td>7</td>
<td>16</td>
<td>212</td>
<td>1</td>
<td>1</td>
<td>1/203</td>
<td>2.4 ± 0.05</td>
<td>135 ± 8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8</td>
<td>13</td>
<td>171</td>
<td>2</td>
<td>1</td>
<td>0/163</td>
<td>2.6 ± 0.01</td>
<td>134 ± 2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9</td>
<td>6</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>0/77</td>
<td>2.7 ± 0.02</td>
<td>137 ± 6</td>
</tr>
<tr>
<td>Controls (uninjected)</td>
<td>7</td>
<td></td>
<td>7</td>
<td>94</td>
<td>2</td>
<td>0</td>
<td>1/91</td>
<td>2.8 ± 0.02</td>
<td>137 ± 2</td>
</tr>
</tbody>
</table>

P < 0.001, group 2 < group 3 < group 4 < group 5 < group 6 < group 7, one-way analysis of variance

P < 0.01, group 2 < groups 3, 4, 5, 6, 7, one-way analysis of variance
The group with the highest incidence of ‘unturned’ embryos was that group injected with copper sulphate on the 8th day. In fact, it was only in this group that ‘unturned’ embryos were found on the 10th day. This represents a cumulative delay in development of between 12 and 24 h compared to the development of control embryos.

Nearly half (20/42) of these ‘unturned’ embryos were also very abnormal in appearance, and anomalies of neural tube development were commonly encountered (16/42). The neural tube defects observed in the ‘turned’ experimental embryos seemed mainly to result from failure of closure of the neural tube in the head region. In some of the more severe cases this appeared to be due to failure of elevation of the cephalic neural folds, whereas in other embryos the neural folds had apparently elevated normally, but had simply failed to meet and fuse in the midline (Figs. 1 and 2).

Many of these abnormal ‘turned’ embryos, in addition to having closure defects involving a major or minor part of the cephalic region, often displayed a considerable degree of asymmetry. Asymmetrical development of the cephalic region was also observed in a proportion of the ‘unturned’ embryos isolated on the 10th day.

Of the 81 embryos in this group, 24 had, in addition to abnormalities of neural tube closure, gross anomalies of the heart. In a further 14 embryos, only abnormalities of the heart were present.

The most commonly encountered cardiac anomaly consisted of gross enlargement of the whole heart. In several of these abnormal hearts a degree of malrotation of the heart tube was apparent. In the most marked cases, the ventricular region appeared to have failed to descend to its normal position, so that the heart tube appeared to be abnormally prominent laterally. In other cases, the ventricular part of the heart tube was abnormally prominent ventrally. These anomalies occasionally were accompanied by an over-expanded pericardium (Fig. 3). Another cardiac anomaly encountered was dextrocardia, and this was usually associated with gross enlargement of the whole heart as described above.

*Autopsies on the 12th Day.* A total of 23 living fetuses and 6 resorptions were observed in the two females injected with copper sulphate on the 8th day. Eleven of the fetuses were morphologically normal, while the remainder had anomalies involving the head region. The neural tube was closed in the head region in three of the less severely affected fetuses, but the region overlying the fourth ventricle appeared to be collapsed, giving this part of the hindbrain an abnormally flattened appearance. The extent of failure of closure of the neural tube in the head region varied considerably in the nine remaining abnormal fetuses. In the majority, the open region extended from the caudal part of the presumptive midbrain to just rostral to the otocysts. A typical fetus is illustrated in Fig. 4.

*In vitro Studies*

The embryos cultures in serum containing the lowest concentration of copper (5 × 10\(^{-6}\) M) showed no evidence of retarded development after 36 h in vitro,
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Fig. 2A–C. Frontal views of control and experimental embryos isolated on the 10th day.
A Control embryo showing normal appearance of the head region
B Embryo treated with copper sulphate in vivo on the 8th day, showing failure of closure of the neural folds in the upper fore- and midbrain regions. Note also the asymmetrical development of the exposed neural tissue.
C Embryo incubated in $5 \times 10^{-5}$ M copper sulphate showing failure of elevation of the neural folds. The exposed neural tissue (arrow) is in the presumptive thalamic region. In this embryo the open neural tube extended caudally beyond the level of the otic pits. Scale bar: 100 µm

Fig. 1A–D. Side views of control and experimental embryos isolated on the 10th day. (A) Control embryo of approximately 20 somites, showing normal appearance of the head. The region overlying the fourth ventricle is closed. (B) Experimental embryo at a comparable stage of development to the control embryo (A) isolated from female injected on the 8th day with copper sulphate. The most striking feature of this embryo is the failure of elevation of the cephalic neural folds. The neural tube is open from the presumptive forebrain region to a point just rostral to the midline bleb (region between two arrows). (C) Slightly less advanced embryo in which the neural tube is open in the mid- and hindbrain regions. The neural folds, although elevated, are somewhat everted. Note the presence of midline bleb (arrowed) which marks the most caudal extent of the open neural tube. Embryo exposed to copper sulphate in vivo on the 8th day. (D) Embryo at developmental stage similar to that illustrated in C. This embryo was incubated in medium containing $2.5 \times 10^{-5}$ M copper sulphate. The neural tube is open in the regions indicated. In the mid- and upper hindbrain regions the neural folds are minimally elevated and everted. The heart region also appears to be abnormally large. Scale bar: 100 µm
Fig. 3A-D. Frontal views of control and experimental embryos showing heart and neural tube anomalies. A Control embryo at approximately 10–12 somite stage, showing normal heart and cephalic neural fold development. B Embryo at same developmental stage as A, previously exposed in vivo to copper sulphate on the 8th day. Note gross enlargement and abnormal shape of the heart, and asymmetry of the cephalic neural folds. C Control embryo at approximately 20–25 somite stage showing normal appearance of the head and heart regions. D Experimental embryo at approximately the same developmental stage as C, which was incubated in medium containing $2.5 \times 10^{-5}$ M copper sulphate. Note particularly the malrotation of the heart, and failure of fusion of the neural tube in the head region. Scale bar = 0.5 mm.
compared to control embryos. On gross inspection at the end of the culture period all the embryos in this experimental group appeared to be completely normal.

Embryos incubated in $2.5 \times 10^{-5} \text{ M}$ concentration of copper sulphate (the 5X group) had a significantly higher incidence of gross anomalies compared to the controls. These anomalies usually resulted from failure of closure of the neural tube in the cephalic region, although cardiac defects were occasionally observed in embryos in this group.

When embryos were cultured in serum containing the highest concentration of copper sulphate tested ($5 \times 10^{-5} \text{ M}$, the 10X group), failure of elevation of the cephalic neural folds was the most common anomaly observed. Severe malformations of the head region in these embryos resulted. Despite the presence of these malformations, almost all the embryos in this group appeared to be viable at the end of the culture period, with a beating heart and functioning yolk sac circulation. However, their overall appearance suggested that they probably would not have survived an additional 12-h culture period.

The protein content of embryos in the '10X' copper-treated group was significantly less than the control level. These embryos were also slightly smaller than the controls as gauged by their crown-rump lengths, although this difference was not significant. Sixteen embryos cultured in serum containing $5 \times 10^{-5} \text{ M}$ magnesium sulphate (as an additional control for any possible deleterious effect of the sulphate ion) were morphologically normal when examined after 36 h in vitro. Details of the protein content and crown-rump lengths of control and copper-treated experimental embryos are presented in Table 2.
Table 2. Influence of various concentrations of copper sulphate on embryonic development in vitro. Early somite embryos isolated on the morning of the 9th day and maintained in culture for 36 h

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number embryos explanted</th>
<th>Heart-beat present</th>
<th>Yolk sac circulation present</th>
<th>Forelimb bud present</th>
<th>Crown-rump lengths (±SE) mm</th>
<th>Protein content (μg/embryo (±SE))</th>
<th>Total delayed embryos</th>
<th>Neural tube anomalies</th>
<th>Heart anomalies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper sulphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^{-6}$ M (X)</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>17</td>
<td>$2.6 \pm 0.04^a$</td>
<td>$126 \pm 7$</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$2.5 \times 10^{-5}$ (5X)</td>
<td>21</td>
<td>19</td>
<td>19</td>
<td>17</td>
<td>$2.3 \pm 0.04$</td>
<td>$129 \pm 5$</td>
<td>5</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>$5 \times 10^{-5}$ (10X)</td>
<td>19</td>
<td>16</td>
<td>14</td>
<td>14</td>
<td>$2.2 \pm 0.04^b$</td>
<td>$84 \pm 5^a$</td>
<td>3</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Controls (C)</td>
<td>25</td>
<td>24</td>
<td>22</td>
<td>18</td>
<td>$2.4 \pm 0.06$</td>
<td>$133 \pm 5$</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Combined group$^d$ (CG)</td>
<td>11</td>
<td>11</td>
<td>10</td>
<td>9</td>
<td>$2.4 \pm 0.09$</td>
<td>$-$</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a P < 0.05$, $X > 5X$, 10X, C, CG, one-way analysis of variance

$^b P < 0.01$, 10X<X, 5X, C, CG, one-way analysis of variance

$^c P < 0.001$, 10X<X, 5X, C, one-way analysis of variance

$^d$ Embryos isolated on day 9 from females injected with copper sulphate on day 8, and incubated in copper-free medium for 36 h.
Combined in vivo and in vitro Groups

When pregnant females were injected with 0.1 ml of 0.06 M copper sulphate solution on the 8th day, and the embryos isolated 24 h later and explanted into roller tubes containing serum alone, relatively normal, although slightly retarded, embryonic development was observed after 36 h in vitro. No obvious developmental retardation was observed at the time of explantation of the embryos into the roller bottles.

Discussion

This study demonstrates that during the early post-implantation period in the mouse, copper may be either teratogenic or embryolethal, and that the stage of embryonic development at the time of injection determines both the nature and the extent of the effect. Thus, the most sensitive period for the production of anomalies of the neural tube and heart is a fairly circumscribed one, occurring somewhere between the morning of the 8th and 9th days.

When embryos were isolated early on the 9th day and incubated in medium containing various levels of copper sulphate, a clear dose-response effect both on embryonic development and on the incidence of anomalies was observed. The fact that early somite embryos are sensitive to copper sulphate in vitro, but apparently insensitive to it in vivo, is at first rather difficult to understand. Two hypotheses may be advanced to explain this apparent paradox: (1) That the high ‘teratogenic’ concentration of copper in the culture medium is greater than the concentration of copper present in the embryonic environment following an injection of copper sulphate on the 9th day. (2) That in vivo, copper gradually accumulates in the embryonic compartment, and that only after an ‘effective’ level of copper has been reached can lethal or teratogenic effects be observed. Support for the latter hypothesis might be that neural tube closure occurred in embryos previously exposed to copper sulphate in vivo on the 8th day which were subsequently explanted into culture medium on the 9th day.

The neural tube defects observed were of several types, and may have resulted from abnormal proliferation of mesenchyme or neuroepithelial cells, defective intracellular matrix production, or interference with the normal cell shape changes which occur within the neuroepithelium during neural tube closure.

Interference with normal cell shape changes may also be important in the production of the cardiac defects observed in the present study, as Manasek et al. (1972a and b) have described changes in cell alignment and apical surface area thought to be important in cardiac looping and rotation.

The response of the mammalian embryo to copper seems to be quite variable and may be species specific, and may be determined, at least in part, by the mode of administration of the copper. Although similar defects to those reported in the present study have been observed in the hamster (Ferm and Hanlon, 1972), Webb and Coppola (1976) have reported an absence of embryonic anomalies when developmentally more advanced rat embryos were cultured under similar experimental conditions. To date, no conclusive evidence of copper-
induced teratogenicity has been reported in man, even in infants who have been born to mothers bearing copper IUDs throughout pregnancy (see Guillebaud, 1976). (For a recent review of the role of copper in reproduction, see Oster and Salgo, 1977.) Ultrastructural analyses and copper binding studies are being carried out to investigate the mechanism of production of these anomalies in the mouse.

Acknowledgements. We wish to thank Dr. D.L. Cockcroft for valuable discussion, and Mr. W. Mouel for his assistance with the scanning electron microscopy. Serum copper analysis was kindly performed by Dr. J.M. Walshe. This work was supported by the Chhia Tsio Project, the Peel Medical Research Trust, and the H.E. Durham Fund.

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Received December 16, 1978; Accepted in revised form March 26, 1979
Cephalic Neurulation and Optic Vesicle Formation in the Early Mouse Embryo

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ABSTRACT The overall pattern of cephalic neurulation and the concomitant early development of the optic vesicles in mouse embryos were examined by scanning electron microscopy. Paraffin-sectioned specimens were also examined.

The overall pattern of closure of the cephalic neural folds accords well with earlier observations of this process. The earliest indication of optic placode formation was seen in histological sections of embryos at the 4-somite stage, while optic pit formation was first observed at the 5- to 6-somite stage. The upper halves of the optic vesicles were formed in 10- to 15-somite embryos by the fusion of the neural folds at the junction between the mesencephalon and prosencephalon, while closure of the lower halves was associated with the closure of the rostral neuropore, and was usually completed by about the 20-somite stage.

By the 25- to 30-somite stage, a rapid increase in the volume of the forebrain was observed, so that the optic vesicles were displaced laterally. An overall increase in the volume of the optic vesicles and decrease in the diameter of the optic stalks were also observed at this time. This account of cephalic neurulation and optic organogenesis provides useful baseline data relevant to the study of the normal early development of the mouse. A comparison is made between similar events in the rat, the hamster, and the human embryo.

It has been known since 1817, following the studies of Pander (1817) on the chick embryo, that the optic vesicles are outgrowths from the neural tube. Since that time a great deal of work has been done on the development of the eye, particularly the mammalian eye. These studies have mostly considered its development following optic vesicle formation. Indeed, very little work has been carried out on the earlier stages of optic organogenesis. To date, much of the information available on the development of the mammalian eye up to the formation of the optic vesicles has been derived from studies of serial sections of early human embryos (for a review of this material, see O'Rahtilly, '66).

The aim of the present article is to discuss optic vesicle formation in the mouse, and its temporal interrelationship with cephalic neurulation. Attention will also be drawn to the major differences between the overall pattern of cephalic neural tube closure observed in the mouse and that seen in the human embryo, and the consequent differences between optic vesicle formation in these two species.

Detailed histological studies of the closure of the neural tube have been well documented for various mammals, but the overall pattern of closure, which differs markedly between species, has received much less attention. A detailed histological study of neural tube closure in the cephalic region of the mouse embryo has recently been presented by Geelen and Langman ('77), and the description given here of the general pattern of closure agrees well with their findings and the brief description of these events presented by Waterman ('76). Observations of this process in hamster embryos (Marin-Padilla, '70; Keyzer, '72; Shenefelt, '72; Waterman, '72, '74, '75, '76) and rat embryos (Adelmann, '25; Bartelmez, '62; Christie, '64; Edwards, '68; Keibel, '37) indicate that the same general pattern of approximately simultaneous closure at various points in the neural tube occurs in both species.
sites is followed in all three species. This is in marked contrast to the pattern of closure of the cephalic part of the neural tube in human embryos (Streeter, '42; O'Rahilly, '66) which is similar to the pattern of closure observed in the macaque embryo (Heuser and Streeter, '29) where fusion of the neural folds proceeds rostrally as a continuous process from the cephalic region of the embryo to the rostral or anterior neuropore.

Further observations on optic vesicle formation in the human embryo may be found in Barber ('55), Duke-Elder and Cook ('63), and Mann ('64). Essentially, these various studies have shown that in the human embryo the fusion process proceeds rostrally from the cephalic region to the region overlying the upper part of the forebrain. This stage is achieved by the 14- to 16-somite embryo, and the rostral neuropore, which is located at the level of the optic pits, closes by about the 20-somite stage, or shortly thereafter.

In order to understand how the optic vesicles develop from the optic primordia in the mouse, and the overall pattern of closure of the cephalic part of the neural tube, it is essential to appreciate the 3-dimensional morphological changes which take place in the external form of this region of the embryo during the period when the embryo progresses from the early somite to about the 20 to 25-somite stage. To facilitate the present investigation the surface topography of mouse embryos was studied by scanning electron microscopy. Representative scanning electron micrographs of the cephalic region are presented here, as these provide an overall picture of the gross changes which take place during the period studied. These micrographs are complemented by representative histological sections through the head region of embryos at similar developmental stages. The present study serves as a detailed introduction to an earlier report by Pei and Rhodin ('70) in which the development of the mouse eye from the optic vesicle stage to term was described, and complements the less detailed account of eye formation in the mouse by Rugh ('68) and Theiler ('72).

MATERIALS AND METHODS

Throughout the present study 6- to 8-week-old randomly-bred CFLP (Anglia Lab.) female mice were used. These were mated overnight with fertile CFLP males and examined the next morning. Those with a vaginal plug were isolated and caged together in small groups. The day of finding a vaginal plug was considered the first day of pregnancy. Pregnant females were killed at frequent intervals between the morning of the ninth and the afternoon of the tenth day of gestation, when embryos would be expected to be developing from the pre- or early somite stage to about the 25-somite stage. The precise timing of fertilization was not important in this study since the primary concern was to examine in detail representative samples of embryos that demonstrated sequential changes in cephalic neural fold closure.

Pregnant females were killed by cervical dislocation, the uterine horns dissected out, and individual decidua isolated into phosphate-buffered saline (pH 7.2). Embryos were dissected out of the decidual swellings with fine watchmaker's forceps under the 6 × 12 × magnification of a Wild M5 dissecting microscope.

Representative embryos were selected, and those that were to be examined histologically were transferred, usually within an intact amnion, to half-strength Bouin's solution and kept in this for 12 to 24 hours. Embryos were then transferred to 70% ethanol, in which they were stored prior to dehydration, embedding and sectioning. In order to facilitate orientation of the smaller embryos in the paraffin wax, embryos were pre-stained with eosin. Sectioning was carried out at a nominal thickness of 6 μm, and the sections were subsequently stained with haematoxylin and eosin. Light micrographs were taken with a Zeiss photomicroscope.

A second group of embryos was fixed, dehydrated, and sputter-coated using a modification of the technique described by Eager et al. ('76) for scanning electron microscopy. The fixative solution contained 1% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2; osmolarity 600-700 mOsmol). After approximately two hours at 4°C, embryos were transferred to 0.1% sodium cacodylate buffer (pH 7.2) to which sucrose had been added to adjust the osmolarity to approximately 500 mOsmol. After approximately 24 hours, embryos were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for one hour, washed rapidly in distilled water, then transferred through a graded acetone series into 100% acetone. Embryos were then
dried via CO₂ in a Polaron E3000 critical-point drying apparatus. The embryos were attached by double-sided tape to an aluminum stub and coated to a thickness of 40-50 nm in a Polaron Sputter Coater (E 5000) using a gold target. The embryos were subsequently viewed in a Cambridge S600 scanning electron microscope operated in the secondary mode at 15 kv.

The various terms used to describe the curvature of embryos, namely whether they were “untuned,” “partially turned,” “early turned,” or “completely turned,” are diagrammatically illustrated in figure 1 (for a description of this process, see Snell and Stevens, '66). A general guide to the incidence of these developmental stages in CFLP × CFLP matings during the ninth and tenth days of gestation is presented in table 1.

The terms used to describe the various regions of the developing brain prior to its closure to form the cephalic part of the neural tube have been chosen with care and have followed common embryological usage. The regions whose fate is not clearly determined at a particular stage have been left unnamed. The main advantage of this conservative approach is that it does not need to presuppose knowledge of the developmental origins of different regions of the brain where this is, in fact, not available.

RESULTS

a. The pattern of closure of the neural tube in the cephalic region of the mouse embryo

In pre- and early somite embryos a symmetrical pair of elevations were present in the cephalic region of the neural plate (see Tamarin and Boyde, '76). These were the neural folds, and were separated by the neural groove. Initially there was no clear line of demarcation between the neuroepithelium and the presumptive surface ectoderm. However, the neuroepithelial cells were principally columnar, while the surface cells were generally squamous in character. By the 2- to 3-somite stage a marked forward growth and considerable degree of lateral expansion was evident in these neural folds (figs. 2, 3), and resulted in the formation of a short headfold region into which the foregut extended (figs. 4, 5).

One feature of note at the 5- to 6-somite stage was the sharp ventral bend which developed at the rostral end of the neural plate, and resulted in its most anterior part being directed almost at right angles to the more cau-
dal part (fig. 6). It was in this rostral region of the prosencephalon that the optic primordia developed (figs. 5-7).

By about the 7-somite stage the neural folds in the cervical region of the embryo came into direct contact with each other at about the level of the fourth and fifth somites and fused to form the neural tube in this region (fig. 8). By about the 9- to 10-somite stage, about the time that the process of "turning" was being initiated in many embryos, the fusion process proceeded in a cephalic direction until it reached a level just caudal to the otic placodes. Further closure in the direction of the hindbrain was a rather gradual process and only reached a level slightly rostral to the otic pits by the time the embryo was at about the 15- to 20-somite stage (figs. 9, 10). Most embryos by this time had completed the process of "turning." By about the 25-somite stage the neural tube in the cephalic region had either completely closed or was in the final stages of closure (fig. 11).

During the 7- to approximately 12-somite or even more advanced stages, the neural folds in the rostral part of the cephalic region came progressively closer and eventually fused at a point near to the sharp ventral bend initially observed at the 5- to 6-somite stage (figs. 12-14). In the more advanced embryo this approximately corresponded to the most caudal part of the prosencephalon close to its junction with the mesencephalon.

In a few 10- to 15-somite embryos it was possible to observe a second quite limited site of fusion which was located at the most ventral aspect of theforebrain in close proximity to the buccopharynx (fig. 12). From about the 15-somite stage the primary site of ventral fusion extended quite rapidly rostrally and caudally (fig. 15) so that by about the 20-somite stage only a small part overlying the forebrain remained unfused. This region lying between the primary and secondary sites of fusion, overlying the presumptive forebrain, was the final part to close on the ventral aspect of the head (figs. 16, 17).

The corresponding caudal extent of fusion from the primary ventral site then proceeded as far as the rostral limit of the rhombencephalon. Thus, the last part of the neural tube to close overlay the region of the fourth ventricle (fig. 10).

b. Observations on the development of the optic vesicles

The earliest indication of optic placode formation was seen in histological sections of the most anterior and ventrally facing part of the cephalic neural plate of embryos at about the 4-somite stage. Initially, only a rather diffuse area of thickening of the neural ectoderm was seen in this region. However, by about the 5- to 6-somite stage, an indentation appeared in the central part of the optic placode (fig. 18); these were the optic pits (or foveae). In a few embryos the two optic pits appeared to be loosely linked together in the midline by posteromedial extensions of the optic sulci (fig. 7). These grooves, which appeared to unite the optic pits in the midline, are normally observed in hamster embryos at this stage of development (Waterman, '72), and are, but to a much more marked degree, also present in human embryos. This has led various authors to associate this region with the ultimate location of the optic chiasma (for example, see Streeter, '42; O'Rahilly, '66).

It should be noted that, unlike the distinct boundary zone already present separating the

<table>
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<tr>
<th>Day</th>
<th>Period</th>
<th>Approximate somite number</th>
<th>&quot;Unturned&quot;</th>
<th>&quot;Partially turned&quot;</th>
<th>&quot;Early turned&quot;</th>
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<tr>
<td>9</td>
<td>9 A.M.-midday</td>
<td>Pre-somite to 3</td>
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<td>10</td>
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<td>4 A.M.-9 A.M.</td>
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<td>9 A.M.-midday</td>
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1 Day of finding vaginal plug = day 1 of pregnancy. CFLP × CFLP matings.
surface and neural ectoderm (fig. 6), there appeared to be no corresponding line of demarcation between the presumptive optic neural ectoderm and the rest of the prosencephalon during this stage of development. With the gradual approximation and fusion of the neural folds in the most caudal part of the prosencephalon, the first stage of the development of the optic vesicles was completed. Subsequent closure over the more rostral part of the prosencephalon resulted in the formation of the upper halves of the optic vesicles. Development of the lower halves was slightly different, and was associated with the closure of the rostral neuropore, as discussed in the previous section.

While the overall pattern of neural tube closure in the prosencephalon is best understood by reference to the relevant scanning electron micrographs, it is necessary to complement these with representative histological sections through this region in order to fully understand the final stages of optic vesicle formation.

The first histological evidence of optic pit formation was observed in embryos at about the 5- to 6-somite stage (fig. 18). A considerable degree of anteroposterior flattening of the cephalic region was also observed at this time. By about the 8- to 10-somite stage, the general shape of this region became considerably modified so that the cephalic region now appeared to be flattened from side to side (figs. 19-23).

Between the 5- and 10-somite stages a considerable change in the shape of individual neuroectodermal cells was also apparent, from a relatively low to a high columnar form. By about the 10-somite stage the optic pits had considerably deepened and were directed both laterally and to a lesser extent posteriory. The first evidence of bulges on the surface of the region overlying the optic pits was also apparent at this stage (figs. 12, 20, 22).

With the formation of the ventral bridge at the junction between the mesencephalon and the prosencephalon, the caudal parts of the optic vesicles were produced (fig. 15). A gradual rostral extension of this fusion process resulted in closure of the neural tube overlying the point of maximum depth of the optic pits (figs. 24-26). Subsequent closure in the region of the rostral neuropore completed the process with the formation of the definitive optic vesicles (figs. 27-30).

By about the 10- to 20-somite stage the optic pits became directed both posterolaterally and superiorly. This change in direction was most apparent by about the 15- to 20-somite stage (figs. 27-30, 31-34). At about this time, when the rostral neuropore finally closed, the neural canal in the forebrain region was almost completely flattened from side to side (figs. 27-30). However, by about the 25- to 30-somite stage a rapid increase in the volume of the forebrain became apparent, so that the optic vesicles were eventually located a considerable distance from the ventral midline.

The overall volume of the optic vesicles also increased, and a corresponding reduction was observed in the diameter of the optic stalks connecting them to the forebrain (figs. 32, 36). The distance between the neural ectoderm of the optic vesicles and the surface ectoderm gradually decreased until there was no intermediate layer of mesenchymal tissue (figs. 35, 37). The lateral wall of the optic vesicles and the surface ectoderm overlying it subsequently becomes markedly thicker, to form the retinal discs and the lens placodes, respectively (see Pei and Rhodin, '70; Wrenn and Wessells, '69).

**DISCUSSION**

In this paper the overall pattern of optic vesicle formation in the mouse has been described in detail, as has its temporal interrelationship with cephalic neurulation. A major feature of interest is the close similarity between optic primordium formation in the mouse compared to the rat (Bartelmez, '62) and hamster (Waterman, '72, '74, '75, '76). Bartelmez ('62), for example, has reported that optic placode formation and the first appearance of the optic pits occurs between the 4- and 6-somite stages in the rat.

The present study has also clearly demonstrated that in the mouse the rostral neuropore is located below the lower halves of the developing optic vesicles. As indicated earlier, the overall pattern of cephalic neurulation and optic vesicle formation in the mouse is dissimilar to that seen in the human embryo, primarily because of the different pattern of closure of the neural tube overlying the forebrain region. It therefore seems likely that a different pattern of congenital anomalies might be expected to result from primary non-closure of the neural tube in the cephalic region in mouse and human embryos.
While the present study has attempted to demonstrate the normal sequence of events associated with cephalic neurulation, it is of interest that in some instances experimentally induced anomalies of neural tube closure may serve to accentuate the normal pattern of events. For example, when pregnant female mice were exposed to acetaldehyde during the early post-implantation period, primary non-closure of the neural tube was induced in some embryos. An example of an embryo in which the teratogenic insult occurred after the neural folds had fused in the region overlying the junction between the forebrain and midbrain is illustrated in O'Shea and Kaufman ('79: fig. 3A). In this embryo the neural folds rostral and caudal to the fusion site are everted.

The actual mechanism of fusion of the neural folds in the different regions of the embryo has been studied in numerous mammalian and non-mammalian species (for example, see Di Virgilio et al., '67; Marin-Padilla, '70; Schroeder, '70; Karfunkel, '74; Waterman, '76; Geelen and Langman, '77), and the interested reader should refer to these studies for further details. All the above evidence seems to suggest that both optic vesicle formation and neurulation are essentially similar processes involving complex morphogenetic cell movements, shape changes, and cellular interactions. In the present study an attempt has been made to describe and analyze the normal pattern of events in optic organogenesis and its relation to cephalic neurulation in the mouse. It is hoped that the information presented here may provide a source of useful baseline data relevant to the study of the early development of this species. The similarities and differences between the development of the mouse and that of the rat, the hamster, and particularly the human embryo have also been indicated.

ACKNOWLEDGMENTS

The author thanks the following for their technical assistance, Mrs. S. C. Barton (histology), Mr. C. F. Burton (photography), Mr. W. Mouel and Mr. K. Thurley (electron microscopy). The work was supported by grants from the Peel Medical Research Trust, and the C'h'ia Tsio Project (Downing College, Cambridge).

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Figs. 2-17 are scanning electron micrographs which illustrate the process of cephalic neurulation in the mouse and the early stages in the development of the optic primordia. The scales presented in all these figures provide an indication of the dimensions of the embryos following fixation, dehydration, and sputter-coating.

PLATE 1

EXPLANATION OF FIGURES

2 Anterior view of the headfold region of a 3- to 4-somite embryo. The prominent neural folds (N) are separated by the neural groove. There is no indication at this stage of the location of the optic primordia. Scale bar = 100 \( \mu \)m.

3 Dorsal view of the same embryo shown in figure 2. The considerable degree of lateral expansion of the neural plate in the cephalic region is apparent. The preotic sulcus is just evident (arrows). No distinct boundary between the presumptive surface and neural ectoderm is seen at this stage. Scale bar = 100 \( \mu \)m.

4 Anterior view of a 5- to 6-somite embryo. Flattened areas are just apparent in the most anterior and ventrally facing parts of the cephalic neural plate. These rather diffuse areas indicate the location of the optic placodes (OP). Note also the exposed presumptive midgut region (M), and the foregut pocket (F). Scale bar = 100 \( \mu \)m.

5 Dorsal view of the same embryo shown in figure 4. The ventrally and laterally facing direction of the optic placodes (OP) is seen. Note that the preotic sulcus (arrows) is now clearly seen, and that the neural folds are still widely separated at this stage. Scale bar = 100 \( \mu \)m.

6 Antero-lateral view of a 5- to 6-somite embryo. This embryo clearly shows the sharp ventral bend which develops in the rostral end of the neural plate. It is generally thought that the anterior facing region rostral to this bend represents the presumptive forebrain region or prosencephalon, and the region caudal to it represents the presumptive midbrain or mesencephalon. A pair of indentations in the prosencephalic region is clearly seen. These are the optic pits (O) which at this stage are still directed posteriorly and medially. A distinct boundary zone (B) between the surface and neural ectoderm is clearly seen in the mesencephalon. Other features of note are the torn edge and squamous appearance of the amnion (A), the exposed primitive heart tube (H), and the foregut pocket (F). This embryo displays the normal degree of dorsal lordosis seen in “unturned” embryos at this stage of development. Scale bar = 100 \( \mu \)m.

7 Anterior view of a 6- to 7-somite embryo. The forebrain neural folds are more elevated than in the embryo illustrated in figure 6, and the optic pits directed posterolaterally. Two grooves, the optic sulci, are directed medi ally from each of the optic pits. Note the exposed heart tube (H) is now S-shaped. Scale bar = 100 \( \mu \)m.
OPTIC VESICLE FORMATION IN THE MOUSE EMBRYO
Matthew Kaufman

PLATE 1
PLATE 2
EXPLANATION OF FIGURES

8 Posterior view of a 9- to 11-somite embryo. Note the extension of the process of neurulation to a level just rostral to the otic placodes. Scale bar = 100 μm.

9 Posterior view of a 14- to 16-somite embryo. Note the two slight indentations of the otic pits (arrows), and neurulation in the region of the mesencephalon. Scale bar = 100 μm.

10 Posterior view of a 15- to 17-somite embryo showing the last part of the neural tube on the dorsal aspect of the head which still remains open. This region overlies the rhombencephalon. The otic pits are clearly seen (arrows). Scale bar = 100 μm.

11 Postero-lateral view of a 25- to 27-somite embryo. Note that the cephalic part of the neural tube has closed, whereas in the tail region the caudal neuropore (C) has yet to close. By this stage the otic pits are no longer visible, having sunk beneath the surface to form the otocysts. The second branchial arch is now seen (2), as is the prominence overlying the heart (H). Scale bar = 200 μm.
OPTIC VESICLE FORMATION IN THE MOUSE EMBRYO
Matthew Kaufman
PLATE 3
EXPLANATION OF FIGURES

12 Anterior view of a 9- to 11-somite embryo. Note the obvious optic eminences (OE) and the location of the first branchial arch (B). In the upper part of this figure note the gradual approximation of the cephalic neural folds at the junction between the forebrain and the midbrain, and that a limited degree of fusion has already been initiated in the most ventral part of the forebrain close to the buccopharynx (region between two arrows). Scale bar = 100 μm.

13-15 Anterior views of a series of 10- to 15-somite embryos showing progressive approximation (figs. 13, 14) and eventual fusion of the neural folds in the region overlying the junction between the forebrain and midbrain (fig. 15). It is just possible to see in figure 13 that it is the neural ectodermal cells in this region that first meet in the midline, and that the medial boundaries of the surface ectoderm of the two sides are still some distance apart. In figure 15 the closure process has extended over the forebrain region to form the upper halves of the optic vesicles. The open region on the ventral aspect of the forebrain is the rostral neuropore (arrow). Scale bars = 100 μm.

16 Anterior view of an 18- to 20-somite embryo. At this stage the roof overlying the preoptic forebrain is quite thin and easily distorted by the preparative procedures. Scale bar = 200 μm.

17 Anterior view of a 26- to 28-somite embryo. Note the small laterally situated optic eminence (arrow), and the mandibular arches that have yet to meet in the middle. Scale bar = 200 μm.
OPTIC VESICLE FORMATION IN THE MOUSE EMBRYO
Matthew Kaufman

PLATE 3

12

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PLATE 4

EXPLANATION OF FIGURES

18 Transverse section through the cephalic and primitive streak regions of a 5- to 6-somite embryo. The neural ectoderm is thickened in the prosencephalic region, and the indentations representing the early stage of optic pit formation (arrows) are visible. The cephalic mesenchyme (CM) is loosely packed at this stage. Scale bar = 200 μm.

19 Transverse section of the cephalic region of a 6- to 8-somite embryo through the middle of the optic pits. This is an early developmental stage showing elevation of the forebrain neural folds. The optic pits are clearly seen (arrows). The upper half of the photomicrograph shows the neural ectoderm in the rostral part of the hindbrain. Note the foregut pocket (F) which extends into the headfolds. Scale bar = 100 μm.

20 Transverse section of the cephalic region of an 8- to 10-somite embryo, through the middle of the optic pits, and at the level of the origin of the first branchial arch (B). A greater degree of forebrain neural fold elevation is observed here than in the embryo illustrated in figure 19. An increase in the antero-posterior width of the head and average height of the neural ectodermal cells is also apparent in this figure. The extension of the foregut pocket (F) is clearly seen in this section. Scale bar = 100 μm.

21-23 Transverse sections through a 10- to 12-somite embryo. These sections illustrate the typical appearance observed slightly above (fig. 21), at about the middle (fig. 22) and ventral to the optic pits (fig. 23). By this stage the optic pits have deepened considerably and are now directed both posteriorly and to a slight extent superiorly. The area of the presumptive prosencephalon occupied by the optic neural ectoderm is relatively reduced and more localized than in the 8- to 10-somite stage (fig. 20). The neural folds at the junction between the prosencephalon and mesencephalon now almost meet in the midline (fig. 21, arrow). At the most ventral aspect of the forebrain the small secondary site of fusion of the neural folds is evident in figure 23 (arrow). In the hindbrain region the tips of the two opposing neural folds are beginning to move towards each other. This medial movement of the neural folds is particularly evident in the caudal part of the rhombencephalon (fig. 23). The optic eminences (OE) and the first branchial arches (B) are well seen at this stage, as is the clear line of demarcation between the surface and the neural ectoderm in both the forebrain and hindbrain regions. Same scale for figures 21-23. Scale bar = 100 μm.
OPTIC VESICLE FORMATION IN THE MOUSE EMBRYO
Matthew Kaufman

PLATE 4

18 19 20

21 22 23

CM F OE
PLATE 5
EXPLANATION OF FIGURES

24-26 Transverse sections through the cephalic region of a 12- to 14-somite embryo which had almost completed the process of “turning,” but had not yet reached the “early turned” stage illustrated in figure 1, at approximately the same stage as the embryo illustrated in figure 15. Note the rostral extension of the primary fusion site beyond the middle of the optic pits (figs. 24, 25), and the smaller secondary site of forebrain neural fold fusion (fig. 26: arrow). A gradual lateral flattening of the head occurs at about this time, with a corresponding reduction in the relative volumes of the forebrain and midbrain cavities. The rostral rhombomeres are seen in figure 24. Two features of note in figure 25 are the anlage for the fifth cranial nerve ganglion (GV), and the sheath of neural crest cells derived from the lateral crest primordium which surround the optic vesicles at this stage (arrows). The crest cells are present in this location in 10- to 12-somite rat embryos (Bartelmez, '62), and are present, though less clearly seen, in figures 22 and 23 surrounding the neural ectoderm of the optic evaginations. The most rostral part of the anlage for the VII-VIII cranial nerve ganglia is seen in figure 26 (GVII-VIII). Same scale for figures 24-26. Scale bar = 100 μm.

27-30 Transverse sections through the cephalic region of a 15- to 17-somite embryo which has “completely turned” (as evidenced by the direction of sections through the tail region). In this embryo the process of cephalic neurulation is completed with the fusion of the neural folds in the region of the rostral neuropore, and in the region overlying the fourth ventricle (4: fig. 28). The cavity of the forebrain is still markedly flattened, while the optic vesicles, which are now directed both posterolaterally and superiorly (fig. 28), are enlarging. The anlagen for the fifth and VII-VIII cranial nerve ganglia are clearly seen in figures 28 and 30 (GV and GVII-VIII, respectively). The first branchial (mandibular) arches, containing their arch arteries, surround the buccopharynx (P: fig. 30). Same scale for figures 27-30. Scale bar = 200 μm.
PLATE 6

EXPLANATION OF FIGURES

31-34 Transverse sections through a 26- to 28-somite embryo. The main features of note in embryos at this stage of development are the very considerable increase in the volumes of all of the brain vesicles. The dilation of the preoptic forebrain region or telencephalon (T) and fourth ventricle (4) are well illustrated in figures 31 and 32. As a result of the increase in the volume of the forebrain, the optic vesicles become located a considerable distance from the ventral midline. The optic vesicles and optic stalks (arrows) are now directed laterally and superiorly, the latter being apparent from their appearance in these representative sections. The mantle of neural ectoderm and surface cells overlying the fourth ventricle is extremely thin and squamous in character. In this region there is no intervening layer of mesenchymal cells. These sections also cut through the tip of the tail, and show the open neural folds in the region of the caudal neuropore (N).

The extensive area of surface ectodermal thickening in the ventral aspect of the forebrain in figures 33, 34 represents the olfactory placodes (O) which are first apparent at about the 25-somite stage. The anlage for the fifth cranial nerve ganglion (GV) is particularly well seen in figure 32. Same scale for figures 31-34. Scale bar = 300 μm.

35 View of the optic vesicle close to its mid-point. The section is from the same embryo featured in figures 31-34, and is located between the sections illustrated in figures 31 and 32. Even in this region where the optic neural ectoderm is in closest proximity to the surface ectoderm, there is still a small gap separating the two layers. The optic neural ectodermal cells are of the high columnar type, and their nuclei are predominantly located distant from the cavity of the optic vesicle. The surface ectodermal cells are of the low columnar type. There was no evidence to suggest the initiation of lens placode formation in this embryo. Scale bar = 50 μm.

36 Transverse section through the optic vesicle, optic stalk and telencephalon of a 28- to 30-somite embryo. The pseudostratified nature of the neural ectodermal cells is clearly seen. Scale bar = 100 μm.

37 Close-up view of a part of figure 36 showing the direct apposition of the optic neural ectoderm (the retinal primordium) and the overlying surface ectoderm. Even at this stage there appears to be no evidence of surface ectodermal thickening consistent with lens placode formation. Note the large number of mitotic figures (arrows) located adjacent to the cavity of the optic vesicle in the ependymal layer. The embryo is surrounded by a sheet of squamous cells which constitutes the amniotic membrane (A). Scale bar = 50 μm.
Copper-induced Microtubule Degeneration and Filamentous Inclusions in the Neuroepithelium of the Mouse Embryo

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Summary. The neuroepithelium of embryos exposed either in vivo or in vitro to copper sulphate was examined by transmission electron microscopy. Exposure to copper sulphate generally resulted in failure of elevation of the neural folds, and consequent development of neural tube defects. These defects appeared to result from the failure of individual cells of the neuroepithelium to change from a low to the high columnar form necessary for normal closure to take place.

Ultrastructurally, the neuroepithelial cells showed a considerable reduction in the number of microtubules present, or contained microtubular remnants. Occasionally, deposits of approximately 100Å filamentous material were also observed in the middle regions of neuroepithelial cells devoid of microtubules. The overall morphology of individual cells appeared otherwise unchanged.

This study supports the concept that microtubules are required for neuroepithelial cellular elongation, an essential step during the process of neurulation. It also suggests that copper, like other microtubule-active agents, may induce the production of characteristic fibrillary deposits that are also observed in a number of neuropathological conditions.

Key words: Copper — Microtubule degeneration — Neuroepithelium — Mouse

Two major components of the cytoskeleton, the microtubules and microfilaments, have been observed in a number of cell types. Because of their importance in maintaining cell shape, it is generally considered that these intracellular components probably play an essential role in many morphogenetic processes, as these often depend on cellular migration or changes in cell shape.

Formation of the embryonic neural tube (neurulation) appears to be one such process (Karfunkel, 1974: review). During neurulation, the cuboidal cells of the neural plate first undergo cell elongation, a process which is microtubule-dependent. This is followed by an active microfilament-mediated narrowing of the cell apices. As a result, individual cells become wedge-shaped. This series of events normally gives rise to the neural folds, which become elevated and apposed, and finally fuse in the midline to form the neural tube.

Exposure of early neural plate stage embryos to agents that are known to depolymerize microtubules might be expected to produce neural tube defects by interference with the cell shape changes indicated above which occur within the neuroepithelium. We have previously reported that exposure of embryos to copper sulphate produced a high incidence of neural tube defects, which appeared to form as a result of insufficient elevation of the neural folds (O'Shea and Kaufman, 1979). The current investigation was therefore undertaken to examine at the ultrastructural level, the neuroepithelium of embryos exposed in vivo or in vitro to copper sulphate.

Methods

Female CFLP mice (Anglia labs) were mated with males of the same strain, and isolated on the morning of finding a vaginal plug (designated the 1st day of pregnancy).

On the morning of the 8th day of pregnancy females were injected i.v. (tail vein) with 0.1 ml of 0.06 M copper sulphate solution (0.8 mg copper/ml) in sterile saline, or 0.1 ml of saline alone (controls). On the afternoon of the 10th day, females were killed by cervical dislocation, embryos were isolated from the uterus, examined externally, and the presence or absence of gross anomalies noted. Embryos were then fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (containing 10% sucrose) at room temperature for 2 h.

After fixation, embryos were washed briefly in 0.1 M sodium cacodylate buffer and post-fixed in 1% osmium tetroxide containing...
5% sucrose for 1 h. They were then routinely incubated in a solution of saturated aqueous uranyl acetate for 1 h. Embryos were dehydrated through a series of graded alcohols and embedded in resin (Spurr, 1969). Thin sections were cut, double-stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed in a Phillips 300 electron microscope.

On the morning of the 9th day of pregnancy, additional groups of embryos were isolated as described above, and explanted into roller culture bottles (New et al., 1973) containing either $2.5 \times 10^{-5} \text{M}$ (1.6 μg copper/ml) or $5 \times 10^{-5} \text{M}$ (3.2 μg/ml) copper sulphate in 2 ml rat serum prepared as described by Steele and New (1974). Distilled water (0.1 ml) alone was added to control cultures. To provide a
control for the possible deleterious effects of the sulphate radical, other embryos were incubated in serum containing a final concentration of $5 \times 10^{-7} \text{M}$ magnesium sulphate added in a 0.1 ml volume of distilled water. The bottles were gassed with 5% CO$_2$ in air for 10 min at 12 h intervals throughout the 36 h culture period. At the end of this period, embryos were examined, and processed for TEM as described above.

Results
A high proportion of the embryos exposed in vivo or in vitro to copper sulphate appeared to be abnormal. The most common defect observed in these embryos was nonclosures of the neural tube, principally involving the cephalic region, but additional defects were also often located in other regions along the neuraxis. These appeared to be due in many cases to failure of elevation of the neural folds. Detailed data on the extent and gross appearance of these defects have been reported elsewhere (O'Shea and Kaufman, 1979).

By the afternoon of the 10th day in vivo, and at the end of the culture period, mouse embryos usually had about 25 somites present. The neuroepithelial cells of control embryos and those exposed to magnesium sulphate, were characteristically wedge-shaped and closely apposed, and held together at the luminal surface by tight junctions. Microtubules were commonly observed in the middle and basal portions of the cell (Fig. 1a, b) oriented perpendicular to the lumen, and microfilament bands could be seen to link the tight junctions at the cell apices.

The neuroepithelium of copper-treated embryos often appeared to be ultrastructurally normal, apart from an almost complete absence of microtubules. This was especially evident in embryos exposed in vitro to the higher level of copper tested. Scattered remnants of microtubules were, however, also observed in the neuroepithelium of embryos exposed to the lower level in vitro, and in those exposed to copper in vivo. The normal orientation of the microtubular remnants was retained, but often the microtubules appeared to have a fuzzy rather than a sharply defined outline, and occasionally gaps were present along their length (Fig. 1c, d). In addition, the cells of the neuroepithelium were usually in tight contact with each other and with the external limiting membrane, and microfilament bands were present at the luminal surface. Other intracellular organelles, such as mitochondria, cell membranes, ribosomes, and polysomes appeared morphologically normal.

Dense deposits of clumped filaments measuring approximately 100 Å in diameter were observed in many of the neuroepithelial cells of these copper-treated embryos (Fig. 2). These inclusions were observed only in the middle regions of the cells, and were only present in those cells in which microtubules were absent, although microfilaments were normally present, and prominent at the luminal surface.

Discussion
These results support the concept that microtubules play an essential role in neuroepithelial cell elongation, and that the deleterious effects of copper sulphate on neural tube closure principally result from its action on this component of the cytoskeleton. Essentially, this agent produces neural tube defects because the cell shape changes that normally facilitate elevation of the neural folds are largely inhibited. These effects appear to be due to the copper ion itself, as similar levels of the sulphate radical (present in the form of magnesium sulphate) had no apparent effect on neuroepithelial architecture.

It has previously been established that microtubule assembly/disassembly is dependent on the concentration of divalent cations present in solution. Magnesium ions, e.g., may stimulate microtubule assembly (Weisenberg, 1972), while calcium appears to induce microtubular depolymerization (Weisenberg, 1972; Weisenberg and Timasheff, 1970; Wilson, 1975). Most metallic cations have a stabilizing effect
(Goode and Roth, 1969), although zinc (Gaskin et al., 1978), copper and nickel (Roth and Shigenaka, 1970) ions may depolymerize microtubules.

The fibrillar deposits observed in the neuroepithelial cells following copper sulphate exposure, reported in this study, have not been noted before. Gaskin et al. (1978) have reported similar inclusions in axons of explanted rat dorsal root ganglia incubated in a solution containing zinc sulphate. However, the inclusions reported by these authors measured approximately 25 Å in diameter, considerably smaller than those observed in the present study. Intracisternal application to rats and rabbits of known microtubule-active drugs, such as vincristine, colchicine, and aluminium paste, also produce neurofibrillary changes (Wisniewski et al., 1969).

Various authors have suggested that these fibrillar inclusions may represent the breakdown products of the microtubules themselves (Gaskin and Shelanski, 1976). Alternatively, an elevation in the intracellular concentration of microtubule precursor elements, following microtubule depolymerization, may lead to their ‘precipitation’ and consequent appearance in this aggregated form. Protein synthesis per se, does not appear to be crucial for this conversion to take place, as filament deposits may also form in the presence of cyclohexamide (Wisniewski et al., 1968).

It is of interest that in certain neurological disorders, regions of the human brain that contain large deposits of copper, such as the substantia nigra and locus coeruleus (Curzon, 1975), are the principal areas in which similar aggregates have been observed. These are characteristically seen in cases of Guam-Parkinsonism and postencephalitic Parkinsonism (Hirano, 1970) and in Alzheimer’s disease (Iqbal et al., 1975). Surprisingly, no similar neuropathological changes have been reported in patients with Wilson’s disease, in which copper levels in the brain are elevated (Cumings, 1948). Gaskin et al. (1978) have suggested that areas of the nervous system in which there is a rapid assembly/disassembly of microtubules may be especially sensitive to changes in the concentration of certain cations. The developing neuroepithelial cells have a similar high rate of assembly/disassembly of these cytoskeletal elements. This may well explain the susceptibility of early neurulae to teratogenic agents during this particularly sensitive period of development (Kalter, 1968).

It is hoped that electron probe microanalysis of similar material will enable the actual site(s) of copper binding in the neuroepithelium to be accurately determined.

Acknowledgement. This study was supported by a grant from the National Fund for Research into Crippling Diseases.

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Received October 19, 1979/Accepted November 20, 1979
X-chromosome inactivation in extra-embryonic membranes of diploid parthenogenetic mouse embryos demonstrated by differential staining

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In somatic cells of female mammals one of the two X chromosomes is genetically inactive and heterochromatic, resulting in dosage compensation for X-linked genes. In marsupials the paternally derived X chromosome is preferentially inactivated. In eutherian mammals, although either X chromosome can be inactivated at random in somatic cells, preferential inactivation of the paternally derived X chromosome has been demonstrated cytologically in mouse and rat yolk sacs and mouse chorion and biochemically in mouse yolk sac, chorionic ectoderm and trophoblast. In mouse yolk sac the non-random element has been shown both biochemically and cytologically to be confined to the endodermal layer in which there is almost total paternal X-chromosome inactivation. We have therefore looked at X-chromosome activity in the separated yolk sac layers of diploid parthenogenetic mouse embryos in which both X chromosomes are maternally derived. Kaufman et al. have demonstrated X inactivation in somatic cells of diploid parthenogenetic embryos, and we have used a modification of Kanda's method, which renders the presumptive inactive X dark staining, to reveal the inactive X chromosome in both endoderm and mesoderm layers of separated yolk sacs from parthenogenotes. Thus even in tissues in which there is normally total non-random paternal X inactivation, in the absence of a paternally derived X chromosome a maternally derived X can be inactivated.

Kanda identified the inactive X chromosome at metaphase in female mice and other rodents by treating bone marrow cells with hot hypotonic potassium chloride solution before fixation. The inactive X chromosome then stained much more darkly with Giemsa than the other chromosomes in the cell. The modification of the Kanda method for use with embryonic material involved placing the whole embryo and/or embryonic membranes for 90 min in culture medium 199 (Flow Laboratories) to which colchicine had been added (4 μg/ml final concentration) to accumulate metaphases. The embryos were then placed individually in Petri dishes containing 0.5% (w/v) potassium chloride solution at room temperature and incubated in a water bath at 50°C for 15 min, followed by fixation in freshly made 3:1 absolute ethanol/glacial acetic acid solution. After fixation the embryos were isolated, touched lightly to filter paper to remove excess fixative and disaggregated in 0.5 ml of 60% acetic acid for 5 min. Chromosome spreads were made by allowing small drops of the acetic acid mixture to evaporate on slides placed on a 40°C hot plate. The preparations were stained in 2% Giemsa buffered at pH 6.8 for 20 min.

A dark-staining X chromosome was seen in more than 80% of metaphase cells from female embryos at 5½, 6½, 7½ and 13½ days post-coitum (p.c.) (Table 1 and Fig. 1a, b). Strong evidence that the dark staining chromosome is the inactive X chromosome is that it is seen in such a high proportion of XX female cells, but not in X0 female cells nor XY male cells. A dark staining X chromosome was also present in more than 75% of cells from a bone marrow preparation of an adult XX male mouse carrying Sxr, the autosomal gene for sex reversal.

Five experimental diploid parthenogenetic embryos were produced by the method of Kaufman et al. by suppression of second polar body formation, and dissected out of their decidua at a stage equivalent to 9½-10½-day gestation (10-25 somites present). Their yolk sacs were removed, washed in medium 199 and placed in medium 199 with colchicine (4 μg/ml final concentration) for 3 h at room temperature to accumulate metaphases. The yolk sacs were then separated into their endodermal and mesodermal components by partial digestion for 3 h in a mixture of 2.5% pancreatic and 0.5% trypsin (w/v) made up in Ca²⁺ and Mg²⁺-free Tyrode solution at 4°C, followed by dissection with watchmaker's forceps. The cleanly separated cell layers were then individually treated by the modified Kanda method, as described above. Chromosome spreads from the two yolk sac components from normal fertilized zygotes at the same developmental stage were also obtained. The slides were all stained in 2% Giemsa at pH 6.8 for 20 min, randomized and coded, and cells at metaphase were scored for the presence or absence of a dark staining X chromosome (Table 2). A dark staining X chromosome was seen in 76 out of 94 metaphases in parthenogenetic yolk sac endoderm (Fig. 1c) and in 255 out of 328 metaphases scored from parthenogenetic yolk sac mesoderm (Fig. 1d). We therefore conclude that X-chromosome inactivation has taken place in both endodermally and mesodermally-derived layers of yolk sac from diploid parthenogenotes.

Cooper has suggested that passage of the X chromosome through male gametogenesis or fertilization led to imprinting of the paternal X chromosome and was an important factor in paternal X inactivation in marsupials. It has been further suggested that imprinting also occurs in eutherian mammals and results in paternal X inactivation in those tissues of the embryo that differentiate early, such as yolk sac endoderm which is derived from the primitive endoderm, but that cells of the embryo proper escape from imprinting before the time of X-chromosome differentiation so that in them X inactivation is random. Our results show that even in a tissue such as yolk sac endoderm in which primary non-random inactivation of the paternal X is thought to occur, a maternal X chromosome can be inactivated in the absence of a paternal one. In our parthenogenetic material neither X chromosome could have been imprinted, either by passage through male gametogenesis, fertilization or re-entry of the polar body, and so we conclude that imprinting cannot be essential to the mechanism of X inactivation in the tissues in which non-random paternal X inactivation normally occurs. One cannot, however, assume that absence of an imprinted X chromosome has not affected X inactivation in any way; for example, is the onset of X inactivation in these tissues delayed? Homozygous diploid uniparental mice, produced by the removal of one or other pronucleus after

Virgin female mice of the 3H1 strain (F1, hybrids between two inbred strains, C3H/HeH and 101/H) were injected with 5 μl pregnant mare's serum gonadotropin (PMS) followed 44 h later by an injection of 5 IU human chorionic gonadotropin (HCG) to synchronize ovulation, and were mated to 3H1 males. Females were checked for vaginal plugs early the next morning, and the day of the plug was designated day 1. Pregnant females were killed by cervical dislocation at various stages of pregnancy, and the embryo was dissected out under a dissecting microscope. Whenever possible Reichert's membrane was removed to ensure that there was no contamination by maternal cells. The 13½-day embryos were sexed by dissecting out the gonad, which has visibly differentiated by this stage, and the earlier embryos were sexed cytologically.

Table 1

<table>
<thead>
<tr>
<th>Age of embryos (days p.c.)</th>
<th>No. of female embryos</th>
<th>Total metaphases</th>
<th>Metaphases with a dark X chromosome</th>
<th>Per cent with a dark X chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>5½</td>
<td>8</td>
<td>221</td>
<td>192</td>
<td>86.88</td>
</tr>
<tr>
<td>6½</td>
<td>13</td>
<td>1,188</td>
<td>1,019</td>
<td>85.77</td>
</tr>
<tr>
<td>7½</td>
<td>6</td>
<td>545</td>
<td>455</td>
<td>83.49</td>
</tr>
<tr>
<td>13½</td>
<td>8</td>
<td>598</td>
<td>495</td>
<td>82.78</td>
</tr>
</tbody>
</table>

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Table 2  Staining of metaphases from yolk sacs

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. embryos</th>
<th>No. metaphases scored</th>
<th>No. metaphases with a dark X chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parthenogenones</td>
<td>Controls</td>
<td>Parthenogenones</td>
</tr>
<tr>
<td>Yolk sac endoderm</td>
<td>5</td>
<td>14</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>74</td>
</tr>
<tr>
<td>Yolk sac mesoderm</td>
<td>5</td>
<td>14</td>
<td>328</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>255</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>558</td>
</tr>
</tbody>
</table>

This table shows the number of cells at metaphase from parthenogenetic separated yolk sac endoderm and mesoderm exhibiting a dark-staining X chromosome after treatment with the modified Kanda method, compared with controls of fertilized material which have received the same treatment.

Fig. 1 Metaphase cells showing an inactive dark-staining X chromosome after hypotonic treatment at 50 °C. a, Metaphase from a normal S½-day p.c. embryo; b, metaphase from a normal 7½-day p.c. embryo; c, metaphase from the endoderm layer of diploid parthenogenetic yolk sac; and d, metaphase from the mesoderm layer of diploid parthenogenetic yolk sac.

fertilization, followed by diploidization in cytochalasin B, are viable 21, which suggests that X inactivation in all tissues has occurred normally. However, as no live-born mammalian parthenogenones have been produced so far, one cannot rule out the possibility that some abnormality of X inactivation is involved in their premature death.

We thank Mrs A. Burling for technical assistance.

Received 7 July; accepted 17 September 1980.

Phospholipase C-induced neural tube defects in the mouse embryo

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Summary. Mouse embryo neurulae were exposed in vitro to phospholipase C to examine the role of carbohydrate-rich extracellular material (ECM) during neurulation. Exposure of embryos to this agent for 12 h resulted in failure of closure of the neural tube. Ultrastructural examination revealed an absence of ECM from regions of the neural tube which failed to close.

Cell sorting and tissue interactions involved in the complex process of morphogenesis probably depend on the recognition of specific surface characteristics. Thus, changes in the nature of the extracellular material (ECM) present at the cell surface, or alterations in the morphology of the cell surface itself at critical periods of development, may well determine the nature and timing of cell-cell and tissue interactions.

Carbohydrate-rich material is present at the cell surface during the morphogenesis of a number of tissue types. ECM has been demonstrated on apposing cell surfaces prior to palatal shelf fusion, preceeding fusion of the nasal processes and on the apical surface of the neural folds prior to neural tube closure in the chick, amphibian embryos.

Despite the temporal correlation between the deposition of the ECM and epithelial fusion, the role of this material in the fusion process itself is unclear. In the present study ECM was removed from the apical surfaces of the neural folds with phospholipase C to examine the effect of this treatment on the course of neurulation in the mouse.

Methods. Random-bred CFLP (Anglia labs) female mice were mated to males of the same strain, and the morning of finding a vaginal plug designated the 1st day of pregnancy. Embryos were removed from the uterus early on the morning of the 9th day, and explanted into roller bottles containing 1 x 10^{-3} mg/ml phospholipase C (Sigma Chemical Company, P7633) and 2 ml of heat inactivated rat serum prepared as described by Steele and New. 0.1 ml of distilled water alone was added to control serum.

Embryos were exposed to this agent for a total of 12 h, either immediately after explantation (0-12 h culture period) or after 24 h (24-36 h culture period). The bottles were gassed with 5% CO_{2} in air for 10 min at 12-h intervals throughout the 36-h culture period.

At the end of the culture period, embryos were washed in phosphate buffered saline, fixed at room temperature in 2% glutaraldehyde solution, containing 3% sucrose and 1% acetic blue 8GX (G.T. Gurr, London), and again in buffer, dehydrated through a graded alcohol series and embedded in resin. Thin sections were cut and examined unstained in a Philips 300 electron microscope. Additional embryos were fixed for 2 h at room temperature in 2% glutaraldehyde (without acetic blue) and dehydrated through a graded alcohol series. These were then critical point dried, sputter coated and subsequently viewed and photographed in a Cambridge S600 scanning electron microscope.

Results. When embryos were exposed to phospholipase C for the first 12 h after explantation, the neural folds were observed to elevate normally, but failed to fuse in the midline in the majority (15/16) of these embryos (figure, C). When phospholipase was introduced 24 h after the beginning of the culture period, at a stage just prior to fusion of the neural folds in the cephalic region, approximation of the folds occurred, but in all 12 embryos treated in this way, the neural folds failed to fuse. In all other respects, overall embryonic development did not appear to be impaired compared to controls.

Ultrastructural examination of controls revealed a dense deposition of lanthanum-positive material on the surface of the neuroepithelium, particularly over the prospective fusion zone (figure, C). In phospholipase-treated embryos, A SEM photomicrograph of a 10-15 somite control embryo showing the extent of neural tube closure after 12 h in vitro. The neural tube is closed in all but the most cephalic and caudal regions. Note the presence of somites (arrowed), and the first branchial arch (1). Scale bar = 200 μm. B Postero-lateral view of an embryo isolated after exposure for 12 h (0-12 h period) to phospholipase C. The neural tube has failed to close throughout its entire length, and the neural folds are everted, especially in the upper 'spinal' region (arrowed). Scale bar = 200 μm. C TEM photomicrograph of lanthanum staining material lining the neural groove in the upper 'spinal' region of a control embryo. Scale bar = 0.5 μm. D Photomicrograph of a cross-section of the neuroepithelium near the cell surface in a similar region to that illustrated in figure C. This embryo was exposed to phospholipase C for the 1st 12 h in culture, and isolated and stained immediately following. Note that the lanthanum positive material is almost completely absent. Scale bar = 0.5 μm.
no lanthanum staining material was observed (figure, D), although fragments of staining material were occasionally observed along the neural groove.

**Discussion.** The ultrastructural observations presented here confirm Sadler’s light microscopical study of ECM accumulation along the neural groove of the mouse embryo. It seems likely that this material plays an important role during neurulation, as its removal with phospholipase C inhibited neural tube closure. Similar observations have been made by Lee and his colleagues, who exposed chick neurulae to concanavalin A, and found it to inhibit neural tube closure. These authors suggested that concanavalin A was probably bound to the ECM rendering the cell surface too ‘rigid’ to participate in the changes required for neural tube closure.

Rice and Moran using electron probe microanalysis of amphibian neurulae pretreated with lanthanum, demonstrated the presence of both phosphorus and bound lanthanum along the neural groove. They suggest that the lanthanum in the stain had replaced calcium in the ECM to render it electron dense, and further suggested that the ECM may act as a storehouse for bound calcium. The phosphorus observed in the ECM is probably a normal component of the cell membrane phospholipid matrix, and the phospholipase employed in the present study may have acted at the membrane lipid bilayer. It appears that the ECM may be required in maintaining apposition of the neural folds prior to normal neural tube closure. Further investigations into its biochemical nature may shed light on its role in cell-cell interaction during morphogenesis.

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1. This study was supported by a grant from the National Fund for Research into Crippling Diseases.

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Neural Tube Closure Defects Following In Vitro Exposure of Mouse Embryos to Xylocaine

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ABSTRACT Early somite mouse embryos were exposed in vitro to xylocaine (Lignocaine hydrochloride) to examine its effect on neural tube closure. When embryos were exposed to $2.4 \times 10^{-4}$ mg/ml xylocaine for the entire 36-hour culture period, cephalic neural tube closure did not take place. When examined by SEM, the neuroepithelial cells of these embryos were incompletely elevated, and depressions in their lateral surfaces were often observed. TEM observations on the neuroepithelium of similar embryos indicated that the microtubules and apical microfilament bundles were absent. The role of calcium in the maintenance of neuroepithelial cell asymmetry and in the production of these defects is considered.

Recent theories of the mechanisms involved in neurulation have focused on the role of the cytoskeletal elements in producing the neuroepithelial cell-shaping changes required for neural tube closure. Low columnar cells of the early neural plate undergo microtubule-mediated elongation and then become wedge-shaped (following microfilament constriction of the cell apices) (cf., Karfunkel, '74, for review). Thus, a flat plate of cells tightly connected by apical junctions "rolls up" to form a cylindrical tube.

Recently, several investigators have shown that when calcium was displaced from the neural folds following papavaranin treatment of early neurulae, microfilament function was disrupted, the neuroepithelial cells failed to become wedge-shaped, and neural tube closure did not progress (Rice and Moran, '77; Lee and Nagele, '79). Application of Ionophore A23187, an agent thought to mobilize bound calcium, reversed the alterations in cell shape, and neural tube closure proceeded (Moran, '76).

The current investigation was initiated to examine the effects of in vitro exposure of early mouse embryos to xylocaine, a cationic local anesthetic which displaces membrane-bound calcium (Seeman, '72).

MATERIALS AND METHODS Female CFLP (Anglia labs) mice were mated with males of the same strain, and the morning of finding a vaginal plug was designated the first day of pregnancy. Early on the morning of the ninth day, females were killed and embryos explanted into a roller culture system (New et al., '73). Each roller bottle contained 2 or 3 early somite (Theiler stage, TS 12, '72) embryos, 2 ml serum prepared as described by Steele and New (74) to which xylocaine (lignocaine hydrochloride, Astra, Watford, U.K.), to a final concentration of $2.4 \times 10^{-4}$ mg/ml or 0.1 ml distilled water (controls) alone had been added.

Some (n = 10) of these embryos were removed from serum containing xylocaine and placed in control serum for an additional 6 hours to determine if the defects could be ameliorated by further culture in the absence of xylocaine. Table 1 summarizes these treatments.

The culture bottles were gassed with 5% CO$_2$ in air at 12-hour intervals during the 36-hour culture period. At the end of this time the embryos were examined for gross anomalies, then fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 3% sucrose, at room temperature, for 2 hours. They were then either dehydrated and gold-coated for SEM, or en bloc stained with a saturated solution of aqueous uranyl acetate, postfixed in 1% osmium tetroxide for 1 hour, and then embedded in resin (Spurr, '69). Thin sections were cut and stained with uranyl acetate and lead citrate and viewed in a Phillips 200 electron microscope.
RESULTS

During the 36-hour culture period, control embryos progress from the 1–2 somite stage of development (TS12), to the 26–28 somite stage of development (TS15, approximately the tenth day in vivo). During this period the embryos "turn" (undergo yolk sac inversion), and the neural tube closes in all regions of the neuraxis but the posterior neuropore, which closes slightly later. Figure 1A illustrates the normal appearance of the frontal region of a control embryo.

When embryos were cultured in medium containing xylocaine, the anterior neural folds failed to fuse in the midline (19/22 embryos). In many of these embryos the neural folds had not elevated normally, nor were they approximated in the midline. In fact, although these embryos were "turned" and had 22–24 somites (TS15), the cranial neural folds had the appearance of a much earlier stage of development (Fig. 1D, Table 1).

When cross-section fractures were made through the cephalic region of the neural tube and examined by SEM, the neuroepithelial cells of control embryos were characteristically wedge-shaped, and most cells extended from the basement membrane to the lumen. Rounded (ie., mitotic) cells were also seen near the lumen. The lateral surface of the neuroepithelial cells was smooth and the lumen relatively even (Fig. 1B).

In xylocaine-treated embryos, the cells of the neuroepithelium appeared to be only partially elevated, although occasional wedge-shaped cells were observed. A few of these cells also had scattered depressions in their lateral surface (Fig. 1E).

Embryos exposed for 30 hours to xylocaine and then placed in control serum for the final 6 hours appeared morphologically and ultrastructurally normal. However, the 6-hour "recovery" period was not sufficient to reduce the growth retardation of these embryos.

The neuroepithelium of control embryos appeared ultrastructurally normal. Except for cells in division, the nuclei were located in basal regions of the cells, Golgi apparatus, cell-cell junctions, and mitochondria were normal. Microfilaments and microtubules were present, and normal amounts of "zeiotic" extrusions were present at the lumen (Fig. 1C).

Both microfilament bundles and microtubules were absent from neuroepithelial cells in the embryos exposed to xylocaine. In addition, remnants of shed plasma membranes and cellular debris were often extruded into the
A. SEM frontal view of a control embryo (TS 15) illustrating the normal appearance of this region. The anterior neuropore is completely closed at this stage of development. (Scale bar = 200μm.)

B. Cross section of the neuroepithelium in a control embryo. The neural tube was fractured through the hindbrain region. The cells are wedge-shaped, processes connecting them to the lumen (L) and basal regions of the neural tube. Mitotic cells (m) can be seen at the apex of the cells. (Scale bar = 5μm.)

C. TEM photomicrograph of the neuroepithelium of a 26-28 somite control embryo. The cross section shows the normal irregular configuration of the luminal surface; zeoitic protrusions (Z) are present, and microfilament bands (mf) connect apical junctions. Nuclei (N) are normally situated in middle to basal regions of the cells; mitochondria (mit) are numerous. (Scale bar = 2μm.)

D. Frontal SEM view of an embryo exposed to 2.4 × 10^{-4} mg/ml xylocaine for 36 hours in vitro. The neural folds have not approximated in the midline. (Scale bar = 200μm.)

E. Cross-sectional fracture through the neuroepithelium of an embryo exposed to xylocaine. The cells are somewhat disorganized and incompletely elevated. There also appears to be some pitting of their lateral surfaces (arrow). L = lumen. (Scale bar = 5μm.)

F. TEM photomicrograph of the neuroepithelium of a xylocaine-exposed embryo. The intercellular junctions (J) appear to be normal, except that there are no microfilaments associated with them. Mitotic cells are located near the lumen, and mitochondria are present in their usual quantity. Note the presence of membranous remnants (arrow), along with other cell debris in the lumen. (Scale bar = 2μm.)
lumen of these embryos (Fig. 1F). Mitochondria and Golgi apparatus appeared to be normal, and there was little evidence of the lateral surface pitting seen using SEM. Intercellular junctions, although devoid of microfilaments, appeared otherwise normal.

DISCUSSION

These results confirm earlier reports that anesthetic agents may be teratogenic to early somite embryos (Kaufman and Steele, '76; Basford and Fink, '68). It also appears that the observed neural tube defects probably result from alterations in the cytoskeletal elements of the neuroepithelium, following changes in the cell membrane.

Local anesthetics affect other processes that appear to be mediated by the cytoskeleton, including: axonal transport (Byers et al., '76), cell spreading, and locomotion (Gail and Boone, '72; Poste et al., '75). Ultrastructural investigations have confirmed that exposure of other cell types to local anesthetics may affect the microtubules and microfilaments, and the membrane lipid bilayer has been suggested to be the site of action of these compounds (Rabinovitch and DeStefano, '76; Nicolson et al., '76).

Little is known about the ionic requirements of the embryonic neuroepithelium. Moran and Rice ('75) and Lee and Nagele ('79) have shown that calcium appears to be required for neural tube closure in amphibian and chick embryos. Barth and Barth ('72) studied the uptake of 45Ca+ into Rana neurulae and found cephalic incorporation to be greater than that in caudal regions of the neuraxis. Hughes ('75) has reported that early chick embryos with open neural tube defects (following ouabain treatment) have a four-fold increase in sodium/potassium ratio compared with controls, and suggested a failure of extrusion of sodium to be the basis of these defects.

Chick embryonic neural tube defects produced by cytochalasin B have been suggested by Messier ('76; see also Spooner, '73) to result from similar surface membrane alterations in the neuroepithelium. Both treatments (xylocaaine, cytochalasin B) produced lateral surface pitting and microfilament disassembly, but only xylocaaine affected microtubules as well. It appears that alterations in the configuration of the cell membrane may result in uncoupling of microfilaments and microtubules from the inner aspect of that membrane.

Study of the uptake of labelled calcium into xylocaaine- or cytochalasin B-treated embryos is currently in progress to study these effects in detail.

ACKNOWLEDGMENTS

The authors are grateful to M.A. Webb for excellent technical assistance. This study was funded by grants from the National Fund for Research into Crippling Diseases (U.K.) NIH Grant HD-07097 and NSF RIASSER 77-06922.

LITERATURE CITED

Effect of acetaldehyde on the neuroepithelium of early mouse embryos

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(Accepted 14 July 1980)

INTRODUCTION

The putative teratogenicity of ethyl alcohol has been a topic of medical concern since ancient times (cf. Warner & Rosett, 1975). Recently, there has been increased concern for infants born to mothers with a high alcohol intake during pregnancy. These infants exhibit a characteristic pattern of intrauterine growth retardation, certain craniofacial, ocular and cardiovascular features (Jones, Smith, Ulleland & Streissguth, 1973). The CNS also appears to be involved, as many of these infants are mentally retarded, and structural abnormalities of the brain have also been reported (Clarren et al. 1978).

Attempts to find an animal model to study these effects in more detail have produced conflicting reports which may result from differences in the mode of administration of the alcohol, as well as on strain differences in alcohol metabolism. These discrepancies, and the fact that affected infants have been born to mothers who drink small amounts of alcohol during pregnancy, while some females with an extremely high alcohol intake give birth to apparently healthy infants, have led investigators to suggest that the breakdown products of alcohol may be primarily responsible for these effects (Hanson, Streissguth & Smith, 1977).

We have previously reported that acetaldehyde (the primary metabolic product of alcohol oxidation) produced growth retardation, central nervous system and cardiovascular anomalies in mouse embryos exposed to it during the early post-implantation period of development (O'Shea & Kaufman, 1979). The present study was initiated to examine the possible relationship between the time of exposure of embryos to acetaldehyde and the extent and location of the resulting neural tube defects. In addition, SEM observations have been made on the morphological appearance of the neuroepithelium which may provide clues as to the underlying mechanism of production of the acetaldehyde-induced neural tube defects.

METHODS

Ten to twelve weeks old CFLP (Anglia Labs) female mice were mated with males of the same strain, and isolated on the morning of finding a vaginal plug (the first day of pregnancy). Food and water were available to the animals ad libitum and the colony was maintained under controlled conditions of humidity, temperature, and lighting (dark phase from 7 pm to 5 am).

Pregnant mice were injected intravenously into the tail vein with 0.1 ml of acetaldehyde (2% in saline) on either one, or on several days during the early post-

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Table 1. Observations following injection of acetaldehyde between the sixth and the ninth day of gestation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Groups</th>
<th>Injection day</th>
<th>Autopsy day</th>
<th>Total females</th>
<th>Total implants</th>
<th>Total resorptions</th>
<th>Embryos dead</th>
<th>Total abnormal</th>
<th>Average crown/rump*</th>
<th>Protein content†</th>
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<td>2.3±0.06</td>
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<tr>
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<td>0</td>
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<td>0/42</td>
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<td>1/92</td>
<td>1/91</td>
<td>2.8±0.02</td>
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* P < 0.01, Group 2 < Groups 5, 12 < Groups 1, 6, 7, 10, 13 < Group 3 < Group 11 < Group 14, one way analysis of variance.
† P < 0.05, Groups 1, 2, 3, 5, 6 < Groups 9, 10, 11, 12, 13, 14, one way analysis of variance.
implantation period. Control females received an equal volume of saline alone, or were not injected. During the early afternoon of the tenth or twelfth day of gestation, females were killed by cervical dislocation and embryos dissected from the decidua into phosphate-buffered saline (PBS, pH = 7.2). At this time, crown-rump lengths, developmental stage, and presence of any gross anomalies were recorded.

Embryos to be examined histologically were fixed in 50% Bouin’s solution overnight, and post-fixed and stored in 70% ethanol prior to dehydration, paraffin embedding and sectioning. Embryos were routinely sectioned at 6 µm and stained with haematoxylin and eosin.

Embryos to be examined by transmission or scanning electron microscopy (SEM) were fixed in 2% glutaraldehyde in sodium cacodylate buffer containing 3% sucrose, washed in cacodylate buffer (plus sucrose) and subsequently dehydrated through a graded ethanol series. Embryos for SEM were critical point-dried and sputter-coated, and subsequently viewed and photographed in a Cambridge S600 Stereoscan microscope. After fixation, additional embryos to be examined by transmission electron microscopy (TEM) were post-fixed in a 1% solution of osmium tetroxide containing 5% sucrose for one hour. They were subsequently dehydrated and embedded in resin (Spurr, 1969). Thick and thin sections were cut, stained, and examined by light microscopy or TEM.

In a number of control embryos, the roof plate of the neural tube was incised with a fine glass needle for a length up to 1 mm prior to fixation.

Other embryos were washed in PBS, and stored at -4 °C prior to protein determination. Whole embryo protein content was determined by the Miller (1959) modification of the Lowry technique. Because of the considerable variation in size and morphology of the embryos that had failed to adopt the characteristic fetal position (i.e. were still ‘unturned’) by early in the afternoon on the tenth day of gestation, the protein content of these developmentally retarded embryos was not determined, and has therefore not been included in the data presented in Table 1.

RESULTS

Developmental parameters

When acetaldehyde-treated embryos were examined on the tenth day of gestation they were uniformly smaller than control embryos as measured both by crown-rump length as well as by whole embryo protein content. The experimental group of females injected with acetaldehyde contained more resorption sites and more embryos that had failed to adopt the fetal position (i.e. were still ‘unturned’) than saline-injected or uninjected controls. These results are summarized in Table 1.

Injection of acetaldehyde on several successive days had an even more dramatic effect on embryonic development than a single injection. For example, when females were injected on the sixth, seventh and eighth days of gestation, all 59 of the living embryos examined had failed to adopt the fetal position. The incidence of resorptions following this treatment schedule was 8.8%. Injection on the seventh, eighth and ninth days also resulted in a high incidence (47.2%) of resorptions. In this latter group 7/54 of the living embryos were apparently healthy but ‘unturned’, and 9 of the 47 ‘turned’ embryos had a gross neural tube defect.

When embryos were treated with acetaldehyde on the seventh and eighth days and examined on the twelfth day of gestation, the incidence of neural tube defects
Fig. 1. Scanning electron micrographs of acetaldehyde-treated embryos showing sites of localized neural tube defects. (A) Side view of early 10th day embryo with mid-line open defect in the midbrain region (arrowed). The small indentation dorsal to the branchial arches represents the otic pit (O). (B) Frontal view of the head region of an embryo at similar developmental stage as the embryo illustrated in (A), with open mid-line neural tube defect in the forebrain region (arrowed). Note, in addition, the asymmetry of the head. (C) Side view of embryo showing mid-line neural tube defect in the upper cervical region (arrowed). Note its location between the otic pit (O) and the presumptive forelimb bud (F). (D) Side view of embryo isolated on the afternoon of the 12th day with open neural tube defects (large arrows) (i) in the midbrain and (ii) just caudal to the forelimb bud (F). Note the large number of mid-line blebs (small arrows) and the collapsed appearance of the hindbrain region (H). This embryo is developmentally somewhat retarded, and has morphological features consistent with an embryo of about the 11th day of gestation.
Acetaldehyde and neuroepithelial morphology

was very low (1/61). When injections were given on the sixth, seventh and eighth days inclusive, a slightly higher incidence was observed (1/24). Despite the fact that the incidence of resorptions was low, the average number of implants per female was similar to that observed on the tenth day (Table 1).

One of the three grossly abnormal embryos isolated on the twelfth day is illustrated in Figure 1D. This embryo has both a midbrain and high thoracic open neural tube defect, and a considerable number of mid-line subectodermal blebs. The latter were apparent only when this embryo was examined by SEM, and it therefore seems highly likely that many of the apparently normal acetaldehyde-treated embryos were similarly affected, and should therefore also have been included in the abnormal series.

Defects of the neural tube

Location of the defects

Treatment with acetaldehyde during the egg cylinder to the primitive streak stage of development frequently resulted in closure defects of the neural tube at differing levels along the embryonic neuraxis. The most frequent locations of these defects are illustrated in Figure 1. Injection on the sixth day produced no defects caudal to the forelimb buds. Defects consisted mainly of low cervical/high thoracic openings (40%) with additional defects located in the head region. Injection on the seventh day yielded predominantly hindbrain (70%) and midbrain (20%) defects, with only a relatively few (10%) neural tube defects associated with the ‘spinal’ region of the embryo. Multiple daily injections on the seventh, eighth and ninth days resulted in neural tube defects with no clear predominance in any one location. These data are summarized in Table 2.

The head region of one embryo isolated from an uninjected control female appeared to be abnormally flattened from side to side, and this embryo has been included in the abnormal group.

Observations by scanning electron microscopy

When the neuroepithelium of the acetaldehyde-treated embryos was examined by SEM, it was found to be very atypical in appearance. The cell surface was irregular, and groups of cells were often connected together by spiny intercellular processes. Individual cells were often markedly rounded-up, and had numerous small bleb-like protrusions. The typical appearance of the neuroepithelial cell surface of an acetaldehyde-treated embryo is illustrated in Figure 2 (D–F). This appearance is in marked contrast to the neuroepithelial cell surface from a similar region of a control embryo in which the dorsal roof of the neural tube was incised longitudinally with a glass needle prior to fixation and preparation for SEM (Fig. 2A–C).

In several of the ‘unturned’ embryos from the experimental series, the neuroepithelial cell surface was similar in appearance to that observed in the exposed open regions of the neural tube of the more advanced acetaldehyde-treated embryos illustrated in Figure 2 (D–F). In fact, the characteristic surface appearance of the neuroepithelial cells shown in Figure 2 (D–F) was observed at all stages examined (Fig. 3B–D).

Histological observations

Acetaldehyde-treated embryos, both with and without open defects of the neural tube, as well as control embryos, were examined histologically. The neuroepithelium
Table 2. Incidence and location of open neural tube defects, and abnormal flattening in the hindbrain region, following injection of acetaldehyde between the sixth and the ninth days of gestation.

<table>
<thead>
<tr>
<th>Injection day</th>
<th>Total embryos</th>
<th>Embryos with a neural tube defect</th>
<th>Treatment</th>
<th>Open neural tube defects</th>
<th>Forebrain</th>
<th>Midbrain</th>
<th>Hindbrain</th>
<th>Flattening</th>
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<td>9</td>
<td>Controls</td>
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<td>0</td>
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of normal embryos isolated from controls on the tenth day of gestation had a characteristic pseudostratified appearance. The cells were wedge-shaped, in close contact with each other, and were tightly apposed at their luminal surface.

The neuroepithelial cells of all embryos with an open defect, as well as those from some of the experimental embryos without an obvious surface opening, were dis-
Fig. 3. Control and experimental embryos showing typical appearance of the neuroepithelium following acetaldehyde treatment. (A) Dorsolateral view of control embryo (isolated on the 9th day) showing smooth appearance of the neuroepithelium in the headfold region (HF). (B) Acetaldehyde-treated embryo isolated on the 10th day. Note the considerable degree of developmental retardation (the embryo is still 'unturned') and the characteristic rounded-up appearance of the individual neuroepithelial cells, as observed in Fig. 2(F). (C) Side view of early 'turned' acetaldehyde treated embryo showing extensive open defects involving the cranial and caudal extremities of the neural tube. Note the considerable degree of eversion of the neuroepithelium over the surface epithelium, and characteristic cell surface appearance. (D) Frontal view of embryo illustrated in (C) showing localized site of neural tube closure in the junctional zone between the fore- and midbrain. The degree of neuroepithelial eversion is particularly well seen in this view.
Fig. 4. Transverse section through the head region of an acetaldehyde-treated embryo, showing the abnormal appearance of the neuroepithelium in the fore-(F) and hindbrain (H) regions. Note that many of the cells extend into the lumen of the neural tube (arrows). The neuroepithelium in the region of the optic vesicle (O) is also abnormal. No external defect was apparent in this embryo.

organized, and had lost their normal radial orientation. Often the cells were rounded-up, and whole cells or groups of cells were extruded into the lumen, disrupting the normal smooth luminal surface (Fig. 4).

In some regions of the neural tube, the neuroepithelial cells from either side were adherent to each other in the mid-line, either partially, or in some cases completely obscuring the lumen. In the region of the open defect, the neuroepithelial cells had often lost their normal connections, and in some locations gave the impression of having been pulled apart.

Commonly, remnants of the surface epithelium were visible on one side of the
open neural tube. The surface epithelium was often separated from the dorsal portion of the neural tube, a space or fluid-filled bleb separating the neural tube and surface epithelium.

**Ultrastructural observations**

As previously observed by light microscopy, the neuroepithelial cells from the acetaldehyde-treated embryos were rounded-up and contained condensed nuclear and cytoplasmic material. The most severely affected neuroepithelial cells, while not pyknotic, appeared to be very unhealthy.

Areas just distal to the openings were less severely affected. In these regions, although the cells had often lost their wedge-shaped appearance and had become rounded-up, they had not yet become so condensed that their ultrastructure could not be examined. In many cases, the neuroepithelial cells were tightly apposed, but there was minimal evidence of desmosome-like structures. These junctions were also devoid of microfilaments, although filamentous clumps could often be seen in the immediate vicinity in the cytoplasm, and lighter areas often marked their usual location.

Numerous cellular processes and a number of small rounded protrusions were located at the cell surface, which probably correspond to the spikes and blebs observed by SEM. These processes may have been extruded from the cells as they became rounded-up.

**DISCUSSION**

These results demonstrate that neural defects may be produced at different sites along the neuraxis, and that their location is related to the developmental stage of the embryo at the time of exposure to the teratogen. Thus, closure defects of the neural tube observed in the present study were most frequently located in the high thoracic or low cervical region when pregnant females were injected with acetaldehyde on the sixth day of gestation, i.e. at the time when embryos would be expected to be at the early egg cylinder stage of development. However, closure defects involving mainly the mid- and hindbrain regions commonly resulted when females were injected with acetaldehyde on the seventh day of gestation, i.e. at the time when embryos would be expected to be at the late egg cylinder stage of development. There also appears to be a considerable degree of compensation or reversibility regarding these defects, since a much lower incidence of neural tube defects (without an increased incidence of resorptions) was seen when embryos were examined on the twelfth rather than on the tenth day of gestation.

Many authors have suggested that formation of the neural tube requires that the neural plate acts as an integrated epithelial sheet, and that disruption of the integrity of this layer of cells itself might lead to neural tube defects (cf. Theodoses & Fraser, 1978).

Acetaldehyde appears to have a disrupting influence on the normal epithelial configuration, ostensibly resulting in various types of neural tube defects. However, these have certain fundamental features in common, which are primarily related to problems associated with the normal configuration of intercellular junctions, and an inability of individual neuroepithelial cells to undergo the morphogenetic cell shape changes required for neurulation to occur. These changes may be brought about either by factors which lead to alterations in the morphology of the junctional complexes which normally bind adjacent neuroepithelial cells together, and/or via dis-
ruption of the cytoskeletal elements, whose function is to maintain a degree of neuroepithelial cell asymmetry during neurulation.

Various compounds have been shown to disrupt the shape of individual and related groups of neuroepithelial cells. For example, Messier (1976) has reported that exposure of chick embryos to formamide resulted in cell extrusion into the lumen and failure of the neural tube to close in affected regions, while Klika & Jelinek (1963) have shown that mechanical disruption of the surface integrity of the neuroepithelium resulted in closure defects of the chick neural tube.

It has generally been concluded that the neural tube defects resulting from exposure to these various agents was due to primary non-closure of the neural tube. The findings reported in the present paper seem to suggest that acetaldehyde also may act in a similar manner. It is, of course, of interest that acetaldehyde treatment prior to and during different stages of neurulation resulted in a spectrum of damage to the neuroepithelium. The typical cell surface appearance was observed both in affected embryos with obvious neural tube defects, and in apparently normal experimental embryos in which complete closure of the surface epithelium overlying the neural tube had occurred.

Because acetaldehyde is the primary metabolic produce of ethyl alcohol oxidation and highly toxic, it seems likely that while the anomalies of the central nervous system reported here are not identical to those produced by administration of ethyl alcohol to other strains of pregnant mice (Kronick, 1976; Randall & Tabakoff, 1979), nor to those reported in children thought to suffer from the fetal alcohol syndrome (Jones et al. 1973), acetaldehyde may be one of the agents responsible for some of these teratogenic effects.

Considering the extent of the abnormal neuroepithelial features observed on the tenth day of gestation, it remains to be determined whether the postnatal viability and subsequent fertility and behaviour of the apparently normal embryos resulting from acetaldehyde treatment are impaired in any way.

**SUMMARY**

Female mice were injected intravenously with acetaldehyde on single (seventh, eighth or ninth), or on multiple (sixth to eighth, seventh to ninth) days, and examined on the tenth or twelfth day of gestation. Exposure to acetaldehyde on multiple days resulted in a high incidence of embryonic resorptions. However, when females were injected on single days and examined on the tenth day, a high incidence of neural tube defects was encountered in surviving embryos. The neural tube anomalies were located at a number of sites along the neuraxis. When examined by scanning electron microscopy, the individual neuroepithelial cells in acetaldehyde-treated embryos exhibited a characteristic rounded-up appearance with small surface blebs and spiny processes. These characteristic cell surface features were seen in acetaldehyde-treated embryos at all stages of development examined.

When additional females were examined on the twelfth day, a much lower incidence of open neural tube defects was observed. When embryos at this stage of development were examined in more detail by SEM, many had numerous subectodermal blebs along the dorsal mid-line, which were not initially apparent on gross inspection. The neuroepithelial morphology was also found to be abnormal in embryos with no obvious external anomalies.

The results confirm and elaborate on previous observations on the teratogenicity of
acetaldehyde, stressing the ultrastructural changes that are induced in the cells of the neuroepithelium, and the possible relationship between the damage induced by this agent and certain features of the fetal alcohol syndrome.

The authors would like to thank Lisa McGee for typing the manuscript. This study was supported by a grant from the National Fund for Research into Crippling Diseases, NIH Grant HD-07097 and NSF RIAS SER 77-06922.

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1 Parthenogenesis: a system facilitating understanding of factors that influence early mammalian development

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INTRODUCTION

The term parthenogenesis was first employed by Richard Owen (1849) to describe 'procreation without the immediate influence of a male'. More recently it has been defined as 'the production of an embryo, with or without eventual development into an adult, from a female gamete in the absence of any contribution from a male gamete' (modified after Beatty, 1957).

This simple definition excludes two closely related phenomena, (a) where the egg is stimulated to complete the second meiotic division and to undergo further development by a spermatozoon, which, however, does not contribute any genetic material to the developing embryo (gynogenesis), and (b) where the egg is stimulated to develop by a spermatozoon, but where the male genome alone takes part in subsequent development (androgenesis).

Interest in experimentally induced mammalian parthenogenetic development goes back to the early 1930s, with the work of Pincus and his collaborators. In the 1940s and 1950s similar experiments on the activation and subsequent pre-implantation development mainly of rabbit eggs were carried out independently by Thibault and Chang. At about the same time, Austin & Braden activated eggs of the hamster and of various rodent species, and quite commonly observed early cleavage stages. Because of the low activation rates generally achieved, and disappointing development of these embryos, interest in this subject waned (for reviews of this early work see Beatty, 1957; Graham, 1974; and also, Austin & Walton, 1960; Austin, 1961).

However, interest in this aspect of experimental embryology was rekindled by the publication of two papers in 1970 in which two completely different methods were described that were each capable of activating a high proportion of mouse eggs to develop parthenogenetically. Thus Graham (1970) treated unfertilized mouse eggs with the enzyme hyaluronidase, and successfully obtained development from the one-cell stage to the blastocyst completely in vitro, while Tarkowski and his colleagues (Tarkowski, Witkowska & Nowicka, 1970) activated mouse eggs in vivo by stimulating the oviduct of an anaesthetized female with an electric shock. In the latter paper, development beyond implantation was achieved, the most advanced embryo developing to about the eight-somite stage (for further details, see Witkowska, 1973a, b).

Since 1970, the attention of most workers in this field has focused on achieving high rates of parthenogenetic activation, and subsequent development of various classes of haploid and diploid embryos (for recent general reviews see Graham, 1974; Kaufman, 1975a; Tarkowski, 1971, 1975; and for review of methodology, see Kaufman, 1978a). It should be noted that while relatively high rates of activation and subsequent
development are regularly achieved when mouse eggs are activated in vivo, this experimental approach precludes any assessment of the development potential of different classes of parthenogenones. This applies equally to the embryos obtained from LT/Sv strain mice (Stevens & Varnum, 1974; Stevens, 1975a, b) in which a proportion of the ovulated eggs becomes activated spontaneously within the oviduct, and may, after implantation, progress to the egg cylinder stage.

If oocytes from certain F₁ hybrid mice are activated in vitro, a high proportion of the various classes of parthenogenones can be successfully cultured to the blastocyst stage completely in vitro (Kaufman & Sachs, 1976; Kaufman, unpublished). For development beyond the blastocyst stage, it is necessary to transfer embryos to suitably prepared recipients. Diploid embryos regularly develop to advanced somite stages, equivalent to about the tenth or eleventh day of normal gestation (Kaufman, Barton & Surani, 1977) whereas, under similar conditions, haploid parthenogenones have regularly developed to advanced egg cylinder stages (Kaufman, 1978b), but only rarely to the primitive streak stage (Kaufman, unpublished).

When blastocysts have been transferred to ectopic sites, for example, beneath the kidney capsule or to the testis, disorganized development rapidly ensues, often followed by teratoma formation (Graham, 1970; Graham, McBurney & Iles, 1975; Iles, McBurney, Bramwell, Deussen & Graham, 1975). Similar, but spontaneously, occurring, teratomas are also commonly present in the ovaries of LT/Sv strain mice, arising from the parthenogenetic stimulation and subsequent in situ development of ovarian oocytes (Stevens & Varnum, 1974; Stevens, 1975a). Indeed, a high proportion of benign ovarian teratomas are of parthenogenetic origin, derived from spontaneously activated oocytes that have already completed the first meiotic division and continued to develop in situ (mouse: Eppig, Kozak, Eicher & Stevens, 1977; human: Linder, Hecht, McCaw & Campbell, 1975, Linder, McCaw & Hecht, 1975, Linder & Power, 1970).

Because of the considerable difficulty in achieving reasonable rates of development to the blastocyst stage and beyond, relatively few studies have been carried out in which the development potential of haploid and diploid parthenogenetic embryos, and chimeras between parthenogenones and fertilized embryos, have been examined in detail. For the same reason, very few studies are available that have analysed either their ultrastructure or their protein profile, that would allow their morphology and biochemical activity to be compared with fertilized embryos at similar stages of development. Indeed, until quite recently, very few attempts had been made to examine the biochemical and morphological changes associated with the process of activation in parthenogenetically-stimulated mammalian ova. Several reports have now been published (for review, see Whittingham, Siracusa & Fulton, 1978) that have attempted to rectify this omission, and studies along these lines should eventually allow a comparison to be made between the initial changes that take place at and shortly after activation in mammalian and non-mammalian species.

After outlining present information regarding the activation process per se, I shall describe the various types of parthenogenone that may be encountered shortly after activation. Some of the general principles involved that determine which initial pathway of development is taken will then be considered. The ultrastructural changes observed in the oocyte shortly after activation will also be discussed in detail. Reference will then be made to present information on the development potential of different classes of haploid and diploid embryos, and to current thoughts on the possible factors that influence their pre- and early post-implantation development. I
Parthenogenesis

shall then consider some of the factors that may influence the short- and long-term survival of parthenogenones, and parthenogenetically-derived cells and tissues. Finally, I take this opportunity to speculate on possible future goals of this work, drawing attention to areas in which our knowledge is particularly scanty, and to consider briefly some additional aspects that I believe could fruitfully be pursued in depth.

Activation of the Mammalian Oocyte

Biochemical aspects

A considerable amount of information has recently been published that gives insight into the underlying mechanism of activation in the mammalian oocyte. This suggests that the simple displacement of Ca$^{2+}$ (and not other cations) from the vitelline surface is sufficient to initiate oocyte maturation (Whittingham, Siracusa & Fulton, 1978). It has been suggested that the effectiveness of certain local anaesthetics and phenothiazine derivatives in inducing activation in vitro (Siracusa, Whittingham, Codonesu & De Felici, 1978) may result from their ability to displace Ca$^{2+}$ associated with membrane phospholipids (Hauser & Dawson, 1968; Papahadjopoulos, 1970, 1972). Further, alterations in the stability of the plasma membrane of the oocyte by these agents may displace Ca$^{2+}$ ions that are thought to be involved in maintaining the unfertilized egg at metaphase II, until it is stimulated by sperm penetration, or other activating stimuli.

A sudden increase in the intracellular level of Ca$^{2+}$ may also be induced in hamster eggs by the ionophore A23187, in medium lacking in Ca$^{2+}$ and Mg$^{2+}$ salts (Steinhardt, Epel, Carroll & Yanagimachi, 1974), though others have achieved similar rates of activation in Ca$^{2+}$ and Mg$^{2+}$-free medium in the absence of the ionophore (Whittingham & Siracusa, 1978; see also Surani & Kaufman, 1977). The direct iontophoretic injection of Ca$^{2+}$ into mouse oocytes also induces high rates of activation (Fulton & Whittingham, 1978). Adequate controls were provided to exclude the effect of the injection itself, as this stimulation is capable of activating hamster oocytes, but only in the presence of extracellular Ca$^{2+}$ (Uehara & Yanagimachi, 1977).

It is likely that the release of intracellular Ca$^{2+}$ is, at least in recently ovulated eggs, involved in cortical granule breakdown (Vacquier, 1976). However, no information is yet available regarding other possible immediate sequela, such as a change in intracellular pH, or a rapid increase in metabolic activity, as undoubtedly occur in sea-urchin eggs (Johnson, Epel & Paul, 1976). Alterations in the electrical potential of the egg membrane no doubt also occur at activation, and are probably associated with changes in both the rate of influx and efflux of mono- and divalent cations. The role of calcium, in particular, in maintaining the stability of the microtubule-microfilament system in the oocyte, and the biochemical trigger for its release, remains to be determined.

Morphological aspects

It is relevant in the present context to consider what information is available on cortical granule exocytosis, as this is one of the earliest events which takes place in response to the activation stimulus. During fertilization, it is now well established that the cortical granules are principally involved in preventing polyspermy by altering the biochemical and morphological properties of the zona pellucida (Adams & Hertig, 1964; Szollosi, 1967) following evacuation of their contents into the perivitelline space.
(Yanagimachi & Chang, 1961). This change in the properties of the zona pellucida had earlier been termed the ‘zona reaction’ (Braden, Austin & David, 1954). However, sperm fusion with the vitellus per se is apparently insufficient to induce cortical granule exocytosis. This event only occurs in oocytes that have reached or passed beyond metaphase I, as prior to this stage of maturation the cortical granules are located rather deep in the cortex (Usui & Yanagimachi, 1976).

Ultrastructural analysis of aged non-activated and spontaneously activated hamster eggs (Longo, 1974; for additional references to spontaneous activation in the hamster, see Austin, 1956a; Chang & Fernandez-Cano, 1958; Yanagimachi & Chang, 1961) has confirmed that a loss of cortical granules does occur in both classes of egg. However, cortical granule loss is never completed, and a considerable number may be observed even in activated eggs isolated up to 60 h after ovulation. In addition, it was observed that the decline in the number of cortical granules was probably largely due to budding of the surface of the egg (with underlying intact cortical granules) into the perivitelline space. Only on rare occasions were cortical granules observed releasing their contents into the perivitelline space, in a manner similar to that described for the fertilized hamster egg (Szollosi, 1967).

Analysis of unfertilized rabbit eggs aged in vitro (Gulyas, 1974) indicated that a reduction in the number of these organelles beneath the plasma membrane also took place. However, unlike the situation in the hamster, cortical granules were observed to have migrated away from the surface of the egg, and were scattered throughout the cytoplasm.

Several detailed studies of the ultrastructural changes occurring in experimentally activated rabbit eggs have been carried out following cold-shock treatment in vitro (Flechon & Thibault, 1964; Gulyas, 1974; Longo, 1975) and electrical stimulation in vitro (Gulyas, 1976). Following both types of stimulation, activated eggs had a similar ultrastructural appearance. The density of the cortical granules at the periphery of the eggs was almost unaltered, though some appeared to be clumped together, while others were found to be dispersed in the cytoplasm. The few cortical granules that were discharged into the perivitelline space had an intact limiting membrane.

In the most complete study of the ultrastructural appearance of cortical granules in ageing mouse eggs, Szollosi (1971) observed that these organelles tended to migrate away from the cell membrane into the central ooplasm, and that the number remaining at the periphery of the egg was inversely proportional to the post-ovulatory age of the egg. However, in ageing mouse eggs no firm evidence of extrusion of their contents has been observed (Szollosi, 1967) unlike the situation in hamsters where a build-up of periodic acid–Schiff-positive material thought to be of cortical granule origin has been described (Yanagimachi & Chang, 1961).

Gulyas (1976) has reported that following electrical stimulation of mouse eggs in vitro most, but not all, of the cortical granules had disappeared from the surface of the egg by about an hour after activation. Similarly, Zamboni, Patterson & Jones (1976) observed loss of the majority of the cortical granules and almost total absence of Golgi complexes following electrical stimulation of mouse oocytes in vivo. These reports therefore complement an earlier observation that it took about this time for 100% of in-vitro electrically stimulated eggs to undergo a complete block to sperm penetration into the vitellus, though in a high proportion of cases the zona was impenetrable to sperm after only 8 min (Gwatkin, Williams, Hartmann & Kniazuk, 1973).

Indirect evidence for the release of a limited amount of cortical granule material into the perivitelline space following electrical stimulation, and so-called 'partial zona
reaction', comes from the work of Mintz & Gearhart (1973). With the prior knowledge that the zona becomes more resistant to digestion with proteolytic enzymes following fertilization (Smithberg, 1953; Chang & Hunt, 1956) these authors compared the 'zona lysis time' of groups of parthenogenetic and fertilized embryos. They found that, as a population, parthenogenetic mouse embryos induced by electrical stimulation showed an intermediate lysis time in the period taken to dissolve the zona pellucida with dilute pronase solution, lying somewhere between the relatively short period taken by unfertilized eggs and the rather more prolonged period taken by fertilized eggs.

Additional evidence that the zona reaction may not function normally has been provided by Chalmel (1962), who successfully fertilized rabbit eggs previously activated by cold-shock treatment. Similar observations have also been made by Austin & Braden (1954 and Austin (1956b, c) with rat eggs previously activated by cold shock, and by various anaesthetic agents.

The observations presented in this section demonstrate that the morphological changes that take place involving the cortical granules largely depend on the location of these organelles at the time of activation, and that this is clearly related to the post-ovulatory age of the oocyte and the species studied.

**Observations on the Various Pathways of Development That Activated Oocytes Might Take**

Because a degree of unfamiliarity exists, even amongst embryologists, with the various types of haploid and diploid embryos that may be induced to develop parthenogenetically, a brief classification of the various classes that may be encountered shortly after activation will be presented here. It should be noted that all of the following discussion applies to early mouse development, unless another species is specifically mentioned.

For ease of description, it is assumed that activation is carried out in vitro, and that the eggs are retained in culture for at least 24 h; further, that in order to obtain uniform populations of eggs, mice have been superovulated with exogenous gonadotrophins. It is also assumed that all eggs are ovulated as secondary oocytes, at metaphase of the second meiotic division, and that the first polar body has been extruded at the completion of the first meiotic division. If, for example, anomalies of meiotic segregation occur, such as failure of extrusion of the first polar body, a considerable number of additional pathways of development may potentially arise. As these events are encountered so rarely, they will not be considered further (for discussion of these additional theoretical pathways of development, the interested reader should consult Beatty, 1957, 1967, 1972).

The chromosomal status of individual oocytes may also be considerably complicated if autosomal or sex chromosome non-disjunction occurs during either of the meiotic divisions. This, of course, may regularly occur in certain mutant strains of mice, and in the oocytes of females bearing balanced translocations, but it is outside the general scope of this discussion (for example of analysis of this type of material, see Phillips & Kaufman, 1974).

Under normal experimental conditions, five types of parthenogenone may be distinguished within about ten hours of activation. In order to segregate these accurately, it is necessary initially to separate the activated from the non-activated eggs at the earliest convenient time after activation has been induced. This step is
usually carried out within about four to six hours of activation, in order to allow oocytes to complete the second meiotic division, and to develop one or more pronuclei.

Depending on the experimental conditions, activated eggs of four types will be distinguishable by this time. These four types are illustrated in Fig. 1.1. The various classes encountered are defined according to whether a second polar body has been extruded or not, the timing and nature of the cell division (unequal or equal cytoplasmic cleavage), and whether it occurs before or after a pronucleus or pronuclei are apparent.

Four initial classes may be distinguished.

1. Eggs that develop a single (haploid) pronucleus after extrusion of the second polar body. Embryos derived from eggs of this type contain a single clone of cells each of which is genetically identical. This is therefore a form of 'uniform' haploid development.

2. Eggs may undergo 'immediate cleavage' (Braden & Austin, 1954a). The oocyte divides into two equal-sized blastomeres each of which contains one haploid pronucleus, each derived from one of the two products of the second meiotic division. One blastomere is therefore, in a sense, the equivalent of the second polar body. Embryos derived from eggs that undergo immediate cleavage (IC) possess two clones of cells that are genetically dissimilar (the extent of dissimilarity being related to the chiasma frequency). This is therefore an example of 'mosaic' haploid development.

Other eggs may either fail to extrude a second polar body, or its extrusion may be inhibited, at the completion of the second meiotic division. One of the following classes of embryo may be formed, depending on the number of pronuclei that develop, and on whether an anomalous late cleavage division occurs after normal polar body extrusion, but before the first cleavage division.

3. A single diploid pronucleus may form, containing an amalgamation of the two products of the second meiotic division. Assuming crossing-over takes place during the first meiotic prophase, embryos that develop along this pathway will contain a uniform population, or single clone, of genetically identical heterozygous diploid cells, the degree of heterozygosity once more depending on the chiasma frequency.

4. Two haploid pronuclei may develop, each containing one of the two products of the second meiotic division. This population, the proportion depending largely on the genetic background of the strain of mice employed, may either (a) progress as one-cell eggs until the first cleavage division occurs (usually about 15 h after activation), when the chromosomes of the two pronuclei amalgamate on the first cleavage spindle, to give rise to a diploid embryo, which is genetically identical to the type discussed in group 3 above, or (b) cell division may occur within a few hours of pronuclear formation (see 5, below). In the former case two pronuclear chromosome sets are in a uniform state of condensation at prometaphase of the first cleavage division (Kaufman, 1973a), unlike the situation in fertilized eggs where the two prometaphase groups of chromosomes show different degrees of condensation prior to 'syngamy' at metaphase of the first cleavage mitosis (Donahue, 1972). These embryos will contain a uniform population of genetically identical heterozygous diploid cells.

5. A proportion of the eggs that initially develop two haploid pronuclei may cleave a few hours later, usually within seven to ten hours of activation. Because of the potential presence of this additional group of embryos, it is imperative that the first selection procedure occurs before this late cleavage event takes place. Segregation of this group from the two-pronuclear eggs that do not undergo this 'late' or 'delayed'
cleavage can take place at any time between about 10 and 24 h after activation. Thus, in the latter case, this group may be easily distinguished by the fact that they have four blastomeres after the first cleavage division has occurred, whereas the heterozygous diploid embryos (group 4, above) have only two blastomeres at this time. This class was first observed by Graham (1971), and termed 'delayed immediate cleavage'. This has subsequently been abbreviated to 'delayed cleavage' (DC) (Kaufman & Sachs, 1976). Embryos of this type are genetically identical to the IC embryos (group 2, above), although their development potential is significantly different. Possible reasons for this difference are discussed in detail later.

The major pathways of development discussed here are illustrated diagrammatically in Fig. 1.1.

FACTORS THAT INFLUENCE THE PATHWAYS OF DEVELOPMENT TO BE TAKEN BY ACTIVATED OOCYTES

For most of the commonly employed in-vitro activation techniques, the rate of activation is principally controlled by the post-ovulatory age of the oocyte. Once activation has been induced, it is then possible to vary the incidence of the different
classes of parthenogenone, by modifying the culture conditions either during the immediate post-activation period, or over a more extended period of possibly 3–5 h, during which time activated eggs are completing the second meiotic division and developing one or more pronuclei. For example, when oocytes that have been ovulated and retained in the oviduct for about 6 or 7 h are released into culture medium containing hyaluronidase (Kaufman, 1973b), a high proportion (about 60–70%, but this varies considerably between strains of mice, see Graham & Deussen, 1974; Tarkowski, 1975) become activated. Virtually all of these eggs extrude a second polar body, and shortly thereafter develop a single haploid pronucleus (class 1, previous section). This class is essentially (at least, morphologically) identical to the female component of the zygote shortly after fertilization. The unequal segregation of cytoplasm presumably results from the peripheral location of the spindle and its intimate relationship with the plasma membrane at the time of completion of the second meiotic division.

However, when eggs are released from the oviduct into medium containing hyaluronidase about 13 h after ovulation, a similar activation rate is achieved, but a high incidence of IC embryos are now obtained (Kaufman, 1973b). This dramatic change from a very high incidence (95–99%) of one-pronuclear embryos to a high proportion (about 60%) of IC embryos is likely to be due to age-related changes in oocyte membrane stability. One of the manifestations of the gradual loss of membrane stability is the 'release' from the periphery of the post-ovulatory aged egg, and subsequent central migration of its second meiotic spindle (Szollosi, 1971).

Indirect evidence suggesting that the second meiotic spindle is, at least initially, intimately related to the oocyte plasma membrane, possibly by arrays of cytoskeletal elements passing from the spindle to the periphery of the egg, comes from a recent study by Modlinski (1975). He observed a considerable difference in the development potential of fertilized eggs from which one or other of the pronuclei had been withdrawn mechanically. When the male pronucleus was withdrawn, little detrimental effect on subsequent cleavage was observed. However, removal of the female pronucleus had a profound effect, severely restricting further development. This was evident even in androgenetic eggs with an XX genotype. Modlinski (1975) therefore concluded that a greater degree of mechanical disruption must have occurred during the microsurgical removal of the female rather than the male pronucleus. More recently, Tarkowski (1977) has clearly demonstrated that haploid androgenetic embryos, produced by bisection of one-cell fertilized eggs, that carry a Y rather than an X chromosome can cleave twice, but did not appear to survive beyond the four-cell stage.

Additional indirect evidence bearing on the possible importance of the stabilizing role of the cytoskeletal elements associated with the plasma membrane of the one-cell embryo, comes from a recent observation by Hoppe & Illmensee (1977). These authors observed that long-term survival rate of experimentally manipulated fertilized eggs was increased significantly by preincubating them in the presence of cytochalasin B for 1 h before one or other of the pronuclei was removed microsurgically. Enucleated haploid embryos were subsequently incubated overnight in cytochalasin B to obtain homozygous diploid embryos. That a proportion of these embryos survived to birth suggests that the stabilizing effect of preincubation in cytochalasin B prior to microsurgery may have played a considerable role in facilitating the survival of these embryos.

Attempts to use cytochalasin B to diploidize haploid parthenogenetic embryos have
Parthenogenesis been less successful. In the most extensive series in which parthenogenetic material has been used, Balakier & Tarkowski (1976) inhibited second polar body extrusion in embryos activated in vitro by heat shock. Following this treatment, a high incidence of two-pronuclear eggs was obtained, and also embryos that developed a single diploid pronucleus. However, of those transferred to pseudopregnant recipients, only two healthy-looking, but developmentally retarded, egg cylinders were obtained on the eighth and ninth day of gestation.

If only heat-shock treatment is applied to oviducts containing ova, either in vivo (Braden & Austin, 1954a, b) or in vitro (Komar, 1973; Balakier & Tarkowski, 1976), most activated eggs either extrude a second polar body and develop a single haploid pronucleus, or undergo immediate cleavage. The short pulse of heat involved in these techniques, lasting at most 5 or 10 min, and the even shorter duration in the case of electrical stimulation of the oviduct in vivo (Tarkowski et al., 1970), is presumably of insufficient duration to produce complete and/or irreversible denaturation of the meiotic spindle apparatus. The relatively high incidence of the one-pronuclear and IC classes of embryos, and low incidence of eggs in which second polar body extrusion was completely suppressed, seems to bear out this point. The application of heat shock to recently fertilized mouse eggs in vivo produced a significant increase in the number of eggs exhibiting suppression of second polar body formation (Braden & Austin, 1954a, b), and was the commonest cause of triploidy following this treatment.

Several experimental situations may facilitate the production of a high incidence of parthenogenetically activated eggs in which second polar body extrusion is suppressed. This has generally been achieved by modifying the culture conditions during and immediately following activation. The modifications that have most regularly been investigated all probably produce their effect by acting on the meiotic spindle apparatus and associated cytoskeletal elements. Thus, for example, a high proportion of two-pronuclear eggs are regularly obtained when activated eggs are incubated in extremely low osmolar medium (Graham, 1971, 1972; Graham & Deussen, 1974; Kaufman & Surani, 1974). A smaller but still significantly increased incidence of IC embryos (compared to control values when eggs are activated by hyaluronidase treatment) is also obtained following exposure to low osmolar conditions. During the immediate post-activation period, when eggs were incubated in low osmolar medium, protein synthesis, as monitored by the uptake of labelled amino acids by the egg, fell to a very low level. Rapid recovery to normal levels of incorporation were, however, observed shortly after eggs were transferred to standard embryo culture medium. A concomitant delay of similar duration to the time spent in low osmolar medium was also observed in the time of entry of activated eggs into the first cleavage division (Kaufman & Surani, 1974).

Similar, though rather more rapid, cytological changes have been observed when recently fertilized mouse eggs (isolated approximately 6–8 h after induced ovulation, or, following spontaneous ovulation, early in the morning on the first day of pregnancy) were incubated for between 2 and 6 min in distilled water (Opas, 1977). In about half of the eggs examined, permanent incorporation of the second polar body nucleus into the egg cytoplasm occurred, accompanied by rapid and intense swelling of the egg vitellus. This resulted in the build-up of extreme pressure within the perivitelline space, compression of the second polar body, and its subsequent fusion with the cytoplasm of the egg.

A significant increase in the incidence of embryos in which second polar body extrusion was suppressed, and IC embryos obtained, was also observed following
activation and culture in medium lacking Ca\(^{2+}\) and/or Mg\(^{2+}\), whether the latter was of long (4–6 h) or relatively short (0.5–1 h) duration (Surani & Kaufman, 1977; Kaufman, unpublished).

Activation and initial incubation of eggs (at least for a few hours) in the absence of Ca\(^{2+}\) and Mg\(^{2+}\) does not appear to be detrimental in the long term, as embryos whose development has been initiated by this means have progressed at least as far as the fore-limb bud stage, equivalent to about the 10th day of normal development (Kaufman et al., 1977). It may be that the potentially harmful effect of releasing eggs into Ca\(^{2+}\)/Mg\(^{2+}\)-free medium may be reduced by the presence of the small volume of follicular fluid that inevitably passes into the culture drop with the cumulus mass. The follicular fluid, though rapidly diluted, presumably provides sufficient quantities of these ions necessary to stabilize both the cytoplasm of the oocyte, and its plasma membrane.

The activation frequency following incubation in Ca\(^{2+}\)/Mg\(^{2+}\)-free medium is related to the post-ovulatory age of the oocyte at the time of activation, in a similar manner to that observed following activation by handling alone, or hyaluronidase treatment (Kaufman, unpublished). This technique thus provides a most convenient means of obtaining approximately equal proportions of both haploid (IC) and diploid parthenogenetic embryos (for additional technical details, see Kaufman, 1978a).

In a brief study, Miyamoto & Ishibashi (1975) showed that capacitated sperm were unable to penetrate through the zona pellucida in calcium-free medium. In addition, the absence of this ion from medium is known to decrease the rate of cleavage of two-cell mouse embryos (Wales, 1970) and to prevent the development of eight-cell mouse embryos (Whitten, 1957). Miyamoto & Ishibashi (1975) also noted that a high proportion of both mouse and rat eggs became activated and developed pronuclei in the calcium-free medium. However, the incidence of the various types of parthenogenone observed were not detailed. It is of interest that in this study the cumulus clots were washed repeatedly in order to remove the majority of the follicular fluid.

The other in-vitro activation technique that tends to increase the proportion of activated eggs that fail to extrude a second polar body, is heat shock (Komar, 1973), though at all temperatures tested, one-pronuclear haploid eggs predominated over diploid. As indicated earlier, Balakier & Tarkowski (1976) investigated the effect of cytochalasin B on eggs previously activated by heat shock, and successfully suppressed second polar body extrusion in over 90% of the activated eggs, with the majority developing two pronuclei.

It should be noted that the activation technique employed may influence (in absolute terms) the development potential of individual classes of parthenogenone, though in all of the systems tested, diploids tend to develop (for example, from the one-cell to the expanded blastocyst stage) more favourably than mosaic haploid embryos, and all of the former groups generally develop better than uniform haploid embryos. Thus, when oocytes from (C57BL × CBA) F\(_1\) hybrid mice were activated following hyaluronidase treatment, and retained in culture for up to 120 h, a high proportion developed to the expanded blastocyst stage (Kaufman & Sachs, 1976). In this study, 58% of diploid parthenogenones (2-pronuclear type), 55% of the IC (mosaic) haploids, and 21% of the one-pronuclear (uniform haploid) class developed to the expanded blastocyst stage.

When genetically similar oocytes were activated in Ca\(^{2+}\) and Mg\(^{2+}\)-free medium (Kaufman, unpublished) the proportion of activated eggs that develop was even higher, with about 80% of the diploids (2-pronuclear type), and approximately 35% of
Table 1.1. Development of parthenogenetic eggs to the blastocyst stage in culture

<table>
<thead>
<tr>
<th>Type</th>
<th>Ploidy</th>
<th>Total activated eggs</th>
<th>Blastocysts (%) By day 5</th>
<th>Blastocysts (%) By day 6</th>
<th>Total Blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One-pronucleus + 2PBa</td>
<td>Haploid</td>
<td>63</td>
<td>0</td>
<td>7 (11.1)</td>
<td>7 (11.1)</td>
</tr>
<tr>
<td>Immediate cleavage</td>
<td>Haploid</td>
<td>181</td>
<td>49 (27.1)</td>
<td>15 (8.3)</td>
<td>64 (35.4)</td>
</tr>
<tr>
<td>Delayed cleavage</td>
<td>Haploid</td>
<td>40</td>
<td>14 (35.0)</td>
<td>5 (12.5)</td>
<td>19 (47.5)</td>
</tr>
<tr>
<td>Two-pronuclei – 2PBa</td>
<td>Diploid</td>
<td>169</td>
<td>128 (75.7)</td>
<td>7 (4.1)</td>
<td>135 (79.9)</td>
</tr>
</tbody>
</table>

The overall activation frequency in these experiments was 60.9%. Activation was carried out in medium lacking Ca²⁺ and Mg²⁺ salts.

a Second polar body.

the IC type developing to the expanded blastocyst stage in culture (see Table 1.1). About half of both the haploid and diploid blastocysts had formed by approximately 100 h after activation, early on the fifth day of culture. A high proportion of the remaining blastocysts were obtained on the morning of the sixth day. However, the most rapidly developing diploid embryos were often encountered as early as 80–84 h after activation, late in the afternoon on the fourth day. Occasionally, and this was particularly evident in the one-pronuclear embryos, apparently morphologically abnormal morulae developed a blastocele cavity between the sixth and seventh day in culture. The development potential and morphological characteristics of these developmentally retarded blastocysts has yet to be determined.

ULTRASTRUCTURAL CHANGES

Effect on meiotic spindle apparatus and cytoskeletal elements

One of the earliest indications of activation following electrical stimulation of mouse eggs was the movement of the second meiotic spindle from the tangential to the radial position (Gulyas, 1976). This was often associated with an accelerated migration of the spindle from the periphery to the centre of the egg, comparable to the central migration of the spindle that has been observed following oocyte ageing within the oviduct (Szollosi, 1971). By contrast to the situation normally following fertilization, and to a lesser extent as a result of ageing, there was often evidence of damage to the spindle apparatus. However, usually within an hour of electrical stimulation, somewhat disorganized microtubular arrays appeared, reforming portions of the spindle. The considerable intra- and inter-species variability in the response of rabbit, hamster and mouse oocytes activated in vitro by electrical stimulation observed by Gulyas (1976) probably reflects real differences in local conditions within individual oocytes in response to the very short electrical pulse employed.

In the rabbit, microfilaments within microvilli became distorted a very short time after electrical stimulation, in a manner typical of vitelline membrane changes associated with fertilization. The changes observed also resembled those seen in hamster eggs aged for more than 20 h in vivo following ovulation (Longo, 1974).

Little direct ultrastructural evidence is available regarding the effect of modifying
the environmental temperature on the cytoskeletal system of mammalian oocytes, apart from the observations of Longo (1975) in this regard. He activated rabbit oocytes by cold shock, and examined the fine morphology of the meiotic spindle apparatus. The most obvious effect of this treatment was a reduction in the number of microtubules making up the spindle fibres of the meiotic apparatus, and a concomitant local accumulation of small vesicles. This observation is consistent with previous studies using other biological systems in which temperature changes have been shown to alter the equilibrium between microtubular protein in the polymerized and in the monomeric condition (Inoue, 1964; Inoue & Sato, 1967; Roth, 1967; Tilney & Porter, 1967).

As has been indicated earlier, the susceptibility of oocytes to parthenogenetic activation increases with post-ovulatory age. Only very strong stimuli, such as electric, heat or cold shock, are effective in activating recently ovulated eggs. Such stimuli may damage a considerable proportion of the treated eggs, and these often degenerate immediately or shortly thereafter. As a direct consequence of irreversible damage to the cytoskeletal elements of the spindle apparatus, abnormal nuclei may form that do not contain either a complete haploid or diploid genome. This situation arises particularly following stimuli that tend to inhibit second polar body extrusion. In addition to apparently normal pronuclear development, one or more subnuclei may be formed, each containing one or a few chromosomes that have become detached from the spindle apparatus. Alternatively, aneuploidy may arise as a result of unequal segregation of the two products of the second meiotic division (i.e due to non-disjunction). This may not be important in the long term if all of the meiotic products remain in the egg and reaggregate on the first cleavage spindle, though the chances of losing at least one chromosome following subnuclear formation must be considerable (for observations on subnuclear formation, see Tarkowski, 1975).

Graham (1974; and see Graham & Deussen, 1974) has suggested that unequal segregation of the two meiotic products often, in his experience, followed immediate cleavage. This should lead to the production of 'complementary' hypo- and hyper-haploid clones of cells. However, karyological analysis of IC-derived blastocysts and egg-cylinder-stage embryos have only demonstrated euploid chromosome complements (Kaufman & Sachs, 1976; Kaufman, 1978b), though it is likely that aneuploid populations of cells would tend to die during early cleavage.

It may also be argued that eggs that have aged for a considerable period prior to activation may have an increased susceptibility towards aneuploid development following activation, comparable to the situation observed following the fertilization of aged oocytes (Marston & Chang, 1964). However, this has not been the experience of this author who has carried out karyological analysis of possibly thousands of pre- and a few early post-implantation parthenogenetic embryos. While it is likely that all of the hypo-haploids would have been eliminated during early cleavage (for observations on pre-implantation development of haploid and aneuploid embryos see Kaufman & Sachs, 1975), had they been present some would surely have been observed in metaphase plates of the first or second cleavage division, as many of these spreads were prepared to examine this very point (Kaufman, unpublished).

**Effect on surface features of the cell**

The surface topography of unfertilized and pre-implantation stages of fertilized mouse eggs and embryos has been studied by transmission and scanning electron microscopy (for a recent atlas of these stages, see Van Blerkom & Motta, 1979), and provides
Parthenogenesis

insight into some of the earliest morphogenetic changes that take place during embryogenesis. Thus Szollosi (1971) and others (Eager, Johnson & Thurley, 1976; Gulyas, 1976; Nicosia, Wolf & Inoue, 1977) have observed a pronounced asymmetric al distribution of microvilli between the region of the vitellus overlying the spindle and the rest of the oocyte, and between the two products of the first meiotic division. The surface of the oocyte distant from the spindle apparatus is covered by short microvilli, whereas the 20% or so of the surface in close proximity to the first polar body, and the surface of the polar body itself, is covered by a smooth oolema. The sub-cortical zone underlying the surface in the region of the spindle is also devoid of cortical granules. It has also been observed that sperm entrance sites were almost always confined to the cortical granule-rich region away from the area overlying the meiotic spindle apparatus (Johnson, Eager, Muggleton-Harris & Grave, 1975).

Observations on pre-morula stage fertilized mouse embryos (Ducibella, Ukena, Karnovsky & Anderson, 1977) indicated that microvilli were uniformly distributed over the entire cell surface of each blastomere. During early compaction, however, the microvilli became restricted to an apical region of the cell, and at the basal zone of intercellular contact. By the early morula stage, a dense zonular localization of microvilli was present in the intercellular furrows, while a relatively sparse population covered the rest of the cell surface facing the zona pellucida.

No comparable detailed observations are available on the surface topography of early mouse parthenogenones. This information would be of great interest, considering the variety of pathways that may be taken by parthenogenetic embryos, and remembering that the fate of individual oocytes is probably largely controlled by the state of their cytoskeletal elements shortly after activation.

Preliminary ultrastructural observations on IC embryos examined by transmission electron microscopy prior to the completion of cytokinesis (Kaufman, unpublished) have not been able to demonstrate any very obvious differences between the surface morphology of the two blastomeres. Possibly the central movement of the spindle from the periphery of the oocyte shortly after activation in some way released the surface previously overlying the spindle from its local inhibitory effect, thus enabling it to form microvilli. Alternatively, the presence of a functional nucleus within each blastomere may, in some way, have influenced the surface of both blastomeres to form microvilli in the areas overlying the spindle apparatus.

If it is assumed that one blastomere represents the second polar body, it might have been expected that its surface would be smooth (as this is the case following fertilization, see Eager, Johnson & Thurley, 1976), or at least that it would have a considerably reduced number of microvilli compared to that present on the other blastomere. Alternatively, since both products of the second meiotic division are presumably equipotential, it might be reasonable to expect that both cleavage products might also be identical.

In this context, it is interesting to compare the above observations with the findings of Wassarman, Ukena, Josefowicz & Karnovsky (1977) where an anomalous division (termed 'pseudocleavage', Wassarman, Albertini, Josefowicz & Letourneau, 1976) of the germinal vesicle stage oocyte into two equal-sized compartments was induced following prolonged treatment with cytochalasin B. Characteristically the anucleate 'blastomere' had a microvillous surface (the surface of the nucleate 'blastomere' was smooth), and mitochondria were localized at the periphery of the anuclear, but not the nucleate compartment. The authors suggested that the dissimilar surface features of the two 'blastomeres' may indicate that some form of communication (presumably
biochemical or cytoskeletal in nature) exists between the germinal vesicle and the oocyte surface during meiotic maturation.

Other changes in the properties of the egg surface that may be induced by parthenogenetic activation may closely resemble that seen following fertilization. For example, mouse eggs activated by hyaluronidase treatment agglutinated in the presence of very low concentrations of Concanavalin A, as did fertilized eggs, whereas unfertilized eggs required much higher concentrations (Pienkowski, 1974).

**General observations on cellular morphology of parthenogenones**

Information regarding the initial morphological changes that take place, particularly in mouse eggs, following other types of parthenogenetic stimulation than those referred to above are lacking. It is therefore unclear whether the range of effects described here, typically take place in response to parthenogenetic stimulation, or are specific to the stimuli that have been investigated. In addition, the considerable variation in the response reported between different species makes it difficult to extrapolate from one to another.

The ultrastructural changes that have been observed following parthenogenetic stimulation are in many respects quite different from those seen after fertilization. However, the significance of these differences is unclear. For example, cortical granules were presumably evolved to prevent polyspermy via the biochemical action of their contents on the zona pellucida, thus inducing the zona reaction. Following parthenogenetic activation, does it really matter whether the cortical granules remain intact, migrate away from the plasma membrane, or indeed whether they discharge their contents into the perivitelline space? Probably not, unless they play an additional, and as yet undetermined, role in initiating other changes to take place within the vitellus.

What of the changes that have been observed in the spindle and cytoskeletal architecture of the cell following parthenogenetic stimulation? These changes, and this particularly applies to the rotation and central migration of the spindle, and possibly transient damage to the cytoskeletal elements, are likely to be both species specific, and related to the type of stimulation applied. This presumably explains the diverse, though generally fairly consistent, response of populations of activated eggs to different forms of stimulation, and post-activation conditions. In fact, it would be quite reasonable to expect that the gross effects observed following electrical stimulation in vitro may be dissimilar to that observed following heat or cold shock, culture in low osmolar medium, or following hyaluronidase stimulation.

Ultrastructural observations on pre-implantation parthenogenetic embryos, activated by electrical stimulation in vivo and following hyaluronidase treatment in vitro, are also available (Van Blerkom & Runner, 1976). In this study, development to the blastocyst stage was uncommon, most eggs failing to develop beyond the two cell stage. Despite these difficulties, a considerable number of parthenogenetic embryos were examined, and the general conclusion drawn that following both types of stimulation the morphological appearance of these embryos was quite similar to that of normal fertilized embryos at comparable stages of development (Calarco & Brown, 1969; Hillman & Tasca, 1969; Van Blerkom & Motta, 1979). The only difference observed between fertilized and parthenogenetic blastocysts was the comparative rarity in the cytoplasm of the latter of crystalloid bodies. However, the significance of this observation in relation to the development potential of parthenogenones has yet to be determined. The eggs isolated following in-vitro activation were undoubtedly
haploid, and of the one-pronuclear type. However, the ploidy of the in-vivo activated eggs is unclear, as these were isolated some considerable time after the first cleavage division had taken place. This study also confirmed the earlier observation of Biczysko et al. (1974), that the expression of the information for the development of virus-like particles does not require fertilization. However, it failed to confirm the finding of Solter et al. (1974) that the ultrastructure of early cleaving parthenogenones was often severely disturbed. In retrospect, it seems likely that the embryos examined by the latter group had only a limited viability, and would not, in any case, have developed beyond the eight-cell stage.

Observations on the histological appearance of a considerable number of diploid 20–25 somite stage parthenogenetic embryos, and a few even more advanced embryos (Kaufman et al., 1977; Kaufman, unpublished) would seem to confirm that parthenogenetic development per se does not inhibit a proportion of eggs activated in this way from progressing at least to a stage equivalent to the tenth or eleventh day of normal fertilized development. Haploid embryos activated in Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free medium have progressed to the late egg-cylinder stage (Kaufman, 1978b) and also appear to be morphologically normal.

All of these parthenogenones were activated and allowed to develop for 4–5 h in media lacking Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, conditions which undoubtedly induce transient modifications to the normal cytoskeletal architecture of the cell. Whittingham & Siracusa (1978) have also transferred eggs activated in Ca\textsuperscript{2+}-free medium to pseudo-pregnant recipients. A few of the implantation sites contained giant cells, while others contained evidence of disorganized embryonic tissue.

The other stimuli that have given rise to parthenogenetic embryos that have been capable of at least some degree of post-implantation development, in addition to electrical stimulation in vivo (Tarkowski et al., 1970; Witkowska, 1973a, b), are avertin anaesthesia induced in spontaneously ovulating and superovulated pseudo-pregnant females at appropriate times after ovulation (Kaufman, 1975b), and eggs previously activated in vitro by hyaluronidase treatment (Kaufman & Gardner, 1974).

In addition to the experimentally induced parthenogenones indicated above, Stevens & Varnum (1974; see also Stevens, 1975a, b) have observed the occasional presence of egg-cylinder stage embryos both within the ovaries and in the uteri of LT/Sv strain mice in which ‘spontaneous’ activation and development of oocytes has taken place either within the ovary, or following ovulation.

The extent of development achieved by parthenogenetic mouse embryos in utero is considered in more detail in the next section.

THE EXTENT OF NORMAL DEVELOPMENT ACHIEVED BY PARTHENOGENETIC MOUSE EMBRYOS

Various attempts have been made to determine the extent of development that may be achieved by parthenogenetic mouse embryos. For example, Tarkowski et al. (1970; for further details see Witkowska, 1973a, b) examined the fate of oocytes activated in vivo by electrical stimulation, and observed that a high proportion of the embryos that implanted failed to develop beyond the egg-cylinder stage. In fact, only a single, but apparently quite normal, eight-somite embryo was observed in this study. In another in-vivo study, female mice were anaesthetized with avertin at various times after ovulation (Kaufman, 1975b). A proportion of the activated oocytes developed beyond implantation, though the most advanced embryos observed only progressed to the
egg-cylinder stage. In both of these studies, the ploidy of the most advanced embryos was not determined, though in the latter study they were almost certainly derived from IC embryos.

This relatively poor rate of post-implantation development observed following in-vivo activation has proved to be a considerable problem, even in LT/Sv strain mice where spontaneous activation and development of ovulated oocytes occurs quite regularly (Stevens & Varnum, 1974; Stevens, 1975a, b; Eppig, 1978). When attempts were made to increase the number of implants, for example, by superovulating the LT/Sv females and mating them with vasectomized males, embryonic development beyond the egg-cylinder stage was still an extremely rare occurrence. The most advanced relatively normal development so far reported in this strain was a dead embryo that had 'about six pairs of somites, a wavy neural tube, and heart muscle present' (Stevens, 1975a).

Chromosomal analysis of the advanced embryos obtained from these in-vivo and 'spontaneous' parthenogenetic studies, even if available, would provide very little information on the initial ploidy and pathways of development taken by these activated oocytes.

Similar, rather poor rates of development have also been achieved when in-vitro activated embryos were transferred to pseudopregnant recipients, and observations made on the eighth day of 'pregnancy'. Parthenogenones transferred at the pronuclear stage developed to the blastocyst stage, and 50-56% of diploids and 35-36% of haploid embryos evoked a decidual response (Kaufman & Gardner, 1974). Very few embryos in this study developed to morphologically normal egg cylinder-like structures (Kaufman, unpublished).

In an early attempt to increase the likelihood of obtaining development at least to the egg-cylinder stage, parthenogenetic blastocysts were flushed from the uteri of pseudopregnant recipients, and re-transferred to the uteri of asynchronous recipients (Kaufman & Gardner, 1974). Embryos equivalent to about day 3½ of normal fertilized development were transferred to the uteri of day 2½ pseudopregnant recipients. Whilst very high rates of implantation were achieved by this manoeuvre, no significant increase in the development of embryos to the egg-cylinder stage was achieved.

Various possible explanations for the universally poor rates of development must be considered. Firstly, the degree of synchrony between parthenogenones and the uterus of the host may be such that embryos may only be sufficiently developmentally advanced to evoke a decidual response when the uterus is no longer in an optimal receptive phase. The results of the asynchronous transfers just described would seem to argue against this being the complete explanation. In any case, very high rates of implantation were achieved in these studies. The latter observation suggested that the trophectoderm cells, essential in evoking the decidua response, were probably adequate in number and functionally competent.

A second possible explanation is that the inner cell mass cells might in some way be deficient. For example, their immediate derivatives might simply not provide enough stem cells from which an embryo could be formed. This second hypothesis has been tested by two different experimental approaches, the principal aim of each being the production of a considerable increase in the inner cell mass population within the embryo at the time of implantation. In the first experimental attempt to study this problem, parthenogenetic diploid blastocysts were held in 'delay' (i.e. a state of embryonic diapause was achieved) before implantation was allowed to occur (Kaufman et al., 1977).
In order to obtain ‘delayed’ parthenogenetic blastocysts, embryos that had developed completely in vitro, and were therefore of known genotype, were transferred to pseudopregnant recipients that were ovariectomized at the time of transfer (Kaufman et al., 1977). The pseudopregnant state was maintained by daily injections of progesterone. During this period, blastocyst attachment did not occur. When, however, a single combined injection of oestrogen and progesterone was given, implantation followed approximately 12 h later.

In this study, about 80% of the transferred blastocysts eventually implanted after a ‘delay’ period of between seven and ten days. About 40% of the implantation sites examined up to the equivalent of between the fifth and eighth days of fertilized development contained morphologically normal egg-cylinder stage embryos. When development was allowed to proceed further, up to 25% of the implantation sites contained somite embryos. The most advanced embryos obtained in this and in several later studies were apparently normal and healthy, and contained on average about 25 somites. On histological examination, one of these quite advanced embryos from the first series of experiments was found to have an extensive midline open neural-tube defect located caudal to the fore-limb buds (Kaufman, unpublished). In the same series, one set of monoamniotic monzygotic twins was obtained, and several disorganized teratoma-like growths with various distinct cell types present. In a subsequent series of experiments (Kaufman, unpublished), one recipient contained four embryos equivalent to about the eleventh day of normal fertilized development. These embryos contained both fore- and hind-limb buds, but were morphologically abnormal. The principal feature all had in common was the considerable reduction in the neural tissue, both in the developing brain and spinal cord, compared to that normally present in fertilized embryos at a similar stage of development. Additional embryos at a similar stage of development have subsequently been examined, and, though dead at the time of autopsy, they seemed to be morphologically quite normal (Kaufman, unpublished).

In a later study, using a similar experimental approach (Kaufman, 1978b), IC-derived presumptive haploid blastocysts were transferred to recipients, and reasonable rates of development to the egg-cylinder stage achieved.

An alternative means of increasing the total number of cells in parthenogenetic embryos at the time of implantation, and effectively increasing the total inner cell mass population at the same time, may be achieved by the production of aggregation chimeric embryos. Thus, Stevens (1978) aggregated up to four parthenogenetic 4-8 cell embryos derived from LT/Sv strain mice which were then transferred to pseudopregnant recipients. Out of a total of 55 such aggregates transferred, only five were recovered after implantation. One recipient contained two morphologically abnormal egg cylinders, while the second recipient contained two early, but apparently unhealthy, egg cylinders, and a single very abnormal primitive streak-stage embryo.

More recently, Kaufman and Handyside (unpublished) have aggregated groups of two or three early compacted (C57BL × CBA)F₁ hybrid diploid parthenogenetic morulae. These subsequently developed in culture into ‘giant’ blastocysts, which were then transferred to the uteri of suitable recipients. In a preliminary experiment, three such aggregates were transferred to a single recipient, and all successfully implanted. At autopsy on the morning of the eleventh day of pseudopregnancy, one implantation site contained a fore-limb bud-stage embryo with about 20-25 somites. This embryo appeared to be morphologically normal, but had obviously died shortly beforehand. The second implant contained a rounded mass of tissue which, on histological
examination, turned out to be a disorganized teratoma-like tumour. However the constituent cells appeared to be healthy. The third implant only contained a few embryonic giant cells.

**Observations on the Development Potential of Parthenogenetically-Derived Cells and Tissues**

*In vivo*

Various studies have been carried out in which haploid and diploid parthenogenones have been transferred to ectopic sites, and numerous differentiated cell types observed in the teratomas that subsequently developed (Graham, 1970; Iles et al., 1975; Graham et al., 1975). Indeed, the only cells that were commonly observed following the transfer of fertilized, but not parthenogenetic, embryos to ectopic sites were embryonal carcinoma cells. Graham et al. (1975) have suggested that the apparent absence of these cells might have been related to their origin from primordial germ cells, as cells of this type are first clearly observed in fairly advanced somite embryos. The point was also made that parthenogenetically-derived tumours were usually more slowly growing than similar tumours derived from the ectopic transfer of fertilized embryos (Graham et al., 1975). The ploidy of these tumours also tended to alter with their growth. This was particularly the case with teratomas that had been derived from presumptive haploid embryos. What was most remarkable was the considerable degree of cytodifferentiation achieved in many of these tumours.

The apparent ability of parthenogenetically-derived diploid cells to develop into numerous well-differentiated cell types in these tumours strongly suggested that it should be possible to extend the developmental potential of parthenogenetic embryos beyond the early somite period.

In order to investigate this possibility, chimeras have been made by aggregating diploid parthenogenones with fertilized embryos. To date, a reasonable number of these chimeras have survived beyond birth, and their fertility has, in some cases, been tested. The approach taken by Stevens, Varnum & Eicher (1977) was to aggregate eight-cell parthenogenetic with eight-cell fertilized embryos. Out of a total of 456 aggregates transferred to recipients, six live-born chimeric offspring were obtained. In one female chimeric mouse, breeding studies clearly demonstrated that chimerism extended to the gonads, with the production of functional germ cells (Stevens, 1978). Surani et al. (1977), using a different experimental approach, also obtained chimeric development to term following the microsurgical transfer of inner cell masses isolated from diploid parthenogenetic blastocysts into intact fertilized blastocysts. The reconstituted blastocysts were subsequently transferred to pseudopregnant recipients. A high proportion of these embryos implanted, and the incidence of detectable chimerism was extremely high (see Fig. 1.2).

In both of these studies, the extent of chimerism within individual foetuses and live-born mice was considerable, and virtually all of the tissues examined contained parthenogenetically-derived cells. The obvious conclusion to be drawn from this, and the earlier studies on teratoma induction, seems to be that parthenogenetically-derived cells appear to be capable of differentiating normally, but require some form of 'support' from fertilized cells in order to manifest their complete development potential.

It has yet to be determined whether their contribution to all of the organs and tissues tested was a real one, in that they were providing differentiated functional cells.
that freely intermixed with similar cells of fertilized origin. The possibility has to be considered that on some locations their contribution may have been in the form of less well-differentiated cells, for example, mesenchymal or connective tissue elements, such as are found in all organs and tissues.

in vitro

The developmental potential of parthenogenones has mainly been studied in vivo. This approach suffers from the disadvantage that a proportion of embryos transferred to recipients fail to implant, or die shortly thereafter and are lost. A more direct approach to this problem is to examine the development of groups of isolated cells e.g. inner cell masses, ectodermal cores, or complete parthenogenetic embryos, in vitro. In this way, potential information concerning the development of different cell types can be analysed alongside attempts to isolate cell lines.

Preliminary experiments (Kaufman and Handyside, unpublished) have shown that it is possible to culture diploid (and to a lesser extent haploid) parthenogenones to proamniotic cavity egg-cylinder stages in vitro. This approach should facilitate the production of enough material for, for example, enzyme analysis, or cytogenetic studies to be carried out. Furthermore, using techniques established with fertilized pre-implantation stage embryos (Sherman, 1975), it may be possible to isolate diploid and haploid cell lines.

In a recent series of experiments (Kaufman and Handyside, unpublished) the development potential of inner cell masses isolated immunosurgically from diploid and haploid parthenogenones was investigated. Inner cell masses were isolated from fully expanded fertilized blastocysts, and from diploid and haploid parthenogenetic embryos at a similar stage of development. Up to six or seven inner cell masses of each type were aggregated together and allowed to develop in vitro. Under suitable culture
conditions (medium supplemented with serum, amino acids and vitamins), attachment occurred during the first 12 h following explantation. Over the next seven days, a considerable degree of cellular outgrowth was normally observed, and at least two distinct classes of cells could be easily recognized. The earliest cells to appear were fibroblast-like, and probably endodermal in origin, and were the only cell type observed in the explants derived from fertilized inner cell masses. In the parthenogenetic explants, typical giant cells were commonly observed. These were similar to those seen when intact fertilized blastocysts were allowed to develop in supplemented medium. In the latter case, it is thought that the giant cells are almost certainly trophectodermal in origin (Gardner & Papaioannou, 1975). The development of giant cells in parthenogenetic inner cell mass explants is therefore of considerable interest, as it suggests that the parthenogenetic inner cell masses contain cells whose fate is not yet determined by the expanded blastocyst stage, and which are still potentially capable of developing into typical trophectodermal derivatives.

The nuclei in the presumptive haploid outgrowths were also considerably smaller in diameter than the nuclei observed in similar cells derived from diploid parthenogenones, indicating that, at least initially, the giant cells observed in the haploid cultures were still haploid. During prolonged culture, these giant cell nuclei in the haploid explants increased in diameter, but were always considerably smaller than similar nuclei observed in diploid parthenogenetic outgrowths (see Fig. 1.3). In addition to attachment and outgrowth, the haploid and diploid parthenogenetic and control fertilized embryonic aggregates developed large embryoid body-like structures with a clearly recognisable outer ‘shell’ of presumptive endodermal cells (Hogan & Tilly, 1977; Hsu, 1978).

In a subsequent series of experiments, single inner cell masses from fertilized embryos and from diploid and haploid parthenogenetic expanded blastocysts were allowed to develop in vitro, and the extent and cellular morphology of the outgrowths, and the presence or absence of embryoid body-like structures was determined. Typically, a very high proportion of inner cell masses isolated from fertilized embryos developed embryoid body-like structures, and this was usually accompanied by a considerable degree of cellular outgrowth. Embryoid body-like structures were observed in only about half of the cultures derived from the diploid parthenogenetic inner cell masses, and similar structures were only rarely obtained from haploid embryos.

Attempts to determine the total number of cells comprising the inner cell masses of these parthenogenetic blastocysts were only rarely successful, though all of the expanded blastocysts examined on the fifth day contained at least a few inner cell mass cells. Of the few diploid parthenogenetic inner cell masses that were successfully disaggregated, most contained about 20 cells. It also appeared that the developmentally retarded population of haploid and diploid blastocysts, isolated about 120 h after activation, generally contained slightly fewer inner cell mass cells than were normally present in blastocysts isolated 24 h earlier. However, there was a considerable variation between individual parthenogenetic embryos in the total number of inner cell mass cells present. The inner cell masses that contained only a few cells generally developed poorly in culture.

Because both the degree of cellular outgrowth and embryoid body-like development achieved from single inner cell masses grown under these conditions was often poor, various attempts were made to accentuate any possible difference between the different classes of embryo.
One technique that was found to be particularly useful in this respect, was based on the observation that blastocysts that entered a period of embryonic diapause, or 'delay' (for a recent review of methods for inducing delayed implantation, see Bergstrom, 1978), before eventually implanting, had a higher total cell count at the time of implantation than 'non-delayed' blastocysts. The constituent cells of 'delayed' embryos often continue to divide during the first 36 to 48 h after transfer to pseudopregnant ovariectomized recipients. This enables generally one to two, or occasionally up to three cleavage divisions to take place before the blastocyst's metabolism falls to a basal level, and no further mitotic activity is observed. In this way, 'delayed' blastocysts may occasionally contain up to 250 to 300 cells.

Parthenogenetic and fertilized blastocysts were transferred to the uterine horns of either day 2½ pseudopregnant recipients that were ovariectomized at the time of transfer, or to day 2½ pregnant recipients that were both ovariectomized and had their oviducts removed. Control studies had previously indicated that in the case of the pregnant females, the recipients' own embryos were still present within the oviduct at the time of transfer. 'Delayed' blastocysts were subsequently flushed from the recipients' uterine horns 48 h later. About half of the transferred diploid embryos,
and substantially less of the haploid embryos, were recovered from the recipients. Recovered blastocysts were transferred to drops of supplemented culture medium, and their subsequent growth in vitro was found to be considerably enhanced by this means. All of the haploid and diploid ‘delayed’ blastocysts attached, and in addition to the formation of large embryoid body-like structures containing hundreds, and in some cases possibly several thousand cells, gave rise to extensive cellular outgrowths.

Immunosurgically removed inner cell masses from recently isolated ‘delayed’ blastocysts often demonstrated that they had already developed an inner core of cells, surrounded by an outer shell of presumptive endodermal cells. Hogan & Tilly (1977; see also Atienza-Samols & Sherman, 1979) have performed a second round of immunosurgery to remove the outer endodermal layer, and this manoeuvre has allowed the differentiation of a wide variety of adult cell types to develop in culture. It would be of interest to know whether this technique is equally applicable to parthenogenetically-derived embryoid body-like structures grown in culture, as well as to the enlarged inner cell masses obtained from ‘delayed’ blastocysts.

THE DEVELOPMENT OF DIFFERENT CLASSES OF HAPLOID EMBRYOS DURING THE PRE-IMPLANTATION PERIOD

It has yet to be determined why the IC and DC embryos have a considerably better development potential than the one pronuclear ‘uniform haploid’ embryos that only receive one of the two products of the second meiotic division. The latter class developed from the one-cell stage to the blastocyst in vitro more poorly than the ‘mosaic’ haploid embryos (a), when oocytes were activated by hyaluronidase treatment (Kaufman & Sachs, 1976) and (b), following activation in medium lacking calcium and magnesium ions (Kaufman, unpublished, see Table 1.1).

When one-pronuclear and IC embryos (from (C57BL × A2G) F1 hybrid females that regularly failed to develop in vitro beyond the first cleavage division) were transferred to day-1 pseudopregnant recipients within six to ten hours of activation, similar implantation rates were obtained (see Kaufman & Gardner, 1974). However, a slightly higher incidence of non-pregnant females was obtained in the one-pronuclear transfers compared to the IC series (i.e. 28% and 20% non-pregnancy rate, respectively).

Two general hypotheses have been proposed in an attempt to explain why the uniform haploids should universally tend to develop more poorly than the mosaic haploids. One hypothesis suggests that a possible sub-optimal nuclear–cytoplasmic ratio may be present in the uniform haploid class, where, at the pronuclear stage of development, the ratio is N:1 compared to the situation in fertilized embryos and diploid parthenogenones where the ratio is 2N:1, and in mosaic haploids, where the ratio is N:0.5. This explanation is likely to be an over-simplification, as Modlinski (1975) has demonstrated that the development potential of fertilized mouse eggs, from which one of the two pronuclei had been withdrawn, depended on whether the remaining pronucleus originated from the male or female gamete. In this case, the resultant nuclear–cytoplasmic ratios were similar, but the development potential was clearly not so. However, this may depend on the timing of the operation (see Hoppe & Illmensee, 1977).

Alternatively, as the mosaic haploids contain two genetically dissimilar clones of cells, the resultant heterozygosity, even when distributed between two separate clones of cells within the embryo, may confer a developmental advantage compared to the
situation when only a single clone of cells is present, namely the progeny of only one of the products of the second meiotic division. Gene products from the two clones of cells may diffuse from one to the other, so as to compensate for missing products, or to balance the effect of deleterious genes which would be expressed in the uniform haploid state.

There seems little doubt that the pre-implantation development of parthenogenones is under genetic control. Indirect evidence bearing on this point comes from a study in which the development potential of uniform haploid embryos from inbred and random-bred hamsters was investigated (Kaufman, Huberman & Sachs, 1975). This study indicated that uniform haploid embryos from an inbred strain of hamsters had a better development potential than a similar type of embryo from random-bred females. It was suggested that this was possibly due to the reduction in the number of deleterious genes occurring during the process of selection associated with inbreeding. This conclusion is also likely to be an oversimplification, as few lethal genes are known to be expressed during the early pre-implantation period.

The DC class, while apparently morphologically and genetically bearing a very close resemblance to the IC class, appears to have benefited in some way from its short period of development as a two-pronuclear class embryo (see Table 1.1). One possible explanation for this might be that a degree of ‘polarity’ exists during the first few hours after activation, when the egg is completing the second meiotic division. During this initial period there may be a non-random segregation of organelles throughout the cytoplasm. This situation may gradually change, so that all of the intracellular components may become diffused throughout the cell. If this is the case, this presumably occurs by the time the ‘delayed’ cleavage event takes place.

It should, of course, be possible to test this hypothesis by various means. For example, the zona pellucida of both IC and DC embryos may be removed, and the two constituent blastomeres separated prior to the first cleavage division. The development potential of the clones of cells subsequently developing in vitro from each blastomere could then be tested, and their biochemical and ultrastructural features examined.

Other evidence that regionalization is not present at the late pronuclear stage is available. When one-cell fertilized eggs were bisected so that one half contained the male pronucleus and the other the female pronucleus (Tarkowski & Rossant, 1976), one quarter of the resultant ‘blastomeres’ would be expected to lack an X-chromosome, and would probably die after a few cleavage divisions (Morris, 1968; Tarkowski, 1977). However, that seven of 12 operated embryos developed to the blastocyst stage would seem to suggest that at the time of operation (at the late pronuclear stage) these eggs did not contain specialized morphogenetic regions that were capable of affecting development to the blastocyst stage.

Observations on Some of the Factors That May Influence the Survival of Parthenogenetic Embryos

At a well-defined time during cleavage, pre-implantation rodent embryos undergo a marked change in morphology, from a cluster of spherical cells to a compact ball in which individual cell outlines are no longer visible. This change, termed compaction, delineates the beginning of the morula stage, and involves a number of processes including cell flattening (Calarco & Brown, 1969), and intercellular junction formation; initially gap and focal tight junctions are formed (Ducibella, 1977). At the
same time, cell polarization occurs with asymmetrical localization of microvilli (Calarco & Brown, 1969) and antibody binding sites, that are mainly located on the outer surfaces of the outside cells (A. H. Handyside, personal communication).

In fertilized mouse embryos, compaction normally occurs at the eight-cell stage, and is followed approximately two cleavage divisions later by fluid accumulation and blastocyst formation. Since the timing of these events is unaffected by experimental manipulation of cell number, it is thought that it is probably related either to chromosomal replication, or nuclear–cytoplasmic ratio (Smith & McLaren, 1977).

The study of the timing of these early events in different types of in-vitro activated parthenogenones in which various parameters differ from fertilized embryos, provides an additional means of analysing the factors that control early development (for comparison of the timing of the first cleavage mitosis in fertilized and parthenogenetic embryos, see Kaufman, 1973a). For instance, the absence of second polar-body formation results in a reduced nuclear–cytoplasmic ratio (Kaufman & Sachs, 1976), while the occurrence instead of a symmetrical division (IC and DC types) leads to the production of twice the number of blastomeres compared with other classes of parthenogenone, and fertilized embryos, at each successive ‘cleavage’ division (i.e. after the first cleavage division, IC and DC embryos contain four blastomeres, whereas other embryos at this stage have only two blastomeres).

Present evidence based on the recombination of genetically or physically marked cells, followed by analysis of their fates, suggests that the early differentiation of the outer trophectoderm and inner cell mass lineages of the blastocyst is initiated in response to their position within the morula. Thus, outside cells develop as trophectoderm, whereas enclosed inside cells develop as inner cell mass (Tarkowski & Wroblewska, 1967; for review see Johnson et al., 1977). The way in which a cell recognizes its position at this stage is not known at the present time, but it is thought to be related to the differences between inside and outside cells generated by events associated with the process of compaction (Johnson, 1979).

Under phase-contrast microscopy, the early cleavage of parthenogenones of all types appeared to be normal, although there were minor variations in rate (Kaufman & Sachs, 1976; Kaufman, unpublished), and for the reasons indicated above, the IC and DC types had twice the normal number of cells at each cleavage division. At the morula stage, however, haploid parthenogenones often failed to compact normally, and in some cells division was retarded (see Fig. 1.4), whereas diploids were morphologically indistinguishable from fertilized controls (Handyside and Kaufman, unpublished).

Labelling of the outer surfaces of morulae by indirect immunofluorescence (‘immunolabelling’), prior to disaggregation by enzymic or non-enzymic treatments and subsequent analysis of the distribution of the label amongst the individual cells, has allowed various parameters related to the state of compaction to be examined (A. H. Handyside, personal communication). This technique provides information on intercellular permeability (dependent on closeness of apposition of cells and intercellular junction formation), and cell polarization. It also enables outer cells (that become labelled) to be distinguished from inside cells (that remain unlabelled), and, in addition, allows cells to be assigned to cleavage divisions on the basis of their overall cell size. Furthermore, the cells remain viable, and can be recovered and develop into blastocyst-like structures following reaggregation in culture.

Using this technique, it was found that a significant proportion (35%) of haploid parthenogenetic morulae contained cells spanning three cleavage divisions. In the
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Fig. 1.4. Normal looking immediate cleavage-derived haploid morulae, a, and blastocysts, b, c. Slightly more advanced haploid morulae to those illustrated in a. Note the abnormal, almost 'decompacted' appearance quite frequently observed in haploid but not diploid parthenogenetic or fertilized embryos at this stage of development. d. Late morula and early cavitating haploid blastocysts each showing considerable degree of cellular asynchrony. The two large blastomeres clearly seen (arrowed) in these embryos are probably products of the second or third cleavage division.

case of the mosaic haploid embryos, the relationship between the various cleavage products and the two original blastomeres has yet to be determined, but may prove to be of considerable interest from the point of view of cell-lineage studies. This cellular asynchrony was never observed with fertilized or diploid parthenogenetic morulae (Handyside and Kaufman, unpublished) in which cleavage is relatively synchronised (Smith & McLaren, 1977). Also, fewer unlabelled inside cells (presumptive inner-cell-mass cells) were detected in haploid compared with diploid parthenogenones or fertilized embryos derived from the same strain combination.

There are several possible explanations for these observations. Haploid parthenogenones may be relatively more sensitive to in-vitro culture conditions. Diploidization of certain cells may take place. This is known to occur in some cells at later stages of development (Kaufman & Sachs, 1976; Kaufman, 1978b). A third possibility that has to be considered, is that a switch to dependence on the embryonic genome may occur (Monk & Harper, 1978), thus allowing the expression of deleterious recessive mutations (for discussion, see Graham, 1974).

For whatever reason, the basic effect of retarded cell division and/or delayed compaction (which in some cases resulted in the exclusion of cells from the blastocyst) is generally thought to be the reduction or absence of the inner cell mass component of the embryo (Tarkowski & Wroblewska, 1967). This was confirmed in the case of parthenogenones using the technique of immunosurgery (Solter & Knowles, 1975; Handyside & Barton, 1977; Handyside, 1978) to selectively lyse the trophectoderm of various types of parthenogenetic blastocysts. Whereas diploid blastocysts always


contained inner cell masses, the inner cell masses from haploid blastocysts were generally reduced and in a few cases seemed to be entirely absent, especially in those that only achieved the blastocyst stage by the sixth, rather than the fifth, day of culture (Kaufman, 1978b; Handyside & Kaufman, unpublished).

Since the embryo proper is derived from the inner cell mass (Gardner & Papaioannou, 1975), this partly explains the low incidence and possibly also the early post-implantation developmental arrest of haploid parthenogenones, as these were generally retarded during the second half of the pre-implantation period compared to diploid parthenogenones cultured under similar conditions. In support of the latter hypothesis, a small series of haploid parthenogenetic blastocysts derived from aggregated morulae (a procedure known to increase the size of the inner cell mass, see Garner & McLaren, 1974 were transferred to pseudopregnant recipients. This procedure led to the development of one apparently normal haploid implant as far as the primitive-streak stage (Handyside and Kaufman, unpublished).

The reason(s) why the majority of haploid parthenogenones fail to blastulate, despite the attainment of large numbers of cells, is not known. One possibility may be that these embryos contain a larger proportion of ‘retarded’ blastomeres (i.e. products of the second and third cleavage divisions), while Hansmann, Gebauer & Grimm (1978) have demonstrated that mouse parthenogenones show impaired gene activity for 18S and 28S rRNA during early embryonic development. However, the significance of the latter observation has yet to be determined.

The development of the inner cell mass of diploid parthenogenones appeared to be normal. Thus, the relatively late post-implantation arrest observed with these embryos is unlikely to be due to a deficiency in the number of embryonic precursor cells. Furthermore, chimeras formed by the injection of diploid parthenogenetic inner cell masses into intact fertilized blastocysts, which survive to term after transfer to pseudopregnant recipients, have demonstrated that the parthenogenetically-derived cells were capable of contributing to all the tissues so far tested (see previous section).

It is possible, therefore, that the defect(s) preventing the development of diploid parthenogenones beyond limb-bud stages may arise in the extraembryonic tissues of the conceptus, upon which it is increasingly dependent during post-implantation development. In this respect, it may be significant that arrest occurs at the stage that the embryo becomes dependent on the chorio-allantoic placenta.

The extraembryonic membranes and placental tissues are predominantly derived from the trophectoderm and primitive endoderm lineages (Gardner & Papaioannou, 1975) in which the paternal X-chromosome (absent in parthenogenones) is selectively inactivated in fertilized rodent embryos (Takagi & Sasaki, 1975; Takagi, Wake & Sasaki, 1978).

Recent observations on tissues derived from advanced diploid parthenogenones have demonstrated that whereas X-inactivation appeared normal in the embryonic components (Kaufman, Guc-Cubrilo & Lyon, 1978), as has also been shown to be the case in parthenogenetically-derived benign human teratomas (McCaw & Latt, 1971), fewer inactive X-chromosomes could be detected in the intact yolk sac (Rastan, Kaufman, and Handyside, unpublished). Since the yolk sac is a bilaminar structure composed of layers of endoderm (extraembryonic) and mesoderm (embryonic derivative), one possible interpretation is that X-inactivation had not occurred in the endodermal component. This hypothesis will be tested in due course, by separating the two components with mild enzyme treatments prior to chromosomal analysis.

An alternative approach to the direct examination of possible genetic defects in
extraembryonic tissues of diploid parthenogenones, is to examine the development of chimeric combinations of parthenogenetically-derived embryonic and fertilized-derived extraembryonic components. This would be a novel approach, as previous studies of chimeras between fertilized and parthenogenetic embryos, have made no attempt to reduce or eliminate the embryonic components of the host fertilized blastocyst. The effect of replacing (or supplementing) the trophectoderm, and/or the primitive endoderm, of parthenogenones should therefore be undertaken. This experimental approach may, in addition, allow a predominantly parthenogenetic embryo to survive to term.

It is interesting to speculate on the genetic implications of the recent report of the development to term of completely homozygous diploid mouse embryos (Hoppe & Illmensee, 1977) in the light of the difficulties encountered in obtaining advanced parthenogenetic embryonic development.

Modlinski (1975) originally described a microsurgical technique that enabled a single pronucleus to be removed from a fertilized egg. Diploidization of the resultant haploid genome was achieved following cytochalasin B treatment prior to the first cleavage division (see also Markert & Petters, 1977). Treated embryos that had developed to the morula or blastocyst stage were then transferred to pseudopregnant recipients. A total of seven live female offspring were obtained, five derived from the maternal genome (by gynogenesis) and the remaining two inherited only the paternal genome (androgenesis). Six of the seven females proved to be fertile, giving birth to progeny corresponding only to the pronuclear genotype of the mother.

The unequivocal demonstration that complete homozygosity is compatible with viability following both gynogenetic and androgenetic development, suggests that the expression of lethal genes should not be a limiting factor in all diploid parthenogenones. The recent genetic evidence that a proportion of human hydatidiform moles are androgenetic in origin (Kajii & Ohama, 1977; see also Szulman & Surti, 1978a, b), following activation of an egg by a diploid sperm, or by diploidization prior to the first cleavage division following activation by an haploid sperm, suggests that homozygosity per se (and androgenesis), at least in man, does not appear to give rise to viable embryos. These authors suggested that the high malignancy rate in this type of hydatidiform mole might be explained by the expression of a recessive mutation of a gene (or genes) which controls cell growth. The mouse data discussed here do not support this hypothesis, as neither the homozygous diploid embryos produced by Hoppe & Illmensee (1977), nor the chimeras between diploid parthenogenones and fertilized embryos have shown any evidence of malignancy.

The present X-inactivation evidence (albeit preliminary, see earlier in this section) would tend to suggest that the post-implantation development of parthenogenones may be limited by their inability to form a normal placenta. However, there is a strong possibility that the extra-embryonic membranes of the completely homozygous diploid embryos produced by Hoppe & Illmensee (1977) will also prove to have an abnormal pattern, with two active X-chromosomes in the gynogenones, and two inactive X-chromosomes in the androgenones! That the pattern in human androgenones may also be complex is evidenced by the fact that three of the 47 moles studied by Kajii & Ohama (1977) were X-chromatin-negative, presumably because neither of the two X-chromosomes present was inactivated.
It must be obvious from the scope of the material presented in this chapter, that a considerable volume of information has been amassed, principally over the last ten years, regarding both the biochemical events and the ultrastructural changes that take place at and very shortly after activation. Further, that there is some direct, and a considerable amount of indirect, evidence to suggest that the state of the cytoskeletal system of the recently activated oocyte in large measure controls its immediate fate, and consequently dictates its long-term development potential. In addition, the large number of studies in which the pre- and early post-implantation development of different classes of haploid and diploid (particularly mouse) parthenogenones have been investigated, provides insight into the influence of certain genetic factors on early embryogenesis. However, it must be equally apparent that large gaps exist in our present knowledge of the factors that control the early development both of parthenogenetic and indeed fertilized embryos.

Possibly the relatively slow progress being made at the present time in parthenogenesis research is in part due to the general lack of availability of mammalian parthenogenetic material. However, it is also significant that most embryologists have failed to appreciate fully the fundamental value of this material in providing a new experimental approach that might facilitate the investigation of major topics of current interest in the field of developmental biology. One of the principal advantages of working with parthenogenetic material must surely be that, theoretically at least, genetically identical fertilized eggs and embryos are readily available, that could act as ideal controls for certain in-vivo and in-vitro studies. At the same time, any information obtained on the factors that control the development of parthenogenones almost inevitably provides insight into the role of spermatozoa, and the reproductive significance of fertilization.

With the limited space available here, it will only be possible to indicate in very general terms the areas in which future work with parthenogenetic embryos, or parthenogenetically-derived material, might be expected to produce important basic information.

Much of the mouse work described in this chapter has utilized a few inbred strains, and F1 hybrid mice, principally because it has been possible to achieve reliably both high rates of activation, and development at least to the blastocyst stage of haploid and diploid embryos from these sources. Development beyond implantation has also been regularly achieved, and allows more advanced parthenogenones to be studied, and their features compared with fertilized embryos at similar stages of development. However, it would undoubtedly be useful to activate and examine the early stages of development of other inbred and randomly-bred strains of mice. In particular, it would be of great interest to activate oocytes from strains bearing known mutant genes, in order to investigate their development potential in the haploid and diploid state.

The use of mosaic haploid embryos is possibly the only means whereby both products of the second meiotic division may be examined independently. This has been of value in the analysis of a mutant strain of mice in which X-chromosome non-disjunction regularly took place (Phillips & Kaufman, 1974). A similar type of analysis would be equally applicable in other translocation-bearing strains in which the two products of the second meiotic division are not necessarily genetically similar. Equally, it should be possible to investigate (indirectly) the role of the X chromosome
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in early development, by studying haploid parthenogenones with X-nullisomy derived from XO female mice.

The recent demonstration that the addition of EDTA to embryo culture medium largely enables embryos from certain inbred strains of mice to overcome the two-cell block (Abramczuk, Solter & Koprowski, 1977), if applicable to parthenogenones, should greatly facilitate this area of research, as complete development in vitro from the one-cell stage to the blastocyst is, at present, only reliably achieved in certain F1 hybrid strains of mice. In other groups of inbred and randomly-bred mice, the only way to achieve development beyond the two-cell stage (or the four-cell stage in the case of mosaic haploids) involves the transfer of pronucleate eggs to the oviducts of suitable recipients. This is both time-consuming, and can act as a severely limiting factor when, despite this manoeuvre, overall rates of development may, in any case, be quite low.

It would be of particular interest to investigate in more detail the influence of ploidy on early development, and further, to establish why mosaic haploids almost invariably have a better development potential than uniform haploid embryos. Equally, it would be of interest to compare the development of the two-pronuclear heterozygous diploid class with one-pronuclear embryos that have been diploidized prior to the first cleavage division. As has been indicated in a previous section (p. 24), the regulation of cell division appears to be impaired in a substantial proportion of haploid embryos, giving rise to an obvious degree of cellular asynchrony at the morula stage. However, it has also been observed that a moderate proportion of these morulae are capable of developing into morphologically apparently normal blastocysts (Kaufman, unpublished). At the present time, it is unclear how the large and presumably developmentally retarded blastomeres regulate, so that they may be accommodated into the blastocyst. Certainly, the events associated with the process of compaction, both in fertilized and in particular, in parthenogenones, require a great deal more study.

The use of 'delayed' blastocysts has greatly facilitated studies on the early post-implantation stages of development. When this technique is combined with the formation of appropriate aggregation chimeric embryos, it should considerably increase the potential cellular pool that could be used to establish parthenogenetically-derived haploid and diploid cell lines, and the production of more advanced parthenogenones than are at present available. In addition, the technique of immunosurgery has increased the pool of researchers capable of working with isolated cells and embryonic tissues, compared to former times when only a few were technically skilled enough to carry out such microsurgical manoeuvres as, for example, were required to isolate intact inner cell masses. Equally, the application of numerous enzymatic and related techniques allows all pre- and early post-implantation embryonic stages to be disaggregated, and microassay systems are also being geared to investigate the properties of individual cells and related groups of cells (for example, see Van Blerkom, 1978; Wales, 1978). The ability to isolate single cells and small groups of cells also allows some of the manipulatory studies previously thought to be impossible, or more likely not even considered, to be contemplated.

The production of stable haploid cell lines would be particularly valuable in genetic research (e.g., for chromosome analysis, gene mapping and linkage studies (Eicher, 1978)), and in the study of recessive mutations, and lethal and X-linked genes. These might be obtained either directly from the culture of inner cell mass derivatives, or indirectly via the production of transducible teratocarcinomas derived from the ectopic transfer of presumptive haploid embryos. Cell lines derived from homozygous
diploid embryos would be homozygous at all loci, and would also be useful in this regard. These would almost certainly have a greater degree of intrinsic stability, due to their diploid rather than haploid status.

The many technical advances that have taken place over the last few years encourages me to believe that they will act as a catalyst to stimulate research interest in the use of parthenogenetic material in the traditional areas of experimental embryology outlined here, and hopefully in many additional areas of developmental biology.

I wish to thank Dr Alan Handyside for his helpful comments on the manuscript, and for allowing me to present some of our unpublished observations.

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The role of embryology in teratological research, with particular reference to the development of the neural tube and heart

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Introduction

There are many intriguing questions that the experimental embryologist can attempt to answer which could provide insight into the pathogenesis of many of the commoner types of congenital abnormalities that affect the human population. I should like to draw attention to certain areas of embryological interest, and make some general and personal observations on teratological research. I shall concentrate on two particular areas of interest, namely studies into the pathogenesis of neural tube closure defects and cardiovascular anomalies because these are the topics of my special interests.

Two quite different, but complementary approaches may be employed in experimental teratological research to investigate how certain agents act on specific organs or tissues of the embryo or fetus to induce their abnormal development. The first approach takes cognizance of the nature and chemical structure of the agent under investigation, in order to determine at the biochemical level what the underlying mechanism and principal sites of action are likely to be. The primary effect of the agent may be to disrupt maternal metabolism, and the effect on the embryo or fetus may be secondary to the effect on the mother. In these latter circumstances it is not uncommon for an embryotoxic effect to be produced before any toxic manifestation becomes apparent in the mother.

The second approach takes cognizance of the morphological abnormalities which may be induced by embryonic exposure to a teratogenic agent, in order to determine in the first instance at which stage of gestation the teratogenic insult is likely to have occurred, and, also, its site of action at the cellular and tissue level. In preliminary studies, development may be allowed to proceed for a matter of days, or even to term, before the fetuses are examined, although obviously, depending on the severity of the teratogenic insult, a high proportion of the fetuses may not survive long enough to be examined. By looking at progressively earlier stages, and effectively observing in reverse the pathogenesis of the defect under examination, it may be possible to recognize the direct toxic effects of the agent under investigation, and possibly to determine the extent of any compensatory growth that might have occurred.

It is also instructive to examine the pathogenesis of relatively similar abnormalities involving, for example, the cardiovascular and/or central nervous system, induced by a wide variety of teratogenic agents. The induction of these abnormalities can be compared with the development of similar lesions observed in the offspring of animals bearing specific mutant genes or numerical chromosomal anomalies.

While the concepts of critical or sensitive periods (e.g. see Rychter, 1962) have the advantage of simplicity, they are often inapplicable in the clinical context, as in most instances no history of a single exposure to a teratogenic insult is available. While these acute exposure episodes presumably do occur in man, they often pass unrecognized. Most environmental teratogenic factors probably act over a considerable period of time, and are therefore ‘chronic’ in
nature; e.g. when the embryo or fetus is exposed to the metabolic consequences of the maternal ingestion of alcohol, smoking of cigarettes, and numerous other environmental teratogens. It is similarly naïve to assume that individual malformations are invariably associated with a single teratogenic insult occurring at a specific period of organogenesis. For example, a cleft palate may be induced in the rat by a single exposure to an appropriate dose of X-irradiation from about Days 10–14 of gestation, indicating that closure of the palate is an extremely complex process with many steps, the overall process extending over a period of days rather than hours (Trasler & Clarke Fraser, 1977). Each of the numerous steps in the closure process presumably has its own critical period (Clarke Fraser, 1977).

It seems likely that all developmental processes follow this general pattern, and it is only the overall duration of the critical period which varies from organ to organ and tissue to tissue. In addition, embryos in different locations within the uterus may well be exposed to different amounts of the teratogen depending on variations in maternal blood supply, etc. Since this is the case, it is not altogether surprising that even litter-mates may manifest apparently unrelated abnormalities.

Only gross morphological anomalies are likely to be observed at superficial examination if this is carried out 1–2 days after exposure to the teratogenic insult. However, a more detailed histological, or preferably combined histological and ultrastructural, examination often reveals cellular deviations from the norm that may only be manifest clinically much later in gestation, or possibly only well after birth. More subtle changes that may result from, for example, minor alterations in cellular metabolism within the central nervous system will almost certainly not be revealed during such an early examination. Such biochemical deviations may cause changes in the behaviour pattern of the individual which may only manifest themselves when the individual is subjected to an abnormally stressful situation, either physical or mental. An aberrant behaviour pattern may only be detected if special psychological testing is carried out, thus transferring the effect induced during early gestation into the sphere of the behavioural teratologist.

It is especially hazardous to draw conclusions solely from the examination of post-natal material regarding the specific time of exposure to a teratogenic insult if this occurs during the early embryonic period, as it would be almost impossible to distinguish between (a) the abnormal growth induced by the direct effect of the teratogen, (b) secondary destructive processes involving previously normally formed structures, and (c) the effect of tissue regeneration and compensatory growth.

One possible consequence of a teratogenic insult might be death of the embryo shortly after exposure. If the embryo survives, however, there may be abnormality of a specific organ or system which may or may not be associated with general growth retardation or functional derangement of tissue activity. If such abnormalities are induced they may be manifest only after birth. The long-term survival of the individual would largely be determined by the systemic consequences of the resultant abnormality. If, for example, a severe abnormality of the cardiovascular system has been induced, survival to term may occur, but exercise tolerance will almost certainly be severely affected, and premature death may result.

Because I am more familiar with the morphological and descriptive embryological approach, only this aspect of teratological research will be considered here. Examples will be presented which demonstrate how this approach has been utilized to investigate the pathogenesis of certain abnormalities of the central nervous and cardiovascular systems resulting from exposure of experimental animals to teratogenic agents, or as a direct consequence of the presence of an abnormal genome. No attempt has been made to review the very extensive teratological literature in these areas, although particularly relevant work is cited. The following is therefore more in the nature of an account of a general approach which may be used to examine a range of teratological problems in which a detailed knowledge of normal developmental processes is an essential prerequisite.
Neural tube defects

Major defects involving the derivatives of the embryonic neural tube, such as anencephaly, hydrocephalus and spina bifida, remain one of the greatest causes of human perinatal mortality and morbidity (Carter, 1976). These conditions are especially prevalent in certain regions of the British Isles (Carter, 1974), where the incidence may be as high as 7 affected infants per 1000 live births, compared to a worldwide incidence of about 1 per 1000 (for reviews, see Willis, 1962; Warkany, 1971; Hughes, 1976; Leck, 1977; Miller & Lowry, 1977).

Despite the considerable clinical interest in defects of this type, the underlying factors which give rise to them often remain a mystery. The most commonly encountered abnormalities involving the ‘spinal’ and ‘cephalic’ regions of the neural tube are spina bifida, which may present minimal to very major clinical problems depending on the degree of severity and extent of the defect, and anencephaly, respectively, the latter being invariably fatal within a matter of hours after birth (Leck, 1977). A considerable number of other conditions which also result from failure of normal development of the neural tube are also occasionally seen (Willis, 1962). Of the various types of neural tube defects encountered it is usual to group conditions such as encephalocele, meningocele and myelocoele with spina bifida cystica, and iniencephaly with anencephaly. The equivalent of anencephaly in experimental animals is usually exencephaly, a condition only rarely encountered at full term in man, although this is the commonest anomaly involving the cephalic part of the neural tube during the first trimester of pregnancy, suggesting that in man, at least, exencephaly almost always leads to anencephaly by the second half of gestation (Warkany, 1971).

Various hypotheses have been proposed to account for the high incidence of these defects in certain areas and low incidence in other parts of the U.K. (Carter, 1974, 1976; Leck, 1972, 1974). However, because of the nature of the material and the clinical and ethical problems involved, it has not so far been possible to carry out an acceptable prospective investigation into the factors which may give rise to these defects in man (for observations on surveillance systems, see Weatherall & Haskey, 1976; Miller & Lowry, 1977). However, the recent clinical trials to study the possible effect of a high dietary intake of certain vitamins in women who have previously given birth to children with neural tube defects (Smithells et al., 1980) might justifiably be considered to fall into this category.

The association between chromosome disorders and birth defects is well recognized (for reviews, see Hamerton, 1971; Hsu & Hirschhorn, 1977; and for observations on prenatal diagnosis of these disorders, see Laurence & Gregory, 1976). The incidence of chromosome anomalies at birth is in the order of 1-5 per 1000 births, and Down’s syndrome is the most common cause of severe mental retardation in the U.K. However, the incidence at conception is probably considerably higher (see Boué & Boué, 1973; and observations by Ford, 1975).

Because of obvious difficulties encountered in obtaining suitable human embryonic and fetal material, any comprehensive attempt to study the pathogenesis of neural tube defects necessitates the use of an experimental animal model in which it is possible to produce an approximate phenocopy of the human defect to be studied. By investigating the underlying mechanisms whereby certain environmental teratogens induce the formation of these phenocopies, it may be possible to gain some understanding of the factors which influence the normal process of neurulation in the human embryo, and to determine at which stages during development the embryo is most sensitive to teratogenic agents.

The mouse is one of the most useful experimental animals in this area of research as, in addition to being sensitive to many teratogens that induce neural tube defects, numerous mutant strains exist which also develop abnormalities involving the ‘spinal’ and ‘cephalic’ parts of the neural tube, the skull, and vertebral column (Kalter, 1968; Staats, 1975, 1976).

Despite the vast literature that has accumulated on the cellular events thought to be important in the process of neurulation (for recent review, see Karfunkel, 1974), particularly in
non-mammalian species, only a superficial overall picture of the sequential changes which take place during neurulation in the cephalic region of any mammalian embryo exists at present. Similarly, minimal information is available on the interrelationship between this complex process and the formation of the optic primordia (see, however, Streeter, 1942; O’Rahilly, 1966). Consequently, it is essential to establish this baseline information (Pl. 1, Figs 1–6) before progressing towards the next goal of determining, initially at the gross morphological level and later at the ultrastructural level, the events that lead to the development of abnormalities of the cephalic region of the neural tube. Closure of this part of the neural tube is completed in embryos which possess about 20 pairs of somites (Kaufman, 1979), and is an essential prerequisite before facial development can take place, involving as it does the very complex movements of the various facial ‘processes’ (for standard text-book description, see Hamilton & Mossman, 1972).

**Neural tube defects in the mouse**

One of the main problems associated with experimental studies on neural tube anomalies, and this equally applies to experimentally induced abnormalities of other organ systems, is that the mode of action of individual teratogens may differ from the mechanism which usually leads to the formation of an apparently similar defect in the human fetus. In order to go some way towards countering this objection, the influence of two teratogens known to effect neural tube closure was investigated (O’Shea & Kaufman, 1979a, b, 1980, 1981) and a translocation-bearing strain of mice in which a high proportion of the offspring developed neural tube defects (Snell’s translocation; Snell, Bodeman & Hollander, 1935; O’Shea, 1979) was also studied.

The emphasis of O’Shea & Kaufman’s work has largely been towards understanding the relationship between the gross morphological damage induced, and the changes observed within and between individual neuroepithelial cells. These investigations complement the recent work by Solursh & Morriss (1977; see also, Morriss & Solursh, 1978) who have stressed the part played by the underlying mesenchyme cells and the biochemical components of the intercellular matrix during the process of neurulation (for observations on the role of extracellular components during morphogenesis, see Kochhar & Larsson, 1977).

Two major experimental approaches have been employed, namely the in-vivo approach in which a particular agent is given to a pregnant female during the early post-implantation period, and the in-vitro approach, often carried out in parallel, in which isolated embryos maintained in culture (New, Coppola & Terry, 1973) have been exposed to the same agents over approximately the same period of embryonic development. The teratogens tested were acetaldehyde and copper.

Acetaldehyde, the primary metabolite of ethyl alcohol, was tested as part of an investigation into the teratogenic properties of ethanol, to determine its possible role in the fetal alcohol syndrome (Jones, Smith, Ulleland & Streissguth, 1973), as no previous experimental studies had been carried out to examine the possible effect of acetaldehyde on the developing mammalian embryo (for discussion of possible role of acetaldehyde in ethanol toxicity, see Akabane, 1970; Truitt & Walsh, 1971; Rahwan, 1974; Mulvihill & Yeager, 1976). Many workers have investigated the influence of ethanol on embryonic and fetal development when given by different routes to experimental animals (Papara-Nicholson & Telford, 1957; Sandor & Amels, 1971; Tze & Lee, 1975; Kronick, 1976; Rosman & Malone, 1976; Chernoff, 1977; Randall, Taylor & Walker, 1977; Henderson & Schenker, 1977). There was developmental and/or growth retardation, an increased incidence of resorptions and perinatal mortality, and a range of congenital abnormalities involving particularly the central nervous system. The resultant abnormalities were observed when litters were examined at term and during the early post-natal period. However, in only one of the ethanol studies were mid-gestation embryos examined (Sandor & Amels, 1971).
To study the effect of acetaldehyde free from the effect of ethanol, different groups of pregnant mice were injected during the early post-implantation period with 1 or 2% acetaldehyde in saline (9 g NaCl/l) on Days 7, 8 and 9 of gestation, and the uterine contents were examined on Days 10 or 12 (Day 1 = the day on which a vaginal plug was observed).

The experimental embryos examined on Day 10 had a high incidence of developmental delay (Pl. 2, Figs 7 and 8) and anomalies of closure of the cranial and caudal extremities of the neural tube (Pl. 2, Fig. 11) (for experimental details, see O'Shea & Kaufman, 1979a). More detailed examination showed that major damage had been sustained by the neuroepithelium: the cells appeared to have rounded-up (Pl. 2, Figs 8, 9 and 10). This impression was confirmed by transmission electron microscopy: the microtubules and microfilaments appeared to be partially or completely disrupted (O'Shea, 1979; O'Shea & Kaufman, 1981). Damage to these cytoskeletal elements would almost certainly account for the failure of closure of the neural tube in the ‘cephalic’ and ‘spinal’ regions, and the abnormal appearance of the neuroepithelium in the obviously damaged areas and in the apparently normally closed regions of the neural tube.

Similar studies of the teratogenic effect of copper were carried out (O'Shea & Kaufman, 1979b, 1980) as part of an investigation into the possible consequences in man of conception in the presence of copper-bearing intrauterine contraceptive devices (IUCDs). Copper has been shown to be blastocyst-lethal at concentrations above $2.5 \times 10^{-5}$ \text{M} in the mouse (Brinster & Cross, 1972; Naeslund, 1972), lethal to 15- and 30-somite rat embryos at concentrations higher than $5 \times 10^{-4}$ \text{M}, although lower levels had no observable effect on development (Webb & Coppola, 1976), and teratogenic when administered to pregnant hamsters on Day 8 of gestation (Ferm & Hanlon, 1974). Various types of abnormalities were reported in surviving hamster offspring, many of which involved mid-line closure defects both of the neural tube and of the anterior thoracic and abdominal walls. Gosden, Ross, McGovern & Reid (1979) have described severe developmental retardation in spontaneously aborted fetuses, and cases of intrauterine death in other pregnancies occurring in women with a copper IUCD in situ.

In the first series of experiments, copper in the form of an aqueous solution of copper sulphate was injected intravenously via the tail vein into pregnant female mice on the morning of Day 7, Day 8 or Day 9 of pregnancy (see O'Shea & Kaufman, 1979b). Autopsies were mostly carried out on Day 10, although an additional few females were injected with copper on Day 8 and the uterine contents examined on Day 12 of gestation.

Depending on the stage of embryonic development at the time of injection, the copper was teratogenic or embryo-lethal. The most sensitive period for the production of abnormalities of the neural tube and heart was fairly circumscribed, occurring somewhere between the mornings of Days 8 and 9 of gestation. The neural tube defects observed mostly involved failure of elevation of the cephalic neural folds, and consequently resulted in closure defects involving a major or minor part of this region. In over a quarter of the embryos with abnormalities of the neural tube, abnormalities of the heart were also present, while in an additional 10–15% only abnormalities of the heart were observed.

By Day 12, about half of the surviving fetuses had abnormalities involving the head region. In about a quarter of the less severely affected fetuses, the region overlying the 4th ventricle appeared to be collapsed, whereas in the majority of the remainder an open neural tube defect was present extending from the caudal part of the presumptive mid-brain to just rostral to the otocysts (Pl. 2, Figs 12 and 13).

In a parallel series of experiments, pregnant females were autopsied on the morning of Day 9 of gestation, and embryos which possessed an intact yolk sac and eptoplacental cone were explanted into roller bottles (New et al., 1973) containing heat-inactivated immediately-centrifuged rat serum prepared as described by Steele & New (1974). Various concentrations of copper sulphate had previously been prepared and added to the serum samples. At the end of the 36-h culture period, the embryos were isolated and the presence or absence of gross abnormalities noted. While the lowest level of copper tested ($5 \times 10^{-6}$ \text{M}) had
Scanning electron micrographs showing the changes which take place in the overall appearance of the mouse embryo from the late pre-somite period, on Day 8, to the limb-bud stage, early on Day 12.

**Fig. 1.** Dorsal view of embryonic region of an advanced egg-cylinder containing a late pre-somite stage embryo. The two headfolds (arrowed) are separated by the neural groove. The torn edges of the amnion (A) and extra-embryonic endoderm (E) are also clearly seen.

**Fig. 2.** Antero-lateral view of 5–6-somite embryo. The embryonic membranes have been torn open to expose the cephalic and caudal extremities of the embryo. Note the two headfolds (H) and the allantois (A). The embryo is in the characteristic ‘unturned’ position.

**Fig. 3.** Dorsal view of embryo at a similar stage of development to that illustrated in Fig. 2. The embryo has been ‘flattened out’ to demonstrate that the neural folds, possibly in the low cervical or upper thoracic region, have apposed and are in the process of fusion. Note the relatively advanced state of development of the cephalic region, and poorer degree of differentiation in the caudal extremity of the embryo which abuts onto the allantois (A).

**Fig. 4.** Embryo with approximately 10 somites. A large region of the neural tube has been formed, but the neural folds in the cephalic (A) and caudal (C) extremities of the embryo have yet to fuse. The outlines of the somites (arrowed) are clearly visible just lateral to the dorsal midline. The two ‘bulges’ in the cephalic region represent the area overlying the optic indentation (O), and the first branchial arch (I).

**Fig. 5.** Embryo with approximately 25 somites. By this stage, only the caudal region of the neural tube (i.e. the caudal or posterior neuropore, N) has yet to close. The ‘bulge’ overlying the optic vesicle (V) is clearly seen, as are the first (1) and second (2) branchial arches. The ridge (R) along the side of the embryo from which the limb buds develop is also apparent.

**Fig. 6.** Advanced limb-bud stage embryo isolated early on Day 12. Note that the neural tube has closed along its entire length. The ‘bulge’ overlying the optic vesicle (O) is clearly seen, as is the naso-lacrimal groove (arrowed). The limb buds are spade-like, and the apical ectodermal ridge (R) is clearly seen at the peripheral margin of the hind-limb bud.

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**PLATE 2**

Scanning electron micrographs illustrating the normal appearance of the neural ectoderm (neuroepithelium) and its appearance after embryonic exposure to acetaldehyde.

**Fig. 7.** Control headfold-stage early-somite embryo isolated early on Day 9. Note the smooth appearance of the neural ectoderm, and the presence of the neural folds.

**Fig. 8.** Acetaldehyde-treated embryo isolated on Day 10. Note the considerable degree of growth retardation and the rounded-up appearance of the neuroepithelial cells.

**Fig. 9.** Close-up view of the neuroepithelium of a control embryo.

**Fig. 10.** Close-up view of the neuroepithelium of an acetaldehyde-treated embryo. The cells at the surface of the neural ectoderm have rounded-up and lost their normal contacts with their neighbours. This illustration is at the same magnification as the area of neural ectoderm shown in Fig. 9.

**Fig. 11.** Acetaldehyde-treated embryo at the 12–14-somite and early ‘turned’ stage. Note the abnormal appearance of the cephalic and caudal extremities of the neural tube. In the cephalic region a small bridge (B) has formed between the presumptive fore- and mid-brain regions. The neural folds overlying the fore-brain (F), the mid-brain (M) and hind-brain (H) regions have failed to fuse, and are markedly everted. The primitive heart tube is also clearly seen (arrowed), as is the first branchial arch (I). This embryo would almost certainly have developed exencephaly.

**Figs 12 and 13.** Lateral (Fig. 12) and postero-lateral (Fig. 13) views of the cephalic region of an embryo isolated early on Day 12, having previously been exposed in vivo to copper sulphate on Day 8. Note that the embryo has developed exencephaly mainly involving the mid- and hind-brain regions. The boundary zone between the surface and neural ectoderm is clearly seen (Fig. 12, arrowed). Apart from the abnormality in the cephalic region, the rest of this embryo appeared to be quite normal, although similarly treated embryos developed, in addition to exencephaly, cardiac abnormalities.
PLATE 3

Scanning electron micrographs showing the development of the embryonic mouse heart from the 'cardiogenic plate' stage, observed early on Day 9, to the formation of the primitive four-chambered heart, observed on Day 11. The pericardial cavity and its contents have been exposed in these embryos by removal of the overlying antero-ventral layer of surface ectoderm and the adherent subjacent mesenchyme and layer of parietal pericardium.

Fig. 14. Frontal view of 1–2-somite embryo. The headfolds (H), foregut pocket (F) and pericardial cavity (P) are clearly seen.

Fig. 15. Close-up view of part of the exposed pericardial cavity of the embryo illustrated in Fig. 14. A small collection of presumptive myocardial cells can be identified (arrowed) protruding from the ventral splanchnic wall into the pericardial cavity.

Fig. 16. Frontal view of 3–4-somite embryo. The two cardiac rudiments abut against each other in the midline, and are separated by a deep furrow. They are functionally separate entities each containing a medially-directed blind-ending lumen which is lined by a single layer of endothelial cells.

Fig. 17. Frontal view of 5–6-somite embryo. The two horns of the sinus venosus (V) pass around the foregut pocket (F) to enter at the venous end of the median primitive heart. A very early stage in the formation of the optic pit (arrowed) may be seen.

Fig. 18. Frontal view of 6–7-somite embryo. The enlargement of the ventricular region of the primitive heart causes characteristic 'bulges' to appear on either side of the midline which are separated by a shallow groove. The primitive left atrial dilatation is just visible (arrowed). The right ventricular dilatation (R) is continuous with the bulbus cordis (B). Note also the increase in the depth of the optic pits (O) compared to the situation illustrated in Fig. 17.

Fig. 19. Frontal view of an 8–9-somite embryo, the heart is now clearly S-shaped, with a pronounced ventral curvature. The bulbar region (B) is continuous cranially with the myocardial 'mantle' tissue which embraces the origins of the first aortic arch arteries.

Fig. 20. Frontal view of a 10–12-somite embryo. Note in particular the marked degree of ventricular dilatation and the relative decrease in diameter of the bulbus cordis compared to the situation illustrated in Fig. 19. Ventral fusion of the cephalic neural folds occurs initially at two sites: in close proximity to the buccopharynx, and at the junction between the presumptive fore- and mid-brain (arrows). Note also the obvious optic eminences (E) overlying the deep optic pits, and the location of the first branchial arch (I).

Fig. 21. Frontal view of an advanced-somite embryo isolated on the morning on Day 11. The head has been removed to facilitate observation of the upper ventral surface of the heart. The right and left auricular appendages (A) of the atria are clearly seen on either side of the cranial (distal) part of the bulbus cordis. The proximal part of the bulbar region becomes absorbed into the right ventricle. The origins of the first aortic arch arteries are just visible (arrowed). Note the pronounced bulbo-ventricular sulcus and the location of the fore-limb buds (F).

PLATE 4

Transmission electron micrographs illustrating the 2 principal features associated with the transition of cardiac cells from the 'undifferentiated' myoblast stage to the 'differentiated' myocyte: the laying down of contractile elements within the cytoplasm to form sarcomeres, and the development of intercalated disks. Figures 22–24 are sections taken from the heart region of a 6-somite mouse embryo. The area illustrated in Fig. 25 is taken from the ventricular region of a 14–16-somite mouse embryo.

Fig. 22. Area of cytoplasm proximal to the nucleus of a myocardial cell in which randomly orientated filamentous elements (E) may be observed. Several areas where a degree of organization are apparent are also seen in this field (large arrows). × 14 400.

Fig. 23. Area of cytoplasm towards the periphery of a cell in which a considerable degree of myofibrillogenesis has taken place with the formation of a group of sarcomeres separated by regularly spaced Z-bands (Z, arrowed). The bundle is relatively narrow, and quite close to the cell membrane (M), × 700.

Fig. 24. Bundle of filamentous elements organized into sarcomeres which are in continuity with similar elements in another cardiac cell via a primitive intercalated disk (arrowed), × 700.

Fig. 25. Considerably more organized state of myofibrillogenesis than that illustrated in Figs 22–24. Most of the contractile elements appear to be organized into sarcomeres, separated by well-developed Z-bands. A suggestion of A and I banding may also be observed in the sarcomeres proximal to the intercalated disk (D). A degree of branching of this bundle is also apparent (arrowed), × 700.
no obvious embryotoxic effect, a proportion of the embryos tested with $2.5 \times 10^{-5}$ m copper developed neural tube defects involving the cephalic region. However, with the highest concentration of copper tested ($5 \times 10^{-5}$ m) severe malformations involving the neural tube in the head region were frequently observed. The most common defect appeared to result from failure of elevation of the cephalic neural folds, and consequently resulted in failure of closure of the neural tube in the affected region. Despite the grossly abnormal appearance of the head region, almost all of the treated embryos had a beating heart and functioning yolk-sac circulation at the end of the culture period. The defects observed in these in-vitro experiments were therefore remarkably similar to those observed in the in-vivo experiments, suggesting that in-vivo administration of copper has a direct teratogenic effect on the embryo. The similarity of effects of copper on mouse embryos in vivo and in vitro suggests that the pathological changes observed probably resulted from a direct embryotoxic effect of the copper.

A considerable number of the acetaldehyde- and copper-treated embryos showed gross abnormalities involving closure defects of the 'cephalic' and 'spinal' regions of the neural tube. Acetaldehyde-exposed embryos with 25-30 pairs of somites, which appeared externally to be morphologically quite normal, often showed evidence of neuroepithelial damage involving all regions of the brain and neural tube. Similar changes were also induced by embryonic exposure to copper, although, in addition to defects involving the neural tube, a higher proportion than in the acetaldehyde series developed cardiac abnormalities. Ultrastructural examination of the neuroepithelial cells (O'Shea & Kaufman, 1980) revealed, in addition to closure defects at different levels along the neural axis, a considerable degree of damage to the intracellular cytoskeletal elements. Aggregations of filamentous material were also observed within the cytoplasm of neuroepithelial cells which were otherwise devoid of microtubular elements. It was therefore concluded that damage to these cytoskeletal structures may have played a considerable part in the neural tube damage induced by copper, because these elements are thought to play an important role in neuroepithelial cell elongation, which is an essential step during the process of neurulation (Karfunkel, 1974).

Observations on embryos examined on Day 12 after conception revealed that over 50% of the affected fetuses had neural tube defects involving the cephalic region, ranging from a collapsed appearance of the hind brain to exencephaly involving the mid- and hind-brain regions.

Véghelyi & Osztovics (1978) have demonstrated that an acetaldehyde level above 40 $\mu$m inhibited multiplication of lymphocytes and fibroblasts in culture, and significantly elevated the incidence of sister chromatid exchanges.

Similar neural tube anomalies to those induced by embryonic exposure to acetaldehyde and copper are also observed in certain mutant strains of mice. Observations were made on the offspring of mutant mice bearing Snell's translocation (T(2;4)Sn), a high proportion of which develops exencephaly and open neural tube defects along the spinal axis (Snell et al., 1935). This work has mainly been carried out by Dr O'Shea (O'Shea, 1979), the mice being obtained from the M.R.C. Radiobiology Unit at Harwell, by courtesy of Dr M. F. Lyon and Ms M. K. Kirk.

Male carriers heterozygous for the translocation were mated to normal females, and the offspring examined between Days 9 and 12 of pregnancy. Approximately 50% of the embryos died during the early post-implantation period, or survived but developed a range of neural tube abnormalities. The remaining embryos appeared to be quite normal. Apart from evidence of growth retardation, little morphological evidence of neural tube pathology was apparent when surviving embryos were examined on Day 9. On Day 10 open neural tube defects were observed in about 30% of the living embryos both in the 'cervical' (80%) and 'spinal' (20%) regions of the cord. By Day 11 approximately 20% of the surviving embryos had a neural tube defect, and in an additional 15% the head had a flattened appearance from side to side. By Day 12 the incidence of resorptions had increased from a fairly constant level of 20-25% to almost 40%, while the incidence of neural tube defects in surviving embryos had decreased to just under 20%. The types of 'spinal' defects encountered ranged from moderate to severe spina bifida-like lesions.
at various levels along the neural axis, while the lesions involving the cephalic part of the neural tube ranged from small mid-brain exencephalic defects to detects in which the entire cephalic neural tube had apparently failed to close.

Scanning electron micrographic observations on abnormal embryos showed that even in apparently closed regions the neural tube appeared to be wavy or kinked. In addition, on Day 10, the surface–neuroepithelial junctional zone was always poorly demarcated. The ruffles, blebs and lamellipodia observed in this region in control material were invariably absent in affected embryos. By Days 11 and 12 the neuroepithelial surface was often rough and characterized by the presence of small blebs and protrusions. In the juxtaluminal region there were two types of neuroepithelial abnormalities; (a) very variable degrees of apical constriction of the cells bordering the lumen, possibly due to irregular spacing of junctional complexes in this region, and (b) the presence of junctional complexes well below their usual location, often some considerable distance from the cell apex. Presumably when constriction of microfilaments occurs this would result in the extrusion of large amounts of cellular material into the lumen.

Because of the considerable variability observed between the morphologically abnormal embryos and fetuses, it appears likely that several closely related faults may be present. At this stage, it is only possible to speculate, but it seems likely that a relationship may exist between the type of unbalanced genotype present and the phenotype of these individuals, the former being related to the location of the duplicated segment and the particular chromosome segment deficiency involved. It would, of course, be necessary to carry out a comprehensive karyotypic analysis on these early embryos to determine whether such a relationship exists. Similar neural tube defects have also been observed in mutant mice bearing the lethal allele l9 (T-locus) (Spiegelman & Bennett, 1974). In these abnormal embryos poorly formed junctional complexes were observed between the underlying mesodermal cells, and it was speculated that this may have played a part in the development of these defects. In the loop tail mutant a similar abnormal neuroepithelial appearance was observed, possibly due to a deficiency of the microfilamentous elements (Wilson, 1978).

Failure of closure of the neural tube, particularly in the cephalic region, is such a sensitive indicator of embryonic exposure to a teratogenic insult in the mouse that the presence of exencephaly at mid-term may at best only serve as a general guide to the timing of exposure to a noxious agent. For embryos with an abnormal karyotype, due to a numerical chromosome anomaly or a mutant gene, the presence of this anomaly may only serve to indicate at which stage during development genome unbalance first begins to be manifest. However, if a reliable incidence of this anomaly is achieved following exposure to a particular agent, observations carried out during the early somite period (just before and shortly after the mouse embryo ‘turns’ to adopt the characteristic fetal position) on the ultrastructural damage induced within the neuroepithelium and closely related cells may well provide useful clues as to the underlying mode of action of the agent under investigation.

**Congenital cardiac disease**

The incidence of congenital cardiac abnormalities in man has been estimated as being of the order of 0.5–0.8% of all live births (Carter, 1976; Leck, 1977). A very broad spectrum of congenital cardiac defects is recognized, from the presence of relatively mild lesions which may only be manifest during childhood or even later, by minimally reducing the exercise tolerance of the individual, to severe abnormalities which are incompatible with viability beyond a matter of hours after birth.

In one typical series with well-established diagnoses, Campbell (1961) considered 136 patients with 153 cardiac malformations. He observed that the commonest abnormality present was patent ductus arteriosus (58% of cases), then ventricular septal defect (18%), followed by
atrial septal defect (7%), tetralogy of Fallot (7%) and pulmonary valvular stenosis. The incidence of the various types of abnormalities varies quite considerably between individual series. Thus atrial septal defect, for example, generally accounts for about 17% of the total population with congenital heart disease (Wood, Magidson & Wilson, 1954), whereas in children’s series it is less conspicuous, being fourth in frequency and making up about 7% of the total. Indeed, this condition may not be diagnosed in up to 50% of the cases before the age of 18 years. The generally more severe ventricular septal defects account for between 12% (Nadas, 1957; Lamy, DeGrouchy & Schweisguth, 1957) and 22% (Keith, Rowe & Vlad, 1966) of the cases seen in children’s clinics and hospitals dealing with cardiac patients.

In the majority of individuals with congenital cardiovascular defects no obvious etiological factors can be implicated. In only a relatively small proportion is an adequate genetic pedigree or history of exposure to a teratogenic agent available.

A considerable body of evidence is available to suggest that in some cases a genetic mechanism may be involved in the development of congenital heart disease. Some lesions, e.g. supravalvular aortic stenosis, have resulted from the presence of a dominant gene (Jørgensen & Beuren, 1965), while in some cases of atrial septal defect a typical history of familial inheritance has been observed (Weil & Allenstein, 1961; for review, see Nora, McNamara & Clarke Fraser, 1967). However, even when a familial history is available, it is not always possible to exclude persistent environmental or maternal factors which could be responsible. It is now well established that an association often exists between congenital heart disease and chromosome anomalies. This association has frequently been observed in certain trisomic syndromes, such as trisomy 21, 18 and 13–15. A fairly high incidence is also observed in Turner’s syndrome, but in only a few cases have the cardiovascular anomalies been reliably determined at necropsy. For trisomy 18 and 13–15, with which death commonly occurs within a few months after birth, sufficient necropsies have been carried out to demonstrate both the high incidence of congenital heart disease and the great variability of the pathological findings (Warkany, Passarge & Smith, 1966).

Many observations are available to suggest that non-genetic post-conception or environmental factors can also induce congenital heart disease (see Warkany, 1971). Numerous studies have shown that many adverse situations during pregnancy can result in congenital heart disease in experimental animals, notably Vitamin A deficiency (Wilson & Warkany, 1949), hypervitaminosis A (Kalter & Warkany, 1961), folic acid deficiency (Baird, Nelson, Monie & Evans, 1954), hypoxia (Ingalls, Curley & Prindle, 1952), the administration of Trypan blue (Gillman, Gilbert, Gillman & Spence, 1948) and X-irradiation (Wilson, Jordan & Brent, 1953). In man, maternal rubella, thalidomide damage, and discordant monozygotic twins also point to the influence of non-genetic factors in many cases of congenital heart disease (for discussion, see Warkany, 1971).

Embryological development of the heart

The early stages in the development of the mammalian heart are of considerable interest to the embryologist principally because the transport of oxygenated blood and nutrients from the primitive placenta to the tissues of the embryo is first established during the early somite period, and becomes essential when the mass of the conceptus can no longer be adequately nourished by perfusion alone. Once regular contractions take place, the heart must function continuously throughout the life of the individual. At the ultrastructural level, the events associated with the onset of the first irregular contractions, and later by the development of rhythmicity, are paralleled by myocardial cellular differentiation. The first differentiative events to be observed are associated with the process of fibrillogenesis and sarcomere formation, and slightly later by the production of a functional syncytium through the formation of intercalated disks (Challice & Viragh, 1973; Viragh & Challice, 1973).
An examination of the literature has shown that very little information is available on the earliest stages of development of the normal mammalian heart covering the period from the formation of the pericardial–coelomic cavity, and initial laying down of the cardiogenic plate, up to the earliest stages of heart tube formation. However, once loop formation has been initiated, the overall events which take place leading to the formation of the ventricles and atria are reasonably well documented (see, for example, Patten, 1960; Hamilton & Mossman, 1972), although the embryological terminology used is far from universal, and remains a constant source of confusion (see, for example, discussion in Anderson, Wilkinson & Becker, 1978).

One of the principal ways of investigating the pathogenesis of certain anomalies of human cardiac development involves the reliable production of phenocopies, either by the administration of teratogenic agents at appropriate stages of gestation, or by the examination of the embryonic stages of mutant strains of animals which commonly develop cardiac anomalies (for example, see Layton, 1978; Patterson, 1978; Buchanan, 1978; Van Mierop & Patterson, 1978). Sequential observations may then be made to examine the development of the defect in much the same way as described earlier for the examination of anomalies of neural tube development.

To clarify the exact timing and events associated with the transition from the intra-embryonic coelomic to the pericardial cavity, and the formation of the primitive heart tube in mammals, serial standard histological sections through a representative collection of mouse embryos were examined in detail (Kaufman & Navaratnam, 1981) and an overall descriptive account of the earliest stages of cardiac organogenesis has been prepared. An additional series of embryos was mounted in epoxy resin and semi-thin sections cut at a nominal thickness of 0.5–1 μm. ‘Thin’ sections from appropriate regions of the heart were also taken for subsequent analysis by transmission electron microscopy. In a further group of embryos the body wall overlying the cardiac region was dissected away to expose the contents of the pericardial cavity. This contained the cardiogenic ‘plate’ in the earliest embryos, and the primitive cardiac loop in more advanced embryos. The pericardial contents were subsequently examined by scanning electron microscopy (Pl. 3, Figs 14–21).

It was evident from the initial histological analysis that the presumptive myocardial rudiments were established by differentiation of the mesothelial-like cells lining the pericardial cavity which were adjacent to the endoderm and in close proximity to the foregut pocket (Kaufman & Navaratnam, 1981). At first, the cardiogenic ‘plate’ consists mainly of cuboidal cells which lie on the ventral aspect of the pericardial cavity (Pl. 3, Fig. 15), but later, as a result of the changes which take place in the overall configuration of the embryo, they come to lie on its dorsal wall.

A separate layer of cells forms deep to the primitive myocardium, possibly by differentiation of the underlying mesenchyme. This represents the first evidence of endocardial tissue. By about the time that the first 1–2 pairs of somites have appeared, cavitation occurs within the endocardial tissue, and establishes a bilaterally symmetrical pair of relatively flattened endocardial tubes (Pl. 3, Fig. 16). Slightly later the myocardial ‘plate’ which overlies the endocardial tubes appears to bulge into the pericardial cavity. In embryos with about 4 pairs of somites, the endocardial tubes coalesce across the midline to establish a single median cardiac tube which is now suspended in the midline by a wide dorsal mesocardium (Pl. 3, Fig. 17).

These observations seem to be at variance with the widely prevalent view that the endocardial elements in mammalian embryos differentiate from the myocardial (commonly, though possibly incorrectly termed ‘myoepicardial’ (Manasek, 1969)) cells, and indicate that the endocardial elements are probably formed in situ from the subjacent mesenchyme cells rather than from the myocardial cells as originally proposed by His (1885), an opinion supported by others (see, for example, Mollier, 1906; Yoshinaga, 1921; Davis, 1927). However, the present observations do not rule out the possibility that both the myocardial and endocardial elements migrate independently into the cardiac region in the manner described for the chick embryo by Rosenquist & De Haan (1966).

Once the primitive cardiac tube has been formed, and the heart developed an obvious
venous inflow and arterial outflow tract, termed the sinus venosus and bulbus cordis, respectively, a slight degree of asymmetry now becomes apparent (Pl. 3, Figs 18 and 19). From this stage onwards, the morphology of the embryonic mouse heart closely resembles the general text-book descriptions available of early human heart development (see, for example, Patten, 1960; Sissman, 1970; Hamilton & Mossman, 1972; for time-table of human cardiogenesis, see O’Rahilly, 1971).

This descriptive account of early heart development has been complemented by ultrastructural studies (M. H. Kaufman, unpublished) which have clearly demonstrated that the increasing degree of cytodifferentiation which takes place over this period is closely paralleled by the functional activity of the primitive heart tube (see also studies by Challice & Virág, 1973; Virág & Challice, 1973).

The first differentiative event to be observed at the ultrastructural level is probably the laying down within the cytoplasm of discrete contractile elements (Pl. 4, Fig. 22), and their ultimate organization into characteristic cardiac muscle fibres (Pl. 4, Fig. 23) (myofibrillogenesis). The second important developmental step is the formation of a functional syncytium by the anatomical and physiological linking of individual cardiac cells by intercalated disks (Pl. 4, Figs 24 and 25). The latter process allows the whole heart to act as an integrated unit. The first contractile elements that are laid down within the cytoplasm of the myocardium have been observed in embryos with 1 or 2 pairs of somites. By the time 4–6 pairs of somites are present the various elements have become aligned to form discrete myofibrillar bundles. Even at this early stage evidence of sarcomere formation is already apparent due to the presence of Z-bands (Pl. 4, Fig. 23), although obvious A and I bands are not yet clearly visible (M. H. Kaufman, unpublished). In embryos with about 6 pairs of somites, evidence of primitive disk formation is also seen (Pl. 4, Fig. 24), and the ultrastructural differentiation which is observed within the cardiac cells is clearly linked to the functional requirements of the heart, as the first regular contractions have been observed at about this time (Goss, 1938). This occurs well before the heart receives any form of innervation (Navaratnam, 1965).

The changes which take place between the simple cardiac-loop stage of development and formation of the 4-chambered heart of the fetus necessitates the establishment and continued functioning of complex associations between cardiac muscle cells and the subjacent endo- and epicardial tissues. The cellular interactions which are thought to be involved during these stages of cardiac morphogenesis have been reviewed by Manasek (1976).

It has also been proposed, on the strength of experimental findings, that some, possibly even a considerable proportion, of the changes involved in cardiac morphogenesis are brought about in response to alterations in cardiovascular haemodynamics (see recent review by Jaffee, 1977). Examples of morphological changes which are almost certainly brought about in this way are the development of the spiral septum in the arterial outflow region of embryonic heart, and, much later, the considerable circulatory changes which take place within the heart and in the circulation at birth. Indeed, temporary, reversible changes in the haemodynamic equilibrium which have been induced by hypoxia have been shown to result in the production of malformations of the heart in the chick embryo (Jaffee, 1974).

Influence of teratogens on cardiac development

The large number of individual steps which must take place if cardiac organogenesis is to proceed normally, almost certainly involving multiple sequential inductive interactions, presumably explains why the heart and cardiovascular system are so susceptible to teratogenic insult over such a long period of time. However, no clear correlation usually exists between the timing of the teratogenic insult and the type of cardiac abnormality induced, although it seems likely that severe damage incurred at the late primitive-streak stage or during the early somite period, involving interference with myofibrillogenesis or disk formation may, as a result of
subsequent circulatory failure, be incompatible with viability beyond the early fetal period. However, although this would appear to be a plausible explanation to account for a proportion of early fetal losses, no experimental evidence is available to substantiate this hypothesis. Less severe damage incurred during the early embryonic period is likely to affect looping, and subsequently result in the production of major cardiac abnormalities.

In experimental studies in which early rat embryos were treated with Trypan blue (Monie, Takaes & Warkany, 1966) or by ionizing irradiation (Fox & Goss, 1957) transposition of the great vessels was occasionally produced. In all cases, this condition was associated with abnormalities in looping of the primitive heart tube. This experimental evidence would seem to suggest that transposition of the great vessels probably results from damage induced at the time of cardiac looping, and not considerably later by interference with normal morphogenesis of the outflow tract as has previously been thought (for discussion, see Pexieder, 1978). This would also seem to be a relatively clear instance in which interference with looping produced sequelae involving the derivatives of the branchial arch vasculature, rather than the structural components of the heart itself, although the developmental processes of the two are, of course, intimately related. The validity of looping as a general model of cardiac morphogenesis has been discussed by Manasek, Kulikowski & Fitzpatrick (1978), and the observation made that several differences exist between hearts at the time of looping and hearts at later stages of morphogenesis, particularly in their response to alterations in blood flow; initially, looping proceeds in the absence of blood flow, but later morphogenetic processes may be altered if blood flow is disturbed. However, the analysis of the looping process does provide insight into the factors which influence the shape of developing cardiac muscle cells (Manasek, Burnside & Waterman, 1972). This is of fundamental importance, since these changes in cell shape are responsible for establishing the basic shape of the developing heart.

The morphological and structural changes which are associated with looping are closely correlated with cytodifferentiation of the cardiac muscle cells, as the transition from the 'undifferentiated' myoblast cell to the developing myocyte takes place at this time. That these events are causally related seems clear from the experimental finding that prevention of myofibrillar assembly by various agents, such as inhibitors of protein synthesis and substances which act on 'cytoskeletal' or 'contractile' proteins (for details, see Manasek, 1976), prevent looping. Okamoto (1968) reported a maximal incidence of cardiovascular anomalies (84-6%) in rat embryos, when pregnant females were exposed on the 8th day after conception to a single dose of 130 rads of whole-body neutron irradiation. Transposition of the great vessels was found in 69-2% of these abnormal embryos. In a particularly thorough survey on the effect of neutron irradiation on rat embryos, Satow, Okamoto, Hidaka, Akimoto & Miyabara (1978) studied the ultrastructural changes occurring within cardiac myocytes in the 24-h period after irradiation. Maximal effects were observed at about 6 h after treatment (see also Satow, 1971; Ikeda, Okamoto & Satow, 1972), with pycnosis, dilated rough endoplasmic reticulum and/or mitochondria, and vacuolation. In the embryos which survived up to the end of the 2nd day after irradiation, no evidence of cellular degeneration was observed in the heart, although a slight retardation of embryonic growth and development was noticed in most cases, as were anomalies of looping. However, a range of morphological anomalies was observed when fetuses were examined up to 11 days after irradiation, and it was proposed that these abnormalities probably resulted from 'disorders of the various germ layer derivatives, effects of irradiation on genes, and to a decrease in the number of cardiac cells present as a result of cell death following irradiation treatment'.

After looping has been achieved, and the overall shape of the myocardial cells has been relatively stabilized due to the increasing degree of cytodifferentiation which takes place within and between them, it is likely that abnormalities involving later states of cardiac development result from interference with other morphogenetic mechanisms acting at these times. For example, it is well recognized that 'physiological' or 'programmed' cell death plays an important
part in the later stages of the development of the heart and circulation (chick: Hughes, 1948; Manasek, 1969; Menkes, Dandor & Illies, 1970; Pexieder, 1975; rat: Okamoto & Satow, 1975). Differential tissue growth also plays an important role during the later post-looping stages of cardiogenesis, and this aspect of cardiac growth is closely related to the complex interactions which take place between dissimilar cell types at this time (for extensive discussion on the morphogenetic significance of tissue interactions during cardiac development in the chick embryo, see Manasek, 1976).

It will be apparent from the previous discussion that additional basic descriptive and experimental embryological and teratological work will have to be carried out before a thorough understanding of the morphogenetic factors which influence normal mammalian cardiogenesis can be achieved. Only then will it be possible to understand the sequential changes that occur during the production of anomalies of the heart and cardiovascular system. As with the study of neural tube abnormalities, it is essential that comprehensive accounts of the normal events associated with cardiogenesis should be available to the teratologist. These studies should not only present a picture of the normal sequence of events occurring during cardiac development, but should also indicate the degree of variability which may be encountered between individual embryos, between strains and between different species.

Because of the very limited amount of human embryonic material available covering the earliest stages of organogenesis, it seems likely that, in the foreseeable future, mammalian embryologists will have to be satisfied with the analysis of rodent and possibly non-human primate material. Certainly, during the more advanced stages and possibly even during the earlier stages of cardiogenesis, this mammalian material more closely resembles early human development than the almost universally employed chick model which in many respects bears a stronger resemblance to the reptilian heart. It should also be recalled that some of the early somite-stage human embryos, e.g. after therapeutic termination of pregnancy, might in any case have been aborted spontaneously shortly thereafter, possibly as a result of anomalies of the cardiovascular system. In addition, the very low incidence of spontaneous embryonic and fetal losses in rodents, and the presence of very few gross morphological abnormalities in surviving offspring, would appear to be an additional factor in favour of continuing to concentrate our efforts in the analysis of morphogenetic events in these experimental animals.

Conclusions

By understanding in morphological terms how a range of environmental teratogens act under controlled conditions in experimental animals, and appreciating how certain genetically determined lesions are produced, it may then be possible to draw certain conclusions which may go some way towards assisting our understanding of the pathogenesis of similar lesions which occur in man (Poswillo, 1976; Clarke Fraser, 1977). It is also likely to be by this means that a rational approach to the treatment of certain human congenital abnormalities may be achieved. In other instances, knowledge of their pathogenesis may indicate how certain conditions may be prevented, or their incidence considerably reduced. Because this goal is unlikely to be achieved in the near future, it is, of course, essential to improve the efficiency of early prenatal diagnostic methods capable of recognizing major congenital anomalies (see, for example, Brock, 1976; Laurence & Gregory, 1976; MacVicar, 1976).

While extrapolation from the results of animal experiments to man must only be carried out with extreme caution, I believe that the principal long-term aim of experimental teratology must be towards achieving a reduction in the present high incidence of human congenital abnormalities by prophylactic measures, if at all possible. The ultimate aim must therefore be towards the encouragement of rational forms of treatment in these cases, based on a foundation of sound experimental studies.
The work is supported by a grant from the National Fund for Research into Crippling Diseases.

References


Establishment in culture of pluripotential cells from mouse embryos

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Pluripotential cells are present in a mouse embryo until at least an early post-implantation stage, as shown by their ability to take part in the formation of chimaeric animals¹ and to form teratocarcinomas². Until now it has not been possible to establish progressively growing cultures of these cells in vitro, and cell lines have only been obtained after teratocarcinoma formation in vivo. We report here the establishment in tissue culture of pluripotent cell lines which have been isolated directly from in vitro cultures of mouse blastocysts. These cells are able to differentiate either in vitro or after inoculation into a mouse as a tumour in vivo. They have a normal karyotype.

Previous attempts to obtain cultures of pluripotential cells directly from a mouse embryo have been unsuccessful³⁴, although cells with a similar appearance have been reported to be present transiently⁵⁶. We considered that success might depend on three critical factors: (1) the exact stage at which pluripotential cells capable of growth in tissue culture exist in the embryo; (2) explantation of a sufficiently large number of these precursor cells from each embryo; and (3) tissue culture conditions most conducive to multiplication rather than differentiation of these embryonic cells. These considerations have been discussed at greater length elsewhere⁷. An indication of the optimal stage of embryonic development might be gained by a comparison of the properties of embryonic cells at various stages with established cultures of embryonal carcinoma (EC) cells. Cell-surface antigen expression and the patterns of protein synthesis revealed by two-dimensional electrophoresis have suggested that neither the cells of the 6½-day ectoderm nor those of the 5½-day inner cell mass show homology with EC cells, but that epiblast cells of the early post-implantation embryo at 5½ days post coitum may do so⁸ (the day of finding coital plug is termed day 0). Cells from embryos of an early post-implantation stage seem to be the best candidates for direct progenitors of pluripotential cells in culture. As these embryos are difficult to isolate, and as the cell number in the isolated epiblast is small, we chose an alternative route to obtain embryo cells at this stage of development.

Mouse blastocysts may be induced to enter a state of diapause just before implantation. This delay in implantation depends on the maternal hormonal conditions, and may be induced experimentally by ovariectomy at an appropriate stage³. Embryos in implantational delay hatch from the zona but remain free-floating in the uterine lumen. A gradual increase in cell number occurs³⁴, and the primary endoderm may be formed but no further development takes place until implantation occurs, under the control of hormonal stimuli.

129 Sv/E mice were caged in pairs and examined for mating plugs each morning. They were ovariectomized on the afternoon of day 2½ of pregnancy, injected subcutaneously with 1 mg Depo-Provera (Upjohn), and delayed blastocysts were recovered 4–6 days later. The blastocysts were cultured intact in groups of about six embryos in small drops of tissue culture medium under paraffin oil on tissue culture plastic Petri dishes for 4 days. The blastocysts attached within 48 h and the trophoderm cells grew out and differentiated into giant trophoblast cells. The inner cell mass cells subsequently developed into large egg cylinder-like structures, with a group of small round cells surrounded by endodermal cells growing attached to the Petri dish. The egg cylinder-like structures were picked off the dish, dispersed by trypsin treatment and passaged on to gelatin-pretreated Petri dishes containing mitomycin C-inactivated STO fibroblasts. All culture was carried out in Dulbecco’s modified minimal essential medium supplemented with 10% fetal calf serum and 10% newborn calf serum. The cultures were examined daily and passaged by trypsinization every 2–3 days. Actively proliferating colonies of cells closely resembling EC cells were apparent from an early stage. These colonies were picked out, passaged and mass cultures grown. The cell cultures had the appearance and general growth characteristics of feeder-dependent EC cells (Fig. 1).

The embryos used to initiate these cultures are from normal 129 Sv/E strain mice, that is, from the same strain of mice as many EC cell lines, in particular those grown in this laboratory. Therefore it was important to exclude any possibility of
Embryo and their passages of cells. An XY karyotype of male and female cell lines. Cell cultures are free of contamination of these established embryoid bodies and teratocarcinomas. The network of inter-relationships between the mouse embryo and pluripotential cells derived from it has previously lacked only the direct link between the embryo and cells in culture for completion. We have now demonstrated this (Fig. 3).

Teratocarcinoma cells are now being widely used as a model for the study of developmental processes of early embryonic cell commitment and differentiation. Their use as a vehicle for the transfer into the mouse genome of mutant alleles, either selected in cell culture or inserted into the cells via transformation with specific DNA fragments, has been presented as an attractive proposition. In many of these studies the use of pluripotential cells directly isolated from the embryos under study have great advantages. We have now shown that these EK cell lines are readily established from cultures of single blastocysts and so far have 15 lines of independent embryonic origin, some of which have been isolated from non-129, outbred mouse stocks. We are now studying the chimaeric mice formed from these cells.

We thank Mrs A. Burling for technical assistance and Dr E. P. Evans for advice regarding karyotype analysis. M.J.E. and M.H.K. were supported by the MRC; M.J.E. also received support from the Cancer Research Campaign.

Received 6 February; accepted 14 April 1981.

Early differentiation of the heart in mouse embryos

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(Accepted 6 October 1980)

INTRODUCTION

Many of the original workers in the field of vertebrate cardiac development studied the early embryology of the mammalian heart (e.g. human: Davis, 1927; rat: Goss, 1938, 1952; guinea-pig: Yoshinaga, 1921; rabbit: Van der Stricht, 1895), and the standard descriptions of its development have been based on their analyses of serial sections viewed under the light microscope. Others, however, have examined embryonic chick and duck hearts to provide a descriptive account of early avian development (duck: Yoshida, 1932; chick: Sabin, 1920; Patten & Kramer, 1933), and more recently, in order to provide a more dynamic account of the cellular and tissue interactions involved in avian cardiac morphogenesis (chick: De Haan, 1964, 1965; Argüello, de la Cruz & Gómez, 1975; Manasek, 1976). Indeed, all the recent important work on cardiac morphogenesis has been concentrated on the ultrastructural morphology of the avian heart (for review, see Manasek, 1976). However, fundamental differences exist between cardiogenesis in avians and in mammals, particularly in the timing of the first appearance of the pericardial cavity. In the avian embryo two amniocardioc vesicles first appear at about the 1- to 3-somite stage, and median fusion occurs at about the 9-somite stage. Likewise, in the avian embryo, the presence of the primitive cardiac cells, which are eventually located in the splanchnic layer of mesoderm, are thought to migrate to this location before the splanchnic and somatic mesoderm separate to form the pericardial cavity (for detailed discussion, see De Haan, 1964, 1965; Rosenquist & De Haan, 1966). In mammals, the presumptive pericardial cavity appears at a considerably earlier stage of embryogenesis, and the primitive cardiac rudiments are thought to differentiate in situ (for reviews, see Yoshinaga, 1921; Davis, 1927), but little attention has been paid to this issue for several decades.

Although the mouse is now one of the most useful laboratory animals in embryological research, in the accounts available on mouse embryology (for example, see Snell & Stevens, 1966; Rugh, 1968; Theiler, 1972), these aspects of early cardiogenesis have been discussed in only the most superficial of terms. In the present study we describe the initial stages in the formation of the pericardial cavity, the development of the ‘myocardial plate’ from the cells lining the splanchnic pericardial wall, and the apparent origin of the endocardial rudiments from the subjacent mesenchyme.

These findings complement and extend earlier observations on the formation of these tissues in mammals, and fill a gap in our knowledge of the early embryology of the mouse.

We believe that the availability of modern techniques of analysis renders re-examination of the early embryonic development of the mammalian heart desirable in order to provide essential baseline data for subsequent morphogenetic analysis of...
cardiac development in mammals. The present study is based on scanning electron microscopy supplemented by light microscopy of thin plastic sections as well as of standard histological material.

We have avoided the widely used term 'myoepicardium' for the thickening of the visceral layer of pericardium, on account of the evidence presented by Manasek (1969) that the epicardial lining (in the chick) arises later than the myocardium and from a different source.

MATERIAL AND METHODS

Female CFLP mice (Hacking & Churchill Ltd) were mated with males of the same strain and isolated on the morning of finding a vaginal plug (designated the first day of pregnancy). Between the afternoon of the eighth day and the early afternoon of the ninth day of pregnancy individual females were killed by cervical dislocation and the embryos isolated in phosphate buffered saline (pH 7·3). The embryos were dissected free of their membranes and fixed in 2 % glutaraldehyde in 0·1 M sodium cacodylate buffer containing 10 gm sucrose/100 ml. After about 2 hours the embryos were transferred to 0·1 M sodium cacodylate buffer containing 3 gm sucrose/100 ml. After a period of 3–7 days the ventral wall of the trunk was dissected away to expose the developing heart. The embryos were then transferred through a graded series to 100 % acetone, critical point dried, sputter coated with gold and subsequently viewed in a Cambridge S 600 scanning electron microscope.

To complement the scanning electron micrographs, representative histological sections through the heart region at similar stages of development were also studied. The material was fixed in half-strength Bouin solution for 24 hours, dehydrated, cleared and embedded in paraffin wax. 6–7 μm transverse and sagittal sections were stained with haematoxylin and eosin. An additional group of early embryos (principally presomite and 1–2 somite stages) were fixed in glutaraldehyde as described above and, after approximately 2 hours, the material was washed briefly in 0·1 M sodium cacodylate buffer and post-fixed in 1 % osmium tetroxide containing 5 gm sucrose/100 ml for 25 minutes. The material was then embedded in epoxy resin (Spurr, 1969) and 1 μm transverse or coronal sections were taken from the cardiac region. These were subsequently stained with methylene blue and viewed in a Zeiss photomicroscope.

RESULTS

For convenience of description the embryos have been grouped according to the state of somite differentiation. However, it must be emphasized that some overlap in regard to cardiac differentiation is observed between the groups when this type of classification is employed. No absolute correlation exists between the state of development of the heart and the number of somites present. This applies equally to the degree of development of other systems that are differentiating during this stage of embryogenesis, for example, the degree of elevation and overall appearance of the neural folds in the 'spinal' and cephalic regions, and the early development of the optic primordium (Kaufman, 1979).

(1) Presomite stages

(a) Late primitive streak stage embryos

It was not possible for technical reasons to locate the coelomic/pericardial rudiments in scanning preparations of embryos isolated on the afternoon and early evening of the eighth day of gestation. The headfolds and foregut pocket were at a
very early stage of formation and the pericardial cavity had not differentiated sufficiently.

Examination of 1 μm plastic sections confirmed that splitting of the mesoderm in the presumptive pericardial region began about the same time that invagination of the foregut was initiated. Initially multiple narrow spaces appeared in the mesoderm between the most proximal part of the headfolds and the origin of the amnion in this region. These spaces were lined by uniformly flattened mesothelial cells (Fig. 1a, b).

(b) Advanced headfold stage embryos

In some embryos in this group (typically, those isolated late in the evening of the eighth and very early in the morning of the ninth day) more advanced stages of cardiogenesis were observed than those described in section 1(a), above. In these embryos the ventral lining of the prospective pericardial cavity had become thickened; the cells became plump and showed increased mitotic activity. Mesenchymal cells were often observed in the plane between the pericardial lining and the subjacent endoderm (Fig. 1c, d). However, in other embryos in which the headfolds were well developed, only those features of early pericardial development described in section 1(a), above, were observed (for example of embryo of this type, see Fig. 1a, b).

(2) 1–2-somite stage embryos

The scanning electron micrographs (Fig. 1e, f) showed the frontal view of an embryo isolated early on the ninth day of gestation. The headfolds and foregut were clearly seen. By removing the surface ectoderm and adherent parietal pericardial components of the anterior trunk wall, it was possible to identify the relatively wide pericardial cavity and to view the ventral splanchnic wall from within the pericardial cavity. A small collection of presumptive myocardial cells was identified protruding from this surface.

Examination of 1 μm plastic sections and standard histological material confirmed the presence of a wide pericardial space which was continuous caudally on either side of the foregut pocket. The ventral wall of pericardium was thickened to form the myocardial cells which, in some embryos, were prominent on either side of the midline. The thickened area initially comprised a single layer of cuboidal or columnar cells many of which showed evidence of mitotic activity. However, immediately subjacent to the thickened coelomic/presumptive myocardial epithelium separate cells were present which represented the prospective endocardial elements (Fig. 1g, h). Typically, these cells were more elongated and flattened than the myocardial elements. The endocardial cells were located in groups surrounding small cavities which subsequently coalesced in a predominantly anteroventral direction round the developing foregut pocket. In some of the embryos in this group slightly more advanced stages of cardiogenesis could be observed, in which the endocardial tissue had aggregated to form a pair of blind-ended tubes that approximated to each other but had not yet fused in the mid-line anterior to the foregut pocket.

(3) 3–4-somite stage embryos

The scanning electron micrographs showed considerably enlarged myocardial tubular thickenings which appeared to be attached to the lower part of the dorsal wall of the pericardial cavity. The thickenings were apparently bilateral and the two rudiments, while in cellular continuity, were separated by a prominent fissure (Fig. 2a) as they abutted against each other in the mid-line.
Differentiation of the embryonic mouse heart

Serial histological sections through embryos at this stage of development confirmed that the rudimentary endocardial tubes were bilateral entities which protruded into the pericardial cavity from both the ventral wall and from the lower part of the dorsal wall (Fig. 2b, c). The endocardial tubes were almost completely surrounded by the myocardial mantle tissue, which was continuous across the mid-line, and the cardiac rudiments were ‘suspended’ by the primitive ‘dorsal’ mesocardium (see Fig. 2c).

The apparent dorsal migration of the primitive cardiac tubes observed here was principally due to the gross caudal and ventral movement of the trunk region of the embryo containing the pericardial cavity and developing heart (Fig. 2d, e). This event took place at about the same time, and in a similar manner to that thought to occur in early human embryos, namely during the late pre- and early somite stages of development (Hamilton & Mossman, 1972).

Each endocardial tube was lined by a single layer of flattened cells, between which and the myocardial sleeve there had now appeared a space occupied by acellular material, presumably cardiac jelly.

(4) Embryos with more than 4 somites

The scanning electron micrographs showed that the median fissure between the primitive bilateral heart tubes became progressively filled out (Fig. 2f) and at about the 6-somite stage or slightly later the ensuing single heart tube gradually folded to assume an S-shaped configuration.

Examination of serial sections through the heart region of embryos at the 4- to 6-somite stage showed that the endocardial tubes had coalesced across the mid-line

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Fig. 1. (a) Transverse section through upper headfold region of advanced pre-somite embryo isolated late in the afternoon of the 8th day of gestation. Multiple narrow spaces lined by flattened mesothelial cells are present in the mesoderm in the presumptive pericardial region. In this section the extraembryonic coelom (E) is almost in continuity with the intraembryonic coelomic/presumptive pericardial cavity (P). The amnion (A) separates the amniotic cavity from the extraembryonic coelom. 1 µm section stained with methylene blue.

(b) Higher magnification of presumptive pericardial region. Section from same embryo as illustrated in Fig. 1(a), but from a slightly more caudal level. Mesenchyme cells (arrowed) are shown in close proximity to the flattened mesothelial cells (M).

(c) Transverse section through upper headfold region of a late pre-somite embryo at a slightly later stage of cardio genesis than the embryo illustrated in Fig. 1(a). 6–7 µm section stained with haematoxylin and eosin.

(d) Higher magnification of pericardial region of embryo illustrated in (c). The cells lining the ventral wall of the pericardial cavity are cuboidal, and large numbers of mesenchymal cells (arrowed) are present in the space between these presumptive myocardial cells (M) and the underlying endoderm (E).

(e), (f) Scanning electron micrographs showing frontal view of a 1–2-somite embryo isolated early on the 9th day of gestation. The two headfolds (H) and the foregut pocket (F) are clearly seen. By removing the surface ectoderm and adherent presumptive parietal pericardial layer of cells, a window has been created on the anteroventral aspect of the pericardial cavity (P). A small collection of presumptive myocardial cells (arrowed) can be identified protruding from the ventral splanchnic wall of the pericardial cavity in the close-up view (Fig. 1f). The small number of myocardial cells present (which extend across the mid-line) and the relatively large space available within the cavity are shown.

(g) Transverse section through an embryo at a similar stage of development to that illustrated in Fig. 1(e). The pericardial cavity (P) clearly extends laterally on either side of the foregut pocket (F). Immediately subjacent to the presumptive myocardial cells (M), which extend across the mid-line, multiple groups of flattened cells may be seen; these represent the prospective endocardial elements. 6–7 µm section stained with haematoxylin and eosin.

(h) Higher magnification of part of section illustrated in Fig. 1(g), showing presumptive myocardium and subjacent endocardial cells (arrowed).
to establish a single median tube (Fig. 2g, h); no obviously necrotic cells were observed near the region of coalescence. The heart tube was now suspended by a true dorsal mesocardium which was present in the form of a fairly wide ridge. The space between the endocardial lining of the lumen and the myocardial mantle was also relatively wider than at any previous or subsequent stage of development. Within the dorsal mesocardium, and also directed on either side of the foregut pocket, islands of squamous cells were observed that were morphologically identical to, and in continuity with, the endocardial cells (Fig. 3a, b). These islands of cells probably represented the anlagen of the first arch arteries. These cellular pools appeared to develop in much the same way as the endocardial rudiments described earlier, and almost certainly from the mesenchyme in these locations. At this very early stage of branchial arch formation these vessels passed directly dorsally away from the cranial extremity of the heart, whereas the two horns of the sinus venosus were widely separated, and were located more caudally and anteroventrally in relation to the foregut pocket. The heart at this stage was just beginning to lose its essentially symmetrical mid-line form (Fig. 3c, d, e, f).

**DISCUSSION**

Our observations on early aspects of cardiogenesis in the mouse presented here establish that the pericardial cavity develops in situ at the late primitive streak stage of development by the coalescence of multiple mesothelial lined spaces located within the intraembryonic mesoderm in close proximity to the cranial origin of the headfolds and the amnion in this region. Further, in this species the earliest evidence

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Fig. 2. (a) Scanning electron micrograph showing frontal view of a 3- to 4-somite embryo. The two cardiac rudiments abut against each other in the mid-line and are separated by a deep furrow. A considerable increase may be observed in the number of constituent myocardial cells present compared to the situation illustrated in Fig. 1(f).

(b), (c) Representative transverse sections through an embryo at a similar stage of development to that illustrated in Fig. 2(a). The sections clearly demonstrate that despite their close apposition in the mid-line, the two endocardial rudiments at this stage are separate entities, each containing a lumen lined by a single layer of endothelial cells. The region between the endocardial lining and the myocardial mantle cells contains cardiac jelly. The heart is 'suspended' by a primitive 'dorsal' mesocardium (Fig. 2c, arrow). The section illustrated in Fig. 2(b) is approximately 35 μm rostral to the section illustrated in Fig. 2(c). 6-7 μm sections stained with haematoxylin and eosin.

(d) Approximately median sagittal section through the heart region of a very early somite embryo. The pericardial cavity (arrowed) is located rostral to the headfold (H), and the cephalic mesenchyme cells extend between the myocardial cells (M) and the embryonic endoderm (E). 6-7 μm section stained with haematoxylin and eosin.

(e) Slightly oblique sagittal section through the heart region of a 4- to 5-somite embryo about 35 μm lateral to the median plane. The pericardial region is located on the anteroventral aspect of the cephalic region in close proximity to the foregut pocket (F). The myocardial mantle occupies much of the pericardial cavity in this region. The endocardial tissue is in continuity with the dorsal aorta (D) via a vessel in the dorsal mesocardium, presumably the first arch artery (I). 6-7 μm section stained with haematoxylin and eosin.

(f) Scanning electron micrograph of frontal view of a 4- to 5-somite embryo. There is no median furrow, and the two primitive heart tubes now communicate in the mid-line.

(g), (h) Coronal section through the heart region of a 4- to 5-somite embryo. The two primitive endocardial tubes now communicate with each other in the mid-line and the myocardial tissue is absent for some distance on either side of the ventral mid-line (region between arrows, Fig. 2g). In this location the endocardial tube is separated from the embryonic endoderm lining the foregut pocket only by the occasional mesenchyme cell (Fig. 2h, arrowed). The squamous nature of the endocardial cells (E) and cuboidal appearance of the myocardial cells (M) are more clearly seen in the higher magnification view (Fig. 2h). This embryo is at a similar stage of cardiogenesis to that illustrated in Fig. 2(f). 1 μm section stained with methylene blue.
Fig. 3. (a), (b) Transverse sections through the heart region of a 4- to 5-somite embryo. The section illustrated in Fig. 3(a) is approximately 35 μm rostral to that illustrated in Fig. 3(b). In both sections a relatively wide dorsal mesocardium is present. The endothelial continuity between the endocardial cells and the vessels passing laterally on either side of the foregut pocket, represents the anlagen of the first arch arteries (A). The two dorsal aortae (arrowed) are dorsal relations of the foregut. The bilaminar structure of the trunk wall (W) in the cardiac region is clearly seen. 6–7 μm sections stained with haematoxylin and eosin.

(c), (d) Scanning electron micrographs showing frontal view of a 5- to 6-somite embryo. There is considerable enlargement of the heart compared to the situation illustrated in Fig. 2(f). The vitelline veins (V) pass around the foregut (F) to fuse with the respective horns of the sinus venosus (S). An early stage in the development of the optic pits (arrowed) may be observed, particularly on the anterior aspect of the left headfold.

(e), (f) Scanning electron micrographs showing frontal view of a 6- to 7-somite embryo. The enlargement of the ventricular region causes characteristic bulges on either side of the mid-line which are separated by a shallow ‘interventricular’ groove. The primitive left atrial dilatation is just visible (arrow). Cranially, the right ventricular dilatation is continuous with the bulbus cordis which, at this stage, is a wide mid-line channel. There is also an increase in the depth of the optic pits (O) in this embryo compared to the situation illustrated in Fig. 3(c).
of cavitation in the presumptive pericardial region is seen in advanced primitive streak stage embryos in which the headfolds may be only poorly developed. However, considerable variation was apparent between embryos in this respect, and quite advanced headfold stage (but pre-somite) embryos were observed in which pericardial cavity formation had not progressed further.

Examination of the plastic sections, in particular, enabled detailed observations to be made on the cells of the tissue in the immediate vicinity of the developing and fully developed pericardial cavity. Our observations clearly indicate that, at least in the mouse, the primitive myocardial elements, the pre-cardiac myoblasts, develop in situ by differentiation of the mesothelial cells lining the splanchnic or visceral wall of the pericardial cavity, and that initially, during the pre- and very early somite period, the cardiac myoblasts are represented by a single layer of cuboidal cells which bulge directly into the pericardial cavity. Only subsequently do the endocardial elements appear as discrete mesothelial-lined spaces in the subjacent mesenchyme between the myocardial elements and the ectoderm-endoderm junctional zone at the ventral periphery of the foregut pocket.

This observation on the origin of the endocardial elements does not appear to confirm the widely prevalent view that the endocardial elements in mammalian embryos differentiate from the myocardial plate, as originally proposed by His (1885), an opinion subsequently supported by Yoshinaga (1921) and Davis (1927). These authors emphasized the proximity of the earliest endocardial rudiments to the myocardial plate, and the proliferative activity of the latter. We have confirmed these general observations on the location of the early cardiac rudiments. However, the sequence of appearance of myocardial and endocardial elements observed in the present investigation suggests that while the latter differentiate in close relationship to the myocardial plate they do not take origin from these cells. Certainly there is little or no resemblance between the morphological appearance of these two groups of cells at the time of first appearance of the endocardial rudiments. Whereas the myocardial plate consists of plump cuboidal or columnar cells, the prospective endocardial cells are elongated, flattened and irregular in outline and bear a strong resemblance to mesenchyme cells. Our histological and morphological observations therefore lead us to conclude that the endocardial elements probably differentiate from the subjacent mesenchyme cells, rather than from the myocardial cells themselves as previously proposed.

The spaces lined by endothelium coalesce in an anteroventral direction around the developing foregut to establish bilateral, hollow tubular entities which fuse across the mid-line at about the 4-somite stage. In their studies on the comparable stages of endocardial fusion in the chick embryo, Ojeda & Hurle (1975) found extensive evidence of cell death associated with the fusion process, but we were unable to confirm this feature in the present study.

While we cannot, from our observations, unequivocally rule out the possibility that the presumptive myocardial and endocardial rudiments migrate independently into the pericardial region in the manner described for the chick by Rosenquist & De Haan (1966), our findings strongly suggest that in the mouse these elements are formed in situ by differentiation of the cells of the pericardial wall and subjacent mesenchyme, respectively. If this is indeed the case, it emphasizes that it is not justifiable uncritically to extrapolate data, particularly in the field of cardiac morphogenesis, from a non-mammalian to a mammalian species.

Undoubtedly, as stressed by Manasek (1976), the endocardial and myocardial
elements, even if they arise independently, must interact together during subsequent differentiation of the heart. One of the early products of this interaction is the cardiac jelly, though its exact origin, whether produced by the myocardial elements as claimed by Manasek (1976) from chick evidence, or from the endocardial elements, as claimed by Markwald & Adams Smith (1972) from rat evidence, remains to be established.

Even at the most advanced stages examined in the present study (embryos with 6- to 8-somites present), no evidence of a separate epicardial layer was observed. This is not altogether surprising, since, in the chick embryo (Manasek, 1969) this layer does not appear until about stage 17+ (Hamburger & Hamilton, 1951), in embryos with 29–32 somites.

Scanning electron microscopy has been particularly useful in the present study of the earliest stages of cardiogenesis in the mouse, because this technique enabled a three dimensional picture of the changes taking place within the pericardial cavity to be recorded at the organ as well as at the cellular level. The scanning micrographs considerably facilitated the interpretation of the serially sectioned embryos, and provided insight into the events taking place at and shortly after the formation of the myocardial plate.

This approach has for the first time clearly demonstrated that during the late presomite period the primitive cardiac cells are present in the form of a continuous sheet or monolayer which lines the ventral splanchnic aspect of the pericardial cavity. Furthermore, this layer of cells extends forward around the lateral aspect of the foregut pocket towards the ventral mid-line. During the early somite period, presumably due to differential cell growth, two symmetrical myocardial 'bulges' develop, one on either side of the mid-line and separated by a deep median furrow. Examination of histological sections through this region revealed that myocardial cellular continuity across the mid-line was nevertheless retained. Also at about this time the primitive endocardium develops in the subjacent mesenchyme. As endocardial differentiation appears to progress in a dorsal to ventral direction, it is possible that this may have led to the erroneous impression that two distinct heart primordia form, each with its endocardial and myocardial components, which only later fuse across the mid-line (Yoshinaga, 1921; Davis, 1927). It was hypothesized that fusion was facilitated by the apposition of these anlagen brought about by gross changes in the overall configuration of the embryo, such as the caudal migration of the pericardial region, elevation of the headfolds, and development of the foregut pocket. Undoubtedly, distinct endocardial channels develop within each myocardial enlargement, for frontal sections through this region indeed show that two distinct endocardial systems are present at this time. This latter observation presumably explains how this could have been interpreted as evidence of a stage in the fusion of two distinct cardiac systems. Our observations therefore lead us to conclude that at no time during cardiogenesis in the mouse are there two distinct heart primordia.

It would be of considerable interest, therefore, to know whether, despite undoubted differences in timing of the early events and possible origin of the cardiac precursor elements, a similar sequence of events in fact occurs in avian embryos.
SUMMARY

The differentiation of the heart was examined in mouse embryos, isolated between the afternoons of the eighth and ninth day of gestation, by means of scanning electron microscopy as well as by light microscopy of standard histological material and thin plastic sections. Cavitation within the presumptive pericardial mesoderm commences during the late presomite stage (afternoon of eighth day) and myocardial rudiments are established by thickening of the pericardial lining adjacent to the endoderm near the base of the foregut pocket. At first the thickening lies on the ventral aspect of the pericardial cavity but, as the foregut pocket deepens and the trunk region of the embryo moves en masse caudally and ventrally, it comes to lie on the dorsal wall. A separate layer of cells comprising the primitive endocardium differentiates deep to the myocardial thickening, between the latter and the endoderm. Cavitation of the endocardial tissue occurs bilaterally about the 1–2-somite stage so as to establish bilateral endocardial tubes. These endocardial tubes become enclosed within the myocardial mantle which bulges into the pericardial cavity. A layer of acellular material (the cardiac jelly) appears between the endocardium and myocardium at about this time. At about the 4-somite stage, the endocardial tubes coalesce across the mid-line to establish a single median cardiac tube, and the entire heart rudiment is suspended in the mid-line by a wide dorsal mesocardium. Our observations indicate that, contrary to previous views, at no time during cardiogenesis in the mouse are two distinct heart primordia present which subsequently fuse to form a single median entity. The principal similarities and differences between cardiogenesis in the mouse, and mammals in general, and avians are discussed.

We wish to thank Mrs S. C. Barton (histology) and Mr J. Skepper (plastic sectioning) for their expert technical assistance. This work was supported by grants from the National Fund for Research into Crippling Diseases (to M. H. K) and the British Heart Foundation (to V. N.).

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THE PASSAGE TO THE FOETUS AND LIQUOR AMNII OF ETHANOL ADMINISTERED ORALLY TO THE PREGNANT MOUSE

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Received for publication March 19, 1981

Summary.—The relationship between the time of oral administration of a dilute aqueous solution of ethanol and its concentration in the blood and urine was determined in a group of non-pregnant mice. The ethanol concentration was also determined in the maternal blood, foetuses and liquor amnii in female mice to which ethanol had been administered on the 18th day of pregnancy. In the pregnant females peak ethanol levels were achieved first in the maternal blood, then in the foetuses and slightly later in the amniotic fluid. Unilateral ligation of the uterine vessels markedly delayed the amount of ethanol reaching the foetuses and liquor. The present experimental system may provide a model for the human phenomenon of “binge” drinking.

As the foetal alcohol syndrome (Jones et al., 1973) is now regarded as the third commonest cause of congenital mental deficiency in man (Noble, 1977) it is somewhat surprising that relatively little attention has been paid to the build-up and concentration of ethanol in the tissues of the mother and foetus after oral administration of this agent. Excellent papers on the transport of alcohol do exist, notably those of Nicloux (1899, 1900) and Horton (1978), and it and acetaldehyde, its principal breakdown product, have been shown to be teratogenic in numerous experimental animal studies (ethanol: Papara-Nicholson and Telford, 1957; Sandor and Amels, 1971; Chernoff, 1977; Tze and Lee, 1975; Randall and Taylor, 1979; Randall, Taylor and Walker, 1977; acetaldehyde: O'Shea and Kaufman, 1979, 1981). However, the recent introduction of the Alcolometer (Lion Laboratories Ltd) enables the investigator to measure the ethanol levels in any tissue quickly and extremely accurately.

Although work continues in this laboratory to study the pathways followed by ethanol from the mammalian gut to the brain, liver, kidney and other organs of the adult female and into the foetus, this seems an appropriate time to review our recent findings on the relationship between the time of administration of ethanol to the pregnant mouse, and its concentration in certain maternal tissues and in the foetus and the liquor amnii.

MATERIALS AND METHODS

Eight-to-twelve-week-old C57/InJ (Hacking and Churchill) mice, each in the 18th day of pregnancy (1st day = day of finding vaginal plug) were used in these experiments. The mice were lightly anaesthetized with ether, and 1 ml of a 10% solution of ethanol in distilled water was administered to each mouse orally via a fine plastic catheter passed into the stomach. The ethanol concentrations in the following materials were determined after the animal was killed by cervical dislocation, at periods ranging from 1 min to 1 h after the administration of ethanol: blood, amniotic fluid and macerated foetuses. An additional group of pregnant mice were anaesthetized with ether, and the uterine vessels ligated at the cervical and ovarian extremities of one uterine horn. The abdomen was closed to reduce evaporation of water from the peritoneal cavity and a similar volume of ethanol administered as described earlier. In this group, the animals were all killed 5 min after the administration of the ethanol, and the ethanol concentration determined in the maternal blood, amniotic fluid, and macerated foetuses. Similar amounts of the aqueous solu-
tion of ethanol were given by the identical route to non-pregnant female controls, and the ethanol concentration determined in the blood and urine.

RESULTS

The concentration of ethanol reached in the blood and urine of non-pregnant control females necropsied at intervals of between 10 and 60 min after oral administration of a dilute aqueous solution of ethanol are presented in detail in Table I, and diagrammatically in Fig. 1.

TABLE I.—Ethanol concentration in the urine and blood of non-pregnant control females necropsied at intervals after oral administration of an aqueous solution of ethanol

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>No. of females*</th>
<th>Ethanol concentration (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>6</td>
<td>Urine: 50-6±7-4 Blood: 180-3±15-6</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
<td>Urine: 102-8±16-7 Blood: 177-5±17-4</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>Urine: 191-6±10-9 Blood: 227-1±17-5</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>Urine: 162-2±16-2 Blood: 165-2±9-4</td>
</tr>
</tbody>
</table>

* Mean weight ± s.e. 33-2±0-6 g.

In Table II, the ethanol concentrations present in the maternal blood, foetuses and amniotic fluid at various intervals after the oral administration of ethanol are presented in detail. In this second series of experiments groups of females were necropsied at intervals between 1 and 60 min after the oral administration of ethanol. Samples of amniotic fluid were taken from 3 separate foetal sites, ethanol determinations made from these samples, and the mean amniotic fluid ethanol concentration obtained for each female. Similarly 3 foetuses from each litter were isolated and macerated, and individual ethanol determinations made. The mean value was then obtained. The standard errors presented in Table II were subsequently determined from the analysis of these mean values. The relationship between time after administration of ethanol and its concentration in the maternal blood, foetuses and amniotic fluid are presented diagrammatically in Fig. 2. Not surprisingly, ethanol reaches a higher level in the blood than in the foetuses or liquor amnii, although (and this may be very significant) it takes a slightly longer period of time to reach its peak level in the foetuses than in the blood, and a still longer period to reach its peak in the amniotic fluid than in the foetuses (Fig. 2).

The determinations of the level of

TABLE II.—Ethanol concentration in maternal blood, amniotic fluid and foetuses estimated at intervals after oral administration of an aqueous solution of ethanol

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>No. of females*</th>
<th>Maternal blood</th>
<th>Amniotic fluid</th>
<th>Foetuses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>126-2±22-6</td>
<td>19-1±1-7</td>
<td>21-4±2-6</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>119-8±11-9</td>
<td>27-0±6-0</td>
<td>31-2±1-3</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>194-2±17-4</td>
<td>59-3±12-0</td>
<td>103-3±13-9</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>115-0±4-1</td>
<td>94-0±14-6</td>
<td>95-0±5-7</td>
</tr>
<tr>
<td>60</td>
<td>3</td>
<td>467±8-0</td>
<td>545±5-5</td>
<td>41-5±4-6</td>
</tr>
</tbody>
</table>

* Mean weight ± s.e. 54-6±1-4 g.
PASSAGE OF ETHANOL FROM MOTHER TO FOETUS

180

'140

Log time (mins)

Fig. 2.—Ethanol concentration in (a) maternal blood, (b) amniotic fluid, and (c) foetuses, after oral administration of 1 ml of a 10% solution of ethanol in distilled water to female mice on the 18th day of pregnancy.

Table III.—Ethanol concentration in maternal blood, amniotic fluid and foetuses from uterine horns on which uterine vessels ligated and from non-ligated sides.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>No. of females</th>
<th>Maternal blood</th>
<th>Amniotic fluid</th>
<th>Foetuses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ligated</td>
<td>Non-ligated</td>
<td>Ligated</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>194.2 ± 17.4</td>
<td>43.7 ± 6.9</td>
<td>60.6 ± 8.2</td>
</tr>
</tbody>
</table>

ethanol in the maternal blood and in the foetuses and liquor amnii from the side on which the uterine vessels had been ligated and those on the non-ligated side are presented in detail in Table III, and illustrated diagrammatically in Fig. 3. As with the experimental findings presented in Table II, the values shown in Table III result from the analysis of mean values obtained from the determination of the ethanol concentration in 3 individual amniotic fluid samples and from 3 macerated foetuses from each uterine horn. A total of 6 females are included in this experimental group. Laparotomies were performed on several other pregnant females, but ligations were not carried out as these had less than 3 foetuses in one or other uterine horn. The ligation of the uterine vessels clearly has a marked effect in reducing the amount of ethanol reaching the foetus and, though to a slightly lesser extent, the liquor amnii.

DISCUSSION

Two important findings have emerged from the present experiments. The first
observation—namely, that ligating the uterine vessels, at least in the short term, markedly reduced the build-up of ethanol reaching the foetus and liquor amnii—was expected, presumably because the passage of ethanol into the various maternal and foetal fluid compartments and tissues described here is merely a reflection of its passage via the blood into the tissue fluids. However, the second finding—namely, the brief lag period in the build-up of ethanol in the amniotic fluid compared to levels achieved in the maternal blood and foetuses—was unexpected, and is perhaps physiologically the more interesting. Clearly the ethanol almost immediately passes from the stomach of the mother into its bloodstream, and thence via the placenta to the foetus. The most obvious explanation of the relationship between the levels achieved in the foetus and the liquor amnii would seem to be that the foetus urinates a dilute solution of ethanol into the liquor. This seems a reasonable hypothesis, since it has been established that considerable quantities of urine are produced by the foetus (Campbell, Wladimiroff and Dewhurst, 1973), and that foetal micturition contributes significantly to the amniotic fluid (De Voe and Schwarz, 1975). It is likely that this is the route by means of which other chemicals reach the liquor, and further work will be needed to show whether ethanol does indeed reach the liquor from the urinary tract of the foetus.

The speed and quantity at which ethanol was administered in these experiments relates them closely to the human phenomenon of “binge” drinking (Little, 1979; Little and Streissguth, 1978), which is now almost universally regarded as an extremely important mechanism by means of which the foetal alcohol syndrome can be produced. The relationship in the experiments between the ethanol content of the foetus and that of its liquor amnii, and the comparison of these with the ethanol level in the maternal blood suggests two important principles. First, it is evident that the mother hardly appears to intervene between the site of ingestion of the ethanol and its passage to the foetus.

Second, it would appear that the foetus and the liquor amnii quickly come to an approximate balance as far as ethanol is concerned. Even though it is reasonably clear that the presence of ethanol in the liquor amnii probably relates in the first place to its presence in the foetus, there may be a to-and-fro movement between the foetus and the liquor amnii which inevitably results in the foetus being exposed to this potentially teratogenic fluid for a considerable time.
As similar quantities of ethanol were given to the non-pregnant controls and the pregnant females, the rapid rate of uptake, slightly faster achievement of a maximal level, and more rapid clearance of ethanol from the blood in the latter group is of interest, as it probably reflects the increased metabolic activity of the females in this group. However, despite this increased clearance of ethanol from the blood during pregnancy, a high level of ethanol would still be achieved in the foetal compartment before it could be effectively removed from the maternal circulation.

The very rapid achievement of maximum concentrations of ethanol in the various maternal and foetal compartments observed here may be the principal factor in relation to the teratogenicity of this agent following "binge" drinking, more than the genotype of the individual. The latter probably plays a major role both in the ability of the mother to ingest ethanol and the speed of metabolism of the agent (Chernoff, 1980), which is probably of greater importance in the causation of malformations following the chronic consumption of ethanol.

We thank Mrs Andrea Burling for technical assistance. This work was supported by a grant to one of us (M.H.K.) from Action Research for the Crippled Child.

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Indirect Estimation of Crown-rump Lengths of Incomplete Conceptuses from Analysis of Spinal-cord Dimensions

J. A. D. Brailsford M. H. Kaufman

Introduction

The aim of this study was to determine whether a mathematical relationship exists between certain spinal-cord dimensions and the crown-rump length of the conceptus, as this information potentially could provide a valuable means of estimating the crown-rump length of advanced human embryos and early fetuses when the length could not be measured directly. Crown-rump length provides an accurate means of assessing gestational age (Streeter 1920), but often it is unavailable following therapeutic termination of pregnancy by vacuum extraction, as conceptuses are rarely recovered intact. Indeed, in many cases only the vertebral column and attached rib cage are recognizable.

Therapeutic terminations of pregnancy are a major potential source of material which could be used for the study of relatively early stages of human development. The analysis described here provides a reliable and accurate means of estimating crown-rump length when the specimen is either incomplete or badly damaged. The study also provides baseline data which could be of value in the analysis of intact specimens—for example when an attempt is being made to establish whether the conceptus is likely to have been exposed to a teratogenic insult early in gestation, or when there appears to be a discrepancy between crown-rump length and the morphological appearance or dimensions of the cord and/or gestational age.

Material and method

Our study was based on an analysis of specimens from a collection of serially sectioned human embryos and fetuses collected by the late Professor J. D. Boyd, and housed in the Anatomy Department, Cambridge University. A total of 54 intact, transversely-sectioned conceptuses with crown-rump lengths (measured prior to fixation) ranging from 4mm to 112mm were examined, as these approximately corresponded to embryos and fetuses likely to be recovered from therapeutic termination of pregnancy (i.e. up to about 3 to 3.5 months gestation). However, only 23 of these conceptuses, namely those considered to be sufficiently well-fixed and sectioned almost exactly transversely, were used in the analysis of normal spinal-cord growth and development described here.

Just under half of the selected specimens...
had been fixed in Bouin's solution, and slightly over half in 10 per cent formalin. Two additional fetuses had been fixed in Bodian, and a single specimen by the De Castro technique.

While it is appreciated that dehydration, clearing and wax impregnation inevitably cause alterations in volume (i.e. shrinkage or swelling) of tissues no matter what fixation has preceded processing (Thompson 1966, Drury and Wallington 1967), only those specimens with minimal obvious shrinkage artefacts were included in the present analysis.

The spinal cords of the conceptuses that fitted the above criteria were then examined in detail. Five levels of the cord were selected for analysis, as these levels could always be located in intact specimens, and most could be found even in incomplete specimens. The levels chosen were widely spaced along the cord, in order to provide a gross picture of regional differentiation within the cord. Appropriate measurements were taken from sections through the spinal cord at the following levels:

1. at the level of the second cervical vertebral body;
2. at the level of the most rostral part of the articulation of the first rib with the first vertebral body;
3. at the level of the most rostral part of the articulation of the 12th rib with the 12th thoracic vertebral body;
4. at a point mid-way between levels 2 and 3 above; and
5. at a point mid-way between level 3, above, and the caudal extemity of the cord.

The eight dimensions measured at each of these levels are illustrated in Figure 1. Direct measurements of sectioned cords were made by means of a Wild M5 dissection microscope and a graticule eyepiece. The measurements thus obtained were then compared with previous results obtained from the analysis of photographs of the same sections. Preliminary observations indicated that in all instances virtually identical measurements were obtained. As the former method obviously was far quicker and less technically demanding, that form of analysis was adopted for the rest of the study. Representative transverse sections through the spinal cord of a series of conceptuses ranging between 4mm and 112mm crown-rump length, at the level of the first thoracic vertebral body, were photographed with a Zeiss photomicroscope. The measurements obtained were converted to micrometers (μm) and are presented in that form for ease of subsequent analysis.

Results
Quantitative analysis of spinal-cord dimensions

When the various spinal-cord dimensions indicated in Figure 1 were individually plotted against crown-rump length, it rapidly became obvious that only some of the measurements bore a high enough correlation with crown-rump length to be of potential predictive value. Therefore only these obviously useful data will be presented in full here.

When the maximum spinal-cord widths were plotted against the logarithms of their respective crown-rump lengths, the linear regressions obtained showed the high correlations of 0·973 for the T1 level, 0·965 at T12, and 0·926 for the joint regression of T1 and T12 when all of these values were plotted against the logarithms of the crown-rump lengths.

In order to provide an estimate of crown-rump length, and hence an assessment of the gestational age of conceptuses from spinal-cord dimensions alone, the length must become the dependent variable, since regression analysis then enables the 'best fit' of crown-rump length to be calculated from,
Fig. 1. Typical transverse section through spinal cord in thoracic region to illustrate measurements taken in present study at various levels examined.

Key: 1 = maximum spinal-cord width; 2 = maximum spinal-cord height; 3 = central spinal-cord height; 4 = spinal-canal height; 5 = spinal-canal width; 6 = grey matter, 'upper' width; 7 = grey matter, 'central' width; 8 = grey matter, 'lower' width.
CROWN-RUMP LENGTHS OF INCOMPLETE ABORTUSES

Fig. 2. Growth curve obtained by plotting crown-rump length against maximum spinal-cord width at level of first thoracic vertebral body (T1).

Fig. 3. Growth curve obtained by plotting crown-rump length against maximum spinal-cord width at level of twelfth thoracic vertebral body (T12).

for instance, data on spinal-cord width.

Figures 2 and 3 illustrate the regressions obtained when maximum spinal-cord width at T1 and T12, respectively, are plotted against appropriate crown-rump lengths. When the maximum spinal-cord height is plotted against the logarithm of the crown-rump length at T1, a correlation of 0.85 is obtained. Similarly, the correlation at T12 is 0.824, and 0.823 when the joint regression of T1 and T12 was plotted against the logarithms of the respective crown-rump lengths.

When the product of the maximum spinal-cord width and maximum spinal-cord height is plotted against the logarithm of the crown-rump length, a correlation of 0.936 is obtained for T1, and 0.929 for
Regression obtained by plotting grey-matter width against maximum spinal-cord width levels T1 (•) and T12 (○).

Fig. 4. Regression obtained by plotting grey-matter width against maximum spinal-cord width levels T1 (•) and T12 (○).

T12; that is, a value for the correlation which is lower than for width alone, and higher than when the height alone is plotted against crown-rump length.

The regression of the central spinal-cord height against the logarithm of the crown-rump length produced correlations of 0·60 for T1 and 0·69 for T12; and that of central spinal-cord height against crown-rump length of 0·67 at T1 and 0·45 at T12. These correlations are comparatively low, and therefore are of no practical predictive value.

When the maximum spinal-cord height was plotted against the maximum width, correlations of 0·836 for the T1 level, and 0·86 for the T12 level were obtained; while plotting maximum height against the logarithm of the maximum width gave correlations of 0·86 at both the T1 and T12 levels. However, the regression obtained when the maximum grey-matter width was plotted against the maximum spinal-cord width gave very high correlations, namely 0·99 at T1, 0·987 at T12 and 0·99 for the joint regression (Fig. 4). Indeed, the maximum spinal-cord width and grey-matter width obtained from sections at all levels of the cord from the cervical to the lumbar regions, and in all the conceptuses studied, fitted the regression line for this equation.

Descriptive morphology of the developing spinal cord

The series of photomicrographs in Figure 5 illustrate the over-all changes observed in the morphology of the thoracic region of the late embryonic and early fetal human spinal cord, from shortly after the completion of neural-tube closure to a stage at which the appearance of the cord approaches the adult form.

Initially the neural tube is oval in outline and longer dorso-ventrally than it is wide. The lumen is narrow and slit-like (Figs. 5a, b). By 8mm crown-rump length, it is possible to locate the T1 level
accurately, as it is the source of the most caudal major nerve-trunk which passes into the fore-limb bud. Dorsally at this stage the marginal layer forms oval bundles consisting of fibres passing from the dorsal roots of the spinal nerves (Fig. 5c). The lumen of the spinal canal has widened, a process associated with the formation of the sulcus limitans.

By 13mm crown-rump length, both the T1 and T12 levels are easily located because of the condensation of cartilage at the heads of the first and 12th ribs, respectively (Fig. 5d). The cord is now wider ventrally than dorsally, a feature that persists until about 60mm crown-rump length. The ventral enlargement of the cord is more noticeable at T1 than at T12, and presumably is related to the large number of tracts passing into and from the fore-limb bud. The canal remains narrow ventrally, opening into a dilated dorsal portion.

At 17mm crown-rump length the overall volume of the cord is now markedly greater at T1 than at T12 (Fig. 5e). The shape of the dorsal wall of the canal changes as a result of approximation and apposition of the ependymal layers at the dorsal extremity of the canal, leading to its partial obliteration in this region.

In the 26mm crown-rump length embryo (Fig. 5f), prominent lateral columns are observed in the dorsal part of the basal laminae, and the initial stages of substantia gelatinosa formation may be observed dorso-laterally. Just lateral to the dorsal midline, the oval bundles now form the posterior funiculi. Obliteration of the canal dorsally is pronounced, and this, together with further ventral migration of the sulcus limitans, results in the canal now being broader ventrally than dorsally. By this stage the ependymal layer lining the canal has become much thinner.

At 35mm crown-rump length, the pronounced lateral growth of the cord is reflected in its somewhat squatter appearance. The lateral columns and substantia gelatinosa are now prominent in the thoracic region (Fig. 5g). The posterior median septum is also well defined at this stage, particularly at the T1 level, in contrast to the observations of Malínský and Malínská (1970), who first recorded its presence in fetuses with crown-rump lengths between 55 and 75mm. The central canal is now squatter, and has assumed a pentagonal shape.

In the sections from the fetuses with crown-rump lengths between 46mm and 112mm, the elaboration of cell groups and fibre tracts continues (Figs. 5h-k). The cord becomes broader relative to its height and more oval in outline, largely as a result of the formation of the spinocerebellar tracts. Grey matter surrounds the canal dorsally by 46mm crown-rump length, and ventrally by 90mm crown-rump length. The pentagonal, centrally located spinal-canal becomes smaller both in absolute terms and relative to the over-all size of the cord, so that in the fetus with a crown-rump length of 112mm the canal appears as a tiny cavity with a thin but darkly staining ependymal layer.

To facilitate the location of the spinal cord at T1, representative sections through the upper thoracic region at this level in conceptuses with crown-rump lengths of 13, 33.5 and 90mm are illustrated in Figures 6a to c, respectively. Attention is drawn to the major anatomical landmarks present at this level.

Discussion
The high correlations found in the analyses of some aspects of spinal cord growth, particularly between the width of the cord and the crown-rump length, clearly demonstrate that the present findings provide a new, relatively simple and accurate method of estimating the crown-rump length of sectioned human
Fig. 5a-k. Representative transverse sections through spinal cords of human conceptuses with crown-rump lengths between 4 and 112 mm. Figures 5a and b are through 'upper thoracic' region; Figures 5e-k are at level of first thoracic body. Over-all dimensions (crown-rump lengths) of conceptuses are: a 4 mm, b 5 mm, c 8 mm, d 13 mm, e 17 mm, f 26 mm, g 35 mm, h 46 mm, i 60 mm, j 90 mm and k 112 mm. Sections illustrate considerable change in over-all shape and gradual diminution in volume of spinal canal occurring with fetal growth. Note also changes in relationship between grey and white matter.

Key: a = anterior median fissure; d = dorsal-root ganglion; c = ependymal zone; l = lumen of spinal canal; fc = lateral column in dorsal part of basal lamina; mg = marginal layer; mn = mantle layer; mx = matrix layer; o = oval bundle; p = posterior median fissure; s = substantia gelatinosa.

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Fig. 6a-c. Representative transverse sections through upper thoracic region at level of T1 in conceptuses of a 13mm, b 33·5mm and c 90mm crown-rump length. Considerable degree of flexion (or lordosis) in 13mm embryo, and lesser degree in 33·5mm fetus, accounts for presence of cephalic and facial structures in these sections. At these stages of gestation conceptuses are in characteristic 'fetal' position. Arms were removed from 90mm fetus prior to fixation and sectioning. Note in particular decrease in over-all volume of spinal cord with increased gestational age, relative to over-all volume of thoracic inlet at this level.

Key: 1 = spinal cord; 2 = vertebral body; 3 = first rib; 4 = fore-limb bud; 5 = head of humerus; 6 = oesophagus; 7 = trachea; 8 = clavicle; 9 = scapula; 10 = elements of brachial plexus. Other features in a and b are: RA = right atrium; LA = left atrium; BC = bulbus cordis; E = lens vessel within developing eye; D = diencephalon; M = mandible; T = tongue.
embryos and early fetuses when direct measurement of this length is not possible. Potentially, therefore, this technique permits far more use to be made of, and thus valuable information to be gained from, the large number of incomplete human specimens which may be obtained following modern suction methods of terminating pregnancies. In addition, information obtained by this means should provide a valuable adjunct to gestational-age data, and undoubtedly will be of use both to embryologists and clinicians.

The analysis of spinal-cord development described here produced one particular variable, namely maximum spinal-cord width, which varied with crown-rump length in a highly predictable fashion, despite the fact that its rate of growth decreases with time. While spinal-cord width proved to be a much more reliable guide than height as an indicator of embryonic and fetal development, it is possible that this discrepancy may be artefactual, since obliquity of sectioning almost invariably gives a false impression of height, which would be impossible to detect. However, obliquity affecting spinal-cord width is comparatively easy to detect because of the symmetrical emergence from the cord of the spinal roots (and in the thoracic region, the symmetrical appearance of the heads of the ribs). In fact, it is of interest that only one of the embryos examined was rejected because of obvious dorso-ventral obliquity of sectioning, whereas several had to be rejected because of obvious lateral obliquity. The former problem might be expected to arise occasionally because of the natural curvature of the embryo, though this effect is minimal in the thoracic region.

The selection of the first and last thoracic vertebrae as levels for detailed analysis seems likely to be vindicated, since an extensive preliminary analysis by both authors has shown that one or both of these levels was identifiable in all the incomplete embryos obtained from therapeutic terminations of pregnancy so far studied.

The observation that correlations for width against the logarithm of crown-rump regressions fall slightly if T1 and T12 levels are treated as a single datum set, indicates that the identification of either level will lead to a more accurate prediction than would be possible if the sections being studied were only known to be from the thoracic region. However, the correlation for the joint regression (at 0.926) is sufficiently high for some use to be made of it if this is the only information available.

The ratio of total spinal-cord width to grey-matter width is interesting because of its very high degree of constancy, both in relation to embryonic/fetal development and location within the cord: thus the ratio of maximum width of the spinal cord to maximum width of grey matter is approximately 1.25:1 at all levels of the cord, and in all of the conceptuses examined. This observation is rather surprising, since one might expect that there would be relatively more white matter than grey matter at higher rather than at lower levels of the cord. It seems likely that the observations reported here reflect the fact that our study was concluded before the process of myelination is fully under way. This is likely to be the critical factor, as previous studies have demonstrated that—certainly from about 110mm crown-rump length onward—the volume of white matter increases at twice the rate of grey matter (Lassek and Rasmussen 1939). If the widths of the spinal cord and spinal grey-matter are taken as diameters of circles, then the constant ratio of the two width measurements means that the ratio of the areas of the white and grey matter is
relatively constant over the range of cord growth considered in the present study. The ratio of these widths can also act as an indicator of abnormal development affecting the cell body:fibre ratio. Indeed, when we studied a section through a morphologically abnormal human spinal-cord which even on gross inspection appeared to have anomalies in the relationship of white to grey matter at the T1 and T12 levels, it was found not to fit the regression lines presented here, unlike all the control sections studied.

In addition to the embryonic/fetal ageing technique described, the present study also provides a baseline series of normal values which quantify for the first time the development of an important and vulnerable organ system. If the crown-rump length of an embryo or fetus is available from direct measurement, the normality or otherwise of its spinal-cord development can be established from the various width dimensions described here. Of course this type of analysis also may be performed when the crown-rump length is not measurable: the relative proportions of the grey- and white-matter components of the cord may also be compared, and a value for the crown-rump length obtained from spinal-cord dimensions alone, which can then be compared to any (possibly unreliable) gestational-age data available.

The morphological appearance of the thoracic region of the cord at different developmental stages also enables an estimate of gestational age to be made by direct inspection, though even in the range of 20 to 40mm crown-rump length, where change is most profound, the estimate perhaps would be accurate to ±5mm. Figures 2, 3 and 4 clearly illustrate that while the statistical correlation is extremely high, some caution is necessary in extrapolating directly from these graphs in interpreting the prediction of crown-rump length of particular embryos or fetuses. However, combining a morphological inspection with a quantitative analysis can be expected to produce a quality of estimation far in excess of that previously possible.

The data presented here should serve as useful baseline information for subsequent analyses of normal and abnormal neural-tube development, and should also be of value in the study of the possible harmful effects of environmental teratogenic agents on the early development of the human central nervous system.

Acknowledgements: We are deeply indebted to the late Professor J. D. Boyd for his interest in human embryology, and to his technicians (J. Cash and R. S. Smith) for the high quality of their histological preparations. This study was supported by a grant (to M.H.K.) from the National Fund for Research into Crippling Diseases. Additional support (to J.A.D.B.) was provided by a grant from the H. E. Durham Fund (King's College, Cambridge).

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SUMMARY
The spinal cords of serially sectioned human embryos and early fetuses, ranging from 4mm to 112mm in crown-rump length, were examined in order to determine whether a mathematical relationship exists between certain spinal-cord dimensions and the crown-rump length of the conceptus. This information may be useful in estimating the crown-rump length, and hence the gestational age, of advanced human embryos and early fetuses when direct measurement is impossible. Five widely-spaced levels along the spinal cord were studied, but only those data relevant to the levels of the first thoracic (T1) and twelfth thoracic (T12) vertebral bodies are described in detail. Similarly, although eight distinct
spinal-cord dimensions were measured at each level, only those measurements that bore a high enough correlation with crown-rump length to be of any predictive value are presented here, the most useful being the maximum spinal-cord width and the maximum grey-matter width, both of which increased with gestational age in a highly predictable way. The descriptive account of the morphological appearance of the cord at these levels also enables, though less accurately, an estimate of the crown-rump length of a conceptus to be made by direct inspection of a single transverse section through the thoracic region of the cord. The predictive value of these observations is discussed.

RÉSUMÉ
Estimation indirecte de la longueur tête-fesse de foetus non viables à partir d'une analyse de certaines dimensions de la moelle épinière

Les moelles épinières de sections sériées d'embryons humains et de foetus peu développés, d'une longueur tête-fesse de quatre à 112 mm ont été examinés dans le but de déterminer s'il existe une relation mathématique entre certaines dimensions de la moelle épinière et la longueur tête-fesse du foetus. Cette information peut être utile pour estimer la longueur tête-fesse et de ce fait l'âge de gestation, chez des embryons humains avancés ou des foetus précoce lorsque la mesure directe n'est pas possible. Cinq niveaux largement espacés le long de la moelle épinère ont été étudiés mais seuls ceux qui correspondent au premier (T1) et au douzième (T12) corps des vertèbres thoraciques sont décrits en détail. De la même façon bien que huit dimensions médullaires distinctes aient été mesurées à chaque niveau seules celles qui présentent une corrélation assez élevée avec la longueur tête-fesse, pour avoir une valeur prédictive sont présentées ici, les plus utiles étant la largeur maximale de la moelle épinère et la largeur maximale de la matière grise, ces deux mesures augmentant avec l'âge conceptuel de façon hautement prédictive. Le compte rendu descriptif de l'apparence morphologique de la moelle à ces niveaux permet également quoique de façon moins précise une estimation de la longueur tête-fesse du foetus par une inspection directe d'une seule section transverse de la région thoracique de la moelle. La valeur prédictive de ces observations est discutée.

ZUSAMMENFASSUNG
Indirekte Beurteilung der Kopf-Rumpf-Länge inkompletter Feten durch die Analyse bestimmter Rückenmarksgrößen

Berteilung der Kopf-Rumpf-Länge des Feten, indem man einen Querschnitt im Thorakalbereich des Rückenmarks untersucht. Der prognostische Wert dieser Beobachtungen wird diskutiert.

RESUMEN

Estimacion indirecta de la longitud occipucio-rabadilla a partir de conceptos incompletos surgidos del analisis de ciertas dimensiones de la medula

Se examinó la longitud occipucio-rabadilla de la médula en unos cortes seriados de embriones humanos y fetos jóvenes de 4 a 112 milímetros, con el objeto de determinar si existe una relación matemática entre ciertas dimensiones medulares y la longitud del producto de la concepción. Esta información puede ser útil para determinar la longitud occipucio-rabadilla y con ello la edad gestacional de embriones humanos avanzados y de fetos jóvenes cuya medición directa es imposible. Se estudiaron cuatro secciones de la médula separadas por espacios amplios, pero sólo se describen con detalle los datos relevantes de los cuerpos vertebrales T1 y T12. De forma similar, aunque se midieron ocho dimensiones distintas de la médula en cada nivel, sólo se presentan las medidas que comportaban un alto nivel de correlación con la longitud occipucio-rabadilla, suficientes para constituir un valor predictivo. La medicion mas útil era la de la hanchura máxima de la médula y de la substancia gris; ambas aumentaban con la edad gestacional de una forma altamente predecible. La descripción del aspecto morfológico de la médula a los niveles citados hace posible también, aunque con menos precision, una estimación de la longitud occipucio-rabadilla del fruto de la concepción realizada por simple inspección de una sección simple transversa de la médula realizada a nivel torácico. Se discute el valor predictivo de estas observaciones.

REFERENCES

The chromosome complement of single-pronuclear haploid mouse embryos following activation by ethanol treatment

By M. H. KAUFMAN

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SUMMARY

A technique in which mouse eggs are stimulated to develop parthenogenetically following their brief incubation in 7% ethanol in PBS is described. Very high rates of activation were achieved, and a detailed analysis presented of the class of parthenogenone which develops a single haploid pronucleus following second polar body extrusion.

As preliminary studies on presumptive haploid morulae indicated that a proportion of the metaphase spreads examined had an aneuploid chromosome constitution, the incidence of aneuploidy at the first cleavage mitosis was investigated. In the control groups the level of aneuploidy was about 1%, whereas in the ethanol-treated series the incidence ranged from 13.6–18.8%. Additional pre-treatment of ethanol-activated oocytes in low osmolar medium raised the incidence of aneuploidy to 28.3%. Metaphase groups with 18, 19, 21 and 22 chromosomes present were observed in addition to groups with a normal complement of 20 chromosomes. The possible mode of action of ethanol in inducing parthenogenetic activation of mouse oocytes, and a high incidence of aneuploidy, is discussed in relation to previous knowledge of the action of this agent. Preliminary studies using G-banding indicate that the aneuploidy observed appears to arise as a result of non-disjunction which may involve any of the chromosomes of the complement.

INTRODUCTION

The technique in which mouse eggs may be stimulated to develop parthenogenetically by incubating them for a short time in a 7% solution of ethanol in phosphate-buffered saline (7% ethanol in PBS) was first described by Cuthbertson (personal communication). This technique originated from the observations of Dyban & Khozhai (1980) who found that an intraperitoneal injection of ethanol into mice about 7 h after ovulation induced activation of the oocytes. The majority of eggs activated by the in vitro technique, when eggs are stimulated approximately 17 h after the HCG injection for superovulation, develop a single pronucleus following second polar body extrusion (1 pron + 2PB class, see Kaufman, 1981a).

Optimum activation and culture conditions were established, so that a high

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proportion of the single pronuclear haploids subsequently developed to the blastocyst stage. However, analysis of the chromosome constitution of embryos at the early morula stage, initially carried out to confirm the haploid status of these individuals, revealed that a proportion of the metaphase plates in both of the strains examined ((C57BL × CBA)F₁ and 129/SvE) had an aneuploid chromosome constitution. Activated embryos at metaphase of the first cleavage mitosis were then examined, in order to establish the incidence of aneuploidy shortly after activation. Attempts to investigate whether the aneuploidy arose by non-disjunction or by chromosome aberrations or both were made by G-banding, and these results are reported here.

The possible significance of the present findings regarding the exposure of mouse oocytes during the second meiotic division to ethanol, and their relationship to earlier observations on the potential teratogenicity of this agent and its principal breakdown product (see Kaufman, 1981b) are discussed in detail.

MATERIALS AND METHODS

Eight- to 12-week-old (C57BL × CBA)F₁ hybrid female mice were superovulated with 5 i.u. PMSG followed 48 h later by 5 i.u. HCG. In the majority of the experiments described here, females were autopsied at 17 h after the HCG injection. The oviducts were then removed and the individual cumulus masses containing the ovulated oocytes released into PBS. Cumulus masses from four to six females were pooled together, treated as a single group, and all of the following procedures were carried out at room temperature. In the experimental series, groups of cumulus masses were transferred via a Pasteur pipette to a watchglass containing about 1 ml of a freshly prepared 7% solution of Analar quality ethanol in PBS, and retained in this solution for either 1 min, 3 min, 4½ min or 7½ min. The cumulus masses were then washed through three changes of ethanol-free PBS and finally through two changes of embryo culture medium (Whittingham, 1971). Individual cumulus masses were then transferred to separate drops of culture medium under paraffin oil and incubated for 4–5 h at 37 °C in an atmosphere of 5% CO₂ in air. At the end of this time, the adherent cumulus cells were removed with hyaluronidase (Kaufman, 1978) and the overall activation frequency determined, and the various classes of parthenogenone induced separated into different groups. Depending on whether a second polar body had been extruded or not, and the overall size of the latter — whether equal in volume or considerably less than the volume of the oocyte, and on the number of pronuclei formed, four classes of parthenogenone could be determined at this stage, namely (a) oocytes which contained a single (haploid) pronucleus, having previously extruded a second polar body, (b) oocytes which contained two (haploid) pronuclei in the absence of second polar body extrusion, (c) oocytes which underwent ‘immediate cleavage’ in which two equal-sized blastomeres had formed, each containing a single (haploid) pronucleus, one of the blasto-
Ethanol-induced aneuploidy

Fig. 1. The pathways of development of parthenogenetic eggs and overall incidence of activation following their incubation in 7% ethanol in PBS for 1, 3, 4½ and 7½ min. The eggs were isolated from (C57BL x CBA)F₁ hybrid mice at 17 h after HCG and the analysis was carried out at about 5 h after activation.

meres representing the second polar body, and (d) oocytes in which a single (diploid) pronucleus developed in the absence of second polar body extrusion. Apart from briefly considering how the proportionate incidence of these various classes varied with the different treatments, only the haploid oocytes constituting group (a) will be considered further.

At about 10 h after activation the oocytes in group (a) were transferred to fresh medium containing 1 μg/ml colcemid, and returned to the incubator. After a further 10-12 h, the oocytes were isolated and their chromosome constitution determined by the air-drying technique described by Tarkowski (1966). All preparations were stained with Giemsa. Without exception, the oocytes were inhibited from developing beyond metaphase of the first cleavage mitosis. The total number of chromosomes present in individual metaphase spreads was determined using the oil-immersion objective of a Leitz photomicroscope. If there was any doubt regarding the number of chromosomes present due to the overlapping of chromosomes, the group was excluded from the study. All of the aneuploid groups which contained either more or less than the normal haploid complement of 20 chromosomes were photographed.

In addition to the four experimental groups already described, the chromosome constitution of oocytes from three control groups and a further experimental group were also analysed. The first control group consisted of oocytes
Table 1. Chromosome complement of single-pronuclear eggs analysed at metaphase of the first cleavage division

<table>
<thead>
<tr>
<th>Group</th>
<th>Activation after HCG(h)</th>
<th>7% ethanol in PBS</th>
<th>Total no. of metaphase spreads analysed</th>
<th>Chromosome number</th>
<th>Aneuploid (%)</th>
<th>Percentage of scorable preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Control</td>
<td>17</td>
<td>-</td>
<td>10</td>
<td>18 19 20 21 22</td>
<td>0</td>
<td>71.4</td>
</tr>
<tr>
<td>(2) Control</td>
<td>20</td>
<td>-</td>
<td>42</td>
<td>10 19 20 21 22</td>
<td>0</td>
<td>79.2</td>
</tr>
<tr>
<td>(3) Control (hyaluronidase) activation</td>
<td>20</td>
<td>-</td>
<td>37</td>
<td>1 19 20 21 22</td>
<td>2.7</td>
<td>71.2</td>
</tr>
<tr>
<td>(4) 1 min exposure</td>
<td>17</td>
<td>+</td>
<td>48</td>
<td>3 19 20 21 22</td>
<td>18.8</td>
<td>81.4</td>
</tr>
<tr>
<td>(5) 3 min exposure</td>
<td>17</td>
<td>+</td>
<td>81</td>
<td>1 19 20 21 22</td>
<td>13.6</td>
<td>77.9</td>
</tr>
<tr>
<td>(6) 4½ min exposure</td>
<td>17</td>
<td>+</td>
<td>85</td>
<td>4 20 21 22 22</td>
<td>15.4</td>
<td>82.5</td>
</tr>
<tr>
<td>(7) 7½ min exposure</td>
<td>17</td>
<td>+</td>
<td>52</td>
<td>1 20 21 22 22</td>
<td>28.3</td>
<td>78.8</td>
</tr>
<tr>
<td>(8) 4½ min exposure then 2 h in low osmolar medium</td>
<td>17</td>
<td>+</td>
<td>60</td>
<td>1 19 20 21 22</td>
<td>84.5</td>
<td></td>
</tr>
</tbody>
</table>
isolated, as in the experimental series, at 17 h after the HCG injection. In this group the cumulus masses were retained in ethanol-free PBS for 4½ min, then washed through three rinses of ethanol-free PBS etc., exactly as described earlier. However, as the activation frequency in this group was rather low and the number of haploid oocytes available for analysis correspondingly small, two additional control groups were established.

In the second control group, the only factor which was varied from the first control group was the time of isolation of oocytes. In this group, the oocytes were isolated at 20 h after the HCG injection, as previous studies (Kaufman, 1973, 1978) had shown that increasing the post-ovulatory age of the oocyte at the time of activation could markedly increase the activation frequency. In all other respects this group was treated in exactly the same way as the first control group. Oocytes in the third control group were activated by incubation for 10 min in medium containing hyaluronidase, according to the technique described by Kaufman (1973).

An additional experimental group was examined to investigate the effect of incubating oocytes previously activated by exposure for 4½ min to 7% ethanol for 2 h in embryo culture medium diluted with distilled water in the ratio of three volumes of medium to two volumes of distilled water. The rationale for this group was that it had previously been established that this form of treatment could influence the activity of the second meiotic spindle apparatus (Graham, 1971, 1972; Kaufman & Surani, 1974), and might also increase the incidence of non-disjunction.

All of the preparations were examined without reference to their source, and the chromosome complement of all aneuploid groups confirmed by an independent observer.

In a preliminary study, (C57BL × CBA)F1 and 129/SvE oocytes isolated at 17 h after the HCG injection were activated by incubating them for 4½ min in 7% ethanol in PBS, and the cumulus masses then cultured for 4–5 h as described earlier. The activated oocytes in group (a) were then segregated out and transferred shortly afterwards (at about 15.00–16.00 h) to the oviducts of recipients on the first day of pseudopregnancy (i.e., on the afternoon of the day on which a vaginal plug had earlier been observed, following mating of the female with a vasectomized male).

The recipients were autopsied at about midday on the 4th day of pseudopregnancy, and the recovered embryos transferred to culture medium containing 1 μg/ml colcemid. The majority of the embryos were at the morula stage. After an incubation period of about 3 h the embryos were examined by the air-drying technique (Tarkowski, 1966). The preparations were then stained with Giemsa, and examined under the oil-immersion objective of a Leitz photomicroscope. The total cell number of each embryo was determined, as well as its chromosome constitution and ploidy. However, only the chromosome constitution will be considered here, as their ploidy and pre- and early post-implantation development potential will be discussed in detail elsewhere (Kaufman & Handyside, in preparation).
Fig. 2. Representative air-dried chromosome spreads of the first cleavage metaphase of single-pronuclear haploid parthenogenetic eggs. All oocytes were incubated for about 12 h in medium containing 1 μg/ml colcemid. Metaphase spreads with 18 (a), 19 (b), 20 (c, d), 21 (e, f) and 22 (g, h) chromosomes present. The condensed round object in b, d, e and g is the nucleus of the second polar body. The preparations were stained with Giemsa.
G-banding analysis of first cleavage chromosomes

Oocytes for this analysis were either incubated in medium containing Colcemid, or in the absence of this agent. In both groups, air-dried preparations were made as soon as the pronuclear outline disappeared, as this indicated their entry into the first cleavage mitosis. This event usually occurred within 15–18 h after activation.

G-banding analysis of the preparations was obtained by a modification of the A.S.G. technique described originally for rat chromosomes by Gallimore & Richardson (1973). Banded spreads were photographed and karyograms prepared according to the nomenclature suggested by Nesbitt & Francke (1973).

RESULTS

(1) Observations on the activation rate and proportionate incidence of the various classes of parthenogenone induced under various control and experimental conditions

Observations on the various classes of parthenogenone induced when oocytes isolated 17 h after HCG injection were stimulated by exposure to a 7% solution of ethanol in PBS for 1 min, 3 min, 4½ min and 7½ min are presented in diagrammatic form in Fig. 1. In the control group exposed to ethanol-free PBS for 4½ min but otherwise treated in exactly the same way as these experimental groups, the activation rate was 14·0%, and all 15 activated eggs observed were of the single-pronuclear haploid type (group a). Several points of interest emerge. First, that under control conditions the activation rate is extremely low, but it is clear that the rate increases in relation to the duration of exposure to the 7% ethanol solution, with very high rates achieved after 3 min, 4½ min and 7½ min exposure. Secondly, that the principal class of parthenogenone induced under these conditions is haploid, and contains a single pronucleus which develops shortly after the second polar body has been extruded. In only the 4½ min and more particularly in the 7½ min groups were reasonable numbers of other classes of parthenogenone observed (see Fig. 1).

Because of the diversity of the other groups, these will be considered individually. It is of interest that the results obtained in the two additional control groups, in which oocytes were stimulated at 20 h after the HCG injection, were almost identical. Thus in the second control group, in which oocytes were incubated for 4½ min in ethanol-free PBS the activation rate was 45·2%, whereas in the third control group, in which oocytes were activated in medium containing hyaluronidase, the activation rate was 45·3%. In the second control group 54 single-pronuclear haploid eggs and 2 two-pronuclear diploid eggs were observed, whereas in the third control group 52 single-pronuclear haploid eggs and 6 two-pronuclear eggs were observed.

The results of the additional experimental study in which oocytes isolated 17 h
after HCG were released into 7% ethanol solution for 4½ min, then incubated in medium diluted in the ratio of 3:2 with distilled water was also of considerable interest. The activation rate observed in this group was 97.1%, and out of a total of 102 activated eggs, 89 were of the single-pronuclear haploid class, 9 were of the two-pronuclear diploid class and 4 eggs were observed which underwent immediate cleavage. This result was unexpected, in that there appeared to be little or no obvious effect of the incubation in the low osmolar medium. Previous studies in which activated eggs had been incubated in low osmolar medium directly after activation had usually resulted in a high incidence of eggs in which suppression of second polar body extrusion occurred, with the development of two-pronuclear diploid eggs. A moderate incidence of eggs which underwent immediate cleavage were also commonly observed (see Kaufman, 1978).

(2) Observations on the chromosome complement of single-pronuclear haploid eggs at metaphase of the first cleavage division

A summary of the findings in relation to the three control groups and five experimental groups is presented in Table 1. It is of interest to note that the incidence of scorable preparations, i.e. metaphase plates in which unequivocal chromosome counts could be made, varied between 71.2 and 84.5%. These figures would no doubt have been higher had groups with a minimal degree of chromosome overlapping been included. However, as indicated earlier, where there was any possible doubt about the number of chromosomes present, no attempt was made to score these groups.

In none of the control groups were metaphases observed with more than the normal haploid complement of 20 chromosomes. Indeed, in only one control metaphase plate was a group with only 19 chromosomes present recorded.

Of the first four experimental groups in which the oocytes were incubated in 7% ethanol in PBS for between 1 min and 7½ min, there seemed little difference in the overall incidence of aneuploid chromosome preparations observed.

The incidence of preparations with less than 20 chromosomes was almost exactly balanced by the number of preparations observed in which there were

Fig. 3. Representative air-dried chromosome spreads of single-pronuclear derived morulae. (a) Metaphase group with 21 chromosomes present from a 7-cell F₁ hybrid morula. This embryo contained 5 metaphase spreads each of which contained 21 chromosomes. (b) Metaphase group with 21 chromosomes present from a 19-cell 129/SvE morula. This embryo contained 7 metaphase spreads each of which was haploid. In most of these spreads 21 chromosomes could be distinguished. (c) Metaphase group from a 7-cell F₁ hybrid haploid-diploid mosaic embryo. Six metaphase spreads were present, one of which appeared to contain a giant chromosome (arrowed). (d) Metaphase group from an 18-cell F₁ hybrid morula which contained 6 haploid metaphases. The spread to the left contains 20 normal chromosomes while the partial spread on the right appears to contain a metacentric chromosome (arrowed).
Fig. 4. Two G-banded aneuploid first cleavage chromosome spreads containing 19 chromosomes. (a) Metaphase spread in which chromosome number 4 is absent. (b) Karyogram of this spread. (c) Metaphase spread in which chromosome number 9 is absent. (d) Karyogram of this spread.
more than 20 chromosomes present. However, in the fifth experimental group in which the activated oocytes were incubated for 2 h in low osmolar medium, the incidence of aneuploidy was markedly increased from about 14–19 % as observed in the other experimental groups to 28–3 %. As in the other experimental groups, an approximately similar incidence of metaphase preparations with less than 20 and more than 20 chromosomes present was observed.

Representative first cleavage metaphase spreads with complements of 18, 19, 20, 21 and 22 chromosomes are illustrated in Fig. 2. As a result of the extended colcemid treatment the chromosomes were quite condensed, and would probably not have been suitable for 'banding' analysis, even if they had been appropriately pretreated prior to staining. However, their condensed morphology undoubtedly facilitated the present analysis in which only the total number of chromosomes present was of immediate interest. Neither metacentrics nor other morphologically abnormal chromosomes were seen at this stage of development.

(3) Observations on the chromosome complement of single-pronuclear haploid embryos at the morula stage of development

In the preliminary study, air-dried preparations of 44 morulae from (C57BL × CBA)F1 and 17 morulae from 129/SvE strain mice in which metaphase plates were present were analysed. Of the F1 preparations in which unequivocal chromosome counts could be made, two embryos contained several metaphase plates in which groups of 21 chromosomes were present. In two additional embryos morphologically abnormal chromosomes were observed. In one case a 'giant' chromosome was present, and in another an apparent metacentric chromosome was present in a single group but not in another spread in the same embryo. In yet another embryo, only a single metaphase spread was present, but this contained 25 chromosomes. Of the 129/SvE embryos examined, two morulae contained several metaphase plates in which groups of 21 chromosomes were present. In addition, two morulae each contained a single metaphase spread with only 19 chromosomes present, and a third morula, which also had only a single metaphase spread present, apparently contained a complement of 24 chromosomes. The mean number of cells present in the F1 group was 12-8, and that in the 129/SvE group was 13-6. A selection of the metaphase spreads containing abnormal chromosome complements is illustrated in Fig. 3.

In a proportion of the morulae, due to problems with overlapping chromosomes, only the ploidy could be determined. It is possible therefore that the true incidence of aneuploidy at this stage of development could be considerably higher than reported here.

(4) The results of G-banding analyses of the first cleavage metaphase chromosomes

Attempts to analyse several hundred first cleavage metaphase spreads by G-banding were only moderately successful, as it was frequently found that preparations which banded well often had one or more groups of overlapping
Ethanol-induced aneuploidy

chrromosomes, which made any detailed analysis of the complement impossible. The chromosomes in other preparations which failed to band were often found to be severely contracted, despite only a brief period of incubation in Colcemid.

In 10 groups in which 20 chromosomes were present, all of the autosomes and a single X chromosome were recognized. In 3 additional groups, only 19 chromosomes were present. The chromosomes which were found to be absent in these metaphases were autosome numbers 4, 9 and 16, respectively. The first two of these banded preparations and their karyotypes are illustrated in Fig. 4.

DISCUSSION

The present study has clearly demonstrated that exposure of mouse oocytes to a dilute solution of ethanol is an extremely effective activating stimulus. Maximum rates of activation were achieved after exposure to a 7% solution of ethanol in PBS lasting about 3-4.5 min. However, the most interesting finding to emerge from the present study was the observation that ethanol induced chromosome non-disjunction in almost 19% of the haploid oocytes exposed to this agent. It was also apparent that the incidence of non-disjunction could be substantially increased if oocytes previously activated with ethanol were incubated in low osmolar medium during the time that the oocytes were completing the second meiotic division.

Analyses of the chromosome complements of the first cleavage metaphase in the three control studies presented here clearly demonstrate that in (C57BL × CBA)F₁ hybrid mice the incidence of non-disjunction is extremely low. This observation merely confirms similar analyses (Kaufman & Sachs, 1975) in which the chromosome complement of recently ovulated oocytes and first cleavage metaphase groups from the same F₁ hybrid mice had been determined. In this earlier study, one out of a total of 29 metaphase II groups contained 19 chromosomes, as did two out of 61 first cleavage metaphase groups analysed. As in the present study, none of the control groups analysed contained more than 20 chromosomes. This observation is also in accord with the conclusions of Rohrborn (1972) and Uchida & Lee (1974) that the occurrence of non-disjunction at meiosis I is rare in oocytes from young non-translocation bearing mice. However, even in these stocks there appears to be a sharp increase in the incidence of non-disjunction at meiosis I in aged females (Henderson & Edwards, 1968; Gosden, 1973; Yamamoto, Shimada, Endo & Watanabe, 1973).

In the four experimental groups in which cumulus masses were exposed to 7% ethanol in PBS for 1, 3, 4.5 and 7.5 min, a relatively high incidence of non-disjunction was observed (13.6–18.8%). In the first three of these groups aneuploid preparations were observed which contained 18, 19, 21 and 22 chromosomes, whereas in the fourth group only preparations with 18 and 19 chromosomes were observed. Considering the relatively small number of aneuploid preparations analysed in the present study, it is not clear whether the
increase in the incidence of hypohaploidy which appears to be related to increasing exposure to ethanol is a real effort or not. It is curious that 82% of the aneuploid preparations contained either 19 or 21 chromosomes, and the present findings give no clear indication whether one or several chromosomes are particularly commonly involved in the non-disjunctual event or all of the chromosomes are equally susceptible. The only certain way to distinguish between these two possibilities obviously involves subjecting an additional group of preparations obtained under similar conditions to those described here to appropriate pretreatment, for example, using a technique similar to that employed by Nesbitt & Donahue (1972), and subsequently analysing the ‘banded’ chromosomes obtained. In the preliminary G-banding study reported here, in the three aneuploid metaphase spreads with 19 chromosomes present, a different autosome was involved in each of these groups.

The fact that a dilute solution of ethanol is an efficient activating agent is not altogether surprising. It may well be that the underlying mechanism may be similar to that occurring in the case of exposure of mouse oocytes in vitro to local anaesthetics and phenothiazine tranquillizers as demonstrated by Siracusa, Whittingham, Codonesu & De Felici (1978), and in vivo to Avertin (Kaufman, 1975). Their ability to initiate activation may be related to their effect on several cellular processes which are most probably inter-related, though the exact inter-relationships remain unclear. These processes include displacement of Ca\(^{2+}\) from membrane phospholipids (Nicolson, 1976), changes in intracellular pH (Johnson, Epel & Paul, 1976), the disruption of the cellular cytoskeletal system (Nicolson, 1976), changes in the electrical properties of the egg membrane (Seeman, Chen, Chan-Wong & Staiman, 1974), and finally changes in membrane fluidity (Sheetz & Singer, 1974). A recent study (Ahuja, 1982) has suggested that similar mechanisms may be involved during the activation of hamster oocytes by procaine and tetracaine.

The observation reported in the present study, that a high incidence of aneuploidy may be induced when a population of recently activated oocytes are incubated in a dilute solution of ethanol, may also be explicable in the light of previous knowledge of the action of this and other related agents, for example, on the cellular cytoskeletal system either directly or indirectly via other intracellular changes mentioned above. The ultrastructural studies on the teratogenic effect of acetaldehyde (O’Shea & Kaufman, 1979, 1981), the primary metabolic product of ethanol oxidation, for example, and xylocaine (O’Shea & Kaufman, 1980) on early-somite-stage mouse embryos clearly demonstrated that these agents acted on both the microtubular and microfilamentous components of the cytoskeletal system, though the exact mode of action of these treatments is unclear (for recent review, see O’Shea, 1981).

Studies on the effect of ethanol administration to pregnant mice have also clearly demonstrated the rapidity with which this agent reaches the foetal compartment (Kaufman & Woollam, 1981) and its various teratogenic effects
on embryonic and foetal development (Sandor & Amels, 1971; Kronick, 1976; Brown, Goulding & Fabro, 1979; Randall & Taylor, 1979), though detailed ultrastructural studies are lacking. It should not be surprising therefore that this agent may also interfere with chromosome segregation, as the relationship between the spindle apparatus and the centromere is both complex and finely balanced (Hughes, 1952).

This work was supported by grants from the Medical Research Council and the National Fund for Research into Crippling Diseases. I thank Kamal Ahuja for helpful discussion and Mrs Lesley Cooke for technical assistance. The G-banding and karyotyping studies were carried out by Elizabeth Robertson, and her assistance in this aspect of the work is gratefully acknowledged.

REFERENCES


*(Received 21 December 1981, revised 5 April 1982)*
RAPID COMMUNICATION

Two Examples of Monoamniotic Monozygotic Twinning in Diploid Parthenogenetic Mouse Embryos

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ABSTRACT In experiments in which diploid parthenogenetic mouse embryos cultured entirely in vitro from the one-cell stage to the blastocyst were transferred to suitable recipients and maintained in "delay" for about 3 days before implanting, about 25-40% subsequently developed to somite-containing stages. In all, over 30 such embryos were examined. Most were morphologically normal, and equivalent to fertilized development observed on the 9th-11th days of pregnancy. A few embryos, however, had neural tube abnormalities, but of greatest interest were two sets of monoamniotic monozygotic twins. It is unclear whether the twins in some way resulted from the parthenogenetic condition, or from the "delayed" state. The present examples are discussed in the context of previous observations of monozygotic twinning in the mouse and man.

In recent experiments in which nonaggregated diploid parthenogenetic blastocysts were transferred to ovarectomized pseudopregnant recipients, the incidence of development to somite-containing stages, equivalent to normal fertilized development observed on the 9th-11th days of pregnancy, has generally been in the order of 25-40% (Kaufman et al., '77; Kaufman, unpublished). The embryos involved in these studies had been activated in vitro in medium lacking Ca²⁺ and Mg²⁺ (Surani and Kaufman, '77; Kaufman, '78), and were those that developed two pronuclei in the absence of second polar body extrusion. Embryos of this type were retained in culture to the expanded blastocyst stage (Kaufman and Sachs, '76), then transferred to suitable recipients as described earlier.

Of the 30-35 somite-stage embryos examined, most were morphologically normal, and will only be considered briefly. However, two of the most interesting specimens recovered from separate females, at an interval of more than a year, will be described here in greater detail. Both are examples of monoamniotic monozygotic twins in which the diagnosis was made on histological examination of the intact yolk sacs containing the conceptuses. Some observations are made on the stage of development of monozygotic monoamniotic twinning with particular reference to the etiology in the present cases.

MATERIALS AND METHODS

Eight to 12-week-old (C57BL × CBA)F₁ hybrid female mice were superovulated with 5IU PMSG followed 48 hr later with 5IU HCG. The females were autopsied approximately 18 hr after the HCG injection, the oviducts removed and their contents released into embryo culture medium lacking Ca²⁺ and Mg²⁺ (Surani and Kaufman, '77; Kaufman, '78). After about 5-6 hr the activated eggs were classified according to the number of pronuclei present, whether a second polar body had been extruded or not, and the type of cytoplasmic cleavage induced—whether equal or unequal [for detailed description of methods of activation and classification of parthenogenones, see Kaufman ('78, '81)]. Only the presumptive diploid embryos (which developed two pronuclei in the absence of second polar body extrusion) were used in the present study. The diploid parthenogenones were retained in culture for a further 96 hr, by which time about 80% had achieved the blastocyst stage (Kaufman, '81). The blastocysts were then transferred to the uteri of recipients on the third day of pseudopregnancy (day of finding vasectomized plug termed first day of pseudopregnancy). The recipients were ovarectomized at the time of transfer and given a subcutaneous injection of 1 mg Depo-Provera (Upjohn). After an interval of 3 days—during
which time the blastocysts entered a “delayed” state, the recipients received daily a single combined injection of estrogen and progesterone (Kaufman et al., ’77).

The recipients were autopsied on the equivalent of the afternoon of the 10th or 11th day of pregnancy, and the uterine contents examined. By this time the most advanced embryos had usually 20-25 pairs of somites and a good yolk sac circulation.

A proportion of the resultant embryos were embedded in paraffin wax and serially sectioned, some were embedded in resin (Spurr, ’69), while others were disaggregated either in attempts to establish cell lines from them or to investigate their X-chromosome activity (Kaufman et al., ’78; Rastan et al., ’80). The paraffin-embedded sections were stained with hematoxylin and eosin, and the resin sections with methylene blue. In all, about 30-35 somite-stage diploid parthenogenones have been examined following activation in medium lacking Ca2+ and Mg2+.

Other forms of in vitro activation have also given rise to somite stage embryos following the transfer of singleton blastocysts into the uteri of similar recipients to those described previously. In addition, aggregated eight-cell embryos or morulae transferred to nonovariectomized recipients in which the embryos do not enter into a “delayed” stage also develop to somite stages (Kaufman, ’81). However, in the present study, only those conceptuses that were induced in Ca2+/ Mg2+-free medium and were morphologically easily recognizable as embryos will be considered. This group, which accounts for most of the conceptuses recovered, also included two pairs of monozygotic twins. Each pair within its intact yolk sac was serially sectioned, one being embedded in paraffin wax and the other in resin. The wax-embedded material was sectioned at a nominal thickness of 7 μm, whereas the resin-embedded material was sectioned at 0.5-0.75 μm. Serial reconstructions of the two hearts in the second pair of twins was carried out by tracing the outline of appropriate sections onto thin sheets of modeling wax (De Trey®, AD International Ltd). The wax reconstructions were then compared with scanning electron micrographs of the exposed hearts of fertilized embryos at similar stages of cardiogenesis (Kaufman and Navaratnam, ’81). In the first pair of twins, the oblique orientation of the sections did not allow this procedure to be carried out with confidence in the larger twin, whereas the heart in the smaller twin was not sufficiently advanced for this procedure to be of any value.

RESULTS

The majority of the 30-35 singleton diploid parthenogenetic embryos examined on the equivalent of the 10th or 11th day of pregnancy had between 10 and about 25 pairs of somites present, and appeared to be morphologically normal both on gross inspection and when examined histologically. The most advanced 8-10 embryos were either moribund or recently dead. These embryos were equivalent to fertilized embryos on the 11th day of pregnancy, with fore- and hindlimb buds and well-developed lens placodes (occasionally, the lens vesicle was present). All other embryos with 20-25 or more somites present appeared healthy at the time of examination. These embryos had beating hearts and good yolk sac circulation. However, in 4 of the most advanced embryos in the former group, which were recovered from a single recipient, the cephalic region appeared to be enlarged and/or edematous. On histological examination, both the brain and spinal cord tissue appeared to be considerably reduced in volume. Only one other 20-25 somite embryo was seen with a neural tube abnormality. In this otherwise normal and healthy embryo, the cephalic neural tube was closed, as expected (Kaufman, ’79), but an open neural tube defect was present which extended from the thoracic region caudally.

All of the “unturned” and “partially turned” embryos had beating hearts and appeared to be morphologically normal. However, of greatest interest were the 2 pairs of monozygotic twins. On gross inspection, the first pair consisted of two embryos that shared a common and reasonably well-expanded yolk sac. One of the embryos was unturned, and at the early headfold stage with about 3 pairs of somites present. The larger twin was partially turned with 10-12 pairs of somites and a beating heart. Histological examination revealed that both embryos shared a common amniotic cavity. However, because of the obliquity of the sectioning through both embryos, it was only possible to discern that both appeared to be morphologically normal.

The second pair of twins was contained within a considerably smaller sac. Removal of the single ectoplacental cone revealed the presence of two headfold-stage embryos, which appeared to be facing each other. At this stage of the dissection, the sac and its
contents were fixed in glutaraldehyde and embedded in resin, as this procedure facilitated optimal orientation of the specimen prior to sectioning.

Both embryos were at an almost identical stage of development with four to six pairs of somites present, and fortuitously facing each other at a similar level, as though looking into a mirror. A representative section through the thoracic regions of these two embryos is illustrated in Figure 1. This clearly shows that both embryos share a single amniotic cavity. Reconstruction of the two hearts revealed that these were at slightly different stages of cardiogenesis, but both were rotated normally. A detailed inspection of the sections above and below that illustrated in Figure 1 revealed that the thoracic regions of the two embryos were closely apposed, but not fused together, and that the two embryos were morphologically normal.

DISCUSSION

Because of the extreme rarity of spontaneous twinning of any type in mice, those cases that are recognized justify detailed analysis. In the earliest major report on this topic Gluecksohn-Schoenheimer ('49) described the occurrence of partial and complete twinning in mice that were assumed to be homozygous for a T-locus mutation term Kinky (Ki), in matings between male and female mice that were heterozygotes for this mutation (Ki/+). In this case, the mutation was thought to be lethal in the homozygous state, death being due either directly or indirectly to the duplication-related abnormalities observed. More recently, other T-locus mutants have been described in which partial or complete duplication of axial organs is seen (Theiler and Gluecksohn-Waesch, '56; Bennett and Dunn, '60).

In only two subsequent studies have reports been published in which identical twinning in the mouse has been observed; in one series they were induced experimentally in vivo, whereas in the second series they resulted from the experimental manipulation of embryos in vitro. In the first of these studies, Kaufman and O'Shea ('78) exposed pregnant mice to a single injection of Vincristine during the early post-implantation period, either on Day 7 or 8 of pregnancy, and obtained 5 pairs of monozygotic twins (including 1 conjoined pair) out of a total of 189 living fetuses examined on Days 10 or 12 of pregnancy. An additional group of females was injected with this agent on Day 6, but no twinning resulted. Apart from the conjoined twins which were obviously monoamniotic, at least 3 of the remaining 4 pairs were also monoamniotic, and all 8 embryos from the 4 pairs with 2 singleton embryos each appeared to be morphologically normal.

More recently Hsu and Gonda ('80; see also Hsu, '81) have reported that approximately 1% of all 4-day expanded blastocysts, which they had explanted in vitro, gave rise to monozygotic twins. Whether twinning developed or not appeared to depend on the orientation of the blastocyst at the time of attachment—only those blastocysts in which attachment occurred onto the plastic surface of the culture dishes in the antipolar region of the trophoblast developed in this way. In this study, typically two independent egg cylinders formed following the division of the inner cell mass region shortly after attachment. The monozygotic twins thus formed were, therefore, of the dichorionic diamniotic variety.

In a more recent study, attempts to repeat both the in vivo and in vitro experiments failed to induce monozygotic twinning, although the occasional spontaneous occurrence of dizygotic (presumed) twinning, in which pairs of embryos (in one case, three embryos) within a single decidual swelling shared a single eotoplacental cone, was in fact reported (Wan et al., '82). While the overall incidence of twinning in the earlier studies was significantly higher than in the report by Wan et al. ('82), there can be little doubt that the twinning events observed by Kaufman and O'Shea ('78) and Hsu and Gonda ('80) were not fortuitous or chance events as has recently been suggested by Wan et al. ('82). The failure of the latter group to induce monozygotic twinning could have resulted from their use of different strains of mice, different timing of injection or explantation, variation in culture conditions, etc.

In the present report, two pairs of monoamniotic monozygotic twins are described. These are generally considered to be the rarest of all of the major varieties of identical twins, probably accounting for only 1-3% of all cases of monozygotic twinning in man: approximately 80% of all identical twins in man are of the dichorionic diamniotic variety, and 60-70% of the monochorionic diamniotic variety (Gedda, '61; Strong and Corney, '67; Boyd and Hamilton, '70). While there may be species variation in the absolute incidence of these different classes, there seems to be little doubt that the monoamniotic variety occurs as a result of
Fig. 1. Representative histological section through the heart regions of four- to six-somite-stage diploid parthenogenetic monozygotic monoamniotic twins. Note in particular the yolk sac (Y) with blood islands (B) present, the primitive heart tubes (H), the thin body wall (W) overlying the heart and pericardial cavity, the bilayered structure of the amnion (A), and the neural folds in the thoracic (T) and primitive streak (P) regions. The plastic section is stained with methylene blue.
splitting of the embryonic axis at a relatively late stage of embryogenesis—possibly as late as the primitive streak stage of development (Balmer, '70; Hamilton and Mossman, '72; Fox, '78; Kaufman and O'Shea, '78). There seems to be little support for the earlier idea that this group results from the amalgamation of the two amniotic sacs in diamniotic twins in cases where the intervening amniotic septum breaks down (see Benirschke and Driscoll, '67).

Serial reconstruction of the hearts in the twins with four to six pairs of somites revealed that the primitive cardiac tubes in each case were rotated normally. Indeed, this is about the earliest stage when any degree of asymmetry occurs in the embryo (Kaufman and Navaratnam, '81) when, for example, the earliest evidence of dextrocardia might have been recognized. This condition is thought to arise during the looping stage of cardiogenesis (Fox and Goss, '57; '58; Christie, '61; Monie et al., '66)—possibly due to abnormalities in the shape of the individual primitive cardiac cells (Manasek et al., '72). As dextrocardia may be associated with situs inversus or occur as an isolated anomaly, it has been suggested that these two situations probably represent two distinct etiological entities, although both situations are occasionally encountered in human monozygotic twin pairs (Warkany, '71).

While the incidence of monozygotic twinning in early somite stage parthenogenetic embryos is not particularly high (about 6% of those examined), that they should have been induced at all is certainly curious. It is unclear whether the underlying mechanism of their induction is related to their parthenogenetic origin, or to the “delayed” state. In both of the cases reported here, the two sets of twins were formed from the division of apparently morphologically normal singleton blastocysts that implanted and subsequently developed following a period of several days in “delay.”

In the one instance, both twins were at an almost identical stage of embryogenesis—with 4–6 pairs of somites present. In the second case a moderate degree of developmental disparity was apparent, with the smaller twin having only 3–4 pairs of somites, whereas the larger “partially turned” twin contained about 10–12 pairs of somites. Such a disparity in size and development is not uncommon in monogygotic twins in man. The incidence of fetal mortality in monoamniotic monochorial placenta tion in man is still in the region of 30–50%—principally due to the tendency of the two cords to become twisted and entangled with each other (Fox, '78).

In 15–30% of cases of monochorial diamniotic placenta tion in man, a unidirectional hemodynamic imbalance forms early in gestation with the shunting of arterial blood from one conceptus to the other principally through the inner circulation, giving rise to a condition termed the “transfusion syndrome” (Schatz, 1900; Rausen et al., '65; Strong and Corney, '67; Arts and Lohman, '71; Benirschke, '72; Fox, '78). Curiously, despite the presence of extensive vascular communications, the latter condition is extremely uncommon in monoamniotic twins. No information is at present available on the presence or otherwise of similar vascular anastomoses in the mouse, although anastomoses develop even between the placentas of dizygotic twins in many species (see Gedda, '61; Boyd and Hamilton, '70).

In relation to the present findings, a control series of experiments in which large numbers of delayed fertilized blastocysts were allowed to implant and the resulting conceptuses subsequently analyzed during the early somite period, would be required in order to discover whether or not the delayed state was the cause of the twinning observed in the present study.

That parthenogenetic mammalian embryos should be capable of surviving beyond the early post-implantation period is, of course, of considerable biological interest (for discussion, see Graham, '74; Tarkowski, '75; Whittingham, '80; Kaufman, '81). Most of the somite-stage embryos examined in the present series appeared to be healthy and morphologically normal, so that it is difficult to understand why they should be destined to die shortly thereafter, as recent studies have demonstrated, for example (1) the pattern of X-inactivation in these embryos (Kaufman et al., '78) and in their extraembryonic membranes (Rastan et al., '80; Endo and Takagi, '81) is of the normal female pattern; (2) single cells or groups of parthenogenetically derived cells are capable of differentiating normally in parthenogenetic-fertilized chimeric individuals made by aggregation or inner cell mass injection techniques (Stevens et al., '77; Surani et al., '77), even to the extent of giving rise to viable germ cells (Stevens, '78); and more recently, (3) the parthenogenetic genome is capable of supporting normal development to the adult (Hoppe and Illmensee, '82). Possibly the embryos die at the limb-bud stage because of problems associated with the formation and/or adequate functioning of the chorionic placenta.
thenogenones should die at this stage of development is beyond the scope of the present paper.

ACKNOWLEDGMENTS
I wish to thank Mr. J. Skepper for his expert technical assistance with the plastic sectioning.

LITERATURE CITED


Establishment of pluripotential cell lines from haploid mouse embryos

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SUMMARY

Eggs from 129 Sv/E and (C57BL × CBA)F₁ hybrid female mice were activated parthenogenetically following their exposure to a 7% solution of ethanol in PBS. Only the haploid class which developed a single pronucleus following second polar body extrusion was examined further. These eggs were transferred to suitable recipients and ‘delayed’ blastocysts subsequently recovered. The ‘delayed’ blastocysts were explanted into tissue culture and a total of four haploid-derived pluripotent cell lines established from individual embryos. Chromosome analysis of morulae revealed that over 80% contained only haploid mitoses. However, chromosome analysis of early passage cell lines revealed that all were diploid with a modal number of 40 chromosomes. When transplanted into syngeneic hosts, all lines formed well-differentiated teratocarcinomas. This technique provides a source of homozygous diploid cell lines of parthenogenetic origin.

INTRODUCTION

It has been suggested that the direct isolation of pluripotential cells from early embryos might provide sources of pluripotential cells with a karyotype unchanged from that of the embryo from which it was derived (Evans, 1981). In addition to their relative ease of production from blastocysts, the principal advantage that such EK cell lines have over most currently available embryonal carcinoma (EC) cell lines derived from tumours is that, at least initially, they do have a normal karyotype (Evans & Kaufman, 1981).

Recent studies in which pluripotential cell lines have been established from a considerable number of individual fertilized mouse embryos with both a normal and abnormal chromosome complement (Martin, 1981; Evans, Robertson, Bradley, Handyside & Kaufman, unpublished) have stimulated us to attempt to

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establish similar pluripotential cell lines from haploid parthenogenetically-derived embryos.

MATERIALS AND METHODS

i. Ethanol activation

Eight- to 12-week-old 129 SvE and (C57BL×CBA)F₁ hybrid female mice were superovulated with 5 i.u. PMSG followed 48 h later by 5 i.u. HCG. The females were autopsied 17 h later and the cumulus masses recovered from the oviducts released into a freshly prepared 7 % (v/v) solution of Analar quality ethanol in Phosphate Buffered Saline (PBS) containing both Ca²⁺ and Mg²⁺, and retained in this solution for about 4½ min. Cumulus masses from four to six females were pooled together, treated as a single group, and this and the following washing procedures were carried out at room temperature. The cumulus masses were washed through three changes of ethanol-free PBS and through two changes of embryo culture medium (Whittingham, 1971). Individual cumulus masses were then transferred to separate drops of medium under paraffin oil and incubated for 4–5 h at 37 °C in an atmosphere of 5 % CO₂ in air. The adherent cumulus cells were then removed with hyaluronidase, and the overall activation frequency determined and the various classes of parthenogenone induced separated into different groups (Kaufman, 1978a). Only those activated oocytes that developed a single haploid pronucleus following second polar body extrusion were used in this study. A more detailed description of this ethanol activation technique has been published elsewhere (Cuthbertson, Whittingham & Cobbold, 1981; Kaufman, 1982).

A proportion of the pronucleate-stage 1-pronuclear haploid eggs were transferred to the oviducts of recipients (Tarkowski, 1959) anaesthetized with Avertin on the afternoon of the first day of pseudopregnancy (i.e. on the day in which the vaginal plug had earlier been observed, following mating of the female with a vasectomized male), while others were retained in culture.

The recipients were divided into two groups. The first group was ovariectomized on the afternoon of the 4th day of pseudopregnancy and, while they were still under the influence of the anaesthetic, given a subcutaneous injection of 1 mg Depo-Provera (Upjohn). This group of females was subsequently autopsied 4–5 days later, the uterine horns removed and flushed with PBS in order to recover delayed blastocysts. The second group of recipients was autopsied at about midday on the 4th day of pseudopregnancy and the reproductive tract flushed with PBS. About half of the recovered embryos, which were mostly at the morula stage, were then incubated for about 3 h in medium containing 1 μg/ml Colcemid. These embryos were then examined by the air-drying technique (Tarkowski, 1966), and the preparations stained with Giemsa. It was possible to classify almost all of the embryos with cells in division into three distinct groups, namely i. haploid, ii. haploid-diploid mosaics or iii. 
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diploid, according to the number of chromosomes present in the individual metaphase plates. The embryos from this group of recipients that were not examined by air drying were allowed to develop to the blastocyst stage in culture, then transferred to the uterine horns of other recipients in order to recover delayed blastocysts.

The embryos that were retained in culture from the 1-cell stage were subsequently transferred, at about 74–75 h after activation, into medium containing 1 μg/ml Colcemid for 3–4 h, and air-dried preparations made as described above. The ploidy of this group of embryos was also determined.

ii. Establishment of pluripotent cell lines from delayed blastocysts

Individual delayed blastocysts, many of which contained large clearly delineated inner cell masses (ICMs) (Fig. 1A) were transferred to tissue culture

Fig. 1. A. ‘Delayed’ 129 SvE blastocyst shortly after its explantation into tissue culture medium. Note the large inner cell mass. B. Appearance of ‘implanted’ blastocyst at approximately 60 h after explantation. Note centrally-located clump of inner-cell-mass-derived cells (arrow). C. A group of haploid-derived cells, growing on a feeder layer, shortly after their establishment in culture.
dishes containing Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% foetal calf serum and 10% newborn calf serum (Evans & Kaufman, 1981). After blastocyst attachment, which usually occurred within 48 h of explantation (Fig. 1B), the ICM-derived cell clumps were selectively removed following an additional 4 days of culture. The ICM clumps were disaggregated in 0.25% (w/v) trypsin, 0.04% (w/v) EDTA and replated onto feeder layers of mitomycin-treated fibroblasts. In successful cultures nests of stem cells appeared following two rounds of cell growth and trypsinization. These cells have a distinctive morphology in culture (Fig. 1C) closely resembling other established tumour-derived and embryo-derived pluripotential cell lines. The haploid-derived (HD) lines were subsequently maintained on feeder layers and subcultured at 4–6 day intervals.

iii. Testing of differentiation ability of the cell lines

The differentiation ability of the 129 SvE lines was tested by inducing tumour formation in syngeneic host animals. For each line 10–12 male 129 SvE mice were inoculated subcutaneously with approximately 10⁶ cells. Tumour masses were retrieved after 4 to 6 weeks and fixed in Bouin’s solution, dehydrated and subsequently serially sectioned at a nominal thickness of 7 μm. Alternate slides were then either stained with haematoxylin and eosin or with Masson’s trichrome.

iv. Chromosome analysis of cell lines

Chromosomal analysis was performed on early passage cell lines. This was usually carried out within five to ten passages following the original disaggregation of the ICM-derived cell clumps. The chromosomes were analysed by G-banding (modification of the A.S.G. procedure of Gallimore & Richardson, 1973), and karyograms arranged according to the nomenclature of Nesbitt & Francke (1973).

RESULTS

A. Observations on the activation rate and incidence of the various classes of parthenogenone induced

Observations on the incidence of the various classes of parthenogenone induced when 129 SvE and (C57BL X CBA)F₁ hybrid oocytes isolated at 17 h after the HCG injection for superovulation were stimulated by exposure to a 7% solution of ethanol in PBS for about 4½ min are presented in diagrammatic form in Fig. 2. The data included in this figure are the combined results of all the activation studies carried out over a period of several months involving these two strains of mice, and in all represent the results of isolated experiments carried out on more than 10 separate occasions.

In both strains, the highest proportion of the activated population consisted of haploid parthenogenones which had developed a single (haploid) pronucleus
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Fig. 2. Incidence of different types of parthenogenones induced when eggs from 129 SvE and (C57BL × CBA)F₁ hybrid mice were briefly incubated in 7% ethanol in PBS. Cumulus masses were released at 17 h after HCG and observations made 4–5 h later. The total number of activated eggs examined in the 129 SvE series was 1351, and the activation frequency was 80.0%. In the F₁ series, 1211 activated eggs were examined, and the activation frequency was 95.7%.

following extrusion of the second polar body. The overall activation rate in both strains was high, with about 80–95% of the oocytes exposed to the ethanol treatment being stimulated to develop parthenogenetically. As indicated in the Methods section, only the 1-pronuclear haploid embryos were used subsequently in the present study.

B. Chromosome analysis at about 75–77 h after activation

i. In vivo series

Activated haploid eggs from 129 SvE and (C57BL × CBA)F₁ hybrid females which had previously been transferred to the oviducts of suitable recipients at the 1-cell stage, were isolated at about midday on the 4th day of pseudopregnancy. The embryos, which were largely at the morula stage, were then incubated for about 3 h in medium containing 1 μg/ml Colcemid, and subsequently examined.
by the air-drying technique described by Tarkowski (1966). As only about half of the recovered embryos were used to assess the ploidy, the others being transferred to additional recipients in order to obtain delayed blastocysts, only details of the fixed embryos with cells in division will be presented here (see Table 1). In the haploid–diploid mosaic embryos only one or two diploid metaphases were usually present, and almost all of the mitoses observed in this group were haploid. In the 129 SvE series 82%, and in the (C57BL × CBA)F₁ series 85% of the embryos examined had only haploid mitoses present (see Fig. 3).

ii. In vitro series

In a parallel series of experiments, (C57BL × CBA)F₁ hybrid oocytes were activated in vitro with ethanol and the 1-pronuclear haploid embryos retained in culture until about midday on the 4th day (about 73–74 h after activation), then those that had progressed beyond the 4-cell stage were transferred to medium containing 1 µg/ml Colcemid for 3–4 h. Out of an initial total of 174 1-cell activated eggs, 157 embryos had more than four cells present by the early afternoon on the 4th day, but by this time most of the embryos were at the morula stage of development. Air-dried preparations were made as described above. In 12 of these embryos no cells were in division, in 141 embryos one or more mitoses were present, and in 4 embryos virtually all of the cells were in division and it was considered impossible to make an assessment of the ploidy because of extensive overlapping of mitotic figures. Of the 141 embryos with cells in division, 102 (72%) had only haploid mitoses, 35 (25%) had both haploid and diploid mitoses present, while 4 (3%) had only diploid mitoses present (see Table 1). The mean number of cells (±s.e.) in the haploid, haploid-diploid mosaic and diploid embryos in this series was 18.2 ± 0.6, 16.4 ± 0.9 and 14.0 ± 4.3, respectively, while the mean number of cells in mitosis in each of these groups of embryos was 5.2 ± 0.3, 6.6 ± 0.6 and 4.0 ± 1.7, respectively. Following the 3–4 h period of incubation in medium containing Colcemid, approximately 30–40% of the blastomeres in these embryos were therefore blocked in mitosis at the time of analysis. In the haploid–diploid

Table 1. Chromosome analysis of 1-pronuclear haploid embryos at the morula stage of development

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Total embryos with mitoses</th>
<th>Haploid</th>
<th>Diploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oviduct transfer eggs</td>
<td>129 SvE</td>
<td>17</td>
<td>14 (82%)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(C57BL × CBA)F₁</td>
<td>78</td>
<td>66 (85%)</td>
<td>5</td>
</tr>
<tr>
<td>In vitro culture</td>
<td>(C57BL × CBA)₁</td>
<td>141</td>
<td>102 (72%)</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

1-cell embryos were transferred to the oviducts of recipients on the afternoon of the first day and isolated at midday on the 4th day of pseudopregnancy.
mosaics, only one or occasionally two diploid mitoses were observed, and almost all of the mitoses present were haploid.

C. Recovery of delayed blastocysts

Out of an initial total of 327 129 SvE and 627 (C57BL × CBA)F₂ hybrid pronucleate-stage 1-pronuclear haploid eggs transferred to the oviducts of
recipients on the afternoon of the first day of pseudopregnancy, 64 129 SvE (20%) and 104 F₁ hybrid (17%) delayed blastocysts were subsequently recovered. The delayed blastocysts were then transferred to tissue-culture medium supplemented with serum. After 72–96 h, when the majority of embryos had 'implanted', the inner-cell-mass-derived lumps were either disaggregated in an attempt to determine their ploidy (see Section F), or retained in culture to establish pluripotent cell lines (see Section D).

D. Establishment of cell lines in culture

Four haploid-derived cell lines have so far been established. These lines were derived on three separate occasions over a period of several months from both 129 SvE and (C57BL × CBA)F₁ hybrid delayed blastocysts (Table 2). The origin of the various lines was confirmed by GPI isozyme analysis, as the 129 SvE-derived lines were homozygous for the Gpi-1a isozyme, and the F₁ derived lines homozygous for the Gpi-1b isozyme of glucose phosphate isomerase.

Table 2. Haploid-derived pluripotent cell lines

<table>
<thead>
<tr>
<th>Strain of origin</th>
<th>Lines established</th>
<th>Modal chromosome number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 129 SvE</td>
<td>HD1</td>
<td>40</td>
</tr>
<tr>
<td>2.</td>
<td>HD2</td>
<td>40</td>
</tr>
<tr>
<td>3. (C57BL × CBA)F₁</td>
<td>HD3</td>
<td>40</td>
</tr>
<tr>
<td>4.</td>
<td>HD4</td>
<td>40</td>
</tr>
</tbody>
</table>

E. Differentiation ability of pluripotent cell lines

Both the HD1 and HD2 cells formed typical well-differentiated teratocarcinomas when injected subcutaneously into syngeneic hosts. A wide range of easily recognizable cell types were present (Fig. 4A–F), in addition to nests of undifferentiated embryonal carcinoma cells. In vitro, all four lines formed typical simple and cystic embryoid bodies following suspension culture of cell aggregates. Cells from lines HD3 and HD4 have recently been injected into syngeneic hosts, but the results have yet to be analysed.

F. Chromosome analysis of pluripotent cell lines

Repeated attempts to determine the chromosome constitution and ploidy of the ICM-derived clumps between 72 and 96 h after blastocyst explantation have so far been unsuccessful. Despite prolonged culture in Colcemid (6–12 h), no cells have been observed in division. Parallel observations on fertilized material at similar stages of development have also failed to demonstrate cells in division (authors, unpublished observations). This appears to be a technical problem, either because the cells are not in division at the time of analysis, or because of problems associated with the disaggregation of the small clumps of cells which
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Fig. 4. Representative regions through a well-differentiated 129SvE haploid-derived teratocarcinoma. Sections stained with Masson's trichrome. A. Keratin whorl. B. Cartilage nodule. C. Region showing areas of melanin pigmentation, precartilage nodule and epithelial-lined tubules. D. Detail from wall of large cyst lined by secretory epithelial cells. E. Area showing tissue interspersed with yolk-sac-like material. F. Organized structure formed from folded layers of columnar epithelial cells.

were tightly adherent and failed to separate following standard disaggregation techniques.

Chromosome analysis of early passage cultures revealed that all cells observed
in division at this stage were diploid – no haploid cells were detected. All four cell lines proved to have a modal number of 40, as expected. G-banding studies of 30–35 metaphase spreads from each of the lines examined confirmed that all the cell lines had a normal diploid autosomal complement. Interestingly, all the lines at the time of karyotyping, were characterized by the possession of a deletion of the distal end of the X chromosome. However, in the HD4 line, of the 31 banded spreads which were karyotyped shortly after its establishment in culture, this abnormality was only present in 16 of the metaphase spreads.

Karyograms from the HD4 line are presented in Fig. 5. In Fig. 5A, a normal karyogram is observed, whereas in Fig. 5B the karyogram showing a deletion of approximately 25% of the distal part of a single X chromosome is presented.

DISCUSSION

We have demonstrated that it is technically feasible to establish pluripotential cell lines from haploid embryos. These cells which were derived from haploid parthenogenones from various strains have all the properties expected in that, once established in culture, they can be induced to differentiate both in vivo into typical teratocarcinomas with a wide variety of cell types present, and in vitro.

Previous attempts to establish haploid teratocarcinomas and to derive cell lines from these sources were only partially successful in that while tumours were derived from the ectopic transfer of haploid parthenogenones (Iles et al., 1975; Graham, McBurney & Iles, 1975), no permanent pluripotential lines have been reported. Lines have, however, been established from spontaneous teratocarcinomas occurring in the ovaries and testes of LT/Sv strain mice, but these are undoubtedly diploid (Martin et al., 1978) and some lines appear to be restricted in their differentiation (Gachelin, cited in Nicholas et al., 1976). However, in the only published report in which LT-derived teratocarcinoma cells were injected into blastocysts, Illmensee (1978) reported that in one instance out of eight chimaeric individuals obtained, the tumour-derived cells not only took part in normal tissue differentiation but even contributed to the germ line.

Chromosomal analysis carried out at different stages in the establishment of the lines reported in this paper indicated that 15–18% of the embryonic population at the morula stage contained at least a proportion of diploid cells. In the in vivo series no significant difference was observed between the 129 SvE- and F1-derived embryos in the numbers of haploid vs. haploid–diploid and diploid mitoses. A difference is apparent, however, between the in vivo and in vitro series in this regard, since more diploid mitoses were seen in the latter group (see Table 1). This may be a reflection of the fact that conditions in vitro may be suboptimal compared to those in vivo for the maintenance of haploidy during the early preimplantation period.

Several attempts to determine the chromosome constitution of the delayed blastocysts within 3–4 days after their isolation and explantation into culture...
Fig. 5. Karyograms from HD4 line. A. Showing normal XX euploid chromosome complement. B. Showing deletion of approximately 25% of the distal region of one of the X chromosomes.
were unsuccessful, as no mitoses were observed in the inner-cell-mass-derived cells. In the earliest stages at which the cells were successfully karyotyped (after the establishment of mass cultures), all the cells were found to be diploid.

The chromosome constitution of early passage cultures was normal. However, with subsequent culture, partial deletions of one of the X chromosomes was evident, though this had no apparent effect on their differentiation. The extent of this deletion varies between HD lines, but the observation that the position of the break point is constant within a given line strongly suggests that, firstly, this phenomenon occurs early in their isolation and, secondly, that it does not arise by progressive deletion. It is interesting to note that the ESC stem cell line isolated by Martin (1981) is also reported as having a deletion of a single X chromosome. A more detailed analysis of the cytogenetic characteristics of these and other parthenogenetically-derived EK lines is currently being prepared (Robertson, Evans & Kaufman, 1983).

To date, no haploid mitoses have been observed in the established lines, and we can only speculate at which stage diploidization is occurring. We believe, from the morula studies indicated above, and from previous analyses of intact egg cylinders derived from haploid embryos (Kaufman, 1978b) that at least a proportion of the cells at explantation and shortly thereafter are still haploid.

While the success rate of establishing haploid-derived lines by the technique reported here is rather low, because of inevitable losses at each stage of the isolation procedure, attempts are being made to modify the explantation and cell isolation techniques in order to increase the chance of establishing both homozygous diploid as well as haploid pluripotent cell lines from this source. The HD lines reported here, which have been established from 1-pronuclear ‘uniform’ haploid embryos (Kaufman, 1981), clearly demonstrate that it is now possible to establish homozygous diploid pluripotent cell lineages of parthenogenetic origin which, at least initially, appear to be karyotypically normal, and capable of a full range of cellular differentiation.

We would like to thank Mrs Lesley Cooke for expert technical assistance. The work was supported by the Medical Research Council (M.H.K. and M.J.E.), the Cancer Research Campaign (M.J.E.) and the National Fund for Research into Crippling Diseases (M.H.K.).

REFERENCES


(Accepted 25 July 1982)
Ethanol-induced chromosomal abnormalities at conception

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Preliminary findings have indicated that mouse eggs exposed briefly in vitro or in vivo to a dilute solution of ethanol activate parthenogenetically1,2. Cyogenetic analysis of the first-cleavage chromosomes of haploid parthenogenetic embryos indicated that up to 20% of this population were aneuploid as a result of non-disjunction3. Anaesthetics also can induce parthenogenesis of rodent eggs3-5, and in studies using anaesthetics, colchicine6-8 and colcemid9,10, abnormal chromosome segregation and heteroploidy of rodent embryos have been observed. I now report that when recently mated female mice are given a dilute solution of ethanol by mouth, non-disjunction can be induced in the female-derived, but apparently not in the male-derived, chromosome set of fertilized eggs. Taken together, these findings suggest that ethanol consumption (as well as exposure to other 'spindle-acting' agents11-13) at the time of conception may be the cause of certain types of chromosomal defects commonly observed in human spontaneous abortions.

The initial studies were designed to investigate the chromosome constitution of haploid embryos induced to develop arthenogenetically following ethanol administration by various routes to unmated female mice. The same high levels of activation can be achieved either using a dilute solution of ethanol administered via the intraperitoneal (i.p.) route1 or following oral ethanol administration (M.H.K., unpublished). Maximum rates of activation (50-60%) can be achieved (M.H.K., unpublished) when mice are injected i.p. with 0.3-0.4 ml of a 25% solution of ethanol in phosphate-buffered saline at 17 h after human chorionic gonadotropin (HCG) injection for superovulation. Detailed examination of the first-cleavage chromosomes from embryos in this group revealed that 7.5% of the haploid mitoses were aneuploid. Out of a total of 133 preparations in which unequivocal counts could be made (63% of preparations examined), 123 metaphases had a normal chromosome complement, 5 contained 19 and 5 contained 21 chromosomes.

Activation could also be induced when lightly anaesthetized mice (ether inhalation) were given 1 ml of a 10% solution of ethanol in distilled water through a gastric tube at a similar time after induced ovulation. Out of a total of 42 preparations in which unequivocal counts could be made (100% of preparations examined), 40 had a normal chromosome complement, one group had 19 and another had 21 chromosomes present. Note that parthenogenetic mouse embryos, even with an apparently normal haploid or diploid chromosome constitution, generally fail to develop beyond the early post-implantation period14.

These preliminary results encouraged an investigation of the effect of ethanol exposure shortly after conception. Recently mated mice were given 1 ml of either a 10%, 12.5% or 15% solution of ethanol in distilled water by mouth at 13.5 h after the HCG injection for superovulation (about 1.5-2.5 h after the predicted time of ovulation). Serum levels of ethanol of about 150-200 mg/ml15 are achieved within minutes of its administration16, and maximum levels would be expected in the oviduct shortly afterwards, during the time that eggs are completing the second meiotic division—shortly after the 'activating' stimulus of fertilization. Table 1 shows that in 15% of the eggs, one of the pronuclear sets contained 20 and the other 19 or 21 chromosomes. In this series, unequivocal chromosome counts could be made in 65.3% of the preparations studied.

However, the absence of an appropriate 'marker' chromosome in either the male- or female-derived pronuclear groups precluded the origin of the aneuploid sets being established with any degree of certainty.

Thus, in a second series of experiments, (C57BL×CBA)F1 hybrid females were mated with males that were homozygous for the T6 translocation in which a reciprocal exchange occurs between segments of chromosomes 14 and 15, with the resultant production of a large and a small translocation product. The latter is easily recognisable as it is considerably smaller than the smallest intact autosome, and has been termed the T6 marker chromosome or T6M15-17. In such matings, the sperm-derived pronuclear set would be expected to contain 20 chromo-

Fig. 1 Air-dried preparations of male and female pronuclear chromosome groups analysed at the first cleavage mitosis. After overnight incubation of eggs in culture medium containing colcemid, two discrete sets of chromosomes can be observed. The preparations were stained with Giemsa. a, b, (C57BL×CBA)F1 hybrid females were mated to males that were homozygous for the T6 translocation. The male-derived pronuclear groups containing the T6 marker chromosome (arrowed) are on the right. c, d, Pronuclear chromosome groups from a study in which F1 hybrid females were mated to males of similar genotype. In both cases, the group on the left contains 20 chromosomes whereas the group on the right contains 21 chromosomes. Indirect evidence from parthenogenetic studies and fertilized matings in which the males carried appropriate marker chromosomes (see a and b, above) indicates that in these examples, the groups containing 21 chromosomes were of female pronuclear origin.
Table 1 The chromosome constitution of male and female pronuclei following intragstric ethanol administration

<table>
<thead>
<tr>
<th>Group</th>
<th>% Ethanol</th>
<th>Total no. eggs examined</th>
<th>% Scorable preparations</th>
<th>18:20</th>
<th>19:20</th>
<th>20:20</th>
<th>21:20</th>
<th>% Aneuploid</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>93</td>
<td>63.4</td>
<td>1</td>
<td>7</td>
<td>50</td>
<td>1</td>
<td>15.3</td>
</tr>
<tr>
<td>2</td>
<td>12.5</td>
<td>58</td>
<td>63.8</td>
<td>1</td>
<td>3</td>
<td>30</td>
<td>3</td>
<td>18.9</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>22</td>
<td>77.3</td>
<td>1</td>
<td>7</td>
<td>55</td>
<td>5</td>
<td>5.9</td>
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<td>4</td>
<td>12.5</td>
<td>79</td>
<td>84.8</td>
<td>1</td>
<td>7</td>
<td>55</td>
<td>5</td>
<td>17.9</td>
</tr>
<tr>
<td>5</td>
<td>Distilled water</td>
<td>12</td>
<td>66.7</td>
<td></td>
<td></td>
<td></td>
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<td>6</td>
<td>Haploids</td>
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* All females received 1 ml of a dilute solution of ethanol in distilled water, or distilled water only (group 5).

Actuation induced in vivo 6-7 h after intragstric administration of 12.5% ethanol.

In 8-8 chromosomes was observed 6-7 h later, hybrid females were injected with 5 IU pregnant mare’s serum gonadotropin followed 48 h later by 5 IU HCG to synchronize ovulation, and were mated to F1 hybrid (groups 1-3) or CBA-T6M (groups 4-6) males. Females were checked for vaginal plugs early the next morning, and at 13.5 h after the HCG injection for superovulation those that had mated were lightly anaesthetised with ether so that the test solutions could be introduced via a fine tube passed into the stomach. Most females recovered from the anaesthetic within a few minutes of the intragastric injection. The females were autopsied 6-7 h later, and the eggs examined for the presence of pronuclei. The fertilized eggs were transferred to medium containing colcemid, and air-dried preparations were made the next morning. The unfertilized eggs were examined 5-7 h later for evidence of parthenogenetic activation (the development of a single pronucleus following second polar body extrosion), and the activated eggs then transferred to a separate drop of medium containing colcemid. These eggs were also examined the next morning, and their chromosome constitution determined. Following incubation in colcemid, the chromosomes of the male and female pronuclei in the fertilized eggs revealed two distinct entities, and the chromosomal constitution of each can then be determined. Only in groups 4 and 5 did the presence of the T6 marker chromosome (T6M) enable the male-derived pronuclear set of chromosomes to be established unequivocally.

The normal chromosome complement in an aneuploid individual, and most of these fetuses would either be aborted spontaneously or, with very few exceptions, die shortly after birth20. Indeed, two recent epidemiological studies have shown that a significant relationship exists between the level of maternal alcohol intake during early pregnancy and the risk of spontaneous abortion31,32, and there seems little reason to believe that the pattern of alcohol consumption during early pregnancy is likely to be radically different from that occurring before and shortly after conception. The reason for the higher incidence of spontaneous abortions in the alcohol-consuming groups was not established, but the present findings suggest that a cytogenetic explanation might be applicable in some cases.

While caution should be exercised in extrapolating directly from the present findings, as there are likely to be certain species differences in ethanol susceptibility, the results draw attention to the potential danger to the conceptus of a single episode of heavy drinking by the mother at about the time of conception. They also indicate that any agent which acts on the spindle apparatus, and could therefore influence meiotic chromosome segregation, should be considered a possible causative agent in those cases of spontaneous abortion in man in which a non-mosaic type of chromosomal defect (for example, trisomy, monosomy and certain polyploids) is observed. These groups probably account for at least one-half of all spontaneous abortions in man30.

I thank Mrs Lesley Cooke for technical assistance. The work was supported by grants from the MRC and the National Fund for Research into Cripping Diseases.

Received 25 October 1982; accepted 7 February 1983.

Pluripotential cells grown directly from normal mouse embryos

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Keywords: Pluripotential cells, EK, Parthenogenesis, Mouse Embryo, X chromosome, Teratocarcinoma, Karyotype, Chimaeras

Summary

It is now possible to isolate pluripotential cells into tissue culture directly from normal mouse embryos. These pluripotential cells called EK cells are the normally occurring embryonic cell equivalent to embryonal carcinoma cells of teratocarcinomas. Isolation of these cells is rapid and immediate and neither medium conditioning factors nor 'implantational delay' of the blastocysts is necessary for their isolation. Cell lines have been established in culture from numbers of both inbred and outbred strains of mice and these cell lines carry a variety of genetic markers. They have a karyotype primarily identical to the embryo from which they were derived and are able to recolonize a blastocyst to form normal but chimaeric adult mice with a high efficiency. In addition, cell lines have been isolated from both diploid and from haploid parthenogenones. Although all these parthenogenetically-derived EK lines prove to be diploid it has been found that there is an instability of their X chromosome constitution. The possible reasons for this instability are discussed, and the utility of a series of otherwise euploid XX cell lines each of which bears a specific terminal deletion of one of the X chromosomes is presented. We have attempted to indicate the wide range of experimental situations in which
fertilized embryo-derived and parthenogenetically-derived EK cells may be of value.

I Introduction

There is an intimate relationship between cancer and differentiation. This is not only conceptual in that malignant growth can only be a feature of a multicellular organism where cell multiplication is not coordinated with the requirements of the development, growth or stasis of the integrated cell population of the whole organism, but it is also actual—malignant cells may often display properties normally seen in embryonic cells. This relationship between malignant cells and embryonic cells is nowhere more clearly exemplified than in teratocarcinomas where it is the malignant stem cell population of the tumour which also has the ability to differentiate into many types of cell. Studies with mouse teratocarcinoma cell lines have now demonstrated that not only is this stem cell—the embryonal carcinoma or EC cell—able to differentiate, but that it is very similar in many properties to an early embryo cell, and its differentiation proceeds by a route which is demonstrably that of the early embryo epiblast. All this evidence strengthens the hypothesis that the embryonal carcinoma cell is essentially a normal early embryo cell, some of the embryonic characteristics of which lead to its behaviour as a malignant cell when it is present in an abnormal environment. In particular, the observation that an early embryo experimentally implanted ectopically may give rise to a teratocarcinoma with a high efficiency suggests that the tumour stem cells may arise directly from an outgrowth of particular embryo cells which pre-exist in their ‘malignant’ form in the embryo. To an extent, however, such in-vivo experiments cannot approach the question of the tumorigenicity of normal embryo cells, as the tumour stem cells can be identified only after their passage through the tumour, and the argument thus becomes circular.

It has now become possible to grow directly from early embryos cells which have the properties of embryonal carcinoma cells. They are pluripotential, and also tumour forming. This review discusses the derivation of such cells and describes some of their properties. We also consider the new experimental opportunities which availability of these cells affords.

Teratocarcinomas are malignant and potentially lethal tumours. The tumour biology, i.e. the sites of origin, the histology and the progression of teratocarcinomas, is similar in man and mouse.

Mouse teratocarcinomas depend for their continued growth upon the presence of an undifferentiated stem cell line (embryonal carcinoma, EC cells). These EC cells give rise to all the other differentiated cells in the wide miscellany of differentiated tissues which are characteristic features of these tumours. Embryonal carcinoma cells may be isolated into tissue culture from transplantable mouse teratocarcinomas, and such cultures, which may be repeatedly sub-cloned, will readily form similar tumours upon re-injection into a suitable histocompatible host mouse.

EC cells are not, however, only able to produce tumours, but are also able
to differentiate. Although this ability to differentiate is severely restricted in some cell lines, and is generally inversely related to the overall aggressiveness and rate of growth of the tumour, it is one of the primary properties of these cells.

The major developmental characteristic of EC cells is their ability to differentiate into a diversity of cells; the only normal cells with this property are early embryo cells. EC cells are derived from either early embryo cells or from germ cells. In the latter case derivation from female germ cells is clearly via an embryo, although in the case of derivation from male germ cells there is no overt embryo route. Is it possible, therefore, that EC cells are indeed normal embryonic cells and if so with which cells are they homologous?

The evidence that EC cells are essentially normal cells is circumstantial and to some extent circular. The circumstantial evidence rests upon the pluripotency of EC cells, their embryo-like mode of differentiation, and the close similarity of the properties EC cells display to those of certain early embryo cells. The circular evidence rests upon the ready in-vivo conversion between normal embryos and teratocarcinomas from which EC cells may be isolated, and the ready incorporation of EC cells into mouse embryos where they will participate in normal development with the production of chimaeric mice.

EC cells differentiate into primarily normal non-tumorigenic cell types, and it has now become quite clear that the early stages of this differentiation follow the pattern of cell differentiation of the inner cell mass of the mouse blastocyst. Subsequent differentiation has not been closely observed, but may be presumed to follow the normal embryonic pathway.

Whenever spontaneous differentiation of mouse EC cells is observed in vitro the first differentiated type of cell formed is recognizable as a primary endoderm cell (Evans & Martin, 1975). This process of differentiation is triggered by the formation of a clump of cells with a free surface and it is on this surface that the first endodermal cells appear. The most straightforward method of inducing differentiation of an EC cell line in culture is to keep cell aggregates in suspension, and under these conditions the primary endodermal layer forms to surround a core of as yet undifferentiated EC cells. The product is a ‘simple’ embryoid body, and this structure of primary endoderm on the outer surface of a group of pluripotential cells is equivalent to the endoderm-invested inner cell mass (ICM) of a five-day mouse embryo. These ‘simple’ embryoid bodies will produce a mass of diversely differentiated cells when allowed to re-attach to a tissue culture surface. Lines of pluripotential EC cells which are not too abnormal will also differentiate further to give ‘cystic’ embryoid bodies. The ‘simple’ body cavitates, and a fluid-filled cyst is formed. The cyst wall is made up of an endodermal outer layer and a mesenchymal inner layer which often contains muscle and which may be seen to contract in slow waves. On one side of the cyst a residual knob of differentiated cells is often found. Clearly here, as in the case after monolayer explantation, a complex but disorganized development into normal tissues has occurred. In the early stages of ‘cystic’ embryoid body formation, however, a process of ectodermal and mesodermal formation very similar to that occur-
ring in normal embryos between six and seven days of development may be seen (Martin et al., 1977).

In fact, most EC cell lines will differentiate in vitro to at least a limited extent, and indeed although several ‘nullipotential’ lines have been described (e.g. F9; Nulli SCC2a), none appears to be totally incapable of differentiation under suitable conditions in vitro or with inducing stimuli (e.g. retinoic acid, Strickland & Mahdavi, 1978), and in most cases even with these (most probably unnatural) stimuli endodermal cells are produced.

Embryonal carcinoma cells differentiate as though they were very early embryo cells. In practice the first differentiation step observed in vitro for mouse EC cells is not to trophectoderm but to primary endoderm. This suggests that EC cells are differentiating in the same way as cells of the ICM of a mouse blastocyst.

As has been previously pointed out (Evans, 1981), it is to be expected that there is only one main genetic programme for differentiation, which is that seen during embryonic development, and it is not therefore surprising that EC cells follow a recognizably embryonic pathway when they start to differentiate. There are several possible explanations for this cell behaviour. EC cells may represent some quite aberrant cell type which happens to be able to start to differentiate in response to particular signals. In doing so, it becomes entrained into the normal pathway of embryonic cell differentiation, and its differentiated phenotype changes to that of cells normally present in the embryo. On the other hand, it is possible that EC cells themselves represent an essentially normal early embryonic cell type which has become transformed to a malignant cell phenotype. It is possible that the particular combination of properties that characterize this normally occurring embryonic cell may lead to its malignant phenotype when it grows outside the normal organization within the embryo, and/or when it occurs in greater than normal concentrations. We believe that our results, in which we are able to isolate cell lines equivalent to EC cells directly from normal early embryos, strongly support the latter possibility.

The question of the possible homology between EC cells and early embryonic cells has been approached by investigations of various cell properties and, in particular, cell surface antigens. EC cells have an unusual pattern of expression of cell surface antigens and numbers of antisera, xenogeneic, syngeneic and monoclonal, have been shown to recognize cell surface determinants which are characteristically shared by EC cells, early mouse embryo cells, and germ cells—both primordial germ cells isolated from 12-day foetal ridges and mature sperm. Many of these antigens occur on few if any other cell types, and thus define an area of homology even if, as in the case of the monoclonal reagents, they recognize only a single small specific determinant (reviewed by Jacob, 1979; Solter & Knowles, 1979; Wiley, 1979; also see Brulet et al., this issue).

The simplest supposition, consistent with their mode of differentiation and with their cell surface antigenicity, would be that EC cells are equivalent to the inner cell mass cells of 3.5-day blastocysts; but comparisons of the spec-
trum of protein synthesis by the two cell types analysed by two-dimensional gel electrophoresis show that these are very different (Dewey et al., 1978; Lovell-Badge & Evans, 1980). Ectoderm tissues from later post-implantation stages, however, are very similar to EC cells (Evans et al., 1979) in this respect.

The most dramatic proof that EC cells can behave as embryo cells comes from their ability to become incorporated into an embryo and to develop into a normal animal. This has been shown with EC cells taken from in-vivo embryoid bodies (Mintz & Illmensee, 1975) and from clonal tissue culture cells (Papaioannou et al., 1975). These cells, although tumour-producing when injected into adult mice, can give rise to a proportion of the cells in an otherwise completely normal adult mouse. In some cases the chimaeric mice have developed tumours. These tumours may either be teratocarcinomas or secondary tumours of differentiated tissues. The occurrence of such tumours may well be associated with the accretion of abnormalities in the EC cell lines (Papaioannou et al., 1978). In only a single case so far reported have tissue culture cells given rise to functional gametes (Stewart & Mintz, 1981). One reason for this is that many of the cell lines which have been used successfully until now in forming chimaeric mice have had chromosomal abnormalities.

Mouse embryos at any stage of development between the two-cell stage and the final determination of the three germ layers at about 7.5 days of development following gastrulation will form a teratoma when transplanted to a suitable site in a histocompatible host (see Solter et al., 1975; Stevens, 1981). The earlier embryos are seen to develop in the ectopic site and to become disorganized at a post-implantational stage. Egg cylinder stage embryos may be dissected before transplantation and it is only the embryonic portion, and of that the ectoderm, which is responsible for teratoma formation (Diwan & Stevens, 1976).

When an embryo is transplanted to an ectopic site in vivo, and results in a teratocarcinoma, it develops to an egg cylinder stage which then becomes disorganized, and a tumour subsequently forms (Stevens, 1981). The very ready formation of such tumours, especially from transplanted egg cylinders, suggests that there is no particular step of malignant transformation. On the other hand the various studies which demonstrated that the main factors influencing the formation of malignant tumours were host- rather than graft-related suggest that the stem cells may be transformed to malignancy, or at least selectively stimulated to grow by the host environment. Whatever the factors involved, in-vivo experiments of this kind are subject to the constraint that the cells are primarily present in a tumour, and therefore questions about their tumorigenicity are circular.

To recapitulate, normal embryos will give rise to teratocarcinomas containing EC cells. EC cells can participate in normal embryonic development. The differentiative behaviour of EC cells is similar to that of an embryonic inner cell mass and their cell phenotype would match the cells into which this inner cell mass develops—the embryonic epiblast. It seems very likely that an EC cell is a normal embryo epiblast cell. The alternative possibility is that a
malignant transformation occurs after ectopic embryo implantation during the initiation of the tumour. EC cells grow readily in tissue culture but pluripotential cells, until recently, proved impossible to grow directly from an embryo. We have now shown that this may readily be done.

II  Direct growth of pluripotential cells from embryos

Embryonal carcinoma cells may be readily isolated into tissue culture from teratocarcinomas formed by ectopic transplantation of an embryo (Evans, 1972). These cells have been found to be primarily highly feeder-dependent, although they may be adapted to grow in culture without added inactivated fibroblast cells. When grown without feeders, however, their ability to differentiate declines and their karyotype becomes abnormal (Hogan, 1976; Magrane, 1982). In our experience all primary cultures of EC cells display this feeder dependence but the recent isolate (METT-1) of Stewart and Mintz (1981) is specifically described as feeder independent and is clearly still totipotential.

Many EC cell lines have been derived from transplantable embryo-derived teratocarcinomas and they display a wide range of differentiative capacity, from the practically nullipotential F9 to the complete totipotency described for METT-1. Deviation from normality may occur both during in-vivo passage of the tumour and during in-vitro growth of the EC cell line.

Normal embryos will give rise to tumours and from these tumours transplantable tumour lines may be established from which clonal embryonal carcinoma tissue culture cell lines may be derived. This is a lengthy progression and although it would seem most likely that the EC cells arise directly from the transplanted embryo, malignant transformation and selection of tumorigenic cell lines might be occurring at any stage. For the objective both of obtaining unmodified embryonic stem cells and of testing whether these are primarily tumorigenetic it was necessary to be able to culture pluripotential cells directly from normal embryos.

We approached the isolation of these pluripotential cells from normal embryos with three points in mind (Evans, 1981). Firstly, that cells homologous to EC cells, if present, were to be found in the ICM-ectodermal cell lineage after the stage of the 3.5-day blastocyst and before the 5.5-day early egg cylinder. Secondly, conditions of culture which were most conducive to EC cell growth and maintenance in an undifferentiated state would be needed. Thirdly, that as the low number of cells present in an embryo might present a difficulty in terms of primary plating efficiency, that the cell number in the embryonic epiblast should be maximized.

Embryos at the peri-implantation stage are difficult to isolate from the mouse. We therefore arrived at this stage of development by allowing an intact explanted blastocyst to continue to develop in culture for two–four days prior to its disaggregation and the initiation of monolayer culture. To increase the number of cells in the inner cell mass blastocysts which had been maintained in vivo in a state of ‘implantational delay’ were used. While it is unclear whether the number of inner cell mass cells actually increases, between 120
and 250 cells have been counted in such embryos (Kaufman, unpublished observations).

By using these methods we have been able to isolate cell lines from mouse embryos into tissue culture (Evans & Kaufman, 1981). These cells grow out rapidly and may be first recognized a few days after the primary disaggregation. They grow rapidly and progressively, in characteristically piling nests of tightly packed cells with no sign of any early adaptation to culture conditions or other ‘cell transformation’ event. Cells of early passage cultures show a cloning efficiency in vitro of approximately 10%.

We have called these pluripotent cell lines, which are isolated directly into tissue culture from embryos without prior tumour formation, EK cell lines to indicate their affinity to, but also their distinction from, EC cells.

Subsequently, Martin (1981) published an alternative method for isolation of pluripotential cells from mouse blastocysts. She explanted isolated blastocyst inner cell masses and supplemented her culture medium with a specially prepared fraction from medium in which established EC cell lines had been maintained. This is probably a factor allowing the isolated inner cell mass cells to grow and develop through to the point at which the epiblast cells may be isolated, but the exact role of such a factor is intriguing. We do not, however, find it to be necessary, and there is no reason to assume that any exogenous source of such a factor is necessary for formation of teratocarcinomas from embryos in vivo. As an experiment for the isolation of normal embryo cells it is clearly undesirable to use poorly characterized products from an established embryonal carcinoma tumour cell line which might contain infective or transforming factors.

Exogenously added conditioning factors are unnecessary, but so too is the technique of in-vivo ‘delay’ which in itself might be considered to introduce an abnormal maternal influence on the embryo. We have now found that cell lines may be derived from embryos cultured entirely in vitro from the one-cell stage. When the embryo has developed to a blastocyst it is explanted in the same way as an embryo which had developed in vivo and pluripotential cell lines may be established directly from it (Robertson et al., 1983). Thus, whilst the enlarged ICM-derived component present in ‘delayed’ blastocysts, and the use of ‘conditioned’ medium may facilitate the isolation of pluripotent cells from early embryos, neither are necessary. It is clear that cells may readily be obtained from a normal embryo which are both pluripotential and tumorigenic. The importance of these results is that within a few days from embryo explantation in vitro we can see the appearance of typical teratocarcinoma stem cells and everything points to their being a completely normal early embryo cell type.

III Properties of EK cells

In vitro embryo-derived (EK) lines prove to be very similar to their tumour-derived EC cell counterparts, in terms of their cellular morphology, cell surface antigen expression, and differentiative behaviour both in vitro and in vivo (Evans & Kaufman, 1981; Martin, 1981; Kaufman et al., 1982). Unpub-
lished observations have shown that (like EC cells) they display cell surface antigens recognized by anti-SIKR serum (see Stern et al., 1975), anti-IMA (see Kapadia et al., 1981), the monoclonal anti-forssman reagent MI.22.25 (see Stern et al., 1978) and the monoclonal antiserum 5D4 (P.L. Stern, personal communication). These observations would suggest that the characteristic cell surface of EC cells is also found on EK cells. As, however, differences in cell surface antigenicity between various EC cell lines have been reported (Gachelin et al., 1977) a more extensive investigation and comparison would clearly be worth while. The pattern of polypeptide synthesis of EK cells as seen by two-dimensional gel electrophoresis of the proteins from $^{35}$S methionine-labelled cells is the same as that of undifferentiated EC cells, and those polypeptide spots previously described (Lovell-Badge & Evans, 1980) as characteristic of EC cells are present (Lovell-Badge, personal communication).

EK cells differentiate extremely readily in vitro, indeed considerable care is needed to maintain pure undifferentiated cultures. The mode of differentiation via embryoid body formation is apparently identical to that of EC cells. Intriguingly, giant trophoblast cells sometimes appear in early cultures which were apparently pure EK for several previous passages. These either arise from a separate and morphologically similar proliferating stem cell line or differentiate from these early EK cells. Only a rigorous clonal analysis will resolve the origin of trophoblast cells in these early cultures.

EK cells form tumours in syngeneic mice and histological examination shows that these tumours are teratocarcinomas. They are extremely well differentiated tumours containing a wide variety of tissues, but nevertheless grow progressively to a large size in the host animal.

Over 70% (15 out of 21) of the fertilized embryo-derived EK cell lines established in our laboratory, and which have so far been fully analysed, possess a karyotype which is, at least initially, identical to that of the blastocyst from which they were derived. This may prove to be the single most important feature of these lines, as the possession of a normal euploid karyotype should considerably facilitate their incorporation into chimaeric animals. Of even greater importance, however, is the likelihood that the possession of a normal karyotype may well be essential for the incorporation of EK cells into the germ line of chimaeric mice (Mintz & Cronmiller, 1981).

The situation in EC cells is in marked contrast to that in EK cells, as, with few exceptions, most of the currently available EC cell lines possess chromosomal abnormalities of various kinds. In addition, EC cells have a range of growth characteristics in culture, and vary widely in their ability to differentiate both in vitro and in vivo. Some lines differentiate well both in vitro and in syngeneic host animals, while others differentiate only in the tumour form in vivo, while yet others (termed 'nullipotent' lines) differentiate only poorly under any circumstances.

EK lines are currently being used in our laboratory, and supplied to other laboratories, for the construction of chimaeric mice, and breeding studies are now in progress to test for possible contributions to the germ line in these individuals.
In a series of experiments (carried out in collaboration with R. L. Gardner), out of a total of 76 blastocysts which had been injected with EK B2.B2 cells and transferred to recipients 58 live-born animals were obtained (76%), of which 26 (45%) were overtly chimaeric. Gpi analysis has revealed contributions to the internal organs in chimaeric individuals. None of these animals had tumours of donor genotype throughout their life.

In a second series of blastocyst injection experiments (in which EK B2.B2 cells were also used) 66 blastocysts were transferred, and a total of 40 (61%) live-born young subsequently recovered. Of these, 14 (35%) were overtly chimeric, as assessed by eye and coat colour pigmentation. Six of these chimeric mice have been retained to adulthood (5 females and 1 male), and these are currently being test-bred (injections by A. Bradley).

Gpi analysis of the new-born mice which failed to survive the early neonatal period (this failure was not due, as far as can be discerned, to any abnormalities in these animals) has revealed that in some instances there was an extensive contribution by the EK cells to all of the organs and tissues tested, including the gonads.

It is of particular interest that the head region of these chimaeric animals was extensively pigmented. This feature was especially noticeable in mice that showed low levels of overt chimaerism (see Fig. 1).

Independent experiments carried out by M. G. Stinnakre (personal communication) using EK B2.B2 cells injected into C57BL blastocysts have also shown that it is possible to obtain high rates of chimaerism, and A. H. Handside (personal communication) has demonstrated that chimaera formation by the morula-aggregation method of Stewart (1980) also works effectively with EK cells. These results of a relatively high incidence (>35%) of overt chimerism in blastocyst injection studies are, we believe, extremely promising.

This is in marked contrast to the variable success which has generally been obtained following the injection of EC cells into normal mouse blastocysts (see Papaioannou & Rossant, this issue). One important disadvantage of EC cell lines is that the majority of those used are aneuploid and in particular have been XO. While this does not appear to prevent their participation in normal development, it has proved to be deleterious for the formation of functional gametes. The recent report by Stewart and Mintz (1981) indicates that the use of a perfectly euploid EC cell line not only increases the relative incidence of chimaerism within the somatic cell population, but is likely also to allow functional colonization of the germ line. In their study, a normal euploid 129-derived EC line (METT-1) was described which gave rise to overtly chimaeric animals at a rate of about 13%. More importantly, 1 out of the 9 chimaeric females which were test-bred gave rise to 3 out of 48 offspring of a 129 phenotype. Test-breeding, of animals chimeric from EK B2.B2 injection, has not yet demonstrated the production of functional germ cells from the EK B2.B2 cells, but this study is still in progress. Although EK B2.B2 has proved very successful at embryo colonization it is itself a relatively old cell line, having been cloned twice and maintained in vitro for over 50 cell generations. It has an apparently normal karyotype, but it is quite
Fig. 1, a and b. Chimaeras made by the injection of the 129 SvE-derived B2B2 cell line into albino host blastocysts. The level of chimaerism is high, appearing mainly in the head region, and is particularly striking in the iris of the eyes.
possible that small undetected changes have taken place which might explain its apparent failure to give rise to functional germ cells. One single-cell clone made in parallel to EK B2.B2—EK B2.C5—does have a visible deletion which is of part of one of the two homologues of chromosome 5.

It is clear that further studies are needed with other EK cell lines both of XX and XY constitution.

IV The isolation of EK cells bearing chromosomal, biochemical and developmental markers

Pluripotential lines derived from embryos appear to have a considerable number of advantages over tumour-derived EC cell lines. In addition to their relative ease and rapidity of isolation, there appears to be no restriction on the mouse strains from which such lines may be isolated. These factors thus facilitate the establishment of new genetically-marked pluripotential stem cell lines from both inbred and randomly bred mice. Cell lines from the latter source had not previously been available, as teratocarcinomas could only be induced experimentally by the ectopic transfer of embryos into histocompatible host animals, and tumours containing EC cells fail to grow in immunosuppressed or nude mice (Solter & Damjanov, 1979). We have made use of this ability to establish a number of EK lines with particular genotypes. These include a line homozygous for the prominent 9:19 Robertsonian translocation Rb 163, lines with X chromosomes bearing Cattanach’s translocation, lines homozygous for the unusual glucose phosphate isomerase isozyme variant gpi 1, and a line homozygous (among other markers) for the gene causing polydactylly in mice. The expression of this latter genotype would seem to be unlikely to be detected during differentiation in vitro! Our establishment of cell lines from both haploid and diploid parthenogenetic embryos is discussed below (section V).

The establishment of stem cell lines directly from embryos has the singular advantage that these cells can be used to investigate homozygous lethal developmental mutations which can only normally be maintained in mouse stocks in the heterozygous state. The potential usefulness of this approach has already been demonstrated by the recent report of the isolation of an EK cell line which is homozygous for the t mutation of the t complex (Magnuson et al., 1982; and see Sherman and Wudl (1977) for a review of the t complex). Although these cells are apparently not karyotypically normal, their results show quite conclusively that the cells are homozygous for the t region of chromosome 17 which bears t*, and yet the cells grow well and also differentiate well both in vitro and in vivo. This belies the ideas that t*/t* embryos die either because of cell lethality or from a general block to cell differentiation. Clearly this approach will allow both developmental and molecular biological investigations of the action of various genes of the t-complex to be carried out.

By a similar approach it should be possible to isolate EK cells homozygous for any required developmentally active gene, with the possible exception only of those very few genes known to act before the formation of the
embryonic epiblast. Such cell lines should prove invaluable as tools in the investigation of specific gene action during development.

The alternative approach to obtaining EK cells that carry interesting developmental mutants would be to select such mutant cell lines in vitro. Although this has been done with EC cells (e.g. Hooper, 1982) such studies have not been undertaken as yet with EK cells. Preliminary studies have, however, shown the feasibility of transforming EK cells with exogenous DNA (Lovell-Badge, personal communication).

V Haploid and diploid parthenogenetic embryos, and cell lines derived from them

Having determined the optimal culture conditions and embryonic stage necessary for establishing pluripotential cell lines from fertilized embryos (see Section II), we then attempted to establish similar cell lines from various classes of haploid and diploid parthenogenetic embryos. It was expected that this material might be of interest, firstly, because it provides a possible source for the establishment of stable haploid lines, and, secondly, because it had previously been established from biochemical and cytogenetic analysis of human and mouse ovarian teratocarcinomas (human: Linder & Power, 1970; Linder et al., 1975a; 1975b; mouse: Eppig et al., 1977) that these tumours were of parthenogenetic origin.

As most of our attention has been focussed on the establishment of cell lines from haploid embryos, this aspect will be considered first. However, before discussing this in detail, it is of interest to recall that previous attempts to establish haploid teratocarcinomas and to derive cell lines from these sources were only partially successful in that while tumours were derived from ectopic transfer of haploid parthenogenones (Iles et al., 1975; Graham et al., 1975), no permanent pluripotential lines have been reported. Lines have, however, been established from spontaneous teratocarcinomas occurring in the ovaries and testes of LT/Sv strain mice, but these are undoubtedly diploid (Martin et al., 1978) and some lines appear to be restricted in their differentiation (Gachelin, cited in Nicolas et al., 1976).

Of the two readily available classes of haploid parthenogenones, namely the single-pronuclear ('uniform') haploids, and the immediate cleavage ('mosaic') haploid group, only the former were studied in detail. Only those embryos, therefore, that developed a single haploid pronucleus following extrusion of the second polar body were selected shortly after activation, and allowed to develop to the expanded blastocyst stage. In the single-pronuclear group, only one of the products of the second meiotic division is retained within the egg, the second product being expelled within the second polar body. All of the cells which develop subsequent to the one-cell stage will therefore be genetically identical. This contrasts with the situation in the immediate cleavage group in which the egg divides into two equal-sized blastomeres at the completion of the second meiotic division, each of which retains one of its chromosomal products. Embryos derived from the latter class will therefore possess two clones of cells that are genetically dissimilar—the extent of the
dissimilarity being related to the chiasma frequency. The diploid ‘two-pronuclear’ class employed develops two haploid pronuclei in the absence of a second polar body. In the majority of these embryos, the two-pronuclear contents amalgamate at the first cleavage mitosis, giving rise to an heterozygous diploid embryo (for discussion of the various possible pathways of parthenogenetic development, see Kaufman, 1981).

All of the parthenogenetically-derived cell lines have so far been established from embryos that have been activated in vitro following a brief exposure to a dilute solution of ethanol in phosphate buffered saline (Kaufman, 1982), though it seems likely that the method of activation employed is unimportant, as long as the selection of the various classes of parthenogenone to be studied is carried out within a few hours of activation (see Kaufman, 1978a).

Depending on the strain of embryo employed, parthenogenetically activated eggs may either be retained in vitro to the blastocyst stage or transferred at the two-pronuclear stage to the oviducts of recipients on the afternoon of the first day of pseudopregnancy, and subsequently recovered at the expanded blastocyst stage. Thus the (C57BL x CBA)F₁ activated eggs which do not block at the two-cell stage in culture are therefore usually retained in vitro, whereas activated eggs from most other strains of mice, including those from the 129 SvE strain (which have been used to obtain parthenogenetically-derived lines) have to be transferred to recipients to allow them to develop to the blastocyst stage. The majority of embryos from both sources tend to achieve the expanded blastocyst stage by about 4.5 days following activation. The expanded blastocysts from both strains of mice were then treated in one of two ways:

i In-vivo ‘delay’
Expanded blastocysts were transferred to the uteri of suitable recipients on the 3rd day of pseudopregnancy. The recipients were then ovariectomized, and given a subcutaneous injection of 1 mg of Depo-Provera. Females were autopsied 3–6 days later, and zona-free ‘delayed’ blastocysts recovered. Individual blastocysts were then explanted into 1 cm wells containing a layer of feeder cells, and the procedure used to establish cell lines from them was the same as discussed earlier (see Section II).

ii Direct in vitro culture
In an alternative procedure, activated F₁ hybrid oocytes were retained in culture directly from the one-cell stage to the blastocyst, then explanted directly into supplemented DMEM medium without the necessity of passing through any form of development in vivo. To ensure ‘hatching’ from the zona pellucida, individual blastocysts were briefly exposed to acid tyrode medium, and subsequently transferred to feeder wells as described above.

The best results were obtained when colonies of a stem cell phenotype were identified at an early stage and selectively explanted into fresh feeder wells. In this way, cultures which were free from other embryo-derived cell types (e.g. endoderm) could be obtained. Details of the methodology involved, and
some of the morphological properties of the haploid-derived lines HD1 to 4 are presented elsewhere (Kaufman et al., 1983).

To date, 10 cell lines of parthenogenetic origin exist. They include 8 lines derived from 'single-pronuclear' haploids and 2 from 'two-pronuclear' diploid blastocysts; these have been derived from 129 SvE and from (C57BL × CBA)F1 strains of mice. However, detailed cytogenetic analyses of all of the parthenogenetically derived lines have revealed that all are diploid—even those established from haploid blastocysts (termed HD lines).

We have yet to establish at which stage of the isolation procedure diploidization occurs. Nevertheless, this method has enabled homozygous diploid lines of parthenogenetic origin to be established.

In initial attempts to establish at which stage diploidization is occurring, it was observed that over 80% of the single-pronuclear embryos examined at the morula stage contained only haploid mitoses. Of the remainder, most were haploid-diploid mosaics with at most one or occasionally two diploid mitoses present.

Several attempts to determine the chromosome constitution of the delayed blastocysts within 3–4 days after their isolation and explantation into culture were unsuccessful, as no mitoses were observed in the inner cell mass-derived cells. In the earliest stages at which the cells were successfully karyotyped (after the establishment of mass cultures), all the cells were found to be diploid.

To date, no haploid mitoses have been observed in the established lines, and we can only speculate at which stage diploidization is occurring. We believe, from the morula studies indicated above, and from previous analyses of intact egg cylinders derived from haploid embryos (Kaufman, 1978b) that at least a proportion of the cells at explantation and shortly thereafter are still haploid.

Chromosome analysis has been performed on metaphases collected from early passage cultures of the majority of the parthenogenetically derived EK cell lines. The modal number of chromosomes in the HD lines was 40, with between 80% and 90% of cells in individual cultures possessing this count. Whereas all the lines were found to have a completely normal autosomal complement, the situation with regard to the X chromosomes was unexpected. Occasionally the cells had become XO by loss of a single X chromosome, but, much more frequently, a partial deletion affecting the distal region of one of the X chromosomes was apparent (designated \(X^{\text{del}}\)). The characteristic morphology of the X chromosomes in the parthenogenetically derived EK cell lines is discussed in detail in Section VI, as is the possible significance of these observations. Similarly, apart from the apparent normality of their autosomal complement, the parthenogenetically derived EK cell lines established from diploid embryos (termed DP lines) also had an unstable XX constitution. The DP1 line possessed a bimodal chromosome count, with approximately equal numbers of cells containing either 39 or 40 chromosomes, owing to the presence of both XO and XX genotype cells within the culture population. The DP2 line possessed cells containing either 40 or 41 (73% of spreads) chromosomes, and G-banding revealed that all cells were
trisomic for chromosome 11. Cells of XO, XX and XX\textsuperscript{del} genotype were recorded, though the majority of cells possessed an XX\textsuperscript{del} genotype in which approximately 70\% of the material of one of the X chromosomes was lost.

Parthenogenetically derived EK stem cell cultures closely resemble their fertilized embryo-derived counterparts (Kaufman et al., 1983), as do the lines derived from in vivo ‘delayed’ and normal non-‘delayed’ blastocysts. These lines differentiate in vitro to form embryoid body-like structures in suspension culture, with cells of an endodermal phenotype being the first to differentiate. The 129 S\textsuperscript{v}E-derived lines HD1 and HD2 have been tested for tumorigenicity, and to assess the extent of tissue differentiation occurring when cells have been injected into syngeneic host animals. As expected, all formed typical teratocarcinomas, with a wide variety of tissue types present.

Attempts are being made to modify the explantation and cell isolation techniques in order to increase the chances of establishing both homozygous diploid as well as haploid pluripotential cell lines from the single-pronuclear haploid embryos. The difficulty so far encountered in maintaining haploid cells in culture despite their demonstration, admittedly in relatively small numbers, in egg cylinder stage embryos grown in vivo may be related to the fact that diploidy per se confers a selective growth advantage over haploidy under the conditions at present being used for the isolation of these cells.

VI X chromosome morphology and activity in normal embryos, EC cells and both fertilized and parthenogenetic embryo-derived EK cell lines

Before considering the important topic of X chromosome activity in teratocarcinoma cells, it is of value briefly to review present knowledge regarding the morphological appearance of the two X chromosomes in EC and EK cell lines. It is only with this background information to hand that it is then possible to evaluate the present, somewhat confusing, results to be found in the recent literature regarding X chromosome activity particularly in undifferentiated and differentiated EC cell lines.

Possibly of greatest interest, however, is the recent observation that in both haploid-derived and diploid-derived cell lines of parthenogenetic origin, the XX state is apparently unstable. The latter observation seems to be in marked contrast to the apparent stability of the Y chromosome, which has until now been thought of as being relatively readily lost. The implications of these somewhat diverse findings are discussed in detail in this section, while the possible use of the cell lines with an XX\textsuperscript{del} chromosome constitution is considered in the next section. Only a single example has been reported in which the morphological appearance of the two X chromosomes in an XX fertilized embryo-derived EK cell line has been other than normal. Martin (1981) has reported the presence of a partial deletion of an X chromosome in her EK line termed ESC ICR, and it seems that this line had various other chromosomal anomalies. The presence of a partial deletion involving one of the X chromosomes appears therefore to be extremely uncommon in fertil-
ized embryo-derived EK and EC cell lines. XO cells have been seen in one of three XX fertilized embryo-derived lines so far—the A13 line. Detailed cytogenetic analyses have been carried out on the 13 XY EK lines available, and we have not yet observed the loss of any of the Y chromosomes. Indeed, it is interesting to note that a single subclonal line, B1–2, has an XYY constitution. Similarly, partial X deletions have not yet been observed in any of the 21 EK fertilized embryo-derived lines examined so far, nor have there been any reports of this phenomenon occurring in those established EC cell lines which have been karyotyped.

Many EC cell lines are, however, XO in constitution, and it has often been assumed that these must have arisen from XY lines in which the Y chromosome has been lost during the course of either their in-vitro culture or during in-vivo growth. However, our evidence, from subcloning and long-term culture experiments involving EK lines, suggests strongly that the XY constitution is in fact extremely stable in these lines. Conceivably, the presence of two active X-chromosomes may be deleterious compared with the situation in XY EK cell lines, where little redundant metabolic burden may arise from the presence of the small Y chromosome. The reason why XO lines are found to arise more frequently from XX than from XY lines could be that removal of one redundant X chromosome therefore confers a selective growth advantage on the cell relative to its XX progenitors. It is also relevant to note here that some of the long-established EC cell lines which in fact retain two X chromosomes also have an increased number of autosomes present (McBurney & Adamson, 1976; Martin et al., 1978), and some tend not to be pluripotential (E. J. Robertson, unpublished results). It is possible that the parental origin of the X chromosomes may in some way influence the loss of one or other of them in the undifferentiated state. Clearly, this cannot be the case in the parthenogenones, where both X chromosomes are of maternal origin.

While all but one of the parthenogenetically derived lines possess an apparently normal autosomal complement, contrary to expectation, the XX constitution of these lines appears to be unstable. The most interesting observation in this regard is the almost universal occurrence of a partial deletion affecting the distal region of one of the two X chromosomes. Curiously, the partial deletion occurs in both the HD and DP lines. The extent of the X deletion varies between lines, and in the most extreme cases an entire X chromosome may be lost with the consequent production of XO genotype cells. This has also been observed in both HD and DP lines.

Although a variety of breakpoints are observed in the different parthenogenetically derived lines studied here, it is unclear at the present time whether a progressive loss of the distal segment occurs, with the eventual production of an XO line. The uniform state of the deleted segment in each of the parthenogenetically derived lines so far examined, however, would suggest that this is not the case, but even in the most well-established lines, cells from only relatively early passages have been examined. We have therefore tentatively interpreted our findings in terms of a single random deletion event, which may involve either a segment or a complete chromosome. We believe this event probably occurs early in the isolation of these lines, and
may give a selective growth advantage to those cells carrying the deleted X chromosome. The X chromosome deletion event has not been observed in the many established EC lines in which cells may have passed through hundreds of generations both as in-vivo tumours and in subsequent growth in vitro. Such prolonged passage would certainly tend to favour the selective overgrowth and survival of the cells with the most stable genotype. Indeed, time alone will tell whether the XX$^{del}$ genotype observed in these parthenogenetically derived lines represents an early stage in the production of the (possibly) more stable XO genotype.

If a variety of distal X$^{del}$ conditions are sufficient to confer the postulated growth advantage on the cell as indicated above, it suggests that either the most serious imbalance is due to loci which map at the distal region of the X chromosome, or that deletion of this region removes a controlling locus which affects the activity of the rest of the chromosome.

The X deletion phenomenon observed in parthenogenetically derived lines is undoubtedly associated with X chromosome activity. Previous observations on X chromosome activity in EC cell lines have tended to indicate that both X chromosomes are active in these cells when they are maintained in the undifferentiated state, whereas X inactivation occurs during, or shortly after, their cellular differentiation (Martin et al., 1978; McBurney & Strutt, 1980). Presumably both X chromosomes are also active in XX fertilized embryo-derived EC cell lines, and indeed in such lines no differential Kanda staining (see Rastan et al., 1980) was observed (unpublished observations, A. Stoker).

Although no biochemical information is yet available on X chromosome activity in the various parthenogenetically derived EC lines considered here, there seems no a priori reason why the X chromosomes should behave differently from the situation observed in the tumour-derived EC cells. However, the possibility has to be considered that in EC cell lines the derivation of the X chromosomes, which are of both maternal and paternal origin, may influence the pattern of X inactivation during their cellular differentiation.

In normal development, however, cells in the extra-embryonic tissues, which are derived from the trophectoderm or primary endoderm, undergo selective preferential paternal X inactivation (West et al., 1977; Harper et al., 1982). Clearly preferential paternal X inactivation cannot occur in parthenogenones where both X chromosomes are of maternal origin.

The inactivation of a single X chromosome is known to occur in heterozygous diploid parthenogenetic embryos (Kaufman et al., 1978), and in the extra-embryonic membranes (Rastan et al., 1980). However, while undifferentiated cells are presumably only present for a short time in diploid parthenogenones, the presence of two identical and genetically active X chromosomes may not be tolerated in parthenogenetically derived EC cells; a partial deletion of one of the X chromosomes may occur as a form of compensatory mechanism conferring a developmental advantage on these cells.

The female mammal has twice the relative dose of X linked to autosomal genes of the male, and X inactivation provides a mechanism of dosage compensation (Gartler & Cole, 1981). That there is a dosage imbalance in XX EC cells is born out by the observations of a double specific activity of X linked
enzymes in these cells (Martin et al., 1978; see also Epstein et al., 1978; Monk, 1978). This is to be expected as the EC cell phenotype is probably identical to that of an early embryo epiblast cell. All X linked products are, therefore, overproduced and these cells are in a state of imbalance. Despite this, XX cells not only grow through this stage of development, but may be isolated and maintained as progressively growing cultures of EK cells.

Further studies will obviously be required to clarify many of the points raised in this discussion. Does, for example, the XX state in parthenogenetically derived EK cells progress to the XX\textsuperscript{det} state, which then eventually progresses to the ‘stable’ XO state? Do fertilized embryo-derived EK XX cells eventually progress along a similar pathway? A study of homozygous diploid pluripotential lines established from enucleated fertilized diploidized embryos (Markert & Petters, 1977) might help to clarify some of these issues.

**VII EK cell lines in perspective**

We have attempted throughout the present text to stress the similarities and differences which are apparent between the embryo-derived EK cells and the tumour-derived EC cells. It will by now be apparent that in most respects they are indistinguishable—particularly in terms of their cellular morphology, cell surface antigen expression, and differentiative behaviour both in vivo, and in vitro (Evans & Kaufman, 1981; Martin, 1981; Kaufman et al., 1983). EK cells also share with EC cells the essential feature of pluripotency. Both share an apparent homology with the epiblast of the early post-implantation embryo, and this, of course, has been exploited in the various techniques which have recently been described (see Section II) to establish pluripotential cells directly in vitro from normal and ‘delayed’ blastocysts. However, the principal advantage displayed by EK cells is that, in addition to their relative ease of isolation, they provide a source of pluripotential cells which, at least initially, have a karyotype that is identical to that of the blastocyst from which they are derived. Indeed, analysis of existing EK fertilized embryo-derived cell lines has revealed that over 70% are karyotypically stable, and retain a normal chromosome constitution (Robertson et al., 1983).

As stressed in Section VI, the above observations are equally applicable to EK cell lines of parthenogenetic origin which share the extremely valuable feature that, with the exception of line DP2 which is trisomic for chromosome 11, all have a normal autosomal chromosome complement. This particular feature will probably prove to be their most valuable property, as there seems little doubt that the possession of a normal or near-normal karyotype greatly facilitates their chance of being incorporated in a chimeric association with normal embryonic cells, and, possibly a more critical test of their normality, of colonizing the gonads and producing functional germ cells. The normality of their karyotype is, of course, in marked contrast to the situation present in most established EC cell populations where, though they maintain a near normal karyotype, few are euploid.

Several female EC lines have, nevertheless, been described (Papaioannou et al., 1979; McBurney & Strutt, 1980; Mintz & Cronmiller, 1981), and this
Pluripotent cells grown directly from normal mouse embryos

has tended to confirm the belief that the XX state may be more stable than the XY chromosome constitution. We believe that the clear demonstration of the instability of the sex chromosomes in parthenogenetically derived lines, and their apparent stability in XX and XY fertilized embryo-derived EK lines (see Section VI) may contribute to our understanding of the possible origin of at least a proportion of the XO EC cell lines. Certainly, preliminary evidence from karyotype analysis of 13 fertilized embryo-derived XY EK cell lines has provided no evidence to substantiate the hypothesis that the Y chromosome is more likely to be lost than the X chromosome. Indeed, the opposite appears to be the case.

The situation with regard to the morphology and activation status of the X chromosomes has also been discussed in considerable detail in Section VI. While the presence of a partially deleted X chromosome or its absence altogether appears to have little detrimental effect on in-vitro and even in-vivo differentiation, there seems little doubt that this may well prove disadvantageous in a chimeric association with a fertilized embryo, particularly in those situations where a high incidence of germ cell chimerism is required. However, apart from the latter rather special case, the presence of certain morphological and biochemical 'mutations' can be distinctly advantageous. This, coupled with the relative ease of production and rapidity of isolation of EK cells, has already been put to good use, as there appears to be no restriction on the mouse strains from which such lines may be established.

The ability readily to establish stem cell lines directly from embryos enormously increases the scope for establishing, for example, cell lines from embryos that are homozygous for early postimplantation lethal developmental mutations which, at the present time, can only be maintained in mouse stocks in the heterozygous state. Indeed, the establishment of a cell line from such an embryo has recently been achieved (Magnuson et al., 1982).

There appears to be diminishing evidence that the majority of established EC cell lines provide a useful model for many aspects of normal mouse embryogenesis, though, undoubtedly, they are still extremely valuable tools for analysing specific aspects of cellular differentiation, X chromosome inactivation, cell–cell communication, etc., under controlled experimental conditions in vitro. Equally, EC cells bearing specific chromosomal mutations provide a useful means for analysing the effect of aberrant genes on cellular morphology and biochemistry, as well as enabling their influence on cell surface antigen expression, etc., to be monitored (see Graham, 1977). However, with the important proviso that the inherent diversity of the presently available EC lines may lead to problems if unwarranted generalizations are made about the properties and behaviour of embryonic cells (Evans, 1981), there seems little doubt that EC cells will continue to play an important, though possibly more circumscribed, role than at present, in facilitating our understanding of both normal embryogenesis and tumorigenesis. It seems clear, therefore, that the quasi-important slot previously occupied by EC cells in the former regard can no longer be vigorously justified, and a sound case can be made to slot EK cells into this particular niche.

The parthenogenetically derived lines may also prove to have other uses
than originally envisaged, though vigorous attempts are still being made to establish stable haploid pluripotential cell lines from this source. Equally, the development potential of blastocysts derived from experimentally induced ‘androgenones’ and ‘gynogenones’ produced by the enucleation of recently fertilized pronucleate stage eggs (Modlinski, 1975; Borsuk, 1982) should also be investigated in this regard. For, whatever the cause and mechanism, a series of cell lines which are characterized by the (apparently randomly occurring) loss of a variable but defined segment of a single X chromosome are now available, and there seems no reason to believe that many more lines of an XX\textsuperscript{del} genotype cannot be isolated in the future. Provided that the partially deleted X chromosome is active in the undifferentiated state, and currently available evidence from EC cells strongly suggests that this might indeed be the case, the relative activities of X linked enzymes in XX, XX\textsuperscript{del} and XO cells, which are identical in all other respects, should facilitate detailed mapping of X linked genes. Similarly, DNA from such cells might be used to locate recombinant DNA clones from mouse X chromosome libraries.

In order that the pattern of X inactivation during the various stages in the differentiation of EK lines may be more closely investigated, we are currently preparing EK lines from embryos which are either heterozygous for X linked allozymal variants, or have paternally and maternally derived X chromosomes which are easily identified by the presence of obvious translocation markers.

Clearly, EK cells have their limitations, and these should not be dismissed lightly. However, when the problems associated with the establishment, maintenance, and utilization of these cells are compared with their EC cell counterparts, their limitations fall into perspective—and should not be over-emphasized. There seems little doubt that EK cells will rapidly establish themselves as an essential tool for the experimental embryologist, being eventually located somewhat higher up on the ladder than EC cells (for most purposes), and only marginally lower than the normal embryo, with its limitations of inaccessibility and very specific (and as yet relatively undefined) intr- and extra-uterine developmental requirements.

Much progress has been made since our initial report of the successful establishment of cell lines directly from mouse embryos (Evans & Kaufman, 1981) in the production of EK cell lines from a wide variety of sources. We optimistically believe that in the next few years these cells will facilitate advances in (and inevitably transform) many areas of developmental biology.

**Acknowledgements**

We are very grateful to the Medical Research Council for their provision of a project grant which has supported much of our joint experimental work discussed here. M.J.E. also gratefully acknowledges the continuing support for this work of the Cancer Research Campaign. We would like to express our thanks to Dr. E. J. Robertson for contributing to this work and her helpful discussion during the preparation of this manuscript.
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Pluripotential cells grown directly from normal mouse embryos


(The authors are responsible for the accuracy of the references.)
X-chromosome instability in pluripotential stem cell lines derived from parthenogenetic embryos

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SUMMARY

The karyotype of six pluripotential stem cell lines derived from haploid and two additional lines derived from diploid parthenogenetic embryos is described. All these lines are diploid and possess a normal autosomal complement. The stage at which diploidization of the haploid cells occurs is not yet known. The XX-chromosome complement in these lines is unstable, although in two haploid-derived lines and one diploid-derived line many normal XX-bearing cells are found in early cultures. All of the lines so far examined either become XO (rarely), or a single X chromosome shows a deletion in the distal region. The extent of this deletion varies between lines, but the position of the breakpoint appears to be constant for a given line. We suggest that these cytogenetic findings raise the possibility that a single deletion event occurring at an early stage during the isolation of these lines may confer a selective advantage to those cells carrying the deleted X chromosome.

INTRODUCTION

Although many mouse embryonal carcinoma (EC) cell lines derived from teratocarcinomas maintain a near normal karyotype, few are euploid. Indeed, many have been found to be XO, and it has often been assumed that these were originally derived from XY lines in which the Y chromosome has been lost during their passage either in vivo or in vitro. Several apparently euploid female pluripotential lines have also been reported (Papaioannou, Evans, Gardner & Graham, 1979; McBurney & Strutt, 1980; Mintz & Cronmiller, 1981) and this has suggested that the XX constitution may be more stable than the XY.

Recently, techniques have been described which allow stem cells to be isolated directly from mouse embryos (Evans & Kaufman, 1981; Martin, 1981). The principal advantage of these embryo-derived (EK) cell lines is that, in addition to their relative ease of isolation, they provide a source of pluripotential stem cell lines which, at least initially, have a karyotype which is identical to that of the embryo from which they are derived. Analysis of existing EK fertilized-derived cell lines has revealed that over 70% are karyotypically stable, and retain a

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normal chromosome constitution (Robertson, Bradley, Evans & Kaufman, unpublished).

In the present paper, we describe the analysis of six haploid-derived (HD lines) and two diploid-derived (DP lines) lines isolated from parthenogenetically activated eggs of both the 1-pronuclear (uniform haploid) and 2-pronuclear (heterozygous diploid) classes (Kaufman, 1981). In both the HD and DP lines the XX condition appeared to be unstable in the undifferentiated cell cultures. In one line only a single X chromosome was found in all spreads examined, and two other lines showed some cells with complete loss of one X chromosome. More frequently, a deletion of the distal segment of one of the X chromosomes was apparent. The extent of the deletion varied between lines, but remained constant within a given line. The XX constitution would appear to be unstable both in fertilized-derived and parthenogenetically-derived lines, but we have only observed the phenomenon of X deletion in the parthenogenetically-derived cell lines.

**MATERIALS AND METHODS**

1. **Isolation and culture of parthenogenetically-derived lines**

Eggs from 8- to 12-week-old superovulated 129/Sv//Ev and (C57BL × CBA)F1 female mice were activated parthenogenetically following a brief exposure to 7% ethanol in phosphate-buffered saline (PBS) (for details of the procedure, see Kaufman, 1982). Only those activated oocytes that had developed either a single pronucleus following second polar body extrusion (uniform haploid class, Kaufman, 1981), or two pronuclei in the absence of second polar body extrusion (heterozygous diploid class, Kaufman, 1981) were used subsequently. The F1 activated eggs were cultured in vitro to the expanded blastocyst stage. Because the 129/Sv//Ev activated eggs generally fail to progress in culture beyond the 2-cell stage, pronuclear eggs were transferred to the oviducts of recipients on the afternoon of the first day of pseudopregnancy (day 1 = day of finding vasectomized plug). The recipients were subsequently autopsied at about midday on day 4, by which time a high proportion of the transferred embryos had achieved the blastocyst stage. The haploid and diploid blastocysts were then treated in one of two ways.

i. in vivo 'delay'

Blastocysts were transferred to the uteri of ovariectomized recipients on day 3 of pseudopregnancy. While anaesthetized, the mice were injected with 1 mg of Depo-Provera. Autopsies were carried out after 3–6 days, and ‘delayed’ blastocysts recovered. The latter were explanted into tissue culture, and pluripotential cell lines established from individual embryos. Details of the methodology involved have been described elsewhere (from fertilized embryos: Evans & Kaufman,
1981; from haploid parthenogenones: Kaufman, Robertson, Handyside & Evans, 1983).

ii. direct in vitro culture

A proportion of the expanded non-'delayed' blastocysts were briefly exposed to acid Tyrode's medium to remove the zona pellucida, then transferred into individual 1 cm wells (Nunc) containing a preformed feeder layer of inactivated fibroblasts (Martin & Evans, 1975), and DMEM medium (Gibco) supplemented with 10% foetal calf serum, 10% newborn calf serum, and $10^{-4}$M-2-mercaptoethanol. Blastocyst attachment occurred approximately 48 h after explantation. After an additional 4-day interval, the inner-cell-mass-derived cell clumps were selectively removed, trypsinized (0.25% trypsin $10^{-4}$M-EDTA in PBS) and replated onto feeder layers. The trypsinization and replating procedure was repeated after 4–6 days and, in successful cultures, nests of stem cells became visible shortly after this second passage (see Evans & Kaufman, 1981).

All the EK cell lines were maintained exclusively on feeder layers as undifferentiated cultures and passaged at 4–5 day intervals by trypsinization. Cells were cultured on feeder layers in order to retain their differentiation ability (Hogan, 1976), and also to minimise alterations occurring in the chromosome complement (Magrane, 1982). Samples of culture populations were routinely frozen at intervals of two passage generations.

2. Chromosome analysis

Karyotype analysis was carried out on well-established culture populations (usually after five to ten passages) in order to ensure that sufficient metaphase spreads were available for G-banding analysis to be performed.

Exponentially-growing cell cultures were exposed to Colcemid (final concentration 0.02mcg/ml) for 50 min. Cells were collected following trypsinization, incubated in hypotonic solution (0.075 M-KCl) for 10 min, pelleted by low-speed centrifugation (500 r.p.m., 5 min) and fixed in 3:1 methanol: glacial acetic acid fixative. The latter was changed a further two times, and spreads prepared immediately by air drying.

The G-banding technique used was a modification of that described for rat chromosomes by Gallimore & Richardson (1973). Slides were rinsed, air dried and examined under oil immersion ($\times650$) using a Ziess photomicroscope.

Between 30 and 60 intact G-banded metaphase spreads were analysed for each of the eight lines studied. In the better preparations an unambiguous identification of all of the chromosomes present could be made. The total number of chromosomes present was scored, and the morphological appearance of the X chromosomes determined. Selected metaphase spreads were photographed and karyograms constructed according to the nomenclature of Nesbitt & Francke (1973).
Table 1. Chromosome analysis of EK cell lines derived from parthenogenetic embryos

<table>
<thead>
<tr>
<th>†Cell line</th>
<th>Modal number (percentage)</th>
<th>Total metaphases counted</th>
<th>Chromosome number 39 40 41</th>
<th>X-chromosome constitution (metaphase scored)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD1</td>
<td>40 (93)</td>
<td>40</td>
<td>3 37</td>
<td>XX</td>
</tr>
<tr>
<td>HD2</td>
<td>40 (98)</td>
<td>45</td>
<td>1 44</td>
<td>XX</td>
</tr>
<tr>
<td>HD3</td>
<td>40 (97)</td>
<td>30</td>
<td>1 29</td>
<td>XX</td>
</tr>
<tr>
<td>HD4</td>
<td>40 (95)</td>
<td>60</td>
<td>2 57 1</td>
<td>XX del</td>
</tr>
<tr>
<td>HD5</td>
<td>40 (77)</td>
<td>31</td>
<td>7 24</td>
<td>XX</td>
</tr>
<tr>
<td>*HD6</td>
<td>40 (83)</td>
<td>35</td>
<td>4 29 2</td>
<td>XX</td>
</tr>
<tr>
<td>*DP1</td>
<td>40 (58)</td>
<td>31</td>
<td>13 18</td>
<td>XX</td>
</tr>
<tr>
<td>*DP2</td>
<td>41 (73)</td>
<td>30</td>
<td>8 22</td>
<td>XX del XX del</td>
</tr>
</tbody>
</table>

* Cell lines obtained by direct culture method from the 1-cell stage.
† HD1, HD2 and HD5 lines isolated from 129 Sv/Ev strain embryos, all other lines from (C57BL × CBA)F₁.
RESULTS

A total of six haploid-derived and two diploid-derived pluripotential lines have been isolated from parthenogenetic embryos. The origin, method of isolation and modal number of these lines are summarized in Table 1.

1. Lines derived from haploid parthenogenetic embryos

Six parthenogenetically-derived lines have been established from the 1-pronuclear (haploid) class of embryos. These have been termed 'haploid-derived' or HD lines. A summary of the detailed karyological analyses carried out on G-banded metaphase spreads collected from early passage cultures are presented in Table 1.

All six lines were composed solely of diploid cells, and hence provide a source of completely homozygous diploid cell lines. The modal number of chromosomes in these lines was 40, with between 77.5% and 98% of cells possessing this count (Table 1).

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Fig. 1. A schematic representation of an X chromosome (based on the nomenclature of Nesbitt & Francke, 1973) to illustrate the approximate position of the breakpoint for each of the HD cell lines.
Between 10 and 30 spreads from each line were karyotyped, and all found to have a completely normal diploid autosomal complement. As the original haploid chromosome complement had been doubled, each cell would be expected to contain two identical X chromosomes. However, changes from the expected XX constitution were apparent in all lines. These changes fell into two groups: very occasionally, the cells had become XO following the loss of a single X chromosome, or, much more frequently, a deletion affecting the distal region of a single X chromosome had occurred.

The presence of a partial deletion involving one of the X chromosomes was recorded in all six HD lines, whether they had originated from the 129/Sv/Ev or the F1 hybrid strain of mice, despite the fact that they had been isolated on separate occasions over a 9 month period. The position of the breakpoint differed considerably between lines, but appeared to be constant for a given line. A summary of the extent of the deleted segment in the various HD lines is presented in Fig. 1, and representative examples of XX\textsuperscript{del}-chromosome pairs from the various lines are illustrated in Fig. 2.

The most extreme form of the deletion is seen in the HD1 line in which approximately 70\% of the chromosome is deleted, with only a small fragment of the X chromosome remaining beyond the centromere. The deletion is least pronounced in the HD4 and HD5 lines in which the deletion involves approximately 35\% and 40\% of the total length of the X chromosome, respectively.

In three of the lines, metaphases were found in which two apparently normal intact X chromosomes were present. These were present in low numbers in the HD5 line (3 out of 12 metaphases scored), and HD6 line (1 out of 13 metaphases scored), but present in approximately half of the cells of the HD4 line (16 out of 31 metaphases scored).

![Fig. 2. X deletion in haploid-derived cell lines. Examples of pairs of G-banded X chromosomes from the HD cell lines.](image-url)
The only haploid-derived line in which XO cells were recorded was the HD5 line (3 out of 12 metaphases scored).

Thus, in all of the lines studied, the majority of cells possessed an XX\textsuperscript{del} genotype (Table 1).

All HD lines retained a normal diploid autosomal component. Figure 3 gives a karyogram from the HD5 cell line, which is of 129/Sv/JEv origin, to illustrate the possession a normal euploid chromosome complement.

2. Lines derived from diploid parthenogenetic embryos

Two parthenogenetically-derived stem cell lines have been isolated from the 2-pronuclear class of embryos. These lines differ from the haploid-derived lines in that they contain heterozygous rather than homozygous diploid cells. Details of their karyotype analysis are presented in Table 1.

Both lines appear to have an unstable XX constitution. The DPI line possesses a normal diploid autosomal complement but has a bimodal chromosome count with approximately equal numbers of cells containing 39 and 40 chromosomes. This is due to the presence of both XO- and XX-genotype cells in the culture population (see Table 1). A karyogram illustrating the XX genotype is given in Fig. 4A. No partial deletion involving one X chromosome was observed in this line. The DP2 line possesses cells containing either 40 or 41 (73 % of spreads)

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Fig. 3. A G-banded karyogram from the HD5 cell line (129/Sv/JEv origin) to show a normal euploid chromosome complement.
chromosomes. G-banding revealed that all spreads were trisomic for chromosome 11. Cells of XO, XX and XX<sup>del</sup> genotype were recorded, though the majority of cells was characterized by the possession of an XX<sup>del</sup> genotype, in which approximately 70% of the material of one of the X chromosomes was lost. A representative karyogram for this line is presented in Fig. 4B.

**DISCUSSION**

This paper is the second in a series in which the establishment and properties

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**Fig. 4.** Karyograms from the DP lines. (A) DP1. Normal diploid complement. (B) DP2. The cell line is characterized by possession of trisomy 11 (recorded in all metaphase spreads examined). This particular metaphase spread illustrates the extent of the deletion of a single X chromosome found in this cell line.
of parthenogenetically-derived pluripotential cell lines are presented. In the first paper (Kaufman et al. 1983), details of the establishment of lines HD1, 2, 3 and 4 from ‘delayed’ blastocysts were presented, as well as preliminary observations on their properties when allowed to differentiate in vivo and in vitro. Minimal information was provided on the cytogenetic findings, as these had yet to be investigated in detail. In the present paper, an attempt has been made to clarify this aspect, with the presentation of details of the karyological analysis of the original four HD lines, as well as two additional HD lines (HD5 and HD6), and two diploid-derived lines (DP1 and DP2).

However, before discussing the cytogenetic findings in detail, we believe it worth stressing the fact that, contrary to our previous report (Evans & Kaufman, 1981) and also to the findings of Martin (1981) it is now possible to establish stem cell lines directly from normal non-‘delayed’ blastocysts in the absence of teratocarcinoma-conditioned medium: lines HD6, DP1 and DP2, for example, as well as various fertilized-derived lines (authors, unpublished) were established in serum-containing tissue-culture medium from embryos that had been maintained completely in vitro from the 1-cell stage onwards. Thus, while the enlarged inner-cell-mass-derived component present in ‘delayed’ blastocysts, and appropriately ‘conditioned’ medium may both facilitate the isolation of EK pluripotential lines, they are not necessary.
It is also of interest that the EK cell lines derived from normal non-"delayed" blastocysts seem to be indistinguishable from those obtained from 'delayed' blastocysts. Similarly, EK lines derived from parthenogenetic material were indistinguishable from pluripotent cell lines established from teratocarcinomas (see Evans & Kaufman, 1981; Kaufman et al. 1983).

Apart from confirming our previous finding (Evans & Kaufman, 1981) that the autosomal complement of EK cells is apparently quite normal, the present study has revealed that, contrary to expectation, the X-chromosome complement in parthenogenetically-derived pluripotent lines does not appear to be stable. Possibly of greatest interest is the observation that in all of the lines so far studied, a partial deletion involving one of the X chromosomes manifests itself in the lines established from both haploid- and diploid-derived parthenogenetic material. Whereas both groups appear to form stable diploid lines, the former are genetically homozygous, while the latter group – which form from embryos which retain both products of the second meiotic division – are likely to be progressively more heterozygous at loci increasingly distal from the centromere (Eicher, 1978). In the most extreme cases, both in the HD and DP lines, an entire X chromosome is lost from the complement, resulting in the production of an XO line. While the complete loss of an X chromosome was only rarely encountered in most of the parthenogenetically-derived lines studied, the presence of a partial deletion of one of the X chromosomes occurred regularly.

While X-inactivation occurs apparently normally both within the embryo (Kaufman, Guc-Cubrilo & Lyon, 1978) and in the extraembryonic membranes (Rastan, Kaufman, Handyside & Lyon, 1980) in heterozygous diploid parthenogenones, no information appears to be available on X-chromosome activity in homozygous diploid embryos.

There is considerable evidence that both X chromosomes are active during early development of female mouse embryos (reviewed by Gartler & Cole, 1981) and this has also been shown to be the case in some XX EC cell lines (Martin et al. 1978; McBurney & Strutt, 1980). Presumably both X chromosomes are also active in XX EK cell lines and indeed in such lines no differential Kanda staining was observed (unpublished observations A. Stoker). Although no biochemical information is yet available on X-chromosome activity in the various parthenogenetically-derived EK lines considered here, there seems no a priori reason why the X chromosomes should behave differently from the situation observed in the tumour-derived EC cells.

The female mammal has twice the relative dose of X-linked to autosomal genes of the male, and X-inactivation provides a mechanism of dosage compensation. That there is a dosage imbalance in XX EC cells is born out by the observations of a double specific activity of X-linked enzymes in these cells (Martin et al. 1978; see also Epstein, Travis, Tucker & Smith, 1978; Monk, 1978). This is to be expected as the cell phenotype is of an early embryo epiblast cell. All X-linked products are, therefore, overproduced and these cells are in
a state of imbalance. Despite this, XX cells not only grow through this stage of development, but may be isolated and maintained as progressively growing cultures of EK cells.

Analysis of 21 EK cell lines derived from fertilized embryos shows that 15 of these are karyotypically normal (authors' unpublished results). To date we have not observed the loss of a Y chromosome from the 12 XY lines available, however the loss of an entire X chromosome has been observed in two of the three (otherwise euploid) XX lines studied with the consequent production of both XX and XO cells in these cultures. Whilst it is clear, therefore, that XX lines can give rise to XO cells, we have not yet observed a partial deletion of an X chromosome in any fertilized-derived EK cell line nor has this phenomenon been reported to occur in any established EC cell line.

It is perhaps relevant that a number of established EC cell lines (which have been derived from tumours made by ectopic transplantation of early embryos) are XO. While it has usually been assumed that these arose from XY lines due to the loss of the Y chromosome, this preliminary EK evidence suggests that this is perhaps more likely to be due to the loss of an X chromosome. Conceivably, in contrast to an XX EK line with both X chromosomes active, an XY EK cell line is carrying little redundant metabolic burden from the small Y chromosome. The reason why XO lines are found to arise more frequently from XX than from XY lines could thus be that the removal of one redundant X chromosome confers a more significant growth advantage on the cell relative to its XX progenitors. It is noticeable that those long-established EC cell lines which do possess two X chromosomes also have an increased number of autosomes (McBurney & Adamson, 1976; Martin et al. 1978) and some tend not to be pluripotential (e.g. Nulli SCC2A, E. J. Robertson, unpublished results).

Possibly the fact that in most teratocarcinomas one of the X's is maternally derived and the other paternally derived has some effect on their inactivation during early cellular differentiation (as is known to be the case in the normal embryo, West, Frels, Chapman & Papaioannou, 1977; Harper, Fosten & Monk, 1982). Their parental origin may also in some way influence the loss of one or the other X chromosome in the undifferentiated state. Clearly, this cannot be the case in the parthenogenones, where both X's are of maternal origin.

Although a variety of breakpoints are observed in the different parthenogenetically-derived lines studied here, it is unclear at the present time whether a progressive loss of the distal segment occurs, with the eventual production of an XO line. The uniform state of the deleted segment in each of the lines so far examined, however, would suggest that this is not the case, but even in the most well-established lines, cells from only relatively early passages have been examined. This is in marked contrast to the situation observed in many EC lines in which cells may have passed through hundreds of generations both as in vivo tumours and in subsequent growth in vitro. The latter situation would certainly tend to favour the selective overgrowth and survival of the cells with the most
stable genotype. Indeed, time alone will tell whether the XX\textsuperscript{del} genotype observed in these lines represents an early stage in the production of the (possibly) more stable XO genotype. If a variety of distal X\textsuperscript{del} conditions are sufficient to confer the postulated growth advantage on the cell it suggests that either the most serious imbalance is due to loci which map at the distal region of the X chromosome, or that deletion of this region removes a controlling locus which affects the activity of the rest of the chromosome. It would be interesting to discover if the X\textsuperscript{del} chromosome is active in these cells.

Whatever the cause and mechanism, a series of X-chromosome deletions are now available, and it seems likely that many more of these apparently randomly occurring deletions could be produced. These may be extremely useful for genetic mapping of the X chromosome. If the XX\textsuperscript{del} chromosomes remain active, the relative specific activities of X-linked enzymes in XX, XX\textsuperscript{del} and XO cells, which are identical in all other respects, should provide mapping information. Similarly, DNA from such cells might be used to locate recombinant DNA clones from mouse X-chromosome libraries.

Further studies will obviously be required to clarify many of the points raised in this discussion. Does, for example, the XX state in parthenogenetically-derived EK cells progress to the XX\textsuperscript{del} state, which then eventually progresses to the ‘stable’ XO state? Do fertilized-derived EK XX cells eventually progress along a similar pathway? A study of homozygous diploid pluripotential lines established from enucleated fertilized diploidized embryos (Markert & Petters, 1977) might help to clarify some of these issues.

We would like to thank Lesley Cooke and Mary Knox for their excellent technical assistance. This work has been supported by a grant from the Medical Research Council.

REFERENCES


X-chromosome instability in pluripotential cell lines


(Accepted 17 November 1982)
Isolation, Properties, and Karyotype Analysis of Pluripotential (EK) Cell Lines from Normal and Parthenogenetic Embryos

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Techniques have recently been described which have made possible the isolation in culture of pluripotential stem cell lines directly from preimplantation-stage mouse embryos (Evans and Kaufman 1981; Martin 1981). Previous to these reports, stem cell lines have been available only from teratocarcinoma tumors. Embryonal carcinoma (EC) cell lines have been isolated from tumors that arise spontaneously in the gonads or, more frequently, from tumors induced by the transfer of early-stage embryos to ectopic sites (reviewed in Graham 1977).

In vitro embryo-derived (EK) lines are indistinguishable from their tumor-derived EC cell counterparts in terms of their cellular morphology, cell-surface antigen expression, and differentiative behavior both in vitro and in vivo (Evans and Kaufman 1981; Martin 1981; Kaufman et al. 1983) and are also capable of participating in normal development in a chimeric association with a fertilized embryo (Evans and Kaufman 1981).

Pluripotential lines derived from embryos would appear to have a considerable number of advantages over tumor-derived EC cell lines. In addition to their relative ease and rapidity of isolation, there appears to be no restriction on the mouse strains from which such lines may be isolated. These factors thus facilitate the establishment of new genetically marked pluripotential stem cell lines from both inbred and randomly bred mice. Cell lines from the latter source had not previously been available, as teratocarcinomas could only be induced experimentally by the ectopic transfer of embryos into histocompatible host animals, and tumors containing EC cells fail to grow in immunosuppressed or nude mice (Solter and Damjanov 1979). The establishment of stem cell lines directly from embryos has the singular advantage that these cells can be used to investigate homozygous lethal developmental mutations which can only normally be maintained in mouse stocks in the heterozygous state. The potential usefulness of this approach has already been demonstrated by the recent report of the isolation of an EK cell line which is homozygous for the $r^3$ mutation of the $t$ complex (Magnuson et al. 1982).
Over 70% (15 out of 21) of the fertilized-derived EK cell lines established in our laboratory that have so far been analyzed possess a karyotype which is, at least initially, identical to that of the blastocyst from which they were derived. This may prove to be the single most important feature of these lines, as the possession of a normal euploid karyotype should considerably facilitate their incorporation into chimeric animals. Of even greater importance, however, is the likelihood that the possession of a normal karyotype may well be essential for the incorporation of EK cells into the germ line of chimeric mice (Mintz and Cronmiller 1981).

EK lines are currently being used in our laboratory and have been supplied to other laboratories for the construction of such chimeric mice, and breeding studies are now in progress to test for possible contributions to the germ line in these individuals.

Since the initial report from our laboratory in 1981, we have established pluripotential cell lines directly from both in vivo "delayed" and, more recently, from non- "delayed" fertilized blastocysts. To date, over 20 EK lines have now been established from individual fertilized embryos from a variety of inbred and randomly bred sources, thus making available for study genetically normal stem cell lines, and those carrying a range of isozymal variants, as well as lines with translocation-bearing "marker" chromosomes.

We have recently extended our studies by establishing similar cell lines from parthenogenetically derived embryos. Haploid and diploid blastocysts obtained following the in vitro activation of eggs with ethanol (Kaufman 1982) have now been used successfully to establish pluripotential cells from this source (Kaufman et al. 1983). At the present time, ten cell lines of parthenogenetic origin exist. They include lines derived from haploid (1-pronuclear class) and two from diploid (2-pronuclear class) parthenogenetic blastocysts (for details of classification, see Kaufman 1981); these have been derived from both 129/SvE and (C57BLX CBA)F, strains of mice. Some of these lines were obtained from in vivo "delayed" blastocysts, and others were established from normal blastocysts maintained completely in culture from the one-cell stage onward. The latter technique has certain obvious advantages, not the least being the minimizing of loss of experimental material (the recovery of embryos following in vivo "delay" is often low).

To date, all the parthenogenetically derived lines have been diploid—even those established from haploid blastocysts (termed HD lines)—and we have yet to establish, at present, at which stage of the isolation procedure diploidization occurs. Nevertheless, this method has enabled homozygous diploid lines of parthenogenetic origin to be established.

Although many EC teratocarcinoma-derived lines maintain a near-normal karyotype, few have been found to be karyotypically completely normal. Indeed, many of these lines have been found to have an X0-chromosome constitution, and it has often been assumed that these were originally XY lines in which the Y chromosome has been lost at some stage during their passage either in vivo or in vitro. Several apparently euploid female lines have been reported, however (Papaioannou et al. 1979; McBurney and Strutt 1980; Mintz and Cronmiller 1981), and this has tended to confirm the belief that the XX may be more stable than the XY-chromosome constitution. We believe that an examination of the stability of the sex chromosomes in parthenogenetically derived lines and in XX and XY fertilized-derived EK lines may contribute to our understanding of the origin of these X0 EC cell lines.

In this paper, we describe in detail the isolation techniques, properties, and karyotype analysis performed on EK cells that have been derived from fertilized and parthenogenetic blastocysts. Preliminary results on the developmental capabilities of a single fertilized-derived EK line are also presented.
EXPERIMENTAL PROCEDURES

Isolation of Pluripotential Cells from Fertilized-derived Blastocysts

Pluripotential cell lines were isolated from blastocysts that had been subjected to a period of hormonally induced in vivo delay. Female mice were ovariectomized 2.5 days postcoitum (p.c.) and, while under the influence of the anesthetic, given a subcutaneous injection of 1 mg of Depo-Provera (Upjohn). "Delayed" blastocysts were recovered from the uterine horns 4–6 days later. Individual "delayed" blastocysts were explanted into tissue culture dishes which contained Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum, 10% newborn calf serum (selected batches), and 10⁻⁴ M 2-mercaptoethanol. The explanted blastocysts attached after about 48 hours culture. Migration of the trophoblast cells was apparent at this time (Fig. 1A), and, following a further 4 days of culture, the inner cell mass (ICM)-derived cell clumps, which had increased considerably in size by this time (see Fig. 1B,C), were selectively removed. The clumps were disaggregated, using 0.25% trypsin, 10⁻⁴ M EDTA in phosphate-buffered saline (PBS), into several smaller cellular aggregates which were then transferred into 1-cm tissue culture wells (Nunc) containing a layer of preformed inactivated fibroblast cells (Martin and Evans 1975). The disaggregation procedure was repeated after a further 4 days and, following this, nests of stem cells became apparent; they were isolated individually and allowed to develop into mass cultures (Fig. 1D). Examples of the morphological appearance of the blastocysts and the various cell types derived from them are illustrated in Figure 1.

EK lines were maintained exclusively on feeder layers, and cellular samples from the cultures were frozen at regular intervals.

Isolation of Pluripotential Cell Lines from Parthenogenetically Derived Embryos

Oocytes from superovulated 129/SvE and (C57BL \times CBA)F₁ hybrid female mice were activated parthenogenetically following a brief exposure to a dilute solution of ethanol in PBS (for details of this activation procedure, see Kaufman 1982). Various haploid and diploid classes of parthenogenones are induced by this method (Kaufman 1981), and their appearance at about 4–6 hours following activation is illustrated in Figure 2. Only those activated oocytes that developed a single pronucleus following extrusion of the second polar body (uniform haploid class; Fig. 2a) or those that developed two pronuclei in the absence of a second polar body (heterozygous diploid class; Fig. 2b) were used in this study.

The F₁-activated oocytes were retained in culture until they reached the expanded blastocyst stage. The majority of embryos achieved this stage by about 4.5 days following activation. As 129/SvE-activated eggs generally fail to progress beyond the two-cell stage in culture, pronuclear stage one-cell eggs were transferred into the oviducts of pseudopregnant recipients on the afternoon of the first day of pseudopregnancy (day 1 = day of finding the vasectomized plug). The embryos were recovered at midday of the fourth day of pseudopregnancy, by which time a high proportion of the transferred embryos had reached the expanded blastocyst stage. The expanded blastocysts from both strains of mice were then treated in one of two ways:

In Vivo "Delay"

Expanded blastocysts were transferred to the uteri of suitable recipients on the third day of pseudopregnancy. The recipients were then ovariectomized and given a sub-
Figure 1
Isolation of pluripotent cells from "delayed" fertilized-derived blastocysts. (A) Appearance of "delayed" 129/SvE blastocyst 48 hr following explantation into tissue culture. (B) Appearance of blastocyst at ~96 hr after explantation. Note centrally located cell clump derived from the inner cell mass. (C) Blastocyst culture at ~140 hr after explantation. The inner cell mass has proliferated to form an "egg cylinder-like" structure. (D) Nest of EK cells, growing on a feeder layer, second passage after their establishment in culture.

cutaneous injection of 1 mg of Depo-Provera. Females were autopsied 3-6 days later, and the "delayed" blastocysts were recovered. Individual blastocysts were then explanted into 1-cm wells (Nunc) containing a layer of feeder cells, and the
Pluripotent Cells from Embryos

Figure 2
The four classes of parthenogenetic eggs which can be distinguished at 4–6 hr after activation, viewed by interference-contrast optics. (a) Single-pronuclear haploid egg with extruded second polar body (uniform haploid). (b) Two-pronuclear presumptive diploid egg (heterozygous diploid). (c) Immediate cleavage embryo with two approximately equal-sized blastomeres (mosaic haploid). (d) Single-pronuclear diploid egg (heterozygous diploid).

procedure used to establish pluripotential cell lines from them was the same as described in the previous section.

Direct In Vitro Culture
In an alternative procedure, which has been used successfully with haploid and diploid parthenogenones, activated oocytes are retained in culture directly from the one-cell stage to the blastocyst. These embryos are then explanted directly into supplemented DME without the necessity of passing through any form of development in vivo.

To ensure hatching from the zona pellucida and subsequent attachment, individual expanded blastocysts were briefly exposed to acid tyrode medium and subsequently transferred to feeder wells as described above. The blastocysts attached after about 48 hours, and, after a further 4 days, the individual ICM-derived
cell clumps were selectively removed and treated as described above. The best results were obtained when colonies of a stem cell phenotype were identified at an early stage and selectively explanted into fresh feeder wells. In this way, cultures which were free from other embryo-derived cell types (e.g., endoderm) could be obtained.

An initial sample was frozen when a sufficient number of cells were available (generally after three to four passage generations), and, subsequently, aliquots were frozen at regular intervals.

Chromosome Analysis
Chromosome analysis has been performed on the majority of the EK cell lines established from fertilized and parthenogenetic embryos. This has usually been carried out after between 5 and 10 passage generations.

The mitotic chromosome spreads were analyzed by G banding by a modification of the ASG technique of Gallimore and Richardson (1973). The modal number was ascertained by counting between 30 and 60 intact metaphase spreads. Only those spreads in which all chromosomes could clearly be distinguished were analyzed in detail. At least ten banded spreads were assessed per line with particular attention being given to the morphology of the sex chromosomes. Selected metaphase spreads were photographed, and karyograms were arranged according to the nomenclature of Nesbitt and Francke (1973).

In Vivo Differentiation
The in vivo differentiation potential of EK cell lines was tested by the subcutaneous injection of cell suspensions into syngeneic host animals. For each line to be tested, approximately 10⁶ cells were injected in 0.1 ml PBS into the flank of three to four male mice. Palpable masses usually developed within 10 days of inoculation; the animals were sacrificed when the tumor masses reached a diameter of approximately 1.5 cm. The tumors were excised, fixed, and subsequently embedded in paraffin wax using standard histological techniques. Sections were then cut from the midplane of the tumors and stained with either Hematoxylin and Eosin or Masson’s Trichrome.

Blastocyst Injection
The developmental potential of a single fertilized-derived EK cell line is currently being tested by blastocyst injection. The line being tested is B2B2, a single cell clonal derivative of the B2 line, and is a normal male XY euploid line of 129/SvE origin (Evans and Kaufman 1981), which carries the agouti coat color and Gpi-I⁺ allele. Host blastocysts were obtained from selected stocks of random-bred CFLP mice which are albino and homozygous for the Gpi-I⁺ allele.

Single-cell suspensions of B2B2 cells were prepared from cultures growing on feeder layers by disaggregation either with 0.25% trypsin-EDTA or using 5 mM EGTA in PBS. Between two and eight B2B2 cells were transferred microsurgically into the blastocoelic cavity of individual blastocysts, and the operated embryos were then transferred into the uterine horns of appropriate recipients on the third
day of pseudopregnancy. Overtly chimeric mice were identified at term by their eye pigmentation, whereas coat pigmentation was usually apparent several days later. Any newborn young that died shortly after birth were autopsied, and their component tissues were analyzed for GPI type by starch gel electrophoresis.

RESULTS

Isolation and Karyotype Analysis of EK Lines Derived from Fertilized Blastocysts

The origin, designation, and karyotype analysis of the various EK lines established from individual "delayed" blastocysts are presented in Table 1. So far, 22 EK lines have been established, and two of these, B1 and B2, which were isolated from a group of "delayed" blastocysts, have now been subcloned, to give a total of 28 new pluripotent stem cell lines.
As may be seen from Table 1, lines have now been established from both inbred and outbred stocks of mice, thus making available for study lines which are homozygous for various allozymal variants. For example, lines which are homozygous for the Gpi-1* (129/SvE-derived lines) and Gpi-1' (Rm-derived lines) alleles are now available. The 5.13 line, established from an inbred stock of the same designation, carries the markers for polydactyly, and fuzzy and leaden coat hair. In addition, translocation-marked lines have also been established: X1, which is a normal euploid XY line, is homozygous for the Rb163H 9/19 autosomal translocation; Y2 is also a normal euploid XX line which is heterozygous for the Cattanach-bearing (T[X;7]1Ct) X chromosome, whereas the DD6 line is heterozygous for the T6 translocation.

A detailed chromosomal analysis is currently being made of existing EK lines. To date, 21 have been karyotyped (including the 6 subclonal lines), and 15 (70%) of these have a normal euploid constitution; 12 are XY and 3 are XX lines. Chromosomal abnormalities have been detected in the remaining 6 lines, 4 lines of which have both autosomal and sex chromosomal abnormalities. Interestingly, the B1-2 line, subcloned from the B1 line, is autosomally normal but has an XYY constitution; the latter abnormality was present in all of the cells examined from this line. The B2C5 line, a single cell cloned line established from the B2 line, has a normal XY complement but is characterized by the presence of a partial deletion of a single chromosome 5.

Whereas most of the karyotype analysis has so far been performed on lines at an early stage in their passage history (normally less than 15 passage generations), the B2B2 line has now been shown to retain a normal XY karyotype after more than 45 passage generations.

### Isolation and Karyotype Analysis of EK Lines Derived from Parthenogenetic Embryos

Using the experimental techniques outlined above, pluripotential cell lines have now been routinely established from ethanol-activated oocytes (Kaufman 1982).

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### Table 2

**EK Cell Lines Established from Parthenogenetically Derived Blastocysts**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Modal number</th>
<th>Status of X chromosomes</th>
<th>Extent of deletion (% total length)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haploid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HD1</td>
<td>129/Sv//Ev</td>
<td>40</td>
<td>XX&lt;sup&gt;del&lt;/sup&gt;</td>
<td>70</td>
</tr>
<tr>
<td>HD2</td>
<td>129/Sv//Ev</td>
<td>40</td>
<td>XX&lt;sup&gt;del&lt;/sup&gt;</td>
<td>45</td>
</tr>
<tr>
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<td>40</td>
<td>XX&lt;sup&gt;del&lt;/sup&gt;</td>
<td>40</td>
</tr>
<tr>
<td>HD4</td>
<td>(C57BL×CBA)F&lt;sub&gt;1&lt;/sub&gt;</td>
<td>40</td>
<td>XX:XX&lt;sup&gt;del&lt;/sup&gt;:X0</td>
<td>35</td>
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<tr>
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<td>129/Sv//Ev</td>
<td>40</td>
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<td></td>
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</tr>
<tr>
<td>DPI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(C57BL×CBA)F&lt;sub&gt;1&lt;/sub&gt;</td>
<td>40 (39)</td>
<td>XX:X0</td>
<td>—</td>
</tr>
<tr>
<td>DP2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(C57BL×CBA)F&lt;sub&gt;1&lt;/sub&gt;</td>
<td>41</td>
<td>XX&lt;sup&gt;del&lt;/sup&gt;:X0</td>
<td>70</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cell lines obtained by direct culture method from the one-cell stage.
although there seems every reason to believe that parthenogenetic blastocysts derived following other forms of activation (see Kaufman 1978) should be equally useful in this regard. A total of eight haploid-derived and two diploid-derived pluripotential lines of parthenogenetic origin have now been established from both 129/SvE and (C57BL × CBA)F1 hybrid strains of mice. The method of isolation, strain of origin, and karyotype analysis of these lines are summarized in Table 2. The isolation of HD lines 1-4 has recently been reported (Kaufman et al. 1983).

Parthenogenetically derived EK stem cell cultures closely resemble their fertilized-derived counterparts (Kaufman et al. 1983), as do the lines derived from in vivo “delay” and normal non-“delayed” blastocysts. These lines differentiate in vitro to form embryoid body-like structures in suspension culture, with cells of an endodermal phenotype being the first to differentiate. The 129/SvE-derived lines HD1 and HD2 have been tested for tumorigenicity and to assess the extent of tissue differentiation occurring when cells have been injected into syngeneic host animals, as described above. All formed typical teratocarcinomas, with a wide variety of tissue types present. Representative regions are illustrated in Figure 3.

**Lines Derived from Haploid Parthenogenetic Embryos**

To date, a detailed karyological (G banding) analysis has been carried out on six of the eight haploid-derived lines presently available, with metaphases collected from early-passage (five to ten passages) cultures. All six lines were composed solely of diploid cells and hence provide a source of perfectly homozygous diploid cell lines. The modal number of chromosomes in these lines was 40, with between 80% and 98% of cells in individual cultures possessing this count. All lines were found to have a completely normal autosomal complement.

As the chromosome complement in these lines had arisen by doubling of the original haploid complement, it would be expected that the cells would contain two identical X chromosomes. However, changes from the expected XX constitution were apparent in all lines. These changes could be divided into two categories: Very occasionally the cells had become X0 by loss of a single X chromosome, or, much more frequently, a deletion affecting the distal region of one of the X chromosomes was apparent. The presence of a partial deletion involving a single X chromosome was recorded in all six HD lines, regardless of their strain of origin and despite the fact that they had been isolated on separate occasions over a 9-month period. The position of the breakpoint differed considerably between lines but appeared to be constant for a given line (see Table 2). Representative examples of the XX<sup>del</sup> chromosomes are illustrated in Figure 4.

The most extreme form of the deletion is seen in the HD1 line, where only a small fragment of the X chromosome remains distal to the centromere. The deletion is least pronounced in the HD4 and HD5 lines and involves approximately 35% and 40%, respectively, of the total length of the X chromosome.

In three of the lines, metaphases were recorded in which two apparently normal intact X chromosomes were present. These were found in low numbers in the HD5 line (3/12 metaphases scored) and in the HD6 line (1/13 metaphases scored) but present in approximately half the cells of the HD4 line (16/31 metaphases scored). Representative karyograms which illustrate typical XX and XX<sup>del</sup> genotypes in the HD4 line are presented in Figure 5.

The only HD line in which X0 cells were recorded was the HD5 line (3/12 metaphases scored). Thus, in all the six HD lines so far studied, the majority of cells possessed an XX<sup>del</sup> genotype.
Figure 3
In vivo differentiation of the HD1 line. Sections through tumors contain a variety of tissue types, including keratin whorl (a), cartilage nodule (b), melanin pigmentation (c), epithelial cells (c), precartilage nodule (d), secretory epithelial cells (d), areas of yolk sac material (e), and folded columnar epithelial cells (f).

Lines Derived from Diploid Parthenogenetic Embryos
Two pluripotential cell lines have so far been established from the 2-pronuclear class of parthenogenetically activated embryos (Fig. 2b). These lines, termed DP lines, differ from the HD lines in that they contain minimally heterozygous rather than homozygous diploid cells, as their diploid complement contains both products of the second meiotic division (Kaufman 1981).

G-banding analysis revealed that both these lines also had an unstable XX constitution. The DPI line possessed a normal diploid autosomal complement but had
a bimodal chromosome count, with approximately equal numbers of cells containing either 39 or 40 chromosomes, due to the presence of both X0 and XX genotype cells within the culture population. The DP2 line possessed cells containing either 40 or 41 (73% of spreads) chromosomes, and G banding revealed that all cells were trisomic for chromosome 11. Cells of X0, XX, and XX<sup>del</sup> genotypes were recorded, although the majority of cells possessed an XX<sup>del</sup> genotype in which approximately 70% of the material of one of the X chromosomes was lost.

**Blastocyst Injections**

In an early series of experiments (carried out in collaboration with R.L. Gardner), out of a total of 76 operated blastocysts transferred to recipients, 58 live-born animals were obtained (76%), of which 26 (45%) were overtly chimeric. GPI analysis has revealed extensive contributions to the internal organs in chimeric individuals.

In a second series of blastocyst injection experiments in which B2B2 cells were used, 66 blastocysts were transferred and a total of 40 (61%) live-born young subsequently recovered. Of these, 14 (35%) were overtly chimeric, as assessed by eye and coat color pigmentation; 6 of these chimeric mice have survived to adulthood (5 females and 1 male), and these are currently being test-bred.

GPI analysis of the newborn mice that failed to survive the early neonatal period has revealed that in some instances there was an extensive contribution by the EK cells to all of the organs and tissues tested, including the gonads.

It is of particular interest that the head region of these chimeric animals was extensively pigmented (Fig. 6). This feature was particularly noticeable in mice which showed only low levels of overt chimerism. These preliminary data, with regard to both the survival to term of operated embryos and the percentage of overtly chimeric individuals, compare favorably with the results obtained by the injection of EC cells into host blastocysts. For example, the normal euploid XX EC cell line METT-1 (Stewart and Mintz 1981) gave rise to chimeric mice with a frequency of 13%. In one out of nine female chimeras tested, a contribution to the germ line was demonstrated.
Figure 5
Representative karyograms from the HD4 line to illustrate normal euploid complement (A) and a deletion of approximately 35% of the distal region of one of the X chromosomes (B).

DISCUSSION
In this paper, we report the experimental procedures that have enabled us to establish new pluripotential cell lines directly from fertilized blastocysts. Pluripotential stem cell (EK) lines have also been established routinely in our laboratory from parthenogenetically activated material.
Figure 6
Chimeric mice produced by injection of EK B2B2 cells into albino CFLP blastocysts. (a) Extensively chimeric female individual. Note abundance of pigmented coat hairs of 129/SvE origin in head region. (b) Male chimera with low level of overt chimerism. Note the localization of pigment in the coat hair to the head region and the pattern of pigmentation in the iris.

Contrary to our earlier findings (Evans and Kaufman 1981) and those of Martin (1981), we have demonstrated that it is now possible to establish stem cell lines directly from non-“delayed” blastocysts in the absence of medium conditioned by EC cells; the parthenogenetically derived HD6, DP1, and DP2 lines were established in serum-containing tissue culture medium from embryos that had been retained completely in vitro from the one-cell stage onward. Thus, although the enlarged ICM-derived component present in “delayed” blastocysts and the use of appropriately “conditioned” medium may facilitate the isolation of pluripotent...
cells from early embryos, neither are necessary. Indeed, both of these methods would appear to have disadvantages for the isolation of normal embryo cells: the "delaying" procedure may confer an abnormal maternal influence on the embryo, whereas the presence of "conditioned" medium may be similarly undesirable because of the possible presence of uncharacterized product(s) from the established tumor cell line.

With few exceptions, most of the currently available EC cell lines possess chromosomal abnormalities of various kinds. In addition, EC cells have a range of growth characteristics in culture and vary widely in their ability to differentiate both in vitro and in vivo. Some lines differentiate well both in vitro and in syngeneic host animals, and others differentiate only in the tumor form in vivo, and yet others (termed "nullipotent" lines) differentiate only poorly under any circumstances.

The majority of the fertilized and parthenogenetically derived EK lines so far examined (> 70%) appear to retain a normal euploid karyotype and differentiate extensively both in vitro and particularly in vivo, which should facilitate their incorporation into chimeric animals. Our preliminary results presented here, in which we describe the relatively high incidence (> 35%) of overt chimerism in our blastocyst injection studies (in which host blastocysts were injected with EK B2B2 cells), are, we believe, extremely promising.

This is in marked contrast to the variable success that has generally been obtained following the injection of EC cells into normal mouse blastocysts, especially with regard to the production of germ line chimera. One important disadvantage of EC cell lines is that the majority of those used are X0. Although this does not appear to prevent their participation in normal development, it is obviously deleterious for the formation of functional gametes. This would seem to be a reasonable hypothesis and is in agreement with the recent report by Stewart and Mintz (1981) which indicates that the use of a perfectly euploid EC cell line not only increases their relative incidence within the somatic cell population, but is likely also to allow functional colonization of the germ line. In their study, a normal euploid 129-derived EC line (METT-1) was described which gave rise to overtly chimeric animals at a rate of about 13%. More importantly, 1 out of the 9 chimeric females which were test-bred gave rise to 3 out of 48 offspring of a 129 phenotype. Test breeding of animals chimeric from B2B2 injection has not yet demonstrated the production of functional germ cells from the B2B2 cells, but we intend to persist with this study. We also intend to utilize this approach to assess the developmental capabilities of other EK lines of both XX and XY genotypes.

We have established a considerable number of pluripotential cell lines from parthenogenetically activated material. All lines so far examined, however, have been diploid, including those isolated from haploid embryos. It is unclear, at present, at which stage diploidization is occurring, although the majority of the cells in the 1-pronuclear haploid embryos are still haploid at the morula stage (> 80%, Kaufman et al. 1983). Haploid mitoses are also known to persist in small numbers in immediate cleavage-derived embryos at least up to the egg cylinder stage of development (Kaufman 1978). We can only speculate, therefore, that diploidy confers a selective growth advantage over haploidy under the conditions used for isolation.

Cytogenetic studies have revealed that, contrary to expectation, while all but one of the parthenogenetically derived lines possess an apparently normal autosomal complement, the XX constitution of these lines appears to be unstable. The most interesting observation in this regard is the almost universal occurrence of a partial deletion affecting the distal region of one of the two X chromosomes. A partial
deletion occurs in both HD and DP lines. The HD lines have a genetically homozygous diploid constitution, having arisen by the doubling of an originally haploid set of chromosomes, whereas the DP lines are heterozygous diploids having been obtained from embryos that retain both products of the second meiotic division (see Kaufman 1981).

The extent of the X deletion varies between lines, and in extreme cases, an entire X chromosome may be lost with the consequent production of X0 genotype cells. This has been observed in both HD and DP lines. In XX <sup>0</sup> genotype cells, the position of the breakpoint appears to be constant within a line, but the extent of the deleted segment varies considerably between lines. We have tentatively interpreted our findings in terms of a single random deletion event, which may involve either a segment or complete chromosome, which probably occurs early in the isolation of these lines, and which we believe may confer a selective growth advantage to those cells carrying the deleted X chromosome.

Of the fertilized-derived EK lines that have so far been karyotyped, we have only very rarely encountered the loss of an entire X chromosome. Of the three XX fertilized-derived lines so far examined, X0 cells have only been observed in a single line, the A13 line. Detailed cytogenetic analyses have been carried out on the 13 XY EK lines available, and we have not yet observed the loss of any of the Y chromosomes. Indeed, it is interesting to note that a single subclonal line, B1-2, has an XYY constitution.

Although partial X deletions have not yet been observed in any of the 21 EK fertilized-derived lines examined, nor have there been any reports of this phenomenon occurring in those established EC cell lines which have been karyotyped, it is interesting to note that Martin (1981) has reported the presence of a partial deletion of a single X chromosome in her EK line termed ESC-ICR.

As mentioned in the introduction, many EC cell lines are X0 in constitution, and it has often been assumed that these must have arisen from XY lines in which the Y chromosome has been lost either during the course of their in vitro culture or during in vivo growth. However, our evidence from subcloning and long-term culture experiments involving EK lines suggests strongly that the XY constitution is in fact extremely stable in these lines. Conceivably, the presence of two active X chromosomes may be deleterious compared with the situation in XY EK cell lines, where little redundant metabolic burden may arise from the presence of the small Y chromosome. The reason why X0 lines are found to arise more frequently from XX than from XY lines could be that removal of one redundant X chromosome therefore confers a selective growth advantage on the cell relative to its XX progenitors. It is also relevant to note here that some of the long-established EC cell lines which in fact retain two X chromosomes also have an increased number of autosomes present (McBurney and Adamson 1976; Martin et al. 1978) and some tend not to be pluripotential (E.J. Robertson, unpubl.).

The X-deletion phenomenon observed in parthenogenetically derived lines is undoubtedly associated with X-chromosome activity. Previous observations on X-chromosome activity in EC cell lines have tended to indicate that both X chromosomes are active in these cells when they are maintained in the undifferentiated state, whereas X inactivation occurs during, or shortly after, their cellular differentiation (Martin et al. 1978; McBurney and Strutt 1980). No information is at present available on the activity status of the X chromosomes in parthenogenetically derived stem cells, but there seems little reason to believe that these cells would behave differently from their EC cell counterparts. Possibly the fact that in EC cell lines the chromosomes are of both maternal and paternal origin
may influence the pattern of X inactivation during their cellular differentiation. In normal development, cells in the extraembryonic tissues, which are derived from the trophectoderm or primary endoderm, undergo selective preferential paternal X inactivation (West et al. 1977; Harper et al. 1982). Clearly, preferential paternal X inactivation cannot occur in parthenogenones where both X chromosomes are of maternal origin.

The inactivation of a single X chromosome is known to occur in diploid parthenogenetic embryos (Kaufman et al. 1978) and in the extraembryonic membranes (Rastan et al. 1980). However, although undifferentiated cells are presumably only present for a short time in diploid parthenogenones, the presence of two genetically active, and identical, X chromosomes may not be tolerated in parthenogenetically derived EK cells; a partial deletion of one of the X chromosomes may occur as a form of compensatory mechanism conferring a developmental advantage on these cells.

A variety of breakpoints have been recorded in the parthenogenetically derived lines, and it is unclear at present whether the X deletion observed results from a progressive phenomenon which eventually results in the production of X0 lines. Our experimental data argue against this view, as the position of the breakpoint appears to be constant for a given line, and suggest that once the distal portion is lost, the cell genotype at least temporarily becomes stable. Experiments involving subcloning of XX/XX<sup>del</sup> genotype lines are currently being undertaken to test the validity of this hypothesis.

The extent of the deletion varies between HD lines, and the minimum deletion recorded (HD4 line) involves the loss of the chromosomal material distal to the XD band. If this deletion is sufficient to confer a selective growth advantage on these cells, it may imply that the most serious imbalance results from the influence of loci which map at the distal region. Alternatively, the deletion may be removing a controlling locus which affects the activity of the rest of the chromosome.

Parthenogenetically derived lines may prove to have potential use for mapping the X chromosome. The position of the breakpoint appears to be random, and there is no reason to believe that many more lines of an XX<sup>del</sup> genotype cannot be isolated from this source, thus providing large numbers of cell lines which are characterized by the loss of a variable but defined segment of a single X chromosome. Provided that the partially deleted X chromosome is active in the undifferentiated state, these lines would be useful for mapping of X-linked genes. Similarly, DNA from such cells might be used to locate recombinant DNA clones from mouse X-chromosome libraries.

In order that the pattern of X inactivation during the various stages in the differentiation of EK lines may be more closely investigated, we are currently preparing EK lines from embryos which either are heterozygous for X-linked isozymal variants or have paternally and maternally derived X chromosomes which are easily identified by the presence of obvious translocation markers.

**ACKNOWLEDGMENTS**

We are grateful to Richard Gardner for allowing us to quote unpublished results. We would like to thank Lesley Cooke, Marlene Dermody-Weisbrod, and Mary Knox for their technical assistance. This work has been supported by grants from the Medical Research Council (M.J.E. and M.H.K.), the Cancer Research Campaign (M.J.E.), and the National Fund for Research into Crippling Diseases (M.H.K.). A.B. is in receipt of an M.R.C. Studentship.
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CHAPTER 2

The origin, properties and fate of trophoblast in the mouse

M. H. KAUFMAN

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1. Introduction

In this chapter the experimental and histological findings which shed light on the origin, properties and eventual fate of the trophoderm cell lineage in the mouse are discussed. The mouse has been chosen because of all the species its pre- and early post-implantation development have been studied in greatest detail (Snell and Stevens, 1966). The two general approaches which have been used to study the early embryogenesis of the mouse and the events occurring during the peri-
implantation period are, of course, complementary. However, only a very limited number of accounts have been written in which both the histological and experimental findings have been presented together to emphasize this point. This therefore seemed an ideal opportunity to present an up-to-date summary of current views regarding the origin and fate of those cells in the pre-implantation embryo which are not involved in the production of the embryo proper. At all stages in the present discussion, the functional and morphological inter-relationship which exists between the inner cell mass and the trophectoderm lineages is stressed. In fact, by the expanded blastocyst stage, the trophectoderm cells have already played an essential role in the establishment of a distinct microenvironment within the embryo, and have become specialized for the task of establishing cellular contact with the uterine epithelium. This occurs shortly after the zona pellucida has been shed. Once implantation has occurred, a proportion of the trophoblast transforms into giant cells, while other cells of this lineage eventually become involved in the formation of the chorio-allantoic placenta.

Because of the difficulties which have been encountered in the past in the interpreting of histological material, particularly in attempts to establish the origin and lineage of certain cell types (for discussion of problems encountered see Rossant and Papaioannou, 1977), the results of the recent experimental studies will be considered first, as it will soon become clear that this provides essential background information necessary to understand the later morphogenetic interactions which take place between the cellular components of the embryo and its surrounding membranes. Some of the most valuable contributions towards our understanding of the events occurring during the early stages of embryogenesis in the mouse are based on microsurgical and micromanipulatory techniques which have been developed over the past ten years or so. These experimental findings are described in detail, as they shed light on the trophoblast cell lineage, and also provide evidence on the timing of critical differentiative events which take place within the early embryo (for recent reviews, see Gardner, 1975a; Gardner and Papaioannou, 1975; Rossant and Papaioannou, 1977; Rossant and Frels, 1981). Information of this nature derived from a wide range and variety of experimental sources has also helped to explain certain developmental features whose precise role had not been entirely clear.

In the second part of this chapter, I will concentrate on the morphological aspects of the role and fate of the trophectoderm cells during the process of implantation and shortly afterwards. The descriptive account presented here is based on an analysis of standard histological sections and ultrathin sections viewed by transmission electron microscopy of early implanting blastocysts, egg cylinders and primitive streak stage embryos. As with the experimental material considered in the first part of this chapter, the principal concern will be to establish the part played by the trophectoderm cell lineage in the normal sequence of events as it occurs in vivo.

During the pre-implantation period, only a rather general impression can be
formed of the origin and fate of the trophectoderm cells from morphological sources alone, and it is necessary to supplement this information by tracing the fate of suitably marked cells experimentally, taking particular care to ensure that during these studies the spatial relationship of a particular cell is identical or at least closely similar to that observed in the undisturbed embryo. Despite these obvious pitfalls, many studies involving these early stages of development have been carried out. However, since the isolation and experimental manipulation of early implanting blastocysts and egg cylinders is technically rather difficult, less experimental information is available concerning these stages of development. However, the results of the morphological and particularly the recent ultrastructural studies have gone a considerable way towards clarifying some of the complex events taking place during the early post-implantation period.

While much of the information, particularly regarding the observations on the origin of the trophectoderm lineage presented here, may well be valid in other species, it was considered that insufficient information was available on the early events occurring in these other species to justify a detailed comparison being made. Where possible, the events are considered in chronological order, so that the starting point for the present discussion will be the events occurring at the 8- to 16-cell stage, and this will be followed by an account of the events occurring during the late pre- and early post-implantation period.

2. Cell lineage studies

2.1. Formation of the inner cell mass and trophectoderm lineages

A considerable literature has recently developed on the events taking place during the brief transitional period between the 8-cell stage, when discrete blastomeres are observed, and the morula stage, when the outlines of individual blastomeres are no longer clearly delineated – during the process of 'compaction' (for observations on cellular changes occurring at this time, see Ducibella, 1977; Van Blerkom and Motta, 1979). This process is briefly considered in the present paper, as it is the first stage of development when the progenitors of two distinct cell lineages become apparent: at this stage an outer 'shell' of cells (the presumptive trophectoderm) surrounds an inner 'core' of cells, the presumptive embryonic cells, or progenitors of the inner cell mass, or ICM. Apart from their obvious inner and outer locations within the morula, the two cell types in the intact embryo already have certain characteristic morphological differences (Enders and Schlafke, 1965; Van Blerkom et al., 1973; Ducibella et al., 1975) as well as biochemical features which enable them to be distinguished; for example, their qualitative pattern of protein synthesis (Van Blerkom et al., 1976; Van Blerkom, 1977), and their sensitivity to [³H]thymidine (Snow, 1973). In fact, at an early stage of compaction, some ICM- and trophectoderm-specific proteins have been recognised in the inside
and outside cells, respectively (Johnson et al., 1977; Handyside and Johnson, 1978). However, in disaggregated embryos the ‘prospective potency’ of an individual cell type may well be quite different from its ‘prospective fate’ during normal undisturbed development, as disaggregation releases the cell from the normal constraints imposed on it by its environment (Weiss, 1939). Behavioural differences between trophectoderm and ICM cells have also been described when these cells have been isolated from their normal environment, and these additional features will be considered in due course. However, at these early stages, the fate of individual cells from one or another location may not be irreversibly fixed, and simple disaggregation and reaggregation studies have been carried out which clearly demonstrate that irreversible cellular commitment has not yet taken place, cells of one apparent lineage still retaining the potential to form tissues normally associated with cells derived from the other lineage. This is seen particularly in studies in which ICMs isolated at different times – from the advanced morula to the expanded blastocyst stage – are allowed to develop in culture, and the ‘prospective potency’ of individual cells examined.

2.2. Appearance and role of inter-cellular junctional complexes in early development

The transition from the 8-cell stage to the morula takes place within the distal extremity of the uterine lumen, embryos generally passing through the utero-tubal junction late on the second or early on the third day post-coitum. One of the most important morphological features to appear in the outer cells of the early compacting embryo is the presence of inter-cellular junctions and junctional complexes (Enders, 1971). These are of critical importance in that they enable the presumptive trophectoderm cells to form a permeability barrier or seal, so that the blastocoelic fluid is initially able to accumulate in the interstices between the cells, and then, once a sufficient amount has entered the embryo, enables the expanded blastocyst stage to be achieved. It is also likely that these complexes (zonular tight junctions) play an additional role in that they may be involved in the regulation of cellular cleavage products, as in the intact embryo the fate of individual cells is related to their position in a similar way to that described at the 8- to 16-cell stage, when the cell lineage ultimately depends on whether it is surrounded by other cells, or is located in an outer position with respect to the whole embryo.

At the 16-cell stage serial reconstructions suggest that there is on average only a single inside cell present, and that by the 64-cell stage there are on average about 11 cells in this location (Barlow et al., 1972). This dramatic difference, which is more than would be expected by synchronous cleavage alone, was explained by a possible faster rate of division of inside compared to outside cells. More recent studies, involving differential labelling of inside and outside cells (Handyside, 1981) indicate that there are probably substantially more inside cells at the 16-cell stage than originally thought. This would be sufficient to account for the numbers observed at the early blastocyst stage. What is clear though, is that by implan-
...tion, nearly 75% of the total cells in the blastocyst are no longer involved in the production of the embryo (Copp, 1978), but have become specialised for the task of establishing contact with the uterine epithelium and, once implantation has been achieved, in the formation of the chorio-allantoic placenta. The more specialised appearance (Enders, 1971) and properties (Gardner, 1972, 1975a) of the trophoderm compared to the ICM cells at the blastocyst stage may reflect the imminence of this functional burden. The permeability barrier formed by the trophectoderm cells enables the formation of a distinct microenvironment within the embryo (Hastings and Enders, 1974; Borland, 1977; Borland et al., 1977), which presumably facilitates the molecular and morphological differentiation of the ICM and its derivatives (Lutwak-Mann, 1971; McMahon, 1974; Ducibella, 1977; Pedersen and Spindle, 1980).

The cells of the ICM retain the essentially spherical form characteristically seen during the early cleavage stages, and only establish focal or macular inter-cellular contacts. Possibly the most important role of these complexes, in addition to their purely physical function of reducing cell to cell movement, is in mediating cell to cell communication (Griep and Revel, 1977; Magnuson et al., 1978; Lo and Gilula, 1979) and thus allowing groups of cells to coordinate their activity at the molecular level. Viewed in the context of the early embryo, the development of these low resistance junctions is of considerable morphogenetic significance, in that it enables the cellular components of the embryo to regulate their developmental fate as a group rather than at the level of the individual cell. However, because the focal junctional complexes do not provide a permeability seal, they allow the fluid transported by the trophoderm to pass between the ICM cells and eventually aggregate in a single loculus to form the blastocoelic cavity.

At the molecular level, it has been established that when compaction takes place in vitro, it is a calcium-dependent process (Wales, 1970; Ducibella and Anderson, 1975). It is thought that compaction is probably associated with a change in the rigidity of the plasma membrane, possibly accompanied by a change in the orientation and distribution of cortical cytoskeletal elements (Ducibella et al., 1977). Compaction is also associated with a major redistribution of surface microvilli from a uniform distribution over the whole surface during the earlier 'pre-compaction' cleavage stages, to a more localised distribution restricted to the apical region of the cell, and to the basal zone of inter-cellular contact (Ducibella, 1977). The functional significance of the redistribution of microvilli observed at this time may be that they play a role in facilitating cell to cell apposition prior to and during the formation of definitive junctional complexes, as both microvilli and other cellular projections have been observed to extend between cells at this time (Calarco and Epstein, 1973).

One of the early properties of trophoderm cells to become apparent is their ability to translocate organic and inorganic solutes and water from the extracellular environment centripetally (Daniel, 1963; Gardner, 1972). The mechanism by which these substances are transported into the embryo has been studied in the
mouse. While the details are beyond the scope of the present discussion it is interesting to note that the morphological basis of the mechanism is thought to be the tight junctions located between individual trophectoderm cells (McLaren and Smith, 1977). Their presence enables a physiological polarity to be established in these cells (Borland, 1977). However, the mechanism by which the mouse blastocyst concentrates elements within the blastocoel to produce its distinct microenvironment has yet to be fully established. In addition to the intra-cellular concentration of these elements, a considerable volume of fluid passes into the inter-cellular spaces. However, by about the 16- to 32-cell stage, as the volume of fluid accumulated within the embryo increases, it tends to aggregate into a single loculus termed the blastocoel or blastocyst cavity. The volume of fluid gradually increases until, when the embryo has about 64 blastomeres, it tends to emphasize the two morphological cell components of the embryo. The trophectoderm cells become flattened and squamous in type, while the ICM cells tend to retain their original rounded form. With each cell division the volume of individual cells diminishes, so that by this stage each ICM blastomere has a diameter of less than 10 μm.

Between the 16- and 32-cell stage, with the establishment of the blastocoel, the cells of the embryo are clearly segregated into trophectoderm and ICM, though at the ultrastructural level one of the only distinguishing features is the presence of tight junctions in the former group (Enders and Schlafke, 1965). Since the blastocyst stage persists for almost 2 days in the mouse, various attempts have been made using ultrastructural criteria, mainly based on the progressive appearance of various types of inter-cellular contacts (tight junctions, desmosomes, focal tight junctions), an assessment of the relationship between individual ICM cells, and ICM and trophectoderm cells, and the presence or absence of proximal endoderm, to subdivide this period into various sub-stages (for example, see Nadjicka and Hillman, 1974).

2.3. ‘Inside-outside’ hypothesis, and the importance of cell position within the embryo

The two distinct but obviously inter-related processes, namely differentiation of the ICM and trophectoderm cells, and formation of the blastocoel, are generally considered to be one of the earliest stages of embryonic morphogenesis. It had been suggested that differentiation into the two components was due to cellular polarity, in that these two lineages were derived from specific regions of the cytoplasm of the fertilized egg which were subsequently distributed among the blastomeres following cytokinesis (Mulnard, 1955, 1961; Dalcq, 1957). However, recent experimental evidence has not verified this hypothesis, and some authorities have even suggested that the earlier cytochemical observations which indicated
the presence of cytoplasmic regionalization may have been artefactual in origin (Solter et al., 1973). In fact, most authorities now favour the idea that during the early cleavage stages all blastomeres are labile (Mintz, 1965), and that at about the 8- to 16-cell stage the component blastomeres differentiate according to their position in the embryo. This ‘inside-outside’ hypothesis (Tarkowski and Wroblewska, 1967) is supported by numerous studies, particularly those involving the use of aggregation chimeras (Mintz, 1965; Hillman et al., 1972) and experiments in which inside and outside cells from 8-cell embryos and morulae were labelled with either injected silicone oil droplets (Wilson et al., 1972) or [3H]thymidine (Hillman et al., 1972 – the cell populations in this study were also genetically distinct), and their ultimate location identified at the blastocyst stage. This view is also supported by the studies in which individual blastomeres from embryos at the 2-cell stage (rat: Nicholas and Hall, 1942; mouse: Tarkowski, 1959a,b; rabbit: Seidel, 1956, 1960) and 8-cell stage (rabbit: Moore et al., 1968) were shown to be capable of development to term. Complete development to term from a single 8-cell blastomere has not been achieved in the mouse (Rossant, 1976a), but 8-cell blastomeres are capable of giving rise to both ICM and trophectoderm derivatives (Kelly, 1975).

It is also now well established that development from the 1-cell stage to the blastocyst stage can take place completely in vitro and thus in the absence of maternal influences. This has been achieved with fertilized eggs (Whitten and Biggers, 1968) and parthenogenetically activated eggs (Kaufman and Sachs, 1976), and both classes of eggs are capable of implanting in the uterus of pseudopregnant recipients.

The exact timing of the determinative event has yet to be established, but it seems likely that this may be a progressive process, with cells becoming determined as they become enclosed (Rossant and Papaioannou, 1977). In this respect it would not be surprising if a whole range of inter-relating factors, including the development of specific types of junctional complexes, and the establishment of new synthetic processes, as manifest by the recognition of ICM- and trophectoderm-specific proteins (Johnson et al., 1977; Handyside and Johnson, 1978), played a critical role at this time.

Other evidence suggesting the importance of cell position during the 8- to 16-cell period comes from recent studies in which the surface of isolated blastomeres was labelled with fluorescent concanavalin A (Con A). These indicated that at the 8-cell stage the development of surface polarity was highly dependent on cell contact. When cells were isolated at the 4-cell stage and allowed to divide in culture, the orientation of the localised Con A binding on the surface of the cleavage products was invariably away from the point of contact (Ziomek and Johnson, 1980), regardless of their previous orientation within the embryo or the plane of cleavage. Thus, at the 8-cell stage, evidence of radial polarity is already apparent. Similar observations on the Con A binding pattern of the cleavage products generated when individual 8-cell blastomeres are allowed to divide in culture have
also been reported (Johnson and Ziomek, 1981). At this stage of embryonic development in most cases the pole of localised Con A binding was distributed to only one of the daughter cells. Parallel scanning electron microscopy studies demonstrated that the area of maximum binding was the region which contained a clearly defined concentration of microvilli (Johnson and Ziomek, 1981). It has been proposed that the apolar population so formed gives rise to the ICM cells, while the polarised cells, which are located at the periphery of the embryo, give rise to the trophoblast (Handyside, 1980, 1981; Reeve and Ziomek, 1981). The dissimilarity in the appearance of the two division products is illustrated in Fig. 1.

By the fourth day, as soon as the blastocoele has developed, two populations of trophoblast may be distinguished: the polar trophoblast overlying the ICM, and the mural trophoblast in association with the blastocoele. At least initially, the mural and polar trophoblast cells have similar properties (Gardner et al., 1973), though once the zona pellucida is lost their individual fate is markedly different, and for this reason their respective lineages are best considered in isolation.

Observations on the timing of blastocoele formation in the intact embryo indicate that this is more closely associated with developmental age than cell number. Experimental manipulation of the total cell number of an embryo, by halving, doubling or tripling it did not substantially affect the timing of this event. Similarly, treatment of 2-cell embryos with cytochalasin-B, to suppress the second cleavage division with the subsequent formation of a tetraploid embryo, had minimal effect on the timing of blastocoele formation (Smith and McLaren, 1977).

At about the 32- to 64-cell stage, the blastocyst sheds its zona pellucida in order to facilitate the direct cellular contact between the trophoblast and the uterine epithelium necessary for implantation to take place. This has been termed the ‘hatching phase’. Various subsequent phases of implantation are described, and their morphological features are considered in detail in Section 3.

2.4. Information gained from microsurgical and immunosurgical studies on blastocysts

Before presenting a more detailed and essentially descriptive account of the events associated with implantation, the results of certain experimental studies which shed light on some of the properties of the trophoblast of the 16- to 32-cell stage embryo will be described. In these studies, blastocysts were bisected micro-

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Fig. 1. Cellular organisation during division of a single 8-cell blastomere in vitro as assessed by scanning electron microscopy. (a) Single 8-cell blastomere prior to division; (b) early 8-cell cleavage furrow formation, microvilli mainly at one pole; (c) late cleavage furrow, note that microvilli now extend over much of one of the newly formed blastomeres; (d) cleavage almost completed, to reveal one large microvillous cell and one slightly smaller cell with few microvilli; (e) two 16-cell blastomeres now formed, with a heavy concentration of microvilli present at one pole of the larger cell. Scale bar = 10 μm. (From Johnson and Ziomek, 1981; reproduced with the permission of MIT Press.)
surgically and two uncontaminated components obtained: pure ICM, after separation of these cells from the partial covering of polar trophoblast, and mural trophoblast. The properties of these tissues were then compared both in vitro, and in vivo following their transfer to the uteri of pseudopregnant recipients (Gardner, 1971, 1972).

The principal feature exhibited by mural trophectoderm fragments retained in culture was that they reformed into small vesicles (trophectoderm vesicles). When such vesicles were transferred to the uteri of pseudopregnant recipients, a similar proportion implanted compared to control blastocysts transferred to the contralateral uterine horns (Gardner, 1972). A limited number of giant cells were recognised at the sites of implantation of the trophectoderm vesicles. In only two out of sixty-eight implants were ICM derivatives present, and were almost certainly due to contamination of the trophectoderm fragments by ICM cells at the time of microsurgery (Gardner, 1972). Microsurgically isolated trophectoderm vesicles are also capable of entering a 'delayed' state in vivo, and will subsequently implant when given an appropriate hormonal stimulus (Surani and Barton, 1977). When retained in culture, the vesicles did not aggregate with similar vesicles or trophectoderm fragments. Isolated ICMs, however, did not form vesicles in culture, but did readily aggregate with other ICMs. Pure ICMs failed to implant when transferred to the uteri of pseudopregnant recipients.

Another property of trophectoderm cells is their ability to phagocytose melanin granules (Gardner, 1975a). This property is not shared by the blastomeres of cleavage stage embryos nor by ICM cells, though blastomeres from advanced cleavage stages can immobilize granules on their surface. Regional differences in phagocytic behaviour were observed, in that the mural trophectoderm showed both heightened phagocytic and intra-cellular digestive activity compared to polar trophectoderm. This property of phagocytosis is particularly evident in the early post-implantation period (Billington, 1971; Owers, 1971) once giant cell transformation has taken place.

Another, and technically less demanding, method for obtaining ICM tissue from blastocysts uncontaminated by trophectoderm is by the technique of immunosurgery (Solter and Knowles, 1975). This technique has the advantage that it also facilitates the isolation of inside cells from the morula stage onwards, and has also been used to investigate the properties of ICM cells. These studies have shown that ICMs isolated from early blastocysts are still capable of giving rise to typical giant cells in vitro (Handyside, 1978; Hogan and Tilly, 1978a,b; Spindle, 1978) and functional trophectoderm derivatives in vivo (Rossant and Lis, 1979). Similar giant cells are also observed when ICMs isolated immunosurgically from expanded diploid parthenogenetic blastocyst stages are retained in vitro (Handyside and Kaufman, unpubl. data, reported in Kaufman, 1981). These results would seem to indicate that at least a proportion of the ICM cells of the blastocyst are still labile.

The division of the trophectoderm at the expanded blastocyst stage (at 4½ days
of development) into a mural and a polar component is fully justified on functional grounds, in that they give rise to two distinct cell lineages. The mural trophoectoderm cells away from the influence of the ICM cells (Gardner, 1971, 1972; Barlow and Sherman, 1972; Ansell and Snow, 1975) stop dividing, and, after implantation, form the primary mononuclear trophoblast giant cells (Dickson, 1963). Biochemical evidence indicates that during giant cell transformation, repeated endoreduplication of the entire genome occurs (Sherman et al., 1972; for review, see Ansell, 1975). The first suggestion of nuclear enlargement occurs at about 117 hr post-coitum in the mouse (Barlow et al., 1972), and Ansell (1975) has calculated from an analysis of hatching and outgrowing mouse blastocysts in vitro that the time taken for the nuclear volume (and therefore DNA content) to double is of the order of 8–9 hr. Barlow and Sherman (1972) had previously calculated that between the 6th and 11th days of pregnancy the ‘cell cycle’ time of the largest class of trophoblast nuclei was in the order of 24 hr.

2.5. Observations on the inter-relationship between the inner cell mass and the trophoblast

Barlow and Sherman (1972) have suggested that nuclear enlargement may be related to the proximity of these trophoectoderm cells to the blastocoele. These authors have also suggested that the ICM may ‘inhibit’ or ‘protect’ the trophoectoderm in the polar region of the blastocyst from this stimulus. Giant cell transformation eventually involves the whole trophoectoderm except those cells in the region immediately overlying the ICM (Dickson, 1966). The polar trophoectoderm, however, continues to divide, possibly as a result of an inductive interaction with the underlying ICM, eventually forming the ectoplacental cone. Gardner et al. (1973), using a similar argument, have proposed that the diploid ectoplacental trophoblast cells located most distant from the sphere of influence of the ICM and its derivatives transform into the secondary giant cell population.

The effects of the presence or absence of the ICM on trophoblast development have been investigated by Ansell and Snow (1975; see also Ansell, 1975). These authors observed the effect of culturing intact and ICM-reduced blastocysts in ‘outgrowth’ medium. A progressive reduction in the volume of the ICM was brought about by culturing mouse embryos from the 2-cell stage to the blastocyst in increasing concentrations of [3H]thymidine. Under these experimental conditions, the trophoblast of control blastocysts proliferates and undergoes giant cell transformation, whereas in ICM-reduced blastocysts giant cell transformation occurs, but proliferation is reduced. However, in the ICM-denuded blastocysts no proliferation occurs, though giant cell transformation is possibly more rapid than normal. These results indicate a possible role of the ICM in trophoblast proliferation, and this conclusion is further supported by the results of transfers of similarly treated embryos to ectopic sites, where proliferation only occurred in controls and in treated embryos containing some ICM cells. These results clearly
demonstrate that the ICM is required for trophoblast development. However, it has yet to be established whether the trophoderm is necessary for ICM development, though isolated ICMs may develop as far as blood island and mesoderm formation (Hogan and Tilly, 1978a,b; Wiley et al., 1978).

2.6. Information gained from the study of interspecific chimeras

Further evidence of the role of trophoderm at implantation comes from the experimental production and analysis of the fate of interspecific chimeras between rat and mouse embryos. Chimeras formed by the injection of rat ICMs into mouse blastocysts are capable of implanting and developing in mouse recipients (Gardner and Johnson, 1973, 1975). However, chimeras formed by the aggregation of rat and mouse morulae (Mulnard, 1973; Stern, 1973; Zeilmaker, 1973; Tachi and Tachi, 1981) implant in the mouse uterus, but do not develop beyond implantation (Rossant, 1976b). In the former case, the trophoderm is entirely derived from mouse cells, whereas in the aggregation chimeras the trophoderm is derived from cells of both species. This species specificity is also seen in interspecific transfers between rats and mice. Rat blastocysts transferred to mouse uteri, but the ICM derivatives that form only progress as far as the early egg cylinder stage, and these tend to be morphologically extremely disorganised, and very little trophoblast proliferation occurs (Tarkowski, 1962; Rossant, 1976b; Surani, 1977). It has been suggested that in these cases the interaction between the trophoderm and the uterine endometrium is abnormal (Tachi and Tachi, 1979).

The successful production of liveborn Mus musculus–M. caroli chimeras following the injection of M. caroli ICMs into M. musculus blastocysts (Rossant and Frels, 1981) and the subsequent transfer of these chimeras into mouse recipients is of great interest, considering the very poor rate of development of M. caroli blastocysts transferred to the uteri of M. musculus recipients (Frels et al., 1980). This, once again, stresses the importance of the trophoblast at and shortly after implantation. In the chimeric study, the M. caroli cells were apparently 'protected' by M. musculus trophoblast, whereas in the transfer study, the cells from the two species interacted and embryonic death generally occurred shortly after the egg cylinder stage was achieved.

2.7. The polar trophoderm lineage

As indicated earlier, shortly after implantation the mural trophoblast cells cease dividing and transform into the primary giant cells. At about the same time, the polar trophoblast cells overlying the ICM continue to divide (Copp, 1978) and provide a stem cell population which gives rise principally to the ectoplacental cone, but also, in all probability, to further mural trophoblast cells at its periphery (Gardner and Papaioannou, 1975). With the considerable degree of cellular proliferation occurring in the ectoplacental cone (Fig. 16), the cells at its periphery
become located some distance away from the sphere of influence of the ICM, and transform into the secondary giant cells. Either because of active migration, or possibly because of the considerable growth in volume of the egg cylinder at this time, the secondary giant cells eventually completely surround the conceptus and its membranes. It is not entirely clear whether the stem cell population is retained within the ectoplacental cone, or maintains its close association with the embryonic derivatives of the ICM, and becomes located in the extra-embryonic ectoderm region of the early egg cylinder. While the trophectodermal origin of the ectoplacental cone and secondary giant cells has long been recognized (Snell and Stevens, 1966; for brief historical survey, see Rossant and Papaioannou, 1977), and eventually confirmed experimentally (Gardner et al., 1973), the origin of the extra-embryonic ectoderm, which is histologically clearly in continuity with the ectoplacental cone (Snell and Stevens, 1966), has been the subject of much debate. However, recent evidence from microsurgical studies involving the production and analysis of interspecific chimeras between rat and mouse embryos (Gardner and Johnson, 1973, 1975; Gardner, 1975b) has clearly demonstrated that this tissue is also derived from the polar trophectoderm via cellular proliferation originating in the ectoplacental cone region of the conceptus (Figs. 15a,b, 16 and 18a,b). In none of the chimeras in which rat ICMs were injected into mouse blastocysts did rat cells make any contribution to either the mural or ectoplacental trophoblast or the extra-embryonic ectoderm.

In more recent studies in which pieces of extra-embryonic ectoderm isolated from mouse embryos up to 8½ days post-coitum were either transferred ectopically or retained in vitro, in all cases with the morphological characteristics of trophoblast giant cells were formed (Rossant and Ofer, 1977). Indeed, if further confirmation was required, this has now been provided by Rossant et al. (1978) who have injected 5½- and 6½-day extra-embryonic ectoderm into 3½-day mouse blastocysts and demonstrated that the former contributed exclusively to the ectoplacental cone and/or trophoblast giant cell fractions, reflecting the trophectodermal origin of these cells.

2.8. Primitive endoderm formation

In addition to the various cell groups already considered, a further region of cellular proliferation is evident on the blastocoelic surface of the mouse ICM either shortly before, or more commonly at about the time of hatching from the zona pellucida (Nadijcka and Hillman, 1974). This is termed the primitive endoderm or hypoblast, and initially appears as a monolayer of cells overlying the ICM and its derivatives (the visceral endoderm) (Figs. 3b, 4b and 7). This rapidly expands at its periphery to spread over the inner surface of the trophoblast, and is then termed the parietal endoderm (Snell and Stevens, 1966) (Fig. 14a,b).

It is now established from both microsurgical (Rossant, 1975) and immunosurgical (Solter and Knowles, 1975) studies that the endoderm is derived from the
ICM alone, and that ICMs isolated by both techniques develop an outer 'shell' of cells which are morphologically indistinguishable from endoderm (Enders, 1971). The proximal (or visceral) endoderm of the 5½-day mouse embryo overlying both the embryonic and extra-embryonic ectoderm subsequently only contributes to the endoderm of the visceral yolk sac (Gardner and Papaioannou, 1975), while the parietal (or distal) endoderm cells, which at no time form a continuous sheet of cells, are primarily involved in the formation of Reichert's membrane (Wislocki and Padykula, 1953; Clark et al., 1975a,b; Enders et al., 1978) (Fig. 14). Somewhat surprisingly, microsurgical studies have also indicated that the definitive gut endoderm arises from the primitive ectoderm (Levak-Svajger and Svajger, 1974). A summary of current views regarding the cell lineage relationships in early mouse embryogenesis is presented in Fig. 2.

3. Morphological studies

3.1. General observations on methodology

The justification for considering in some detail the results of studies involving the experimental manipulation of early embryos, is that these findings provide the clearest indication of the fate of the various derivatives of the pre-implantation embryo. Previously, this information has proved to be difficult or impossible to establish from the analysis of serially-sectioned material alone. In this section, a morphological account of the events occurring at and shortly after implantation in the mouse will be given, in order to provide additional background information
on the fate of the derivatives of the trophoderm during the peri-implantation period.

Obviously, the most accurate indication of the relationship which exists between the various cell types in the in vivo situation can best be achieved by the analysis of well-fixed material. This is particularly important when the morphology of the various stages of implantation is to be studied, and can best be achieved when appropriate material is fixed by perfusion with glutaraldehyde (Sabatini et al., 1963; Karlsson and Schultz, 1965; Schultz and Karlsson, 1965) and embedded in plastic (for early ultrastructural studies of implantation, see Reinius, 1965, 1967). This technique generally gives excellent cellular preservation, which enables the cell-to-cell contacts being established at this time to be examined in detail. While the general relationships which exist between individual tissues at later stages of development can be examined adequately by standard immersion fixation and paraffin embedding, the earlier stages inevitably display shrinkage artefacts. Thus, for example, the trophectoderm of an implanting blastocyst, which should be either apposed or even adherent to the surface of a uterine epithelial cell may, due to shrinkage, have become separated by a considerable distance. This, of course, can also pose considerable problems of interpretation, when the orientation of individual zona-intact and zona-free blastocysts is being studied (Smith, 1980). At the early egg cylinder stage, shrinkage generally results in the separation of the various germ layers.

Most of the illustrations of implantation sites presented in this chapter were obtained by initially fixing the uteri of anaesthetized female mice at appropriate stages of pregnancy by vascular perfusion with a solution of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. The material was then post-fixed with 1% osmium tetroxide in the same buffer, dehydrated through a graded ethanol series and eventually embedded in epoxy resin (Spurr, 1969). Blastocyst-containing sites were identified by serial sectioning, and thin sections cut with a Huxley ultramicrotome, stained with uranyl acetate and lead citrate and viewed in a Philips 300 electron microscope. The semi-thin sections (thickness about 0.5-0.75 μm) were stained with methylene blue and examined with a Leitz photomicroscope.

3.2. The events associated with implantation in the mouse

In the following section the terminology used is in accordance with that suggested by Sherman and Wudl (1976). Accordingly, 'trophectoderm' will be used to describe the outer layer of cells of the expanded blastocyst stage, while the term 'trophoblast' will be used once the adhesion phase of implantation has been achieved when describing these cells and their derivatives.

3.2.1. Hatching phase

Before the expanded zona-intact blastocyst can implant, the embryo must shed the zona pellucida. Several hypotheses have been proposed to explain how this
phenomenon is achieved, and it now seems relatively clear from information gained from both in vivo and in vitro sources that the process is a complex one. That a high proportion of early cleaving embryos can develop to the blastocyst stage in culture and successfully hatch (Whitten, 1971) seems to indicate that this is an intrinsic property of the mouse embryo which can take place in the absence of external signals of maternal origin.

As early as 1935 it had been observed that blastocysts could expand and contract in culture (Lewis and Wright, 1935), and this activity, which has been studied by cinemicrography (Borghese and Cassini, 1963; Cole and Paul, 1965; Cole, 1967), probably plays a part in the mechanical rupture of the zona pellucida. However, the results of various experimental studies indicate that the uterus probably also participates in the hatching process, possibly by the production of a uterine zonalytic factor (McLaren, 1970; Mintz, 1971) which appears to be under the control of ovarian hormones (McLaren, 1968). In all probability, the two processes, namely the mechanical expansion and contraction of the blastocyst, and the activity of the zonalytic factor, are both involved to a greater or lesser degree in the escape of the blastocyst from the zona pellucida. As a reflection of the uncertainty of some authorities as to the primary or subsidiary role of these processes, it has even been suggested that the pulsatile movements observed may merely reflect phases in the uptake of ions and solutes from the external environment (Cole, 1967), and that the zonalytic factor may play a more important role in the initiation of implantation (Pinsker et al., 1974).

3.2.2. Apposition phase

Once the blastocyst has escaped from the zona pellucida, it has to orientate itself in relation to the immediately adjacent uterine epithelial cells as well as in relation to the whole uterus. The few in vivo studies which have examined this stage indicate that it is of relatively short duration, lasting possibly only about 2 hr (Kirby et al., 1967; Bergström and Nilsson, 1971).

In the mouse the ultrastructural relationship which develops between the trophoderm and the uterine luminal epithelium has been described in detail (Potts, 1966, 1968, 1969; Potts and Wilson, 1967; Kirby et al., 1967; Nilsson, 1967; Reinius, 1967; Smith and Wilson, 1974; Poelmann, 1975) (Fig. 8). During this stage, individual trophoderm cells are flattened and have a considerable surface area which enables each of them to make close contact with possibly up to 8 or 10 epithelial cells (Potts, 1968). The luminal surface of the endometrial cells at this stage is covered by large numbers of microvilli, whereas the trophodermal surface of the embryo possesses far fewer microvilli (Reinius, 1967; Potts, 1969; Smith and Wilson, 1974). The blastocyst attaches on the antimesometrial wall of the uterus, with the ICM directed towards the mesometrium (Fig. 3a,b), and various experimental studies indicate that the ultimate control of blastocyst orientation appears to be uterine rather than embryonic (Wilson, 1963; Wilson and Potts, 1970).
The exact details regarding the underlying mechanism of blastocyst orientation within the implantation site have yet to be established, but the early hypothesis of ICM rotation (Kirby et al., 1967; Kirby, 1971), in which an active participation by the blastocyst is proposed, in response to an oxygen gradient within the uterus, is not universally accepted. In favour of this hypothesis is the report by Nadijcka and Hillman (1974) who confirmed the observation previously reported by Kirby et al. (1967) that the desmosomes and focal tight junctions initially established between the ICM and trophectoderm tend to be lost at the time of implantation, and that of Jenkinson and Wilson (1970) who claimed to have observed rotation of the ICM within the trophectoderm. However, the microsurgical experiments by Gardner (1975b) would seem to argue against the validity of this hypothesis. Gardner (1975b) marked trophectoderm cells at various locations in the blastocyst and, following their transfer to recipients, demonstrated that in no case did the position of the marked cell change in relation to its original position vis-à-vis the ICM. While many blastocysts cultured in vitro attach at their abembryonic poles, this is not invariably the case (Cole and Paul, 1965; Sherman and Salomon, 1975). Indeed, it is possible that the whole blastocyst may rotate within the uterine lumen until the embryo achieves a suitable orientation (Sherman and Wudl, 1976).

3.2.3. Adhesion and attachment phase
After the blastocyst has become correctly aligned, the actual process of implantation is initiated, and it is thought that this may involve a change in both the physical and biochemical properties of the apposing cell surfaces. The first site of attachment to the luminal surface is generally in the region of mural trophectoderm at the abembryonic pole of the blastocyst, and it has been observed that the region of attachment spreads fairly rapidly to extend over the whole surface of the blastocyst (Potts, 1966, 1969; Nilsson, 1974). Indeed, it has been estimated that the total duration of this phase is in the region of 10-15 hr (Potts, 1968). At the onset of this phase, the surface of the trophectoderm is relatively smooth, whereas the luminal surface of the endometrial cells contains large numbers of microvilli. During the adhesion phase the microvillous projections largely disappear, thus bringing the two surfaces into intimate contact (Fig. 5b), and facilitating the process of attachment (Fig. 6b) (Nilsson, 1966, 1967; Potts, 1969). Eventually a gap of only 15-20 nm separates the two cell surfaces, and a homogeneous electron-dense extra-cellular deposit appears in this space (Potts, 1966, 1968, 1969). In some species cellular continuity develops between the apposing cell layers (rabbit: Larsen, 1961, 1970; Enders and Schlaflke, 1971), though it is not entirely clear whether this occurs in the mouse or not, as attempts to establish this point were unsuccessful (Nilsson, 1970; Smith and Wilson, 1974).

Most of the in vivo and in vitro evidence so far available indicates that various steroid hormones, such as oestrogen and progesterone, have their principal effect on the uterus rather than on the blastocyst, though the possibility cannot be ruled out that these hormones also act on the embryo (Gwatkin, 1966a; Salomon and
Attempts have also been made using various in vitro model systems to establish the necessary requirements for blastocyst attachment to take place (for reviews, see Sherman and Salomon, 1975; Sherman and Wudl, 1976). In general, these experimental systems simulate relatively few of the dynamic aspects of implantation as it occurs in vivo, but have provided useful information on the metabolic requirements of the embryo at this time (for example, see Gwatkin, 1966a,b; Sherman and Barlow, 1972; Jenkinson and Wilson, 1973; Spindle and Pedersen, 1973; Nilsson, 1974). Other biochemical studies have indicated that the glycoproteins on the apposing cell surfaces may be altered in preparation for implantation (Pinsker and Mintz, 1973; Schlafke and Enders, 1975), possibly as a result of the proteolytic action of the uterine zonalytic factor (Pinsker et al., 1974).

Once blastocyst attachment has occurred, the earliest evidence of a decidual cell reaction (De Feo, 1967) is generally apparent in the stromal cells closest to the uterine lumen (Reinius, 1967). These are the earliest structural signs of the maternal recognition of pregnancy, and are associated with a localized increase in endometrial vascular permeability (Psychoyos, 1971, 1973) and presence of oedema in the deeper sub-epithelial layers. The first histological evidence of a decidual reaction is observed in the antimesometrial aspect of the implantation site (see, for example, Figs. 3a,b, 4a,b and 6a), and is presumably related to the profound influence of the trophoderm cells in this region. The reduced or impaired response in the rest of the implantation site may reflect an inhibitory effect of the ectoplacental cone or inner cell mass on this reaction. In the rat, these changes, as well as the pontamine blue reaction, may be inhibited by the injection of indomethacin, which interferes with prostaglandin synthesis (Kennedy, 1977; Lundqvist and Nilsson, unpubl. data, cited in Enders and Schlafke, 1979), suggesting that this agent may act as an intermediary in this process (see also Kennedy and Armstrong, 1981).

3.2.4. Invasive phase

During this period, the trophoblast cell processes initially insinuate themselves into the uterine epithelial layer almost as far as the basement membrane (Fig. 9) (Finn and Lawn, 1968; Potts, 1968; Smith and Wilson, 1974), and eventually displace the resident epithelial cells in this region (Schlafke and Enders, 1975), phagocytosing a proportion of the epithelial cells in the process (Fig. 9) (Poelmann, 1975).

It has been observed that some of the uterine epithelial cells begin to show signs of degeneration even before zona lysis has taken place, suggesting that the changes may in fact be autolytic in nature (Smith and Wilson, 1974; El-Shershaby and Hinchcliffe, 1975), and possibly even 'programmed' (Finn and Bredl, 1973) as in other examples of morphogenetic cell death (Glücksmann, 1951; Saunders, 1966; Menkes et al., 1970). The degeneration and phagocytosis of the cells would provide ready-made pathways which could facilitate the movement and ingress of
invading trophoblast cells. As the epithelium remains intact mesometrially, this possibly lends weight to the suggestion that these cells provide a form of direction stimulus to the invading cells (Finn and Lawn, 1968; Mulnard, 1970). In addition to the death of the epithelial cells just described, a small number of ICM cells also appear to die at this time. These two groups constitute the W-bodies (Wilson, 1963; Finn and McLaren, 1967), though whether the cellular loss in these two locations is related or not remains to be determined.

The phagocytic nature of the trophectoderm cells has long been recognised (Amoroso, 1952). Smith and Wilson (1974) have claimed that prior to the invasion process, the trophoblast cells only phagocytose dead and dying cells (Fig. 9), possibly for nutritive purposes at this stage (Boyd and Hamilton, 1952), whereas during the invasion phase both living and dead cells may be engulfed (Fig. 13). Digestion of the engulfed cells is well under way after about 10 hr, as evidenced by the presence of increased numbers of Golgi vesicles (El-Shershaby and Hinchliffe, 1975).

Once displacement of the luminal endometrial cells has taken place, the trophoblast cells become located in close proximity to the basement membrane of the uterine epithelial cells (Fig. 9) (Potts, 1969; Smith and Wilson, 1974). During the final part of this phase, the basement membrane is penetrated by the trophoblast cells which then migrate into the uterine stroma (Figs. 10b and 12a,b). The invasion is particularly apparent at the periphery of the ektoplacental cone, where the invading trophoblast cells burrow deeply into the decidual tissue (Figs. 12a,b), and eventually displace the endothelial cells lining the maternal blood vessels, bringing the two circulations into close contact (for detailed histological study, see Amoroso, 1952). Placentation in the mouse is of the haemochorial type as the trophoblast cells come into direct contact with the maternal blood (Boyd and Hamilton, 1952; Schlafke and Enders, 1975).

The invasive phase of trophoblast growth continues until about the 10th day of pregnancy in the mouse (Kirby, 1965). While the evidence is inconclusive, it appears that the trophoblastic invasion which occurs in the normal implantation site is inhibited when these cells make contact with healthy decidual cells (Kirby, 1965; Billington, 1971). Evidence from in vitro sources indicates that, in addition to the possible inhibitory influence of healthy decidual cells, trophoblast cells may be 'programmed' to cease their invasive activity on or about the 10th day of gestation, though death of the trophoblast giant cells occurs several days later (Sherman and Wudl, 1976).

3.3. Cytological and morphological aspects of giant cell formation

At various times in the present text the term 'giant cell transformation' has been used with reference to the eventual fate of a considerable proportion of the trophectodermal lineage. This therefore seems an appropriate point to consider the cytological and morphological changes which take place during this process. In
the zona-intact blastocysts of rodents, mitotic figures are prevalent in the trophoectoderm (rat: Alden, 1948; Bridgman, 1948; Dickson and Bulmer, 1960), and in the early post-implantation period, despite the absence of mitotic figures, various groups have demonstrated that the giant cells surrounding the early conceptus are capable of actively synthesising DNA (mouse: Atlas et al., 1960; Saccoman et al., 1967; rat: Jollie, 1964). By transplanting 34-day mouse embryos ectopically, Avery and Hunt (1969; Hunt and Avery, 1971) obtained relatively pure growths of trophoblast which progressed from small dividing cells to large non-dividing giant cells with nuclear diameters of 80–100 μm. Cytophotometric measurements in individual cell nuclei indicated a massive increase in Feulgen-DNA.

Following an extensive study of trophoblast giant cells in the rat and rabbit, Zybina proposed that DNA replication occurs by a process of endoreduplication, that is, replication of the nuclear material without subsequent mitosis and cell division (Zybina, 1961, 1963; Zybina and Mos’yan, 1967), with the resultant formation of polytene chromosomes (Zybina, 1970). In a similar study in the mouse, Barlow and Sherman (1972) measured the DNA content of trophoblast nuclei between the 5th and 11th days of pregnancy, and observed that measurements on the largest nuclei indicated that they contained up to 850 times the haploid value of DNA. The proportion of trophoblast cells with a DNA content greater than 4C rose steadily, so that on the 7th day 8% of the nuclei examined had a DNA content greater than 4C, and by the 8th and 9th days, 30–40% were in this group. However, no evidence of polytene chromosomes was found. As far as the onset of polyploidization is concerned, these authors found that only a small proportion of the cells of the 5th day blastocyst (just prior to implantation) had DNA contents significantly above 4C, thus confirming the earlier findings of Graham (1971) and Barlow et al. (1972). Examination of the ectoplacental cone region clearly demonstrated that between the 7th and 11th days of gestation diploid cells were always present in excess of polyploids, which accords with the hypothesis that this is the likely source of the trophoblast stem cell population during the early post-implantation period (Fig. 16). Some investigators have suggested that trophoblast giant cell nuclei are formed by fusion of adjacent trophoblast cells (for example, see Jollie, 1964; Schlesinger and Koren, 1967; Saccoman et al., 1967), but direct evidence to support this hypothesis is still lacking.

No distinction was made between the so-called primary and secondary giant cells in these studies, and, in fact, the two groups are indistinguishable as far as their nuclear DNA content and phagocytic behaviour are concerned. However, while the primary giant cells tend to remain mononuclear, the nuclei of the secondary giant cells tend to break up into smaller sub-nuclear fragments (Zybina and Tikhomirova, 1963; Zybina and Mos’y yan, 1967).

No obvious giant cells were observed in the implanting blastocysts isolated from females autopsied during the early morning on the 5th day of pregnancy in the present series (Figs. 3 and 4), whereas individual antimesometrially located trophoectodermal cells in embryos examined during the early afternoon on the 5th
day showed clear evidence of giant cell transformation. In these cells, a very considerable degree of nuclear enlargement was already apparent, as was the presence of large quantities of phagocytosed material (Fig. 9). Indeed, these observations serve to stress the point that the transition from a mitotically active diploid state to the non-dividing state in which polyploidisation of the DNA occurs is a fairly gradual process. The early stages of giant cell transformation, which are first manifest in the mural trophectoderm cells in the abembryonic pole, are, in fact, often more clearly seen under phase contrast optics (Dickson, 1963, 1966) than in standard histological sections or on ultrastructural analysis. However, it should be recalled that giant cells in this location were recognised in histological sections of early implanting rodent blastocysts almost 100 yr ago by Selenka (1883) and Duval (1892). In the study by Dickson (1963) about three-fourths (117 out of 157) of normal (non-delayed) zona-free blastocysts recovered from the uterus and only a small proportion (8 out of 134) of delayed mouse blastocysts showed evidence of giant cell transformation. The first suggestion of giant cell transformation reported by Barlow et al. (1972) occurred late on the 4th day of development (117 hr post-HCG), but the exact timing is likely to vary slightly between strains of mice. In this study, air-dried preparations of blastocysts were examined by microdensitometry.

One of the factors that has to be taken into account when attempting to assess whether giant cell transformation has been initiated, is the location of the individual trophectoderm cell, as the mural cells and their nuclei tend to be considerably more flattened than the polar cells at this stage of development. The morphological appearance of the trophodermal cells in these two locations is illustrated in Figs. 5a and 6a.

Following giant cell transformation in the mural trophectoderm, the next most marked change in the morphology of the primary giant cell occurs once the basement membrane of the endometrial layer has been breached. This stage is associated with an apparent increase both in the nuclear and cytoplasmic volume of the giant cells, particularly in the region of the abembryonic pole.

At the periphery of the ektoplacental cone, a rapid increase in the number of secondary giant cells is also evident. This response is particularly well seen in implantation sites examined on the 6th day of pregnancy (Fig. 10a,b). The secondary giant cells observed on the 7th day generally appear to have a greater cytoplasmic volume and possibly slightly larger nuclear volume than the primary giant cells (Figs. 11 and 12). Similarly, the nuclear outlines of the more peripheral of the former group tend to be more irregular than those of the latter group (Fig. 12), and some show evidence of nuclear fragmentation.

On the 7th day, the histological appearance of individual primary giant cells varies quite considerably. A proportion still remain close to the egg cylinder, attached to the outer (abembryonic) aspect of Reichert's membrane (Figs. 11 and 14). These tend to show less phagocytic activity than the giant cells that have migrated away from the embryo, many of which appear to be particularly voracious (Fig. 13).
On the 8th day, when the embryo is at the primitive streak stage of development, the majority of cells at the centre, but especially at the periphery of the ectoplacental cone, appear to be morphologically abnormal, and it is often extremely difficult to make out the individual cell boundaries (Figs. 15a, 16 and 17). It is not entirely clear from the appearance of the cells in this region what their principal role is at this stage of embryonic development.

As indicated earlier, the cell lineage studies, particularly involving the production and analysis of interspecific chimeras between rats and mice (Gardner and Johnson, 1973, 1975), clearly demonstrated the trophectodermal origin of the extra-embryonic ectoderm. These cells are clearly in continuity with the cells of the ectoplacental cone (Snell and Stevens, 1966), and, at the primitive streak stage of development (Figs. 15a, 16 and 17a,b), with the allantois (Figs. 15a, 17a,b and 18a), all three eventually contribute to the formation of the chorio-allantoic placenta.

4. Conclusion

While the main aim of this chapter has been to establish (largely from the results of experimental studies) the origin and fate of the trophectoderm-derived cell lineages, particularly as they arise and develop in vivo, this has necessarily resulted in the exclusion of a considerable volume of material which was not considered to be strictly relevant to the subject matter under discussion. A case in point was whether a consideration of the ultrastructural features and biochemical properties of the ‘delayed’ blastocyst state (for recent review, see Flint et al., 1981; and for observations on methodology, see Bergström, 1978) should be included here. This material was eventually excluded, principally because it provides relatively little information relevant to the origin and fate of trophectoderm cells. Similarly, the influence of certain mutant genes which affect early embryogenesis in this species, though of great interest to embryologists and developmental biologists (see, for example, McLaren, 1973; Pedersen and Spindle, 1976), was excluded on these grounds. Again, in order to limit the field to be considered in the present chapter, minimal reference has been made to the important biochemical and hormonal factors involved in the interaction between the endometrium and the blastocyst during the peri-implantation period. Excellent reviews on these topics are available (for example, see De Feo, 1967; Beier, 1976; O'Grady and Bell, 1977; Surani, 1977; Glasser and Bullock, 1981). Only those events which occur during normal embryogenesis have been considered in detail here, as the other function of the present study was to provide a sequential series of standard histological and ultrastructural photomicrographs to illustrate the morphological appearance and fate of the trophectoderm cells under normal conditions in vivo in the mouse.
Figures 3, 4, 10, 12, 15, 16 and 18 are 0.5-0.75 μm thickness resin sections stained with methylene blue. Figure 17 is a 6-7 μm paraffin section stained with haematoxylin and eosin. Figures 5-9, 11, 13 and 14 are transmission electron micrographs. The day of finding a vaginal plug is the first day of pregnancy, and the times given refer to time of autopsy.

Fig. 3. 9.15 a.m., 5th day. (a) Section through uterus containing implanting blastocyst to illustrate the extent of the decidual reaction observed at this time. (b) Higher magnification of central region, showing detailed morphology of blastocyst. Note that the primary endoderm (E) only overlies the inner cell mass (ICM). Note also the orientation of the blastocyst with the ICM directed towards the mesometrium (M), and the extent of the decidual reaction (a, arrows) which is principally at the anti-mesometrial pole of the embryo.
Fig. 4. 9.15 a.m., 5th day. (a) Implantation site containing slightly more advanced embryo, which is evoking a correspondingly greater decidual response, than that illustrated in Fig. 3b. (b) Higher magnification of central region showing detailed morphology of blastocyst. Note that the endoderm (arrows) is spreading at its periphery over the inner surface of the mural trophoderm.
Fig. 5. 9.15 a.m., 5th day. (a) Embryonic pole of implanting blastocyst. Note rounded appearance of polar trophoderm cell (P) and decreased endometrial response compared to that illustrated in Fig. 6a. I = ICM cells. Magn. × 3100. (b) Higher magnification view of interface between trophoderm and endometrial cells. The apposing surfaces are smooth and undulating. This is thought to be a slightly earlier stage of attachment than that illustrated in Fig. 6b (see Sherman and Wudl, 1976). The trophoderm cell contains darkly staining inclusions of phagocyted material (arrows). Magn. × 5530.
Fig. 6. 9.15 a.m., 5th day. (a) Early attachment phase of implantation, showing extent of endometrial response in a region approximately midway between the embryonic and abembryonic poles of the blastocyst. Within the endometrial cells (E) are large numbers of vesicles containing homogeneously staining material, possibly oestrogenic in nature. The stromal reaction is quite limited. Note the flattened appearance of the trophectoderm cell. Magn. × 2100. (b) Higher magnification of interface between trophectoderm (T) and endometrial cell (E) in a similar location to that illustrated in Fig. 6a. Note that the apposing surfaces are smooth and flattened. Adjacent trophectoderm cells are connected by a junctional complex (J) and an additional region close to the blastocoelic cavity in which the remainder of the cell surfaces interdigitate (arrows). Magn. × 9900.
Fig. 7. 3 p.m., 5th day. Lateral margin of embryonic pole. The single layer of endoderm cells (arrows) overlying the ICM are rounded, whereas the cell overlying the mural trophoblast is considerably elongated. A few coarse microvilli are present on the blastocoelic surface of the endoderm cells. Some of the endometrial cells contain large numbers of vesicles similar to those seen in the cells illustrated in Fig. 6a. Magn. × 3650.
Fig. 8. 3 p.m., 5th day. Lower magnification view of embryonic pole of the same implanting blastocyst as illustrated in Fig. 7. The considerable endometrial cell response is clearly seen. Note that very few vesicles are present in the stromal cells apposed to the basement membrane (arrows) of the endometrial cells. Magn. x 1500.
Fig. 9. 3 p.m., 5th day. Trophoblast giant cell which has insinuated itself between two endometrial cells and extended as far as the basement membrane (M). The trophoblast cell contains large amounts of phagocytosed cellular material in various stages of degradation (arrows) including what appears to be the nuclear region of an endometrial cell (A). Magn. × 3450.
Fig. 10. 10.15 a.m., 6th day. (a) Section through the centre of an implantation site containing an egg cylinder embryo. The conceptus has induced a considerable decidual reaction. (b) High magnification of the ectoplacental cone region of this embryo to illustrate the increased nuclear diameter of the peripheral (P) compared to the centrally located (C) ectoplacental cone cells. The former enlarge as they become transformed into secondary giant cells.
Fig. 11. 10.15 a.m., 6th day. Primary giant cells (G) associated with the outer (abembryonic) border of the acellular Reichert's membrane (R) at the abembryonic (anti-mesometrial) pole of an early egg cylinder. The nucleus of the larger giant cell is markedly irregular in outline. Note the typical appearance of the parietal endoderm cells which contain dilated cisternae of granular endoplasmic reticulum (E). Magn. × 2100.
Fig. 12. 9.30 a.m., 7th day. Anti-mesometrial (a) and mesometrial (b) poles of an early egg cylinder stage embryo. Large numbers of (possibly primary) giant cells (arrows) are present in close proximity to Reichert's membrane (R) at the anti-mesometrial pole. Note the larger overall size and more irregular nuclear outline of the secondary giant cells (S) at the periphery of the ectoplacental cone.
Fig. 13. 9.30 a.m., 7th day. Secondary trophoblast giant cell containing phagocytosed maternal red cells (arrows). The latter cells are probably recently engulfed as they show no obvious signs of cytoplasmic degeneration. Magn. × 3700.
Fig. 14. 4.30 p.m., 7th day. (a) Trophoblast giant cells (G) associated with the outer (abembryonic) border of Reichert’s membrane. Note the characteristic villous border of the extra-embryonic endoderm cells (E) which typically contain large numbers of membrane-bound vesicles. Magn. × 2600. (b) An enlarged view of the parietal endoderm cell associated with the embryonic surface of Reichert’s membrane. The cytoplasm of this cell contains dilated cisternae of granular endoplasmic reticulum of similar density to Reichert’s membrane (R), and numerous free polyribosomes. Magn. × 1020.
Fig. 15. Midday, 8th day. (a) Median sagittal section through a relatively advanced egg cylinder stage embryo containing a proamniotic canal (P) linking the presumptive amniotic (A) and ectoplacental (E) cavities. The base of the developing allantois (arrow) is just visible. (b) Higher magnification view of the extra-embryonic region located opposite the posterior amniotic fold (F). A few mesoderm cells (M) are located between the outer extra-embryonic endoderm (C) and the inner extra-embryonic ectoderm (D). The microvillous border (arrows) of the former is just visible. Y = yolk sac cavity.
Fig. 16. Midday, 8th day. Higher magnification of ectoplacental cone region of embryo illustrated in Fig. 15a. Note in particular the increased diameter of the nuclei and cytoplasmic volume of the ectoplacental cone cells at the periphery (A), compared to the centrally located cells (B) near to the presumptive ectoplacental cavity (C), and that the extra-embryonic ectoderm cells (arrowed) are in continuity with the ectoplacental cone cells.
Fig. 17. 5 p.m., 8th day. (a) Decidual swelling containing a primitive streak stage embryo at a slightly more advanced stage of development than the embryo illustrated in Fig. 15a. (b) Higher magnification of embryo illustrated in Fig. 17a. Note that the proamniotic canal has closed, and the presence of three cavities, the amniotic cavity (A), the exocoelom (B) and the ectoplacental cavity (C). The base of the allantois is visible (arrow), as is the ectoplacental cone (E).
Fig. 18. Midday, 9th day. (a) Sagittal section through an early headfold stage embryo with 6–7 somites present. The allantois (A) is considerably enlarged compared to the situations illustrated in Figs. 15a and 17a and is morphologically quite distinct. Note the appearance of the neural ectoderm in the headfold region (N) and the presence of somites (S). The bilaminar structure of the amnion (arrows) is just visible. (b) Higher magnification of part of the extra-embryonic region of the embryo illustrated in Fig. 18a. The characteristic appearance of the extra-embryonic endoderm cells (E) with their microvillous border (arrowed) is well seen. A = extra-embryonic ectoderm; M = extra-embryonic mesoderm.
5. Acknowledgements

The author gratefully acknowledges the technical assistance he has received from Mr. J. Skepper in the preparation of the transmission electron micrographs. The work reported here has been supported by a grant from Action Research for the Crippled Child.

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Chromosome Translocation (T(2;4)1 Sn)-Induced Neural Tube Defects in the Mouse Embryo

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(Received December 28th, 1982)
(Revised February 22nd, 1983)
(Accepted March 1st, 1983)

Key words: neural tube defects – chromosome translocation – mouse embryo

SUMMARY

Neural tube closure was studied in embryos obtained from matings of male mice heterozygous for a reciprocal chromosome translocation (T(2;4)1 Sn) with normal female (CFLP) mice. When litters were examined on the 9th to 12th days of gestation, there was a high incidence of resorption, developmental delay and neural tube closure defects in these embryos. SEM observations indicated that the neural tube closure defects ranged in severity from a side-to-side flattening of the midbrain to extensive anomalies in which the entire cephalic neural tube had failed to close. In addition to cephalic defects, a number of embryos exhibited open defects or abnormal subectodermal blebbing in the future lumbosacral region. In spinal regions, even in areas in which the neural tube had previously closed, it often was irregular and folded. These observations are discussed in relation to studies of gene-related defects of neural tube closure.

INTRODUCTION

Study of the numerous neurological mutants of the mouse has provided considerable information on both the normal development and sequelae following abnormal histogenesis of the neuroepithelium. A number of these mutants have defects of neural tube closure1 allowing a detailed examination of the events which precede the formation of severe closure defects. In addition to the many genes which produce dose-dependent anomalies of neural tube closure (Splotch15,16,24; curly tail3,5; loop-tail22,23; etc.), chromosome unbalance18, or trisomy of chromosomes 12, 1411,12, or 19 may also result in neural tube defects.

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0167-7063/83/$03.00 © 1983 Elsevier Science Publishers B.V.
The Snell translocation offers an ideal situation for the study of the development of these neural tube closure anomalies, as genetically unbalanced embryos exhibit extensive closure defects along the neural tube. The original males were termed "semi-sterile" following the observation of a high incidence of resorptions when irradiated males were mated with normal females. In addition, a high frequency of "cleft rhombencephalon" or "cleft diencephalon" was observed in surviving fetuses.

The current investigation was initiated to examine the early development of these embryos to determine the timing and characteristics of the resulting neural tube closure defects.

MATERIALS AND METHODS

Ten male (T(2;4)1 Sn) heterozygous translocation carriers (maintained on a non-agouti brown background) were obtained from the MRC Radiobiology Unit at Harwell, England. They were housed individually and mated with nulliparous CFLP females (Anglia laboratories). The morning of finding a vaginal plug was considered the first day of pregnancy. Additional control material was obtained from matings of other CFLP females with males of the same strain. Food (Dixon’s Lab Diet, 41B, Ware, Hertfordshire) and tap water were available to the animals ad libitum.

On the afternoon of the 9th, 10th, 11th or 12th days of pregnancy, females were killed and uteri removed. Individual decidual swellings were placed in Petri dishes containing phosphate-buffered saline and embryos dissected from decidua and chorion. Crown—rump lengths were measured using an optic micrometer, the incidence of gross anomalies noted, and embryos immersion fixed in a 2% solution of glutaraldehyde in 0.1 M sodium cacodylate buffer (with 3% sucrose) for 2 h at room temperature. They were then washed and stored at 4 °C in a solution of 0.1 M sodium cacodylate with 3% sucrose, prior to processing for SEM.

Embryos were dehydrated through graded alcohols and critical point dried in CO₂. They were then affixed to stubs and sputter coated with gold, examined and photographed in a Cambridge S600 scanning electron microscope, operated in the secondary mode at 15 kV.

Analysis of the statistical significance of crown—rump length was carried out using a one-way analysis of variance procedure (ANOVA). Embryos that were "unturned", i.e. had not yet adopted the fetal position on the 10th day, were not included in the analysis of crown—rump length. Similarly, crown—rump lengths of embryos that were grossly delayed by the 11th or 12th days were not included for statistical analysis.

Embryos obtained from matings with male chromosome translocation carriers will be designated as "Snell", while those from CFLP x CFLP matings will be designated "control" to facilitate description.
RESULTS

From the 9th through the 12th days of development, a number of important morphogenetic events occur in the mouse embryo. In addition to the establishment of a functioning heart and yolk sac circulation, fore and hindlimb buds develop, and the embryo reverses its initial dorsal lordosis to adopt the ventral curvature characteristic of the fetal position. During the same period, neuroepithelial cells undergo a series of alterations which cause the low columnar cells of the early neural plate to elevate, then become wedge-shaped as the neural folds approach and fuse in the dorsal midline, forming the neural tube.

Snell embryos examined on the 9th day of gestation had a 26% incidence of resorption compared to 4% in controls. Of the living Snell embryos, 70% appeared morphologically normal. At this time these embryos had developed 6–10 somites and were characteristically “unturned”, i.e., had not adopted the fetal position. A further 30% of these embryos were less developed, having fewer than 2 somites. Control embryos on the 9th day were slightly more advanced (16–20 somites) and fewer (1%) were developmentally delayed.

When 9th-day embryos were examined using SEM, neural tube closure was observed to be confined primarily to spinal regions of the neuraxis. The only obvious difference between control and Snell embryos was the considerable developmental delay seen in 30% of the latter group.

On the 10th day of gestation, the incidence of resorptions was 27%, and 2% in Snell and control embryos, respectively. Of the Snell embryos, 70% appeared morphologically normal; 9% of these apparently normal embryos were developmentally delayed, i.e. were “unturned”. Control embryos were also slightly larger in crown–rump length (2.8 ± 0.02 mm) than Snell embryos (2.2 ± 0.06 mm). The observed growth retardation (as measured by crown–rump length) was uniform and did not suggest the presence of two distinct groups of embryos.

The neural tube of control embryos had closed by the 10th day in all regions but the posterior neuropore, which closes slightly later on the 11th day. In Snell embryos, 33% had some type of neural tube defect of the cervical (78% of affected embryos) or spinal regions (22%) of the neuraxis. The hindbrain appeared to be the most common site of closure defects in 10th day Snell embryos (Fig. 1A,B), although defects were observed in more caudal regions as well.

Hindbrain anomalies often extended into the midbrain and future cervical region of the cord. The cell surface of the exposed neuroepithelium in these regions was characteristically smooth and unbroken. The surface ectoderm was poorly demarcated from the neuroepithelium and ruffles, blebs and lamellodria characteristically seen in control embryos were not observed at this junction in affected embryos.

The incidence of resorptions in litters examined on the 11th day was 22% and 1% in Snell and control embryos. Embryos from matings with translocation carriers also had decreased crown–rump lengths (4.1 ± 0.5 mm) compared with controls (4.6 ± 0.4 mm).
Fig. 1  A,B: side and dorsal views of an abnormal embryo isolated on the 10th day of gestation. The extensive neural tube defect extends from the caudal midbrain into the future cervical region. C,D: side and dorsal views of an embryo isolated on the 11th day of gestation. The neural folds have failed to fuse in a similar region as in the embryo illustrated in A and B. The neuroepithelium has continued to proliferate and has become everted over the surface ectoderm. Note also the midline bleb at the most caudal extent of the defect (D, arrow). Scale bars = 400 μm.
By the 11th day, neural tube closure defects were observed in 33% of the living Snell embryos and 0/79 of the controls. These defects were located primarily in the cephalic region, and were observed in similar areas as on the 10th day (Fig. 1C,D). The neuroepithelium had continued to proliferate and had often everted over the surface ectoderm. In addition, in contrast to the smooth neuroepithelial surface seen on the 10th day, the exposed neuroepithelium of 11th-day embryos was rough and characterized by small blebs and protrusions (cf. Fig. 1A,B and Fig. 1C,D). Subectodermal blebs often marked the most caudal extent of the open defects (Fig. 1D).

By the 12th day, the incidence of resorptions was increased to 37% compared with 3% in controls, while crown–rump lengths continued to be depressed compared with controls (5.5 ± 0.7, 5.9 ± 0.5 mm). Table I summarizes these data.

In embryos isolated on the 12th day of gestation, the incidence of neural tube closure defects had decreased to 19%. These defects ranged in location and extent from subectodermal blebs near the hindlimb buds (Fig. 2A), slight side-to-side flattening of the midbrain, to extensive open defects in the lumbosacral region (Fig. 2B).

In other embryos, the entire cephalic neural tube had apparently failed to close. In these embryos the neuroepithelium had continued to proliferate and differentiate and

![Fig. 2. A: side view of an embryo isolated on the 12th day of gestation. The entire length of the neural tube has closed, but a small bleb is located just caudal to the hindlimb buds (arrow). B: frontal view of an abnormal embryo also isolated on the 12th day, showing an open neural tube defect (arrow) in the same area as the subectodermal bleb seen in A. This embryo also demonstrates the side-to-side flattening of the midbrain region observed in these embryos. Scale bar = 500 µm.](image-url)
### TABLE I

**INFLUENCE OF THE SNELL TRANSLOCATION ON EMBRYONIC VIABILITY, DEVELOPMENT AND NEURAL TUBE CLOSURE**

<table>
<thead>
<tr>
<th>Gestation day</th>
<th>Group</th>
<th>Number of Litters</th>
<th>Implants</th>
<th>Resorptions</th>
<th>Living embryos</th>
<th>Delayed embryos</th>
<th>Embryos with neural tube defects</th>
<th>Mean crown–rump length (mm ± SE)</th>
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<tr>
<td>9</td>
<td>C</td>
<td>15</td>
<td>196</td>
<td>8</td>
<td>188</td>
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<td>–</td>
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<td>9</td>
<td>TSn</td>
<td>8</td>
<td>83</td>
<td>22</td>
<td>61</td>
<td>18</td>
<td>1/91</td>
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<tr>
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<td>C</td>
<td>7</td>
<td>94</td>
<td>2</td>
<td>92</td>
<td>1</td>
<td>25/75</td>
<td>2.2 ± 0.06</td>
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<tr>
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<td>TSn</td>
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<td>113</td>
<td>31</td>
<td>82</td>
<td>7</td>
<td>0/79</td>
<td>4.6 ± 0.4</td>
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<tr>
<td>11</td>
<td>C</td>
<td>6</td>
<td>80</td>
<td>1</td>
<td>79</td>
<td>–</td>
<td>33/100</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>11</td>
<td>TSn</td>
<td>12</td>
<td>128</td>
<td>28</td>
<td>100</td>
<td>–</td>
<td>0/67</td>
<td>5.9 ± 0.5</td>
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<tr>
<td>12</td>
<td>C</td>
<td>5</td>
<td>69</td>
<td>2</td>
<td>67</td>
<td>–</td>
<td>12/63</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>12</td>
<td>TSn</td>
<td>9</td>
<td>100</td>
<td>37</td>
<td>63</td>
<td>–</td>
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*a Only turned embryos included.

*b P ≤ 0.01, TSn10 < C10 < TSn11 < C11 < TSn12 < C12, one-way analysis of variance.
Fig. 3. A,B,C: frontal, side and dorsal views of a severely affected 12th-day embryo showing an extensive defect in which the neural tube failed to close throughout the cephalic region. The neuroepithelium has continued to develop, resulting in considerable eversion of the neural tissue over the surface ectoderm (B, arrows) and in a well-formed but exposed hindbrain (C). The neural tube appears to have a ‘Z’ configuration caudal to the defect (C, arrows), although the embryo appears otherwise normal. Scale bar = 400 µm.

often appeared to rest cap-like on the surface (Fig. 3). Even in closed regions, many times the neural tube of these embryos appeared to have a ‘Z’ or wavy configuration (Fig. 3C).

DISCUSSION

These results parallel the original report on the development of these embryos18, in which 3 classes of anomalies were reported: forebrain or hindbrain clefts, as well as a “distention” and crumpling of the closed neural tube. In addition, we report a high incidence of defects in the caudal region of the neuraxis, both of closure and of subectodermal blebs.

A number of authors have attempted to relate these anomalies to a specific genotype, with marginal success. Eicher and Washburn4 report that at term, fetuses with gross exencephaly as well as those which were small (but apparently normal) which die in the early postnatal period, are chromosomally 2, 2, 42, 4, i.e. there is a duplication of the end of chromosome 2 and a deficiency for the distal end of chromosome 48. However, in other studies, the other unbalanced situation: 2, 24, 4, 4 has been observed in retarded but apparently normal fetuses, while the 2, 2, 42, 4 unbalanced situation was observed in fetuses with gross exencephaly (M.K. Kirk, personal communication, 1979). Clearly, a comprehensive karyotypic study using several strains is required to resolve the question of the role of genotype in the production of a specific phenotype.
The incidence of these defects appears to be highly influenced by the background of the mice with which carriers are mated. The observed incidence of gross anomalies was 23% in C57Br/cdJ animals and 7% in DBA/2J mice at term, compared with 33% in the current investigation when embryos were examined during and just following neurulation. While the basis for the strain differences is not known, it seems likely, based on the increasing number of resorptions seen between days 11 and 12, that many of the more severely affected embryos were resorbed by the later stages of development examined by previous authors.

Neural tube defects also occur in a high incidence of embryos homozygous for the Splotch gene. While the cell cycle generation time of the neuroepithelial cells has been reported to be increased in these mutants during S, M and G1 phases, whether this alteration is a cause of, or results from the observed neuroepithelial alterations remains to be determined.

A second class of mutant with a high incidence of neural tube closure defects includes those related to anomalous tail bud formation and elongation; curly tail, loop-tail, as well as the lethal t alleles. While in all of these embryos there is a disturbance in mesodermally derived elements, resulting in defective development of the axial skeleton, neural tube closure defects are also found along the neuraxis.

Interestingly, defects of neural tube closure have recently been reported in embryos trisomic for chromosomes 12 and 14. In these embryos, unlike most mutant strains, neural tube defects are confined to the cephalic portion of the neuraxis; in the case of trisomy 12 embryos solely to the midbrain. Similarly, teratogen-induced neural tube closure defects occur most commonly in the cephalic region, perhaps because of the more complex series of events which appears to be involved in neural tube closure in this region.

The association between the subectodermal blebs observed in the lumbosacral region and open defects at similar locations, is unclear. At this time, there is no direct evidence to suggest that this condition precedes (or follows) the development of open defects, although fluid-filled blebs produced by trypan blue treatment have been implicated in the formation of neural tube closure defects. Similar blebs have been observed occasionally at the most caudal extent of an open defect where they have been ascribed to mechanical trauma following eversion of the neural folds.

In addition to providing important data on the timing of these effects, the current study supports the conclusion that neural tube closure defects form from primary non-closure of the neural tube rather than following its secondary re-opening as has been alternatively proposed. This observation suggests that the susceptibility of the neuroepithelium to teratogen- or gene-induced defects occurs during a highly circumscribed period of development.

Ultrastructural investigations are currently in progress to determine if there is a characteristic pattern of alterations which precedes the development of these defects.
ACKNOWLEDGEMENTS

The authors are grateful to Dr. Mary Lyon and Ms. M.K. Kirk for providing carriers of the Snell translocation. This study was supported in part by Grant N-8149 from the Easter Seal Research Foundation.

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The relationship between embryonal carcinoma cells and embryos

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Eight years ago at the second symposium of the British Society of Developmental Biology I discussed work with teratocarcinoma cells in vitro and compared the teratocarcinoma stem cells with pluripotential cells of the pre-gastrulation mouse embryo. I suggested at that time that the teratocarcinoma stem cells might be equivalent to the ectomesodermal cells of the pre-gastrulation embryo and that it might be quite feasible to obtain cultures of pluripotent cells directly from the embryo (Evans, 1975). The stem cells are now identified with the embryonic epiblast, and the culture of pluripotential cells with the properties of teratocarcinoma stem cells has now been achieved directly from early mouse embryos. The converse manipulation is also possible; embryonal carcinoma cells are able to recolonise early mouse embryos to give rise to chimaeric mice.

In 1974 it was realised that the stem cells of teratocarcinomas had, by virtue of their pluripotency, properties equivalent to the undetermined cells of the early mouse embryo, and that the processes of cell determination and differentiation which occurred within the teratocarcinoma as well as in the normal embryo might provide a model system for the study of mammalian development. Two other pieces of information strengthened the parallel between the teratocarcinoma cells and the normal embryo: first, the knowledge that transplantable teratocarcinomas could be produced by the experimental ectopic transplantation of early embryos and, secondly, the observation that both xenogeneic and syngeneic antisera raised against the surface of teratocarcinoma stem cells also reacted against components on the cell surface of preimplantation embryos. Against this hypothesis of equivalence of teratocarcinoma...
stem cells with early embryo cells was the fact that the stem cells (which have subsequently become known as EC cells) when isolated from tumours were karyotypically abnormal and were in any case tumour cells and hence hardly likely to be normal. It was also known that teratocarcinomas could arise from foetal primordial germ cells as well as from early embryos.

Tissue culture cell lines could be grown from teratocarcinomas. That pluripotential stem cells existed in these cultures could be shown by the fact that clonal and subclonal cultures could be established which would give rise to well-differentiated tumours when re-injected in vivo. Differentiation in vitro from clonal cultures had proven more elusive although it had been reported by Jakob et al. (1973) and in retrospect had clearly been observed both by Finch (1968) and by Rosenthal (1968). We also observed differentiation in our clonal cultures but it was only just at about that time that we began to realise that the differentiation was by an embryo-like route (Martin & Evans, 1975).

Since that time the further knowledge of properties and origins of EC cells has amply confirmed the earlier supposition of their similarity to early embryo cells and indeed it seems likely that EC cells are in fact normal embryonic epiblast cells. Any differences which are found in established tumour or cell lines are the result of progression to an abnormal state during passage.

The evidence for this statement falls into four categories: the embryo-like mode of differentiation of EC cells; the phenotype of the EC cell (spectrum of protein synthesis and also cell surface phenotype); the generation of EC cells from embryos in vivo and in vitro; and the re-introduction of EC cells into embryos. These are discussed below.

Mode of differentiation

At first it was difficult to obtain differentiation of EC cells in vitro, because the nature of the growth requirements of these cells led to the establishment of cultures of cells either very poorly capable of differentiation or inhibited from doing so by the mode of culture. Primarily EC cells in conventional culture media need an association in culture with a 'fibroblast' cell type; they are 'feeder-dependent' and they may either be cultured as a balanced mixed population with a cell type which provides feeder properties (e.g.
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SIKR cultures) or by adding mitotically incompetent feeder cells at each culture passage. It is also possible to select feeder-independent lines of cells but these, during selection and particularly on prolonged passage without feeders, become much less readily able to differentiate (Hogan, 1976). This is associated with the accumulation of karyotypic abnormalities (Magranc, 1982).

The earlier cell lines that were isolated were either pure EC cultures which had rapidly lost their ability to differentiate readily in vitro as they had been maintained without feeders (e.g. F9; PCC4; PC13) or were mixed cultures (e.g. SIKR). One effect of the presence of feeder cells is to maintain the EC in exponential growth and to inhibit cell differentiation. Differentiation was thus also not seen in these cultures. The resolution of this problem lay in the development of feeder-independent lines which were still sufficiently normal to differentiate recognisably (PCC3; Nicolas et al., 1976) and in particular the isolation (using added feeders) of feeder-dependent lines which differentiate well on removal from feeders.

Whenever spontaneous differentiation of mouse EC cells is observed in vitro, the first differentiated type of cell formed is recognisable as a primary endoderm cell (Evans & Martin, 1975). This process of differentiation is triggered by the formation of a clump of cells with a free surface and it is on this surface that the first endodermal cells appear. The most straightforward method of inducing differentiation of an EC cell line in culture is to keep cell aggregates in suspension and under these conditions the primary endodermal layer forms to surround a core of as yet undifferentiated EC cells. The product is a simple embryoid body and this structure of primary endoderm on the outer surface of a group of pluripotent cells is equivalent to the endoderm-invested inner cell mass (ICM) of a 5 day mouse embryo. These simple embryoid bodies will produce a mass of diversely differentiated cells when allowed to re-attach to a tissue culture surface. Lines of pluripotential EC cells which are not too abnormal will also differentiate further to give 'cystic' embryoid bodies. The simple body cavitates and a fluid-filled cyst is formed. The cyst wall is made up of an endodermal outer layer and a mesenchymal inner layer that often contains muscle and may be seen to contract in slow waves. On one side of the cyst a residual knob of differentiated cells is often found. Clearly here, as is the case after monolayer explantation, a complex
but disorganised development into normal tissues has occurred. In the early stages of cystic embryoid body formation, however, a process of ectodermal and mesodermal formation very similar to that occurring in normal embryos between 6 and 7 days of development may be seen (Martin et al., 1977).

In fact most EC cell lines will differentiate in vitro to at least a limited extent. Although several 'nullipotential' lines have been described (e.g. F9; Nulli SCC2a) none appears to be totally incapable of differentiation under suitable conditions in vitro or with inducing stimuli (e.g. retinoic acid, Strickland & Mahdavi, 1978) and in most cases even with these (most probably unnatural) stimuli endodermal cells are produced.

Embryonal carcinoma cells are differentiating just as if they were very early embryo cells. The first differentiation observed in vitro for mouse EC cells is not to trophoderm but to primary endoderm, which suggests that they are differentiating in the same way as cells of the ICM of a mouse blastocyst. It is to be expected that there is but one developmental pathway for differentiation built into the genome of a cell and this is the pathway seen during normal embryonic development. It is not perhaps therefore surprising that EC cells follow a recognisably embryonic pathway when they start to differentiate. The question remains, are EC cells part of this pathway or do they move onto it as they start to differentiate?

There are, however, several possible explanations for this cell behaviour. EC cells may represent some quite aberrant cell type which happens to be able to start to differentiate in response to particular signals. In doing so it becomes entrained into the normal pathway of embryonic cell differentiation and its differentiated phenotype changes to that of cells normally present in the embryo. On the other hand it is possible that EC cells themselves represent an essentially normal early embryonic cell type. It could be that this cell type has become transformed to a malignant cell phenotype or alternatively that the particular combination of properties which characterise this normally occurring embryonic cell lead to its malignant phenotype when it grows outside the normal organisation within the embryo and/or when it occurs in greater than normal concentrations. We suggest that our ability to isolate cell lines equivalent to EC cells directly from normal early embryos strongly supports this latter case.
Homology of EC cell phenotype with that of cells in the normal embryo

The major developmental characteristic of EC cells is their ability to differentiate into a diversity of cells; the only normal cells with this property are early-embryo cells. EC cells are derived from either early embryo cells or from germ cells. In the latter case, derivation from female germ cells is clearly via an embryo although in the case of derivation from male germ cells there is no overt embryo-route. Is it possible, therefore, that EC cells are indeed normal embryonic cells and if so with which cells are they homologous?

The question of their possible homology has been approached by investigation of various cell properties and, in particular, cell surface antigens. EC cells have an unusual cell surface antigen expression and numbers of antisera, xenogeneic, syngeneic and monoclonal, have been shown to recognise cell surface determinants that are characteristically shared by EC cells, early mouse embryo cells and germ cells – both spermatozoa, and primordial germ cells isolated from 12 day foetal ridges. Many of these antigens occur on few if any other cell types and thus define an area of homology even if – as in the case of the monoclonal reagents – they recognise only a single small specific determinant (reviewed by Jacob, 1979; Solter & Knowles, 1979). While these homologies between EC cell surfaces and embryonic cells, from cleavage stages through to post-implantation, confirm our suspicions of cell type homology they do not define the embryonic stage of pluripotential cell which might represent an EC cell. The monoclonal reagent M1/22.25 that reacts against a determinant of the Forssman antigen – a carbohydrate chain which in these cells is attached to the cell surface as a glycolipid – was particularly useful. It reacted with ICM cells, but in the 6.5 day embryo it reacted not with the pluripotential embryonic ectoderm cells but with the already determined and differentiated embryonic endoderm. Only at an earlier stage, up to the late 5 day old embryo, was the pluripotential ectoderm positive (Stinnakre et al., 1981). At this stage the embryonic ICM has delaminated an outer layer of endoderm but is still internally composed of rounded cells with only a rudimentary cavity. This is the stage to which a simple embryoid body is said to correspond and, by this test of the M1/22.25 monoclonal antibody,
is the latest embryonic stage at which cells homologous to EC cells are present. Primordial germ cells do not become overtly apparent until later and their cell surface antigenicity has not been explored until the stage at which they may be isolated from the germinal ridges, when they are M1/22.25 positive.

The simplest supposition consistent with their mode of differentiation and with their cell surface antigenicity would be that EC cells are equivalent to the ICM cells of 3.5 day blastocysts; but comparisons of their spectra of protein synthesis analysed by two-dimensional electrophoresis show that these two cell types are very different (Dewey et al., 1978; Lovell-Badge & Evans, 1980). Ectoderm tissues from later post-implantation stages, however, are very similar to EC cells (Evans et al., 1979).

Mouse embryos at any stage of development between two cells and the final determination of the three germ layers following gastrulation, at about 7.5 days of development, will form a teratoma when transplanted to a suitable site in a histocompatible host (see Solter et al., 1975; Stevens, 1981). The earlier embryos are seen to develop in the ectopic site but they become disorganised at a post-implantation stage. Egg cylinder stage embryos dissected before transplantation show that only the embryonic ectoderm is responsible for teratoma formation (Diwan & Stevens, 1973). In some permissive strains of mice (e.g. C3H, AKR) the tumour grows progressively as a malignant teratocarcinoma from more than 80% of the transplanted embryos, but in other strains (non-permissive) (e.g. C57BL) only very infrequently are progressively growing teratocarcinomas formed, and most tumours differentiate fully to a benign type. Solter and his colleagues have investigated extensively the factors involved in this different behaviour. Both graft and host are involved but here – unlike the situation with germinal ridge grafting – the major factor is the host. Non-permissive strain embryos (C57BL) grafted into F1 hybrids between permissive strains (C57BL × C3H) give rise to teratocarcinomas at a relatively high frequency. One intriguing observation made by Solter in his investigation of this phenomenon was that the immune status of the host might be an important factor. Immunosuppression, however, reduced the rate of teratocarcinoma formation and athymic mice do not support teratocarcinoma formation (Solter & Damjanov, 1979). The embryo genotype (and therefore the genotype of the resulting tumour cells) also affects the rate of formation.
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of malignant tumours but another quite unexpected result is that there is an effect of maternal inheritance upon both the embryo and the host (Damjanov & Solter, 1982).

The very ready formation of teratocarcinomas from transplanted egg cylinders suggests that there is no particular step of malignant transformation. On the other hand, the demonstration that the main factors influencing the formation of malignant tumours are host- rather than graft-related suggests that the stem cells may be transformed to malignancy or at least selectively stimulated to grow by the host environment. Whatever the factors involved, experiments in vivo of this kind are subject to the constraint that the cells are primarily present in a tumour and therefore questions about their tumorigenicity are circular.

We approached the isolation of pluripotential cells from normal embryos with three points in mind (Evans, 1981): (1) if cells homologous to EC cells were present, they would be found in the ICM-ectodermal cell lineage after the stage of the 3.5 day blastocyst and before the 5.5 day early egg cylinder; (2) the conditions of culture required would be those most conducive to EC cell growth and maintenance in an undifferentiated state; (3) as the low number of cells present in an embryo might present a difficulty in terms of primary plating efficiency, the cell number in the embryonic epiblast should be maximised.

Embryos at the peri-implantation stage are difficult to isolate from the mouse. We therefore arrived at this stage of development by allowing an intact explanted blastocyst to continue to develop in culture for 2–4 days prior to its disaggregation and the initiation of monolayer culture. The number of cells in the ICM was increased by using blastocysts which had been maintained in vivo in a state of implantational delay. Under these conditions the cells of the ICM undergo a few rounds of division without further development and so the number of ICM cells at explantation is increased.

By using these methods we have been able to isolate cell lines from mouse embryos in tissue culture (Evans & Kaufman, 1981). These cells grow out rapidly and may be first recognised a few days after the primary disaggregation. Once established, these cells grow rapidly and progressively with no sign of any early adaptation to culture conditions or other ‘cell transformation’ event. From the outset they grow in characteristically heaped nests of tightly packed cells (Fig. 1). When lines of such cells are established, cells from
low passage number cultures show a cloning efficiency in vitro of approximately 10%.

These pluripotent cell lines, isolated directly into tissue culture from embryos without prior tumour formation, we have called EK cell lines to indicate their affinity to but also their distinction from EC cells. Lines derived from embryos in vitro (EK cells) are indistinguishable from their tumour-derived EC cell counterparts, in terms of their cellular morphology, cell surface antigen expression, and differentiative behaviour both in vitro and in vivo (Evans & Kaufman, 1981; Martin, 1981; Kaufman et al., 1983) and are also capable of participating in normal development in a chimaeric association with an embryo (Evans & Kaufman, 1981).

Pluripotential lines derived from embryos would appear to have considerable advantages over tumour-derived EC cell lines. In addition to their relative ease and rapidity of isolation, there appears to be no restriction on the mouse strains from which such lines may be isolated, so that the establishment of new genetically marked pluripotential stem cell lines from both inbred and randomly bred mice is facilitated. Cell lines from randomly bred mice had not previously been available, as teratocarcinomas could only be induced experimentally by the ectopic transfer of embryos into histocompatible host animals, and tumours containing EC cells fail to grow in immunosuppressed or nude mice (Solter & Damjanov, 1979). The establishment of stem cell lines directly from embryos has the singular advantage that these cells can be used to investigate homozygous lethal developmental mutations that can only be maintained in mouse stocks in the heterozygous state. The potential usefulness of this approach has already been demonstrated by the recent report of the isolation of an EK cell line homozygous for the $t^{rd}$ mutation of the $t$ complex (Magnuson et al., 1982).

Of the 21 fully analysed EK cell lines derived from post-fertilization embryos in our laboratory, 15 possess a karyotype identical, at least initially, to that of the blastocyst from which they were derived. This may prove to be the single most important feature of these lines, as the possession of a normal euploid karyotype should considerably facilitate their incorporation into chimaeric animals. Of even greater importance, however, is the likelihood that the possession of a normal karyotype may well be essential for the incorporation of EK cells into the germ line of chimaeric mice (Mintz & Cronmiller, 1981). EK lines are currently
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Fig. 1. Development of delayed blastocysts in culture. (a), (b) 1 day in culture. Outgrowths of trophoblast cells. (c), (d) 2 days in culture. ICM clearly visible. (e), (f) 4 days in culture. Well-developed egg cylinders. (g), (h) Early passage EK colonies growing on a fibroblast feeder layer.

being used in our laboratory, and supplied to other laboratories, for the construction of such mice, and breeding studies are now in progress to test for possible contributions to the germ line.
Since the initial report from our laboratory in 1981, we have established pluripotential cell lines directly \textit{in vivo} both from 'delayed' and, more recently, non-'delayed' blastocysts. Over 20 such EK lines have now been established from a variety of inbred and randomly bred sources, thus making available for study genetically normal stem cell lines, carrying a range of isozymal variants, as well as lines with translocation-bearing 'marker' chromosomes (Table 1).

We have recently extended our studies (Kaufman \textit{et al}., 1983) by establishing similar cell lines from parthenogenetically derived haploid and diploid blastocysts, obtained following the activation \textit{in vitro} of eggs with ethanol (Kaufman, 1982). At the present time, 10 pluripotential cell lines of parthenogenetic origin exist. They include lines derived from haploid (1-pronuclear class) and two from diploid (2-pronuclear class) parthenogenetic blastocysts (for details of classification, see Kaufman, 1981); these have been derived from both 129Sv and from (C57BL X CBA) F1 mice. To date, all the parthenogenetically derived lines have been diploid, even those established from haploid blastocysts (termed HD lines), but we have yet to establish at which stage of the isolation procedure diploidisation occurs. Nevertheless, this method has enabled homozygous diploid lines of parthenogenetic origin to be established.

Martin (1981) has published an alternative method for isolating pluripotential cells from mouse blastocysts. She explanted isolated blastocyst ICMs, supplementing her culture medium with a factor isolated from the medium in which established EC cell lines had been maintained. We have investigated the use of such a conditioning factor, but have found that it does not improve the rate of recovery of cell lines from explanted blastocysts, although it did in some experiments appear to have a dramatic effect upon the numbers of growing colonies found after the primary disaggregation. If the aim is to isolate normal embryo cells, it is clearly a disadvantage to use a poorly characterised product from an established embryonal carcinoma cell line which might contain some transforming factor.

Exogenously added conditioning factors are unnecessary, but so too is the technique of delay \textit{in vivo} which in itself might be considered to introduce an abnormal maternal influence on the embryo. We have now found that cell lines may be derived from
<table>
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embryos cultured entirely in vitro from the one-cell stage. When the embryo has developed to a blastocyst, it is explanted in the same way as an embryo that has developed in vivo, and pluripotential cell lines may be established from it (Robertson et al., 1983). Thus, whilst the enlarged ICM in 'delayed' blastocysts and the use of 'conditioned' medium may facilitate the isolation of pluripotent cells from early embryos, neither are necessary. It is clear that cells may readily be obtained from a normal embryo which are pluripotential and tumorigenic – i.e. teratocarcinoma stem cells.

The most dramatic proof that EC cells can behave as embryo cells comes from their ability to become incorporated into an embryo, which then develops into a normal but chimaeric animal. This has been shown with EC cells taken from embryoid bodies in vivo (Mintz & Ilmensee, 1975) and from clonal tissue culture cells (Papaioannou et al., 1975). In some cases the chimaeric mice have developed tumours. These tumours may either be teratocarcinoma or secondary tumours of differentiated tissues. The occurrence of such tumours may well be associated with the accretion of abnormalities in the EC cell lines (Papaioannou et al., 1978). In only a single case so far reported have tissue culture cells given rise to functional gametes (Stewart & Mintz, 1981), perhaps because many of the EC cell lines that have been successfully used to form chimaeric mice have had chromosomal abnormalities.

With few exceptions, most of the currently available EC cell lines possess chromosomal abnormalities of various kinds. In addition, EC cells have a range of growth characteristics in culture, and vary widely in their ability to differentiate both in vitro and in vivo. Some lines differentiate well both in vitro and in syngeneic host animals, while others differentiate only in the tumour form in vivo, while yet others (termed 'nullipotent' lines) differentiate only poorly under any circumstances. The majority of the fertilised and parthenogenetically derived EK lines so far examined (>70%) appear to retain a normal euploid karyotype, and differentiate extensively both in vitro and particularly in vivo, which should facilitate their incorporation into chimaeric animals.

In a series of experiments (carried out in collaboration with R. L. Gardner), 76 blastocysts injected with EK B2.B2 cells and transferred to recipients gave rise to 58 live-born animals (76%), of which 26 (45%) were overtly chimaeric. Analysis of the isozymes of glucose phosphate isomerase revealed extensive contributions to the
internal organs in chimaeric individuals. In none of these animals was there any abnormal tumour formation throughout their life.

In a second series of blastocyst injection experiments (in which EK B2.B2 cells were also used), 66 blastocysts were transferred and 40 (61%) live-born young were recovered. Of these, 14 (35%) were overtly chimaeric, as assessed by eye and coat pigmentation. It is of particular interest that the head region of these chimaeric animals was extensively pigmented; this was especially noticeable in mice with only low levels of overt chimaerism. Six of the chimaeric mice have survived to adulthood (five females and one male), and these are currently being bred (Fig. 2).

Analysis of glucose phosphate isomerase isozymes in the newborn mice which failed to survive the early neonatal period (this failure was not due, as far as can be discerned, to any abnormalities in these animals) has revealed that in some instances there was an extensive contribution by the EK cells to all of the organs and tissues tested, including the gonads.

These preliminary data, with regard to both the survival to term of operated embryos and the percentage of overtly chimaeric individuals, compare favourably with the variable success that has generally been obtained following the injection of EC cells into normal mouse blastocysts. One important disadvantage of EC cell lines is that most are XO in chromosome constitution. While this does not appear to prevent their participation in normal development, it may be deleterious for the formation of functional gametes.

A recent report by Stewart & Mintz (1981) indicates that the use of a euploid EC cell line not only increases the relative incidence of EC-derived cells within the somatic cell population, but is likely also to allow functional colonisation of the germ line. In their study, a normal euploid 129-derived EC line (METT-1) gave rise to overtly chimaeric animals at a rate of about 13%. More important, 1 out of the 9 chimaeric females that were bred gave rise to 3 out of 48 offspring of a 129 phenotype. Although animals chimaeric from EK B2.B2 injection have not yet given progeny demonstrating the production of functional germ cells from the EK cells, we intend to persist with this study, and to utilise the same approach to assess the developmental capabilities of other EK lines of both XX and XY genotype.

Normal embryos will give rise to teratocarcinomas containing EC cells in vivo and will give rise directly to EK cell cultures in vitro.
Fig. 2. (a), (b) Chimaeras made by the injection of the $129$, B2.B2 cell line into albino host blastocysts. The level of chimaerism is high, appearing mainly in the head region, and is particularly striking in the iris of the eyes.
At the moment there is no evidence which would lead one to doubt that EK cells are the normal counterparts of EC cells. The differentiative behaviour of EC cells is similar to that of an embryonic ICM and their phenotype matches that of the cells derived from the ICM — the embryonic epiblast. EC and, in particular, EK cells (in chimaeric combination) give rise to a large part of a normal mouse. The most reasonable conclusion from these studies is that EC cells are not only similar to embryonic epiblast cells — they are epiblast cells. How the relationship between EC cells and germ cells fits into this scheme has yet to be determined.

References


Occlusion of the neural lumen in early mouse embryos analysed by light and electron microscopy

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SUMMARY

A histological account of neural tube occlusion during early mouse embryogenesis is presented here from an analysis of sections taken from plastic-embedded material. Because the overall pattern of neuroepithelial apposition and the duration of luminal occlusion appears to vary slightly from one embryo to another at otherwise similar stages of development, only a general guide to the events occurring in the mouse embryo between about midday on the 9th to late on the 10th day of gestation can be given. The earliest evidence of complete luminal occlusion was seen when the cephalic and caudal extremities of the neural tube were still widely open.

An ultrastructural analysis of the morphological appearance of the closely apposed luminal cells in zones of partial and complete occlusion has demonstrated that occlusion is brought about initially by the interdigitation of processes from closely apposed neuroepithelial cells. This initial event is followed by direct contact over a much more extensive area between the surfaces of apposed cells with a characteristically flattened luminal border. Apposition and luminal occlusion is probably facilitated by the presence of viscous extracellular material. Finally, complete occlusion involving an extensive region of the lumen occurs. The latter phenomenon is a transient event lasting 1 or at most 2 days in the mouse. At no stage were junctional complexes observed between closely apposed neuroepithelial cells in regions in which the neural lumen appeared to be completely occluded, though they were apparent between adjacent neuroepithelial cells.

Observations on the underlying mechanism(s) of cellular fusion are considered in the light of the ultrastructural findings. These results are compared with findings from analyses of various other sites of cellular fusion during embryogenesis. Attention is also drawn to the similarities and differences observed in the timing and overall pattern of events occurring during the early development of the neural tube in mouse and human embryos.

INTRODUCTION

A recent study has clearly demonstrated that occlusion of the lumen of the neural tube – eventually involving up to about 60% of its length – occurs as a normal event during early embryogenesis in man (Desmond, 1982). Furthermore, it was proposed that complete occlusion of the neural canal, which was first apparent at stage 11 (when embryos possessed 13–20 pairs of somites, see Streeter, 1942; O’Rahilly & Gardner, 1979), plays an important part in facilitating enlargement of the brain (Desmond & Jacobson, 1977). Indeed, these authors suggested that the latter would only occur once the neural tube had become a closed compartment filled with cerebrospinal fluid. Evidence from

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chick embryo studies in which the cerebrospinal fluid pressure within the neural system was markedly reduced following intubation of the neural tube (Desmond & Jacobson, 1977), eye (Coulombre, 1956) or myelencephalon (Coulombre & Coulombre, 1958) has clearly demonstrated that these experimental procedures invariably lead to the abnormal morphogenesis of both the brain and eye. Apart from the detailed histological analysis of the human embryonic material presented by Desmond (1982), observations on neural luminal occlusion in the chick embryo (Desmond & Jacobson, 1977), and the isolated example of luminal occlusion in the thoracic region of an early rat embryo illustrated by Freeman (1972), very little appears to be known about the occurrence and possible significance of this phenomenon.

In the present study, a histological account of neural tube occlusion during early mouse embryogenesis is given, detailing the period of development during which this phenomenon may be observed. Unlike the situation in the human embryo where occlusion occurs 'subsequent to the formation of a closed tube' (Desmond, 1982), in the mouse, at least, occlusion may be observed in embryos with only 10–12 pairs of somites present, when the cephalic and caudal regions of the neural tube are still widely open. Similarly, while the situation described in the human embryo suggests that in man the onset and sequential events associated with neural tube occlusion are remarkably uniform from embryo to embryo, the present findings tend to indicate that this does not appear to be the case in the mouse. For the latter reason, the present account can only serve as a very general guide to the events occurring in the early mouse embryo as development proceeds between about midday on the 9th to late on the 10th day of gestation.

Only sections from plastic-embedded material are presented here, as appropriately fixed material embedded in this way shows little in the way of shrinkage artefacts as are commonly observed in conventional paraffin-embedded material. An ultrastructural account of the morphological appearance of the closely apposed luminal cells in zones of partial or complete occlusion is also presented. The present findings of occlusion of an extensive, but variable, segment of the neural lumen in the mouse embryo would seem to confirm the contention that this phenomenon is probably a normal occurrence in many vertebrate species (Desmond, 1982). However, the detailed timing of this event, the overall pattern of neuroepithelial apposition and the duration of luminal occlusion appear to vary from one species to another, and in the mouse at least, from one embryo to another at otherwise similar stages of development.

MATERIALS AND METHODS

Female CFLP mice (Hacking and Churchill Ltd) were mated with males of the same strain and isolated on the morning of finding a vaginal plug (designated the first day of pregnancy). Between the early afternoon of the 9th day to late on the
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10th day of gestation individual females were killed by cervical dislocation and the embryos isolated in phosphate-buffered saline (pH 7.3). A total of 30 embryos were selected for this study, being divided in groups according to the approximate number of somites present, their degree of ‘turning’, and the presence of an obvious forelimb bud (the most advanced group). Between five and ten embryos were assigned to each group, though a small degree of overlap was inevitable using these somewhat arbitrary morphological criteria. An additional group of three embryos was isolated early in the morning on the 11th day of gestation. All of the embryos were dissected free of their membranes and fixed in 2% glutaraldehyde in 0.1 M-sodium cacodylate buffer containing 10 gm sucrose/100 ml. After about 2 h the embryos were transferred to 0.1 M-sodium cacodylate buffer containing 3 gm sucrose/100 ml. After 1 h the material was postfixed in 1% osmium tetroxide containing 5 gm sucrose/100 ml for 25 min. The material was then dehydrated through a graded ethanol series, and eventually embedded in epoxy resin (Spurr, 1969) and semithin transverse sections (thickness about 0.5–0.75 μm) taken and stained with methylene blue. Thin sections at appropriate levels were cut with a Huxley ultramicrotome, double stained with uranyl acetate and lead citrate and viewed in a Philips EM 300 transmission electron microscope.

An additional group of five embryos was used to determine whether extracellular material was detectable on the neuroepithelial cell surface at sites of close cell–cell apposition. The cephalic region of embryos in this group was removed prior to their fixation (for 2 h) in 2% glutaraldehyde in 0.1 M-sodium cacodylate buffer containing 3 mM-Ca²⁺ (pH 7.2). Decapitation was carried out in order to allow the ruthenium red access to the neuroepithelial cells lining the neural lumen in all embryos in this group, whether the rostral neuropore had closed or not. The embryos were then post-fixed in 1% osmium tetroxide either (i) with or (ii) without ruthenium red (2 mg/ml) in 0.1 M-sodium cacodylate buffer. This material was then dehydrated through a graded ethanol series, embedded, and semithin and thin sections taken as described above. The thin sections were viewed unstained in the Philips EM 300 transmission electron microscope orientated at either 20 or 40 kV.

The author’s collection of Bouin- and Susa-fixed paraffin-embedded material covering embryonic development between the 10th–12th days of gestation was also examined, and the incidence of luminal occlusion in this material is also briefly reported. The latter information is included here as it confirms the author’s contention that this phenomenon is most clearly seen in appropriately fixed plastic-embedded material.

RESULTS

1. Histological appearance of neural tube

Because of the considerable degree of variability observed in neural tube
morphology in mouse embryos that appear, at least externally, to be almost identical, it will only be possible to provide an approximate guide to the sequence of events occurring with respect to side-side apposition and occlusion of the neural lumen. For this reason, an overview of this phenomenon in the mouse will be presented here, using representative histological sections through the neural tube at different levels along the neuraxis in a selection of embryos isolated between the early afternoon of the 9th day to late on the 10th day of gestation.

The earliest embryo in which a moderate degree of occlusion was observed, had about 10–12 pairs of somites present, and had been isolated in the afternoon on the 9th day of gestation. This embryo was still 'unturned', but from its overall appearance was likely to have 'turned' to adopt the characteristic foetal position within a matter of hours. Only a small region of complete luminal occlusion is present, though extensive areas of side-side apposition and partial luminal occlusion are evident at various levels along the spinal axis. Representative sections through the neural tube at different levels are illustrated in Fig. 1.

The appearance of the neural lumen in an embryo at a slightly later stage of development, isolated in the evening on the 9th day of gestation, will now be considered. The cephalic neural folds in this embryo had yet to become apposed and fused in the regions overlying the presumptive fore-, mid- and hindbrain. The embryo was partially 'turned' and had approximately 12–14 pairs of somites present. As in the previous embryo, complete luminal occlusion is only evident over a relatively short segment, though side-side apposition and partial luminal occlusion involving extensive regions of the neural tube are clearly seen. Representative sections through the neural tube at different levels along the spinal axis in this embryo are illustrated in Fig. 2.

The next embryo in this series was almost completely 'turned' and had also been isolated in the evening of the 9th day of gestation. The embryo had about

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Fig. 1. Representative transverse sections through the neural tube of an 'unturned' embryo with 10–12 pairs of somites present, isolated in the afternoon on the 9th day of gestation. Sections stained with methylene blue. (A) Section through the neural tube at the midcardiac level. Note that the neural lumen is widely patent, and the notochord (arrowed) clearly seen. (B) Low-magnification view through the 'thoracic' region of this embryo. The section is taken through the caudal one third of the heart, and through the midtail region. Key: a, atrium; v, ventricle; y, yolk sac; m, amnion. (C) Higher magnification view through the neural tube in the 'thoracic' region at a level identical to that illustrated in B. A slight indication of side-side apposition is apparent at this level. (D) Section through the neural tube at the level of the sinus venosus, at the caudal extremity of the heart. A considerable degree of side-side apposition is seen, particularly in the central region of the neural tube, though the neural lumen is still patent at this level. (E) Section through the neural tube about half way between the sections illustrated in D above and F below, some distance proximal to the U-shaped lordotic segment in this 'unturned' embryo. The middle third of the neural lumen (region between arrows) appears to be completely occluded. (F) Section through the neural tube just proximal to the lordotic segment. The dorsal half of the lumen appers to be completely occluded whereas the ventral segment is still patent. Bar = 100μm.
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20 pairs of somites present, and the cephalic neural folds were completely fused. The region of apposition and occlusion in this embryo was also quite extensive, involving the neural tube from the hindbrain region just rostral to the otic pits, at about the level of the middle of the first branchial arch, caudally almost as far as the caudal neuropore. The appearance of the neural tube in this embryo is illustrated in Fig. 3.

The next embryo was isolated at about midday on the 10th day, had obvious forelimb buds and about 25 pairs of somites present. This embryo also had an extensive region of close side-side apposition with intermittent regions of partial
luminal occlusion. At no level, however, did the neural lumen appear to be completely occluded. The appearance of the neural tube in this embryo is illustrated in Fig. 4. The transmission electron micrographs which illustrate the sites of apposition and occlusion in more detail were all taken from this embryo.

The next embryo in this series was also isolated at about midday on the 10th day of gestation, had about 25 pairs of somites and easily recognisable forelimb buds present. A moderate degree of luminal apposition was present in this embryo involving principally the ventral third of the neural canal. The apposed
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Fig. 2. Representative transverse sections through the neural tube of a partially ‘turned’ embryo with 12–14 pairs of somites present, isolated in the evening on the 9th day of gestation. Sections stained with methylene blue. (A) Low-magnification view at the level of the rostral one-third of the heart. Some degree of side–side apposition of the walls of the neural tube are apparent. Key: f, foregut; v, ventricle; w, ‘thoracic’ wall. (B) Low-magnification view of section though the midcardiac level. Note the considerable diminution in the volume of the foregut compared to the situation illustrated in A above, and the almost complete obliteration of the neural lumen. Key: a, atrium, v, ventricle; f, foregut. (C) Low-magnification view through the mid-U region of the neural tube. In the proximal (lower) segment the lumen is virtually occluded, whereas in the distal (upper) segment the ventral half of the lumen is widely patent. Large blocks of somites (arrowed) are apparent on either side of the neural tube. (D) Higher magnification view of section through the neural tube at the level of the rostral one-third of the heart, just distal to the section illustrated in A above. A degree of side to side apposition is apparent. E. Section through the neural tube at a similar level to that illustrated in B above. Apart from a short dorsal segment (above), the majority of the neural lumen at this level appears to be completely occluded. (F) Section through the neural tube just rostral to the lumbosacral region. The neural lumen appears to be completely occluded. Bar = 100 μm.

Fig. 3. Representative transverse sections through the neural tube of an almost completely ‘turned’ embryo with about 20 pairs of somites present, isolated in the evening on the 9th day of gestation. Sections stained with methylene blue. (A) Section through the cephalic region just rostral to the otic pits and slightly distal to the origin of the 1st branchial arches (arrowed). Some degree of dorsal and particularly ventral side to side apposition is apparent in the hindbrain region (h). Key: f, forebrain; g, foregut. (B) Section through the hindbrain at the level of the otic pits (arrowed). Note that the neural lumen is completely occluded. Key: f, foregut; 1, first branchial arch; 2, origin of the second branchial arch. Bar = 300 μm. (C) Section through the ‘thoracic’ region at the level of the distal one-third of the heart. The ventral two-thirds of the neural lumen is occluded at this level. Key: a, atrium, prior to division into right and left sides; v, ventricle. (D) Section through embryo at the level of the two horns of the sinus venosus (s). The neural tube is virtually completely occluded at this level. (E) Slightly oblique section through embryo just above the forelimb bud (arrow). The lumen in the ventral half of the proximal region of the neural tube (upper) is almost completely occluded, while in the distal region (lower) the lumen, though narrow, is completely patent. (F) Section through embryo just below the forelimb bud. The middle two-thirds of the neural tube shown here represents the occluded proximal ventral segment of the lumen illustrated in E above. The paraxial blocks of somites are clearly seen at this level.

Fig. 4. Representative transverse sections through the neural tube of an embryo with approximately 25 pairs of somites present, isolated at about midday on the 10th day of gestation. Sections stained with methylene blue. (A) Section through fore- and hindbrain regions of embryo at the level of the first branchial arch. Key: f, forebrain; h, hindbrain; 1, first branchial arch; t, tail region. (B) Section through ‘thoracic’ region at the outflow of the heart. This section also passes through the hindbrain at the level of the otocysts. Key: o, otocyst, b, bulbus cordis; v, ventricle. (C) Slightly oblique section through embryo at the level of the forelimb bud (arrow). (D) Higher magnification view of neural tube in hindbrain region at the level illustrated in A above. A considerable degree of side to side apposition is apparent in the ventral third of the neural tube. Note that the notochord is adherent to the endodermal lining of the oropharynx at this level (arrow). (E) Neural tube in hindbrain region at level illustrated in B above. The middle segment of the neural lumen is completely occluded. (F) Neural tube proximal to the forelimb bud at level illustrated in C above. Bar = 200 μm. The majority of the ventral half of the neural lumen is completely occluded. (G) Neural tube distal to the forelimb bud at level illustrated in C above. (H) Neural tube in lower trunk region at level illustrated in A above. (I) Neural tube in lower trunk region just proximal to the caudal neuropore.
Fig. 3. For legend see p. 217.
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Fig. 4. For legend see p. 217.
segment extended for a short distance both rostrally and caudally from the level of the forelimb bud. This, and the previous embryo, appeared externally to be at an almost identical stage of development, but the extent and overall pattern of apposition and luminal occlusion in these two embryos were obviously quite dissimilar. The pattern seen in this, the last embryo in this series, however, represents the more typical situation observed at this period of embryogenesis. The appearance of the neural tube in this embryo is illustrated in Fig. 5.

Of three plastic-embedded embryos examined that had been isolated early on the 11th day of gestation, no evidence of occlusion, or even of close side-side apposition was observed.

Reference to the author’s collection of paraffin-embedded material covering the period studied, however, would appear to suggest that the incidence of luminal occlusion observed in embryos fixed on the 9th and 10th days is extremely low: only 3 out of 58 embryos had evidence of partial luminal occlusion. In all three cases, the embryos had about 25 pairs of somites present. Only a short partially occluded segment was evident, being located at the level of the forelimb buds. No evidence of partial occlusion was observed in the material isolated at earlier stages of embryogenesis.

Out of 34 paraffin-embedded embryos isolated throughout the 11th day, 3 showed a limited degree of partial luminal occlusion. In all of these embryos, only a short segment of the neural tube appeared to be involved, being located either proximal to the level of the hindlimb bud, at the level of the hindlimb bud, or between the fore- and hindlimb buds.

Somewhat surprisingly, out of five 12th day embryos examined, two had evidence of partial luminal occlusion with the zone of apposition being located in one embryo at the level of the hindlimb buds, and in the second embryo, between the fore- and hindlimb buds. In all but one of the eight paraffin-embedded embryos in which a zone of side-side ‘fusion’ was present, the lumen was patent dorsal and ventral to the site of apposition. In only one embryo was the dorsal half of the canal completely occluded. Fixation of embryos for 4 h in Susa produced considerably less evidence of shrinkage artefacts, particularly in the more advanced groups studied than 12–24 h fixation in full-strength or half-strength Bouin solution.

It is of interest that a section through the neural tube of a rat embryo with 13–20 pairs of somites present, at approximately the mid- to high-thoracic level, appears to demonstrate that complete neural tube occlusion also occurs in the rat embryo at this site (see Figure 1, E, Freeman, 1972). No additional information on the extent or overall pattern of neural tube occlusion observed in this species is, however, available.

2. Ultrastructural appearance of neuroepithelial cell surfaces at sites of close cell–cell apposition

The observations presented in this section are largely derived from an analysis
Fig. 5. Representative transverse sections through the neural tube of a second embryo with approximately 25 pairs of somites present, isolated at about midday on the 10th day of gestation. Sections stained with methylene blue. (A) Section through the 'thoracic' region at the level of the distal one-third of the heart. Key: h, distal region of hindbrain; f, foregut; a, atrium; prior to division into right and left sides; v, ventricle; t, mid-tail region. (B) Section through embryo at the level of the forelimb bud. Canalization of the midgut is just visible (between arrows). Bar = 500 μm. (C) Section through embryo distal to the forelimb bud. (D) Higher magnification view of neural tube at similar level to that illustrated in A above. (E) Neural tube proximal to the forelimb bud at level illustrated in B above. (F) Neural tube in the lower trunk region just proximal to the segment illustrated in C above.
of the electron micrographs taken at various locations along the neural axis from the embryo illustrated in Fig. 4. Other embryos at slightly different stages of development (both more and less advanced) were also examined, but the representative sections from this embryo serve to illustrate the principal ultrastructural features observed at the neuroepithelial cell surface in embryos in which occlusion of the neural lumen is taking place.

In locations where cellular contact had been made across the midline, a narrow luminal slit was still evident. Initial contacts were usually established between relatively small or occasionally quite large cellular protruberances. Characteristically, the majority of the apposing cell surfaces were either completely flattened or had a few small undulations (see Fig. 6A, B). Even the occasional small cellular protrusion observed appeared to have a relatively wide cross section (see Fig. 6A). At locations of very close apposition (e.g. see Fig. 6C), only a few areas where minimal ‘point’ contact was established were generally seen. At the cell surface, the occasional presence of coated pits was noted, while in the subcortical zone, small numbers of coated vesicles were also apparent.

In areas where extensive cell–cell contact had been established, only small pockets of luminal fluid remained between these sites of neuroepithelial cell–cell ‘fusion’. Despite a detailed search along lengthy stretches where the lumen was completely occluded (see Fig. 6D–E), no convincing evidence of junctional complexes linking the two sides was seen, even though the apical complexes between adjacent neuroepithelial cells were clearly apparent.

Cellular contact was generally established between two non-dividing cells, but was also not uncommonly observed between a non-dividing and a dividing cell (see Fig. 6C–D).

In the ruthenium-red-treated group, a thin layer of positively staining material was usually apparent on the neuroepithelial cell surface in regions of close cell–cell apposition, and extended distally until sites of complete neural tube occlusion were encountered. In those embryos in which there was complete

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**Fig. 6.** Transmission electron micrographs illustrating appearance of neuroepithelial cell surfaces at sites of cell–cell apposition and luminal occlusion. These are representative thin sections from various sites along the neural axis of the embryo illustrated in Fig. 4.

(A) and (B) Apposing neuroepithelial surfaces bridged by relatively large cellular protrusions. Between these sites of initial contact, the cell surfaces are either completely flattened or only slightly undulating. Both micrographs ×2600. (C) Extensive region of close apposition in which a narrow luminal channel is still visible. Note the occasional presence of coated pits (arrowheads) and coated vesicles (arrows). ×3700. (D) Extensive regions in which the neural lumen is completely obliterated, interspersed with small lacunae containing ‘spinal’ fluid. ×3100. (E) Higher magnification view of region in which the neural lumen is completely obliterated. Note the presence of coated pits and vesicles with granular contents (arrowed) in close proximity to the neuroepithelial cell surfaces, and the absence of junctional complexes between the apposed neuroepithelial cell surfaces. In this, as in A–D above, apical junctional complexes are observed between adjacent neuroepithelial cells. ×12 500.
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occlusion of the neural lumen, however, the neuroepithelial cell surfaces in non-occluded regions just distal to the initial sites of occlusion generally remained unstained, presumably because the ruthenium red failed to penetrate beyond the occluded segment. Examples of electron micrographs of the neuroepithelial cell surface in ruthenium-red-stained and unstained control material are presented in Fig. 7.

**DISCUSSION**

It seems evident from this and previous experimental and descriptive studies on neural tube occlusion (chick: Desmond & Jacobson, 1977; man: Desmond, 1982) that there are considerable species differences in the overall pattern of neuroepithelial cell apposition and luminal occlusion along the spinal axis. The underlying morphogenetic processes which eventually bring about this phenomenon are obviously rather complex, and presumably involve an interplay between a series of morphological changes which must occur within the cellular components of the neural tube, and extrinsic cellular and extracellular 'forces' which act on the neural tube from without (see, for example, Schroeder, 1971; Karfunkel, 1974). Once medial migration of the walls of the neural tube has been initiated, close apposition and eventual, albeit transient, 'fusion' may be the inevitable consequence. It is possible that the first contact may be established across the midline by the interdigitation of relatively small diameter projections which protrude from the surfaces of the many neuroepithelial cells whose apices abut on the spinal lumen. The initial contact and adhesion may in addition be facilitated by the presence of viscous ruthenium-red-positive extracellular material at the luminal surface of these cells.

An increase in ruthenium-red-positive material has been demonstrated along apical neural fold borders and on the overlying ectoderm cells in regions immediately prior to and at the time of neurulation (Moran & Rice 1975; Sadler, 1978), and its removal at this time can interfere with neural tube closure (Lee, Sheffield, Nagele & Kalmus, 1976; O'Shea & Kaufman, 1980). The presence of carbohydrate-rich surface coat material is also observed along prospective zones of fusion in the palate (Greene & Kochhar, 1974; Pratt & Hassell, 1975) and during fusion of the medial and lateral nasal processes (Gaare & Langman, 1977;}

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Fig. 7. Unstained transmission electronmicrographs from two embryos with about 15-20 pairs of somites present to illustrate the presence of extracellular material at the surface of neuroepithelial cells in sites of close cell–cell apposition. Both embryos were isolated at about 6 p.m. in the evening on the 9th day of gestation. (A) Ruthenium-red-stained section showing the presence of a thin coating of positively stained material at the neuroepithelial cell surface which apparently fails to penetrate beyond the apical junctional complexes between adjacent cells. X6100. (B) Higher magnification view of ruthenium-red-positively staining material at the neuroepithelial cell surface. X14 250. (C) Appearance of neuroepithelial cell surface in unstained control embryo. X14 500.
Smuts, 1977). In all of these locations, a decrease in the distribution of surface macromolecules is observed after fusion.

Possibly slightly later, after initial side–side contact has taken place, the cells with mound-like and flattened surfaces become more closely apposed, and allow contact to be established over much more extensive, though still localized, areas. Concomitant with the progressive increase which occurs in the surface area of contact, the luminal volume necessarily decreases.

The underlying mechanism(s) which eventually lead to the complete occlusion of the spinal lumen is still far from clear, though the absence of large numbers of pinocytic vesicles in the subcortical region of the neuroepithelial cells tends to suggest that absorption of the luminal fluid probably plays only a minor role in its removal from this site. The presence of moderate numbers of coated vesicles with granular contents in the subcortical zone and coated pits, which may well be their precursors (see Pratten, Duncan & Lloyd, 1980), at the cell surface, may be indicative of their role – possibly facilitating the removal of excess extracellular matrix material at the fusion site – during the final stages of apposition and cell–cell adhesion.

Curiously, despite the presence of obvious junctional complexes between the apical zones of adjacent neuroepithelial cells, no convincing complexes of any type could be discerned at the fusion interface. While this does not unequivocally exclude the possibility that some type(s) of specialized complexes are in fact formed, clearly other techniques e.g. freeze fracture analysis, would be required to demonstrate them.

In the mouse, unlike the situation in the human embryo (see Desmond, 1982), since apposition and fusion is first apparent in embryos with about 10 pairs of somites when both the rostral and caudal neuropores are still widely open, this would seem to be evidence in favour of the hypothesis that at least at this relatively early stage of embryogenesis most of the luminal fluid is probably displaced caudally or cranially (into the amniotic cavity) rather than resorbed locally. However, once the rostral neuropore, in particular, has closed (for timing, see Geelen & Langman, 1977; Kaufman, 1979), and complete luminal occlusion occurred some distance caudally, it seems likely that the production and only limited resorption of cerebrospinal fluid would facilitate the dilation of the brain and optic vesicles. While a situation similar to this appears to occur in man and in the chick embryo, this is obviously a considerable oversimplification, as in the majority of mouse embryos, for example, the neural lumen becomes patent along its entire length just before the caudal neuropore eventually closes (for timing, see Copp, Seller & Polani, 1982). Presumably, once ‘cerebral’ dilation has been initiated, the equalization between the CSF pressure and the amniotic fluid pressure – which occurs after the lumen becomes patent along its entire length, and remains thus at least until the caudal neuropore closes – does not appear to be detrimental in any way, at least in the mouse.

While the above account is undeniably somewhat speculative, at least as far
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as providing a detailed picture of the underlying mechanism of cell–cell fusion in this location, it does appear, to confirm the contention that neural luminal occlusion probably plays an important morphogenetic role in the development of the vertebrate nervous system.

Apart from answering certain questions regarding the timing and overall pattern of events in the mouse, this study raises other important questions. For example, it would be of considerable interest to know whether chemical messengers play a role at any stage in guiding the two morphologically indistinguishable neuroepithelial cell surfaces together. Equally, an analysis of the events occurring when the two sides separate once more late on the 10th or early on the 11th day (in the mouse) would be instructive. Similarly, descriptive and experimental observations on neuroepithelial cell–cell apposition and luminal occlusion in other vertebrate species might enable both the full significance of this phenomenon and its evolutionary history to be established.

This work was supported by a grant from the National Fund for Research into Crippling Diseases. I thank Mr. J. Skepper and Mr. M. Wombwell for their expert technical assistance, and Mr. J. Skepper additionally for his invaluable advice on the interpretation of the electron-micrographs.

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(Accepted 24 August 1983)
Influence of injected pluripotential (EK) cells on haploid and diploid parthenogenetic development

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SUMMARY

A number of pluripotential embryo-derived EK cells were introduced into the blastocoele of haploid and diploid parthenogenetic embryos which were subsequently transferred to suitable recipients. At autopsy on day 10 or 11 of pseudopregnancy 22% of decidua in the diploid series contained somite-stage embryos while an additional 12% contained abnormal egg cylinder-like sacs. In the haploid series, 7% of the decidua contained somite-stage embryos and an additional 5% contained abnormal 'sacs'. In 'injected' diploid and haploid 'controls' in which the zona pellucida were pierced with an empty injection pipette 3% and 0% respectively of decidua in these two series contained somite-stage embryos, while an additional 17% and 3% respectively of decidua in these two series contained abnormal sacs. GPI analysis revealed that the EK cells were incorporated into somite-stage conceptuses in only one third of the diploids and in none of the haploid embryos. Although the presence of EK cells considerably increases the chance of normal embryonic development taking place, a detectable contribution from the EK cells into the resulting somite-stage embryo is apparently not necessary. Possible mechanisms allowing successful early post-implantation development to occur in this study are discussed.

INTRODUCTION

Parthenogenetic mouse embryos generally fail to develop beyond the very early post-implantation period when transferred to pseudopregnant recipients, despite the fact that a high proportion are capable of evoking a decidual response (Graham, 1974; Whittingham, 1980; Kaufman, 1981). Two approaches have so far been successfully employed which enable a proportion of both haploid and diploid parthenogenones to develop to early somite stages. Single blastocysts may be transferred to ovariectomized recipients in which they initially enter the 'delayed' state. They are then allowed to implant and subsequently develop following treatment of recipients with appropriate exogenous hormones (Kaufman, Barton & Surani, 1977). Alternatively, several 8-cell embryos or morulae may be aggregated together to produce a chimaeric individual which then appears to be capable of developing in an 'intact' (non-ovariectomized, non-hormonally treated) recipient (see Kaufman, 1981).
Whatever the reason for the failure of parthenogenetic embryos to complete development, it is not associated with a lack of genetic information for prolonged survival. Thus, for example, teratomas may be induced experimentally following the transfer of blastocyst-stage parthenogenotes to ectopic sites (Iles et al. 1975). These teratomas contain a variety of cell types and indicate the degree to which differentiation can occur. Developmentally, cells derived from parthenogenotes would appear to be completely normal. When parthenogenetically activated embryos are combined with normal fertilized embryos viable live-born chimaeras are produced (Stevens, Varnum & Eicher, 1977; Surani, Barton & Kaufman, 1977). Some of these chimaeras have proved to be functional germ line mosaics (Stevens, 1978). EC cells isolated from ovarian tumours which have been derived from spontaneously activated oocytes are also capable of normal embryonic development when reintroduced into fertilized embryos (Cronmiller & Mintz, 1978; Illmensee, 1978; Fujii & Martin, 1980). In addition, pluripotential EK cells derived from haploid and diploid parthenogenetically activated oocytes grown entirely in vitro (Kaufman, Robertson, Handyside & Evans, 1983; Robertson, Kaufman, Bradley & Evans, 1983) are capable of participating very extensively in normal development in a chimaeric animal (Robertson, Kaufman & Bradley, unpublished observations).

The reason why parthenogenetically derived cells are capable of developing in a chimaeric association with fertilized cells but not in isolation is not known. One possible hypothesis which could explain these observations, namely that most parthenogenetic blastocysts contain an inadequate number of cells which are destined to form the embryo proper has been proposed (Kaufman, 1981). Consequently, most parthenogenotes appear to behave like trophoblast vesicles (Gardner, 1972) which are capable of evoking a decidual response, but fail to give rise to an embryo. In those rare instances in which an embryo does in fact form, it is possible that the number of precursor embryo cells present may have exceeded a certain minimum threshold level (Ansell & Snow, 1975). This hypothesis has obvious limitations in that, for example, it fails to explain why parthenogenotes that successfully develop to the limb-bud stage and indeed appear in most cases to be morphologically normal, should die shortly thereafter. Nevertheless, it does appear to provide a partial explanation for the poor developmental capabilities of the non-'delayed' and non-aggregated groups of parthenogenotes which are transferred to 'intact' recipients.

In order to investigate this topic in more detail, and possibly put the 'threshold level hypothesis' to the test, we decided to examine the effect of introducing a small number of fertilized/embryo-derived pluripotential EK cells (Evans & Kaufman, 1981) into the blastocoelic cavity of both haploid and diploid parthenogenetic embryos.

The EK cells are essentially identical to embryonal carcinoma (EC) cells (Evans & Kaufman, 1981; Robertson et al. 1983), and similar in size to inner cell mass (ICM) cells. Furthermore, they readily become incorporated into the ICM
EK cell injections into parthenogenetic blastocysts

and, under optimal conditions, are also capable of forming a chimaeric association with the embryonic cells (Robertson et al. 1983) so that they might be expected to colonize both the embryo and/or the extraembryonic membranes.

In the present study, small numbers of EK cells were injected into the blastocoelic cavity of either haploid or diploid parthenogenetic blastocysts, and the developmental potential of the resultant chimaeras examined on the 10th or 11th day of pregnancy, at a time when normal (fertilized) embryos would be expected to be at the early forelimb-bud stage of development and contain at least 20–25 pairs of somites.

**Materials and Methods**

Oocytes from superovulated (C57BL×CBA)F₁ hybrid female mice were activated parthenogenetically following a brief exposure to a dilute solution of ethanol in phosphate-buffered saline (PBS; for details of this activation procedure, see Kaufman, 1982). Various haploid and diploid classes of parthenogenone are induced, but only those activated oocytes that developed a single pronucleus following extrusion of the second polar body (uniform haploid class, Kaufman, 1981) were selected following this form of activation for use in this study. Additional groups of oocytes were exposed to a dilute solution of ethanol as described above, but were immediately transferred to medium containing 1 µg/ml Cytochalasin D, and retained in this medium for 4–5 h. This treatment effectively inhibits extrusion of the second polar body, and enables heterozygous diploid embryos (Kaufman, 1981) to be obtained; such embryos develop two pronuclei in the absence of second polar body extrusion.

Both groups of activated oocytes were retained in culture until they reached the expanded blastocyst stage. This stage was usually achieved by about 96–100 h following activation. The haploid and diploid blastocysts thus obtained were treated in one of two ways. They were either assigned to the ‘experimental’ series, in which case they were injected with 129/SvEv derived EK cells, or to the control series. The EK cell lines used in this study were of two distinct types. The B2B2 and CP1 lines were derived from fertilized ‘delayed’ blastocysts and both possess a normal euploid XY chromosome complement. The third line used, termed HD14, is derived from a haploid parthenogenetic embryo and has a normal XX chromosome complement. All of these pluripotential stem cell lines were established (for methodology see Evans & Kaufman, 1981) and propagated exclusively on feeder layers of inactivated fibroblasts. The EK cells were prepared for injection by dissociating the small cellular clumps isolated from tissue culture with EDTA/trypsin solution. Injection experiments were performed using cell lines which ranged between 8 to 15 passage generations since their initial isolation in tissue culture. As the lines have not shown distinct characteristics with regard to the experimental results presented here, the findings in each series have therefore been presented as a single group. All three of
these lines have previously been used in our laboratory to produce chimaeric mice when injected into 'host' fertilized blastocysts with a considerable degree of success (Robertson et al. 1983; Bradley & Robertson, unpublished results). The experimental embryos in the present study into which EK cells have been introduced will be referred to in the rest of the text as the 'injected' blastocysts.

During the injection procedure the blastocyst is immobilized, and all manipulations are carried out in a drop of medium (DMEM+10% Foetal Calf Serum) in a chamber under light paraffin oil at 10°C. Using a Leitz double micromanipulator apparatus, a single sharpened glass injection pipette (internal diameter 15–20 µm) is inserted through the zona pellucida and mural trophoblast layer into the blastocoelic cavity, and a small number of EK cells (generally 5–10) released into this location. Haploid and diploid control groups were established which contained blastocysts which had not at any time been exposed to EK cells. In these control series, the zonas of both haploid and diploid blastocysts were pierced with a glass needle to simulate the injection procedure referred to above, and to facilitate the 'hatching' process which is sometimes impaired in parthenogenones (see Discussion).

Both classes of embryos were allowed to re-expand in culture, and were subsequently transferred to 'intact' (i.e. non-ovariectomized) recipients (F₁ hybrid and CFLP strains) on the 3rd day of pseudopregnancy following mating to F₁ hybrid male mice of proven sterility (day of finding vasectomized plug referred to as the first day of pseudopregnancy).

The recipients were autopsied in the morning of the 10th day, or occasionally on the 11th day, of pseudopregnancy, when fertilized embryos would be expected to have 15–20 or over 25 pairs of somites, respectively. The total number of implants present and their contents were recorded. All of the embryonic material in the experimental series, and most of the material in the control series was analysed biochemically. Several grossly abnormal egg-cylinder-like structures, however, from the control series were examined histologically to investigate their morphology.

The larger embryos that had 'turned' to adopt the 'foetal' position were isolated from their yolk sacs, and the embryonic and extraembryonic samples were analysed separately by one-dimensional gel electrophoresis to determine their respective glucose phosphate isomerase (GPI) allozyme type(s). In the case of the smaller somite-containing 'unturned' embryos and the morphologically disorganized 'egg-cylinder-like' embryos, the entire conceptus including its membranes was examined as a single unit. This form of analysis enabled the origin of their component parts (whether parthenogenetic embryo- or EK-derived, or of mixed origin) to be determined. This was feasible because the parthenogenones resulted from the activation of F₁ hybrid oocytes that were homozygous for the Gpi-1b allozyme, whereas the EK cells were of 129/Sv/Ev origin and homozygous for the Gpi-1a allozyme of glucose phosphate isomerase.
EK cell injections into parthenogenetic blastocysts

Fig. 1. Examples of somite-containing embryos resulting from the injection of pluripotent EK cells into the blastocoelic cavity of haploid and diploid parthenogenetic blastocysts. All the embryos shown here appeared to be morphologically normal and healthy at the time of their isolation. A. headfold-stage embryo with 7–8 pairs of somites present contained within its yolk sac, from the haploid injected series. B. partially 'turned' embryo with about 15 pairs of somites present, from the haploid injected series. C. partially 'turned' embryo with about 15–16 pairs of somites present, from the diploid injected series. D. early 'turned' embryo with about 20 pairs of somites present, from the diploid injected series.
RESULTS

i) Developmental potential of 'injected' versus 'control' embryos

In the present study, a total of 256 out of 335 (76-4 %) of the haploid and diploid 'injected' and 'control' blastocysts successfully implanted (these figures relate to females in which 1 or more decidua were present, and therefore excludes 'recipient failures'). This is similar to the implantation rates achieved in earlier studies in which parthenogenetic blastocysts were transferred to pseudopregnant recipients (see Kaufman & Gardner, 1974; Kaufman et al. 1977; Kaufman, 1978).

Almost 22 % of the decidual swellings examined in the diploid 'injected' series contained somite-stage embryos, as did 7-0 % of the haploid 'injected' series (see Fig. 1). In addition, morphologically abnormal egg-cylinder-stage or possibly slightly more advanced embryos were also observed in these two 'injected' series. No detailed histological findings are available for this group as all embryos were analysed to determine their GPI content. In the diploid controls, examined on the 11th day, two somite-containing embryos, one with ten pairs of somites and the other with 16–18 pairs of somites were observed. In addition, a number of morphologically abnormal embryos were observed (see Fig. 2). The abnormal embryos proved to be particularly interesting, ranging from a single approximately 18–20 somite-stage embryo with a small mass of tissue which connected the head to the tail region (see Fig. 2C–F), to egg-cylinder-like sacs

Table 1. Implantation rate and development achieved by parthenogenetic blastocysts injected with EK cells, and un.injected haploid and diploid controls

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Groups</th>
<th>Embryos transferred</th>
<th>Implants (%)</th>
<th>Abnormal egg cylinder-stage embryos (%)*</th>
<th>Somite-stage embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid 'Injected'</td>
<td>138</td>
<td>101 (73-2)</td>
<td>12 (11-9)</td>
<td>22 (21-8)*</td>
<td></td>
</tr>
<tr>
<td>Diploid Control</td>
<td>83</td>
<td>65 (78-3)</td>
<td>11 (16-9)</td>
<td>2 (3-1)*</td>
<td></td>
</tr>
<tr>
<td>Haploid 'Injected'</td>
<td>72</td>
<td>57 (79-2)</td>
<td>3 (5-3)</td>
<td>4 (7-0)</td>
<td></td>
</tr>
<tr>
<td>Haploid Control</td>
<td>42</td>
<td>33 (78-6)</td>
<td>1 (3-0)</td>
<td>0 (–)</td>
<td></td>
</tr>
</tbody>
</table>

* percent of implanted embryos.
† χ² analysis. p > 0.01

Fig. 2. Representative histological sections through two morphologically abnormal conceptuses from the diploid control series. The material has been stained with haematoxylin and eosin. A, B. two sections through an abnormal egg cylinder-like sac containing healthy, but disorganized, tissues. C–F. intermittent serial sections through the cephalic region of an abnormal embryo with about 18–20 pairs of somites present. A small solid mass arises in the cephalic region (C, arrowed) overlying the forebrain (f). This structure becomes canalized (D, arrowed) and the tube thus formed eventually fuses with the neural canal in the tail region (E, F. arrows).
Fig. 2
which contained very disorganized and unrecognizable arrays of embryonic tissue (see Fig. 2A, B). In the haploid controls, a single morphologically abnormal egg cylinder stage embryo was observed. A summary of these findings is presented in Table 1.

As far as it was possible to judge on gross inspection, all of the other somite-containing embryos appeared to be morphologically normal with beating hearts and (in the more advanced embryos) a yolk-sac circulation.

ii) GPI analysis of injected conceptuses

In the two experimental series reported here, almost three quarters of the somite-stage embryos of diploid origin and all those of haploid origin, appeared to contain cells of Gpi-1b type only. Full details of the GPI results are given in Tables 2 and 3. Interestingly, all but one of the embryos which showed evidence of chimaerism were in the more advanced group, with at least 20–25 pairs of somites present. However, about two thirds of the most advanced embryos did not appear to have any detectable EK contribution. Similarly, there was no apparent evidence of any preferential embryo or yolk sac colonization.

Table 2. Incidence of chimaerism in parthenogenetic blastocysts injected with EK cells

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Stage of autopsy</th>
<th>Abnormal egg cylinder-stage embryos</th>
<th>Somite-stage embryos</th>
<th>Pairs of somites present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2-8</td>
<td>9-12</td>
</tr>
<tr>
<td>Diploid</td>
<td>11th day</td>
<td>12± (2)*</td>
<td>2</td>
<td>2 (1)*</td>
</tr>
<tr>
<td>Haploid</td>
<td>10th day</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate embryos with EK cell chimaerism, as revealed by GPI analysis.
† No result was obtained with five of these embryos.

Table 3. Approximate extent of EK cell contribution in diploid chimaeric embryos and their yolk sacs

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Approximate no. of somites present</th>
<th>EK contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Embryo</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>30*</td>
</tr>
</tbody>
</table>

* Embryo and its yolk sac were analysed as a single sample.
EK cell injections into parthenogenetic blastocysts

The GPI status of the morphologically abnormal egg cylinder-like sacs proved to be technically rather more difficult to determine, presumably because of the very small volume of tissue generally present in these conceptuses (for details of results see Table 2).

DISCUSSION

Several of the findings in this study were unexpected, and consequently raise issues which justify detailed consideration. Firstly, the relatively high incidence of somite-containing embryos in both the haploid and diploid ‘injected’ series was surprising, as previous studies had indicated that unless parthenogenones are induced to enter a period of quiescence (i.e. the ‘delayed’ state) or aggregated together at the 8-cell or morula stage before implantation, the chances of obtaining development beyond the early egg cylinder stage are slight (for discussion, see Kaufman et al. 1977; Kaufman, 1981). Indeed, in the most comprehensive study available, in which embryos were activated and allowed to progress in vivo, the incidence of healthy development to the egg cylinder or early somite stage was 2-3 % when females were autopsied on the 9th or 10th day following electrical stimulation, and the few embryos recovered were retarded in development by approximately 1 day (Tarkowski, Witkowska & Nowicka, 1970). In another series in which in vitro activated eggs were transferred to appropriate recipients, no embryonic development was observed when autopsies were carried out on the 6th or 7th day of pseudopregnancy and the contents of over 130 decidual swellings examined (Kaufman & Gardner, 1974).

In the present study, 21·8 % and 7·0 % of the blastocysts that implanted in the diploid and haploid ‘injected’ series, respectively, progressed to somite-containing stages of development. In the diploid control series 3·1 % of the blastocysts that implanted developed to this extent, while in the haploid control series no such advanced embryos were observed. Even the relatively small number of somite-containing embryos present in the diploid control series was unexpected, as were the morphologically abnormal conceptuses which were mostly at the egg-cylinder-stage, as previous experience with non-‘delayed’ material suggested that embryonic losses almost invariably occurred during the very early post-implantation period. It was therefore all the more surprising to find that the stage of development achieved by many of the somite-containing embryos in the two experimental series was similar to that of fertilized embryos isolated from recipients at a comparable stage of gestation. As far as it was possible to judge on gross inspection, virtually all of the somite-containing embryos appeared to be healthy and morphologically completely normal (see Fig. 1). Those embryos with more than 6–8 pairs of somites present all had a beating heart at the time of their isolation.

One possible explanation for the modest incidence of embryonic development (both normal and disorganized) in the control series was that puncturing the zona
pellucida with a needle facilitated the ‘hatching’ process in these groups. Previous observations on embryos cultured in vitro (M. H. Kaufman, unpub.) have repeatedly demonstrated that the ‘hatching’ process may be considerably impaired in parthenogenones compared to fertilized embryos. Whereas most expanded blastocysts in the latter group might reasonably be expected to ‘hatch’ in serum-containing medium, far fewer parthenogenones (diploids > haploids) appear to be capable of doing so. If any significant delay in ‘hatching’ occurs in vivo, this would almost certainly exaggerate any possible degree of asynchrony present between these embryos and the uterus, and consequently decrease the chance of normal embryonic development occurring. While the eventual exposure of the trophoblast cells to the uterine epithelium would normally be capable of inducing a decidual response, any delay in the appearance of the latter would almost certainly be detrimental to embryonic development. However, the situation in the ‘injected’ group is rather different. It is possible that in these embryos, the enhanced development potential observed was related to the fact that the EK cells rapidly become incorporated into the ICM component of the blastocyst. We believe that the introduction of these cells probably increases its cellular pool, possibly beyond a hypothetical minimum threshold level necessary for embryonic development beyond the early post-implantation period to take place.

It is possible that in those injected blastocysts in this study that implanted but did not form embryos, either insufficient EK cells were injected or, more likely, became incorporated into the ICM, so that the combined EK and ICM cell population was insufficient to enable successful early post-implantation development to take place.

The findings from GPI analysis that the EK cells did not become incorporated into the embryos to form chimaeric individuals in the haploid, and in only one third of the diploid conceptuses was also unexpected, as we have demonstrated that identical EK cells injected into fertilized blastocysts readily gave rise to live-born overtly chimaeric mice (authors, and R. L. Gardner, unpub.). It is unclear whether the injected but apparently non-chimaeric parthenogenetic embryos possessed a low (<5 %), and therefore undetectable EK contribution. The absence of a substantial EK component in two thirds of the somite-stage embryos was all the more surprising because it had been assumed that the haploid parthenogenones in particular would probably act as a ‘passive’ carrier for the EK cells, allowing them to overcome the ‘implantation barrier’ and then take over to form the major component of the embryo. Indeed, only a single ‘injected’ diploid embryo has shown an EK contribution in excess of 50 %.

It is possible that the difference in response observed between the present injection experiments and earlier studies in which similar cells were injected into fertilized blastocysts may reflect subtle differences in the properties and morphology of the ICMs between these two groups. It is relevant to recall that the ICMs of expanded parthenogenetic blastocysts when isolated by immunosurgery
and subsequently allowed to develop in vitro are still capable of producing typical trophoblast giant cells (Kaufman, 1981) at a time when similarly isolated ICM cells of fertilized origin would not be capable of doing so (see Handyside, 1978; Spindle, 1978; Rossant & Lis, 1979). The latter property may be related to the relatively smaller cell population present in the ICMs of parthenogenones compared to fertilized blastocysts (Kaufman, 1978), or may reflect the fact that in parthenogenones, at a comparable stage of morphogenesis, fewer ICM cells are irreversibly committed to form embryonic derivatives. Alternatively, primitive endoderm formation may occur precociously in the parthenogenetic group, and thereby form a barrier which effectively excludes the injected EK cells from forming a significant part of the developing embryo. It is possible therefore that a substantial proportion of the injected EK cells may be relegated to an endoderm lineage, or be excluded completely from taking part in subsequent development.

It seems to us that a more detailed analysis of the fate of the EK cell population during the early post-implantation period in 'injected' parthenogenetic and fertilized blastocysts (using appropriately 'labelled' EK cells) might help to clarify some of these issues. This approach would almost certainly shed light 1) on the transformation of teratocarcinoma cells into normal embryonic cells and tissues, and 2) on the behavioural differences between parthenogenetic and fertilized embryos during this period.

We would like to thank Lesley Cooke for her excellent technical assistance. This work has been supported by grants from the Medical Research Council and the Cancer Research Campaign. A.B. is supported by an M.R.C. Studentship.

REFERENCES


(Accepted 19 December 1983)
RAPID COMMUNICATION

Influence of Ethanol on Chromosome Segregation During the First and Second Meiotic Divisions in the Mouse Egg

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ABSTRACT  This study was carried out to investigate the influence of ethanol on chromosome segregation during the first and second meiotic divisions. Female mice were given a single intragastric injection of a dilute solution of ethanol either just before or at various times after the HCG injection for inducing superovulation. The mice were mated, and the chromosome constitution of fertilized eggs were determined at the first cleavage mitosis. The technique employed allowed the male- and female-derived pronuclear sets to remain as two discrete groups. Exposure from 1.5 h before to 17 h after the HCG injection induced a high incidence of aneuploidy (15–25%) involving in over 90% of cases only one chromosome, so that either 19 or 21 instead of the normal complement of 20 chromosomes were present in one of the two sets (a previous study using a "marker" chromosome has demonstrated that the nondisjunction induced here invariably involves the female set). We suggest these findings draw attention to the susceptibility of chromosome segregation in female germ cells to interference by ethanol and that the mode of action is likely to be via interference with the normal functioning of the spindle apparatus. It is possible that interference with meiotic chromosome segregation by spindle-acting agents such as ethanol might account for a proportion of human spontaneous abortions within similar chromosomal defects where no other obvious cause is apparent.

It has been known for some time that anesthetics and related agents such as ethanol may impair cell division, though little direct evidence for an effect on chromosome segregation has, to date, been reported. These agents presumably act on the spindle apparatus and its associated proteins (see Anderson, '66; Fink, '71) by binding to and changing the physical and chemical properties of the spindle gels (Borisy and Taylor, '67), which essentially consist of orientated microtubules. Following exposure to these agents, the spindle gel appears, but its components are depolymerized (Allison and Nunn, '68; Haschke et al., '74; Hinkley and Telser, '74; Nicolson et al., '76) and its function is consequently impaired. The inhibitor action of these agents on cell division is similar in appearance to the arrest of mitosis induced by colchicine (Levan, '38).

Studies on mitotic cells have indicated that the impaired functioning of the spindle apparatus might be due to the action of these agents on the centrioles (see Brinkley et al., '67). These organelles, however, apparently disappear during the extended period of dictyate arrest, are not present in mammalian eggs after the pachytene stage of oogenesis (they are absent during meiosis I and II), and are also absent during the early cleavage divisions following fertilization (Szollosi et al., '72). Nevertheless, when the chromosome constitution of oocytes isolated from female mice anesthetized with tribromoethanol (Avertin-Winthrop) at intervals during meiosis was determined, a significant incidence of aneuploidy was observed (Kaufman, '77). Highest rates (10–12%) occurred when females were anesthetized at about the time of the HCG injection for inducing superovula-
tion (1.5 h before, and 2 h after the HCG injection; see Edwards and Gates, '59). In a subsequent study in which unfertilized mouse eggs were induced to develop parthenogenetically following a brief exposure in vitro to a dilute solution of ethanol in phosphate-buffered saline (Kaufman, '82; Cuthbertson, '83), up to 20% of the oocytes were shown to be aneuploid. G-banding analysis of these aneuploid preparations indicated that nondisjunction had in fact occurred.

Additional studies (Kaufman, '83a) in which recently mated female mice were given a dilute solution of ethanol in distilled water via an intragastric tube, when their fertilized eggs were in the process of completing the second meiotic division, have also revealed that exposure to ethanol by this route is capable of inducing a relatively high incidence (up to 20%) of aneuploidy. Due to the presence of an easily distinguishable "marker" chromosome in the sperm-derived set, it was possible to establish unequivocally that nondisjunction occurred only in the oocyte-derived set of chromosomes.

The principal aim of the present study therefore was to pursue these observations in more detail by investigating the influence on chromosome segregation of a single in vivo exposure to ethanol, carried out at various times during the first and second meiotic divisions, when the centriolar apparatus was known to be absent from the egg.

To achieve this end, female mice were exposed to ethanol either before or shortly after the HCG injection for superovulation, and the chromosome constitution of these eggs was then determined during their first cleavage mitosis. The same chromosome-spreading technique was employed here that had previously enabled the contents of the two pronuclei to be ascertained separately. The incidence of nondisjunction, in which interference with meiotic chromosome segregation had been induced, was shown to be related to the time of exposure of this agent.

A possible explanation for these findings is presented, and their potential clinical significance briefly discussed.

**MATERIALS AND METHODS**

Eight- to twelve-week-old CFLP (Hacking and Churchill) female mice (mean weight ± SE, 33 ± 1 g) were injected with 2.5 IU pregnant mare's serum gonadotrophin followed 48 h later by 2.5 IU human chorionic gonadotrophin (HCG) to synchronize ovulation.

The mice were caged with (C57BL × CBA)F1 hybrid or CBA-T6T6 males shortly after the HCG injection and checked for vaginal plugs early the next morning. At various times in relation to the HCG injection those females that had mated were lightly anesthetized with ether so that the test solution (either 1.5 ml of distilled water in the case of the controls, or 1.5 ml of a 12.5% solution of Analar quality ethanol in distilled water in the experimental groups) could be introduced via a fine plastic tube passed into the stomach. Most females recovered from the anesthetic effect of the ether shortly after the tube was withdrawn from the stomach. The mice were often very drowsy for 1-2 h after the ethanol administration and were left to recover in the warmth.

The intragastric ethanol administration was carried out at one of the following times, either 1.5 h before, or 1.75 h, 4 h, 13.5 h, 17 h, or 20 h after the HCG injection. Ovulation was assumed to occur at about 11-13 h after the HCG injection (see Edwards and Gates, '59).

The administration of 1.5 ml of this solution of ethanol by the intragastric route gave a serum level of ethanol of about 260-280 mg/100 ml after about 20-30 min. This level was maintained for about 2 h and then rapidly declined, so that baseline levels were usually achieved after 5-6 h.

The females were autopsied following cervical dislocation at about 20-21 h after the HCG injection and the eggs examined for the presence of pronuclei. The fertilized eggs were transferred to medium containing 1 μg/ml of colcemid, and their chromosome constitution was determined early the next morning by the air-drying technique described by Tarkowski ('66). All preparations were stained with Giemsa. The total number of chromosomes present in each spread was determined. If there was any doubt regarding the number of chromosomes present due to the overlapping of chromosomes, the group was excluded from the study. Using this technique, the male- and female-derived chromosomes are almost always present as two discrete sets (see Kaufman, '83a). In those matings in which CBA-T6T6 males were used, the male-derived group is easily recognizable as it contains the T6 "marker" chromosome (see Kaufman, '83a). In the eggs recovered from females mated to F1 hybrid males, it was occasionally possible to distinguish the male from the female pronuclear
set, as the two groups show different degrees
of condensation (Donahue, '72). This a curi-
ous situation, as the chromosomal morphol-
yogy is similar to that observed at the
"chromatid" stage of metaphase of the first
cleavage mitosis, shortly after the two sets
have associated on the equator of the mitotic
spindle. At this stage, the two sets should
show an identical degree of condensation (this
situation is in fact observed when air-dried
preparations are made in the absence of col-
cemid; see Kaufman, 1983b). All of the aneu-
ploid groups that contained either more or
less than the normal haploid complement of
20 chromosomes were photographed.
To test the effect of the intragastric injec-
tion of ethanol on mice of a different strain,
8-12-week-old randomly bred MF1 mice were
lightly anesthetized with ether so that 1 ml
of a 12.5% solution of ethanol in distilled
water could be passed into the stomach. The
MF1 mice received a slightly lower dose of
ethanol because their average weight was in
the region of 23-25 g. In this part of the
study, the ethanol administration was car-
rried out at 1.75 h, 13.5 h, and 17 h after the
HCG injection. Instead of being exposed to
ethanol, an additional group of MF1 females
were injected intraperitoneally with the an-
esthetic agent tribromoethanol (Aver-
tin:Winthrop) at 13.5 h after the HCG
injection. The standard dose for achieving
about 20-30 min of general anesthesia was
employed, namely 0.20 ml/g body weight of a
freshly prepared 1.2% solution of Avertin dis-
solved in 0.9% saline.
No mortalities occurred as a result of the
dosages of ethanol employed, and even the
most drowsy females appeared to have re-
covered completely from the effect of the
ethanol treatment after 3-4 h.
The chromosome constitution of the fertil-
ized eggs from the MF1 females was deter-
mined as described above.

RESULTS
Effect of ethanol administration on
chromosome segregation in eggs of
CFLP mice
The detailed results of the first cleavage
analyses of the fertilized eggs isolated from
CFLP mice that had mated to Fl hybrid and
CBA-T6T6 males are presented in Table 1.
In all of the successful analyses, the chromo-
some constitution of each of the two pronu-
clear sets could be unequivocally determined.
Those groups in which, usually due to over-
lapping of chromosomes, it was impossible to
determine the number of chromosomes pres-
ent in each group were automatically trans-
ferred to the "non analyzable" column.
In all of the experimental groups studied,
with the exception of the females that were
given ethanol at 20 h after the HCG injec-
tion, a relatively high level of aneuploidy
was recorded (i.e., in the groups exposed to
ethanol 1.5 h before, or at 1.75 h, 4 h, 13.5 h,
and 17 h after the HCG injection). While the
incidence varied from 15.3-25.0% in the dif-
ferent groups, these results were not signifi-
cantly different. By contrast, no aneuploids
were observed in the groups exposed to
ethanol at 20 h after the HCG injection, nor
was aneuploidy observed in the single con-
trol group that was given 1.5 ml of distilled
water via an intragastric tube while lightly
anesthetized with ether.

TABLE 1. Chromosome constitution of eggs from CFLP mice analyzed at first cleavage mitosis following ethanol
administration1 at various times in relation to HCG injection for inducing superovulation.

<p>| Time of ethanol  | Total     | Preparations  | Chromosome no. | Percent |</p>
<table>
<thead>
<tr>
<th>administration</th>
<th>relation to HCG injection (±h)</th>
<th>embryos examined</th>
<th>not analyzable (%)</th>
<th>18:20</th>
<th>19:20</th>
<th>20:20</th>
<th>21:20</th>
<th>22:20</th>
<th>Triploids</th>
<th>aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1.5</td>
<td>48</td>
<td>5(10.4)</td>
<td>-</td>
<td>3</td>
<td>36</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>18.3</td>
</tr>
<tr>
<td>2</td>
<td>+1.75</td>
<td>42</td>
<td>37(1.1)</td>
<td>-</td>
<td>2</td>
<td>33</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>15.4</td>
</tr>
<tr>
<td>3</td>
<td>+1.75</td>
<td>52</td>
<td>31(1.9)</td>
<td>1</td>
<td>4</td>
<td>41</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>19.8</td>
</tr>
<tr>
<td>4a</td>
<td>+1.35</td>
<td>26</td>
<td>22(7.7)</td>
<td>2</td>
<td>1</td>
<td>18</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>25.0</td>
</tr>
<tr>
<td>4b</td>
<td>+1.35</td>
<td>59</td>
<td>36(1.1)</td>
<td>4</td>
<td>43</td>
<td>8</td>
<td>-</td>
<td>1</td>
<td>21.8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>+17</td>
<td>67</td>
<td>69(0.0)</td>
<td>4</td>
<td>50</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>+20</td>
<td>22</td>
<td>14(5.1)</td>
<td>4</td>
<td>29</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>+13.5 (control)</td>
<td>34</td>
<td>6(17.6)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1All females received 1.5 ml of 12.5% solution of ethanol in distilled water (groups 1-6) or distilled water only (group 7).
All females mated to C57BL × CBA F1 hybrid males except group 4a, which were mated to CBA-T6T6 males.
The fact that some of the females were mated to F1 hybrid males and others to CBA-T6T6 males did not appear to influence these findings in any way, as in those preparation in which it was possible to recognize the male from the female pronuclear chromosome group, the former invariably contained a euploid set of chromosomes. In 86% of the aneuploid groups in this series only one extra (or missing) chromosome was involved; in the remaining 14%, two chromosomes were involved. The overall incidence of triploidy in this experimental series was 1.2%.

**Influence of ethanol administration in MF1 mice**

The detailed results of the first cleavage analyses involving the MF1 mice are presented in Table 2. A similar level of aneuploidy was observed in the three experimental groups (i.e., in which females were exposed to ethanol at 1.75 h, 13.5 h, or 17 h after the HCG injection). While the incidence of aneuploidy ranged from 15.7–24.0%, these results could not be analyzed statistically because of the small numbers involved. The trend, however, is identical to that observed in the CFLP results (see above). A single pronuclear group with 21 chromosomes present was observed in control series, which received 1 ml of distilled water via an intragastric tube while lightly anesthetized with ether.

**Influence of Avertin anesthesia in MF1 mice**

The results of this study are also presented in Table 2 (group 5). This agent, in the dose employed, appeared to be slightly more effective than the dose of ethanol employed in this strain of mice in inducing aneuploidy. However, despite the apparent trend, the incidence of aneuploidy was not significantly higher in the Avertin compared to the ethanol-treated groups. In this somewhat smaller study than the CFLP results reported above, only preparations in which a single extra (or missing) chromosome was involved were observed.

The overall incidence of triploidy in these two experimental series in which MF1 mice were exposed to ethanol or Avertin was 0.8%.

**DISCUSSION**

In the present study, female mice were given ethanol at various times during meiosis to determine the period during which a significant incidence of aneuploidy might be induced. The sensitive period in this regard was shown to be particularly extensive, with a relatively high incidence of aneuploidy being observed when females were exposed to ethanol from 1.5 h before to 17 h after the HCG injection for inducing superovulation.

When females were given 1.5 ml of 12.5% ethanol by mouth, a peak level of about 260–280 mg/100 ml blood was achieved after 20–30 min. This high level was retained for about 2 h and returned to baseline levels after about 5–6 h. A high concentration of ethanol is therefore maintained in the blood and tissues for a considerable time after its administration (see Kaufman and Wollam, '81).

Exposure to ethanol between 1.5 h before and 17 h after the HCG injection for superovulation extends over the critical periods when oocytes are progressing through metaphase of the first and second meiotic divisions. Because the period over which high blood levels of ethanol are present extends over hours rather than minutes, it is not possible to state unequivocally that oocytes

**TABLE 2.** The chromosome constitution of MF1 mice analyzed at first cleavage mitosis following ethanol or Avertin administration* at various times in relation to HCG injection for inducing superovulation

<table>
<thead>
<tr>
<th>Time of ethanol administration in relation to HCG injection (± h)</th>
<th>Total embryos examined</th>
<th>Preparations, not analyzable (%)</th>
<th>Chromosome No. (21:20)</th>
<th>Triploids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>1.5</td>
<td>38</td>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>13.5</td>
<td>30</td>
<td>5(16.7)</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>13</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Avertin + 13.5</td>
<td>43</td>
<td>6(14.0)</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>Control + 13.5</td>
<td>17</td>
<td></td>
<td>16</td>
</tr>
</tbody>
</table>

*Females received 1 ml of 12.5% solution of ethanol in distilled water (groups 1–3) or distilled water only (group 5).

Females in group 4 received intraperitoneal injection of Avertin (for details, see text). All females mated to C57BL x CBAF1 hybrid males.
are sensitive to the spindle-disrupting properties of this agent only during metaphase of the first or second meiotic divisions. While it is possible that the spindle apparatus and its precursor elements might have been depolymerized at stages of meiosis prior to metaphase, at later stages of the cycle once chromosomal migration has been initiated, aneuploidy is the likely outcome. The fact that aneuploidy results when oocytes are subjected to ethanol at 17 h, but not at 20 h, after the HCG injection for superovulation would also tend to favor this hypothesis. Equally, the observation that aneuploidy results when oocytes are subjected to ethanol 1.5 h before the HCG injection would be explained by the fact that high circulating levels of ethanol would still be present when these oocytes enter metaphase of the first meiotic division.

The present finding that interference with spindle activity can occur at the first as well as the second meiotic division therefore clearly confirms and extends the previous observation (Kaufman, '83a) that ethanol exposure is capable of inducing aneuploidy by interfering with chromosome segregation at the second meiotic division. While the exact mechanism involved still remains to be established, it seems clear that interference with the centriolar apparatus plays no role in the induction of non-disjunction, as this is known to be absent at these stages of oocyte maturation.

Previous studies with the anesthetic tribromoethanol (Kaufman, '77) suggest that this agent also interferes with meiotic chromosome segregation over the same time period and possibly by the same mechanism as ethanol. The additional finding reported in the present study, that the disruptive effect on chromosome segregation is clearly not strain dependent, is particularly interesting. Indeed, since anesthetics and ethanol (and its principal metabolite acetaldehyde) act on the spindle apparatus and on cytoskeletal elements within the cell (O'Shea and Kaufman, '80, '81), it should not be surprising if the effects reported here were applicable to other mammalian species, including man.

The particular relevance of the present findings is that it draws attention to the susceptibility of female germ cells to interference by spindle-acting agents. The effects of ethanol (in the doses employed in the present study) certainly appear to be more subtle than the action of many other spindle poisons (e.g., cytochalasin and colchicine) in the doses usually administered (see Edwards, '54, '56; McGaughey and Chang, '69; for recent reviews, see Niermerko and Opas, '78; Kaufman, '83b). The latter agents almost invariably induce a high incidence of heteroploidy (in about half of the aneuploid embryos examined, a large number of extra or damaged chromosomes were frequently observed), or the complete inhibition of cytokinesis at the completion of the second meiotic division, leading to triploidy.

While triploidy was observed in the experimental groups in the present study, the incidence was very low, and certainly no higher than observed in previous control studies (see Donahue, '72; Kaufman, '73; Fraser et al., '76; Maudlin and Fraser, '77). It appears that the threshold level of ethanol and anesthetics for inducing nondisjunction was only achieved in about one fifth of the oocytes exposed to these agents. Even when these agents were effective in this regard, they only interfered with the normal segregation of one (in about 90% of cases of aneuploidy reported here) or at most two chromosomes. Clearly, extensive "banding" studies would need to be carried out to determine whether particular chromosomes (e.g., chromosome 19; see Martin-DeLeon and Boice, '83) were more susceptible than others to undergo nondisjunction. A detailed analysis of this type would also be necessary to exclude examples of nondisjunction involving two or more chromosomes while the two examples of chromosome "A" migrate to one pole while the other two examples of chromosome "B" migrate to the other pole. In such instances, a genetically unbalanced pronuclear set with, however, the normal number of chromosomes present would result.

We believe that it might be justified to extrapolate from our findings to the situation in man. It is possible that the type of interference with chromosome segregation observed here, which presumably could also be induced by other spindle-acting agents, may account for at least a proportion of the very considerable number of human spontaneous abortions which are known to have a similar type of numerical chromosomal anomaly (see Boué and Boué, '76) where no other obvious cause is apparent. Almost all of the latter must result from errors of chromosome segregation that occurred during gametogenesis or at the time of conception when oocytes are
induced to complete the second mitotic division by the fertilizing spermatozoon. While ethanol may not be the causative agent in
many of these cases of spontaneous abortion, the present and recent studies by Kaufman ('82, '83a) would appear to indicate that in man, exposure to anesthetics, ethanol, and other spindle-acting agents should be avoided during the oocyte maturation cycle leading to ovulation if this is to be associated with conception. The potentially harmful effects of relatively high levels of maternal ethanol consumption during pregnancy are, of course, already well recognized (Ulleland, '72; Jones et al., '73; for recent reviews, see Pratt, '82; Colangelo and Jones, '82, '83; '85).

ACKNOWLEDGMENTS

We would like to thank Mrs. Lesley Cooke for expert technical assistance. The work was supported by the National Fund for Research into Crippling Diseases (M.H.K.).

LITERATURE CITED


Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines

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The recent availability in culture of embryo-derived pluripotential cells which exhibit both a normal karyotype and a high differentiative ability has encouraged us to assess the potential of these cells to form functional germ cells following their incorporation into chimaeric mice. We report here the results of blastocyst injection studies using three independently isolated XY embryo-derived cell lines (EK.CP1, EK.CC1.1 and EKCC1.2) which produce a very high proportion (>50%) of live-born animals that are overtly chimaeric. Seven chimaeric male mice, derived from these three lines, have, so far, proved to be functional germ-line chimaeras.

Although teratocarcinoma-derived embryonal carcinoma (EC) cells have been used to construct chimaeras, they have rarely realized the developmental potential demonstrated by embryonic inner cell mass cells used in a similar combination. Indeed, when introduced into the early embryo many EC cell lines show a low rate of colonization and/or a restricted pattern of differentiation, and many chimaeras develop tumours both pre- and postnatally.

We have recently demonstrated that over 15 of our fertilized and parthenogenetically activated embryo-derived (EK) cell lines of both XX and XY sex chromosome constitutions form normal chimaeras with high efficiency (authors, in preparation). In this study we decided to use fertilized embryo-derived pluripotential XY cell lines, which, unlike most of their tumour-derived EC cell counterparts, readily differentiate in vitro and possess a normal euploid karyotype, an essential prerequisite for the formation of viable gametes. We now present data which clearly demonstrate that such cell lines are capable of forming functional germ-line chimaeras.

Table 1 gives the results of the blastocyst injection experiments and Table 2 the results of the test breeding studies. Figure 1 shows six of the phenotypically male chimaeras which transmitted functional spermatozoa derived from the introduced cells.

It would obviously be desirable to incorporate specific functional genes into the mouse genome. While direct transformation of the zygote has recently shown much promise, an alternative approach is the construction of individuals between 'modified' pluripotential cells and normal embryos. This second approach has the advantage that cultured cells are accessible for genetic manipulation, characterization and selection before their incorporation in vitro. Several EC cell lines have been selected in vitro to carry specific mutations or additional intact 'foreign' chromosomes, and have subsequently been incorporated into chimaeric individuals. It is, however, a necessary prerequisite to demonstrate that germ-line chimaerism can be reliably achieved. Published rates of construction of germ-line chimaeras using EC cell lines have been disappointingly poor. There has been a report of a single XX cell line, maintained completely in vitro, from which two germ-line females have been obtained. In addition to a low rate of chimaera formation (13%), these females produced only four progeny having an EC-derived component.
Clearly, embryo-derived stem cells seem to be particularly efficient at recolonizing the early embryo. This feature, together with the availability of XY lines such as those described here, now allows the routine construction of chimaeric males which are capable of transmitting culture-derived genomes to a potentially limitless number of offspring, and confirms our previous contention of the normality of the genome in these stem cell lines.22.

We thank Pam Fletcher for technical assistance. This work was supported by grants from the MRC (M.E. and M.H.Kj) and the Cancer Research Campaign (M.E). A.B. is supported by a MRC Studentship. The EK..CP1 cell line was isolated by Marie-Therese Schnebelen of the College de France, Paris, while in Cambridge on an EMBO short-term fellowship.

Received 23 January; accepted 20 March 1984.

Table 1 Rates of construction of chimaeras

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. injected</th>
<th>No. born (%)</th>
<th>No. chimaeric (%)</th>
<th>Chimaeras Male: Female: ND</th>
<th>Males Set up Bred</th>
<th>No. of germ-line chimaeras</th>
</tr>
</thead>
<tbody>
<tr>
<td>EK.CP1</td>
<td>254</td>
<td>167 (66)</td>
<td>74 (44)</td>
<td>40: 27: 7</td>
<td>23: 20</td>
<td>35: 7 (20%)</td>
</tr>
<tr>
<td>EK.CC1.1</td>
<td>69</td>
<td>52 (75)</td>
<td>31 (60)</td>
<td>21: 10: 0</td>
<td>13: 8</td>
<td>1: 1</td>
</tr>
<tr>
<td>EK.CC1.2</td>
<td>160</td>
<td>111 (70)</td>
<td>63 (57)</td>
<td>50: 13: 0</td>
<td>21: 7</td>
<td>2: 2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The EK.CP1, EK.CC1.1 and EK.CC1.2 cell lines were isolated from delayed blastocysts. All of these lines are characterized by the possession of black and agouti coat colour markers. The EK.CP1 cell line was derived from a 129/Sv/Ev strain mouse and is homozygous for the GPI-1 allele at the GPI-1 (glucose phosphate isomerase-1) locus. EK.CC1.1 and EK.CC1.2 were derived from a substrate of 129/Sv/Ev which is homozygous for the GPI-1 allele at the GPI-1 locus. All three of these lines possess a normal euploid XY chromosome constitution and have been maintained entirely in vitro on feeder layers of inactivated fibroblasts. The breeding data available for some males are incomplete; these might yet prove to have a low-level culture-derived germ-line component. There is a very evident distortion of the sex ratio in the live-born chimaeras—this deviation in favour of males is highly significant (x², P < 0.001) and probably reflects the conversion of a number of 'host' female embryos to male chimaeras. ND, not determined.

Table 2 Breeding data from the seven functional germ-line chimaeras

<table>
<thead>
<tr>
<th>Male chimaera</th>
<th>No. litters</th>
<th>No. offspring</th>
<th>No. albino</th>
<th>No. black agouti</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP1.3</td>
<td>10</td>
<td>78</td>
<td>0</td>
<td>78</td>
</tr>
<tr>
<td>CP1.5</td>
<td>16</td>
<td>170</td>
<td>163</td>
<td>7</td>
</tr>
<tr>
<td>CP1.11</td>
<td>2</td>
<td>23</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>CP1.34</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>CC1.1.3</td>
<td>1</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>CC1.2.6</td>
<td>1</td>
<td>13</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>CC1.2.8</td>
<td>1</td>
<td>11</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>

Phenotypically male chimaeras were caged with successive virgin CFLP females homozygous for the GPI-1 allele and the albino locus. Resulting litters were scored for the dominant black and agouti phenotypes. GPI analysis of blood samples taken from all the black agouti offspring has shown the predicted GPI-1 allele (EK.CP1) or GPI-1 (EK.CC1.1 and EK.CC1.2) genotype, thus verifying the inheritance of these culture-maintained lines. The chimaeras which appear to be breeding from their XY culture-derived component alone are probably sexual mosaics, although phenotypically they are normal males, as any XX germ cells would not be capable of forming functional spermatozoa.

Fig. 1 Six of the seven germ-line chimaeras described in Tables 1 and 2. a, CP1.3; b, CP1.5; c, CP1.11; d, CC1.1.3; e, CC1.2.6; f, CC1.2.8. Between 8 and 12 embryo-derived cells were introduced into the blastocoeic cavity of host-fertilized blastocysts homozygous for the recessive albino locus. The blastocysts were then allowed to re-expand and were subsequently transferred to the uterine lumen of recipients on the third day of pseudopregnancy. All the conceptuses were allowed to develop to term, and live-born animals were scored for the presence of eye and coat pigmentation at or shortly after birth.
9868

a
b
c
d
e
f

NDK-10848-PRAZLEY-1(MK) SLS
The development potential of ethanol-induced monosomic and trisomic conceptuses in the mouse.

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3

ethanol-induced aneuploidy

Dr M.H. Kaufman
at above address
Tel. No. Cambridge (0223) 68665

ethanol : aneuploidy : triploidy
Abstract

The development potential of fertilized embryos isolated from female mice previously given a single dose of either a dilute solution of ethanol or distilled water (controls) by mouth was studied. Exposure to ethanol occurred at various times during the cycle leading to ovulation and shortly after fertilization. The chromosome constitution of all preimplantation embryos isolated from these females was determined either at the first cleavage mitosis or at the morula stage. The incidence of aneuploidy in the ethanol-exposed groups at these times was approximately 19% and 13.5% respectively, with a similar number of monosomic and trisomic conceptuses observed at these times. In addition, about 2% of all conceptuses examined were triploid. Further females were autopsied on the 10th or 11th day of gestation, though the chromosome constitution of only the morphologically abnormal or developmentally retarded embryos was determined. Eight embryos out of a total of 16 studied in the ethanol-exposed group were either aneuploid or triploid, whereas in the control group only 1 out of 11 examined proved to be aneuploid. The triploids and ethanol-induced aneuploid conceptuses appeared to be capable of surviving to the morula stage, but generally failed to survive to the 10th/11th day. No monosomics were in fact observed in the post-implantation series. The present findings are briefly discussed with reference to the possible pathogenesis of spontaneous abortions in man which often possess similar types of chromosomal anomalies.
Introduction

Many studies have now been carried out which have clearly demonstrated that in experimental animals and in man ethanol (and acetaldehyde, its principal metabolite) may act as a typical teratogen when exposure to this agent occurs during either embryogenesis or organogenesis (ethanol: Ulleland, 1972; Jones, Smith, Ulleland & Streissguth, 1973; for recent reviews, see Pratt, 1982; Colangelo & Jones, 1982; acetaldehyde: O'Shea & Kaufman, 1979, 1981). Less well known is the more recent observation that exposure of female mice to a dilute solution of ethanol during the first or the second meiotic divisions is capable of inducing a relatively high incidence of aneuploidy in the eggs that are ovulated by these females. Cytogenetic analysis of the embryos which resulted from the mating of these ethanol-exposed females to fertile males revealed an approximately equal incidence of monosomic and trisomic conceptuses (Kaufman, 1983a; Kaufman & Bain, 1984). It seems likely therefore that in the aneuploid eggs the ethanol must have interfered with the normal functioning of the meiotic spindle apparatus and its associated proteins. A similar mode of action has been ascribed to ethanol-like agents as well as to anaesthetics, all of which are also thought to be capable of interfering with cell division (see Andersen, 1966; Allison & Nunn, 1968; Fink, 1971; Nicolson, Smith & Poste, 1976).

Because the teratogenic effect of maternal ethanol consumption during pregnancy in man is well recognised, active measures are now being contemplated to propagate information about this potential hazard to the wellbeing of the conceptus (see Lancet, 1983). However, to date, insufficient experimental evidence has so far been produced that might indicate whether steps should be taken to inform prospective parents that ethanol consumption by the mother both prior to and at the time of conception may be potentially even more harmful than ethanol consumption during pregnancy.
To date, the few experimental animal results that are available indicate that exposure to ethanol during the follicular cycle leading to ovulation may in a proportion of cases lead to the production of aneuploid conceptuses.

The present study was, therefore, initiated to investigate the development potential of ethanol-induced aneuploid mouse embryos. Female mice were given a dilute solution of ethanol by mouth during either the first or the second meiotic division and mated with fertile males. The chromosome constitution of embryos isolated from ethanol-exposed females was determined either at the first cleavage division, at the morula stage, or on the 10th/11th day of gestation. In the two pre-implantation stages examined, all of the isolated embryos were analysed cytogenetically, whereas on the 10th/11th day the chromosome constitution of only the retarded and morphologically abnormal conceptuses was determined. The possible clinical significance of these findings is discussed.
Materials and Methods

Eight- to twelve-week-old CFLP (Hacking and Churchill) female mice were injected with 2.5 IU pregnant mare's serum gonadotrophin followed 48h later by 2.5 IU human chorionic gonadotrophin (HCG) to synchronize ovulation. The mice were caged with (C57BL x CBA)F₁ hybrid males shortly after the HCG injection and checked for vaginal plugs early the next morning (day of finding vaginal plug termed first day of pregnancy).

The females that had mated were lightly anaesthetized with ether so that the test solution (either 1.5 ml of distilled water in the case of the controls, or 1.5 ml of a 12.5% solution of Analar quality ethanol in distilled water in the experimental groups) could be introduced via a fine plastic tube passed into the stomach. Most females recovered from the anaesthetic effect of the ether shortly after the tube was withdrawn from the stomach. The mice were often very drowsy for 1-2 h after the ethanol administration and were left to recover in the warmth.

The intragastric ethanol administration was carried out at either 4 h, 13.5 h or 17 h after the HCG injection, and gave a serum level of about 260-280 mg/100 ml after about 20-30 min. This level was maintained for about 2 h and then rapidly declined, so that baseline levels were usually achieved after 5-6 h.

The females were autopsied following cervical dislocation at either 20-21 h after the HCG injection, at about midday on the third day when embryos were at the morula stage, or at about midday on the 10th or 11th day of gestation. The 1-cell stage embryos recovered at 20-21 h after the HCG injection were transferred to medium containing 1 μg/ml colcemid and their chromosome constitution was determined early the next morning by the air-drying technique described by Tarkowski (1966). All the chromosome preparations were stained with Giemsa. Using this technique, the male- and female-derived chromosome sets were almost always found to be present as two discrete groups.
At about midday on the 3rd day of gestation, the morulae were usually located either at the utero-tubal junction or had already passed into the uterine lumen. Both locations were flushed through with tissue culture medium and the recovered embryos were transferred to medium containing colcemid. After about a 3 h incubation period, air-dried preparations were made and the chromosome constitution of the mitotic figures determined. If there was any doubt regarding the number of chromosomes present, due to the overlapping of chromosomes, at either the first cleavage or the morula stage, the group was automatically excluded from the study. In most of the morulae two or more well-spread metaphase preparations were generally available for analysis. This enabled the chromosome constitution of the aneuploids to be confirmed in most instances.

In the post-implantation series, only one time group was studied, namely involving females that had previously been exposed to an intragastric injection of either ethanol or distilled water given at 13.5 h after the HCG injection for inducing superovulation. The ethanol-exposed females were autopsied at about midday on either the 10th or 11th day of gestation, and the uterine contents examined in detail. The autopsies of the controls were all carried out on the 10th day of gestation. All the decidual swellings were isolated into phosphate-buffered saline and the embryos subsequently dissected free from within their extra-embryonic membranes with fine watchmakers forceps under the low power objective of a binocular microscope. All the embryos that were either morphologically abnormal or were considered to be morphologically normal but retarded in their development by more than about 12 h compared to their normal littermates were transferred into tissue culture medium containing 1 μg/ml colcemid. The total number of resorptions as well as normal littermates present was then determined, after which the latter groups were then discarded.

The chromosome constitution of all the morphologically abnormal and developmentally retarded embryos was then determined using a modification of
the technique described by Evans, Burtenshaw and Ford (1972). The embryos were initially incubated for 2-4 h at 37°C in colcemid-containing tissue culture medium, then transferred into a watchglass containing about 1 ml of 1% sodium citrate solution maintained at room temperature. After about 10 min the embryos were fixed by immersion for about 10 min in a 3:1 mixture of absolute ethanol: acetic acid. Individual embryos were then disaggregated in a small volume of aqueous 60% acetic acid, the latter procedure being facilitated by gentle pipetting with a finely-drawn Pasteur pipette. Once cellular disaggregation was complete, small volumes of the aqueous 60% acetic acid solution containing the cell suspension were then dropped onto heated slides on a hot-plate and the drops treated as described by Evans et al., (1972). The slides were then stained with Giemsa. In most cases large numbers of intact metaphase plates were observed. In all of the aneuploid and triploid conceptuses between several and in many cases very large numbers of spreads were available to confirm their abnormal chromosome constitution. Many of these aneuploid and polyploid chromosome preparations were photographed.
Results

i First cleavage analysis

The results of the first cleavage analysis of fertilized eggs isolated from female mice that had previously been given an intragastric injection of 1.5 ml of a 12.5% solution of ethanol in distilled water at 4h, 13.5h or 17h after the HCG injection for inducing superovulation are presented in Table 1 (groups 1a-c). In these three experimental series the overall incidence of aneuploidy was about 20% (range 15.3-22.8%), there being no significant difference between these groups in this regard. An approximately similar incidence of monosomic and trisomic conceptuses was also observed (16:21) at this stage of gestation. In the control series in which females were given 1.5 ml of distilled water at 13.5h after the HCG injection (see Table 1, group 1d), no aneuploid preparations were seen. The overall incidence of triploidy in the experimental series was 1.6%, and the triploid:aneuploid ratio when the results from the three groups are pooled was 1:12.3.

ii Morula analysis

The detailed results of the analyses of the chromosome constitution of the morulae isolated on the third day of gestation from the same three experimental series and the single control series, namely from females exposed to ethanol at 4h, 13.5h and 17h after the HCG injection for inducing superovulation, and females exposed to distilled water at 13.5h after the HCG injection are also presented in Table 1 (groups 2a-d). As observed in the first cleavage analysis, a similar incidence of aneuploidy was observed to be present in all three experimental groups (range 11.1-15.4%). An approximately similar incidence of monosomic and trisomic conceptuses was also observed at this time (15:18). Curiously, while the overall incidence of triploidy in the experimental series was 2.0%, only marginally above that observed at the first cleavage division, the triploid:aneuploid ratio was
almost halved, at 1:6.8, largely due to the slight reduction in the overall incidence of aneuploidy observed at this time from 19.6% of all preparations analysed at the first cleavage division, to 13.8% at the morula stage. While these findings are not statistically significantly different, this overall trend was to be expected.

iii Analysis performed on the 10th or 11th day of gestation

An analysis of the contents of the implantation sites examined in both the ethanol-treated and control females is presented in Table 2. While both series contained only a relatively small number of embryos that were developmentally retarded compared to their normal (and therefore more advanced) littermates, morphologically abnormal conceptuses were only seen in the experimental series. Of the 'retarded' embryos encountered in the control series, virtually all were delayed in their development by a maximum of only about 12h. By contrast, the 'retarded' embryos in the experimental series were a much more varied group. Several of those recovered on the 10th day were still at the egg-cylinder or early primitive streak stage of development. The relationship between the morphological appearance of the abnormal and/or 'retarded' embryos and their chromosome constitution is presented in Table 3. At this stage of development, the triploid:aneuploid ratio in the experimental series was 1:1, largely due to the absence of the monosomic class and the considerable reduction in the incidence of trisomic conceptuses compared to the situation observed in the preimplantation period.

The absence of an appropriate 'marker' chromosome in either the male or female set makes it impossible to determine whether the triploids have resulted from digyny (due to suppression of either the first or second polar body extrusion) or dispermy. The occasional presence of triploids in the control series suggests that their presence is probably unrelated to the ethanol treatment.
Discussion

The present study confirms and considerably extends the initial report that exposing female mice to a dilute solution of ethanol by mouth at about the time of conception is capable of inducing a relatively high incidence of aneuploidy in the resultant zygotes (Kaufman, 1983a). In a subsequent study, a similar effect on chromosome segregation was observed when exposure to this agent occurred during either the first or the second meiotic divisions (Kaufman & Bain, 1984). In both of these studies, fertilized eggs were analysed at metaphase of their first cleavage mitosis, whereas in the present paper cytogenetic analyses were additionally carried out at the morula stage and on the 10th or 11th days of gestation when embryos would be expected to possess rudimentary limb buds.

Whereas all of the control and experimental embryos isolated at the first cleavage and at the morula stage were examined cytogenetically, the chromosome constitution of only the grossly retarded or morphologically abnormal 10th/11th day conceptuses was determined in the present study, as it was thought that these individuals might have the greatest likelihood of being aneuploid (see Gropp, Putz & Zimmermann, 1976). Recently, Gropp (1982) has shown that a proportion of trisomies 12-14, 16, 18 and 19 appear to be capable of survival until or beyond birth. However, he also observed that the genetic background of the trisomic conceptus apparently plays an important part in determining whether it is capable of surviving to mid-gestation or, in some instances, into the early post-natal period.

In the present study, we have clearly demonstrated that a high proportion of both the monosomic and trisomic conceptuses induced by maternal exposure to ethanol are capable of surviving at least to the morula stage. However, the majority of the ethanol-induced aneuploid conceptuses encountered during the pre-implantation period apparently fail to survive to the limb-bud stage. A small number of trisomics but no monosomic individuals were observed at the latter time. However, somewhat unexpectedly, a relatively high proportion of
the triploid conceptuses that had been encountered both at the first cleavage mitosis and at the morula stage, though either developmentally retarded or morphologically abnormal, were apparently still viable on the 10th/11th day of gestation. Other observers have recently reported that the phenotype of triploid conceptuses in the mouse appears to be dependent on their genetic background (see Wroblewska, 1978). In the most extensive studies in which the development of triploid mouse conceptuses is described (see Wroblewska, 1971; Niemierko, 1981) morphologically normal embryos were only rarely encountered beyond the early egg cylinder stage, though even in those few strains in which more advanced development has been observed, they do not survive beyond the 12th day of gestation (see discussion in Niemierko, 1981). The findings, however, appear to be very species specific, as triploid rat conceptuses are capable of surviving to the 12th day (Piko & Bomsel-Helmreich, 1960), rabbit embryos to the 17th day (Bomsel-Helmreich, 1965, 1971), and human fetuses may very occasionally survive to term and beyond (see Niebuhr, 1974; Kaufman, 1983b).

Despite the relatively high proportion of embryos from the control series only whose chromosome constitution was determined on the 10th day, one aneuploid but no triploid conceptuses were observed. This was in marked contrast to the situation found in the experimental series where half of the abnormal/retarded group were either trisomic or triploid. A possible explanation for these findings may be related to the fact that almost all of the 'retarded' embryos isolated from the control series were only delayed in development by about 12 h compared to their more advanced littermates, whereas in the experimental series the degree of retardation observed in the morphologically normal embryos was, in most instances, much more marked.

While it seems unlikely that more than a relatively small proportion of the aneuploid and triploid conceptuses might have been indistinguishable in all respects from their normal (i.e. euploid) diploid littermates on the 10th/11th day of gestation (see, however, Gropp, 1982) further studies will
obviously be required to establish this point unequivocally.

Clearly, if it is valid to extrapolate from the present findings to the situation in man, it might be possible to implicate ethanol and other spindle-acting agents as potential causative agents in at least a proportion of those cases of aneuploidy for which no other adequate explanation has so far been proposed. Obvious groups, for example where one parent bears a balanced translocation (Penrose & Smith, 1966; Hamerton, 1971), and where conception occurs in 'aged' females (Boué, Boué & Lazar, 1975; and for recent review, see Bond & Chandley, 1983) would, of course, not fall into the latter category. However, even if such cases were excluded, their omission would have little effect on the very high incidence of spontaneous abortions with numerical chromosomal defects that almost certainly result from non-disjunction during either the first or the second meiotic division. Decreased fertility and the production of certain developmental anomalies have also been ascribed to ageing changes which take place in the ovum following ovulation but prior to fertilization. Indeed, various cytoplasmic and spindle-related changes have been observed in mammalian eggs that are thought to underly these age-related effects (Szollosi, 1975).

It would be of the greatest interest to determine whether some members of the chromosome complement are more susceptible than others to undergo non-disjunction (see Martin-DeLeon & Boice, 1983), as this factor alone would tend to influence the incidence of particular types of aneuploid conceptuses. Clearly, monosomies have a considerably reduced viability compared to trisomic individuals in which the same autosome is involved (Ford, 1975), and a third factor which is equally likely to affect these findings is the influence of the genotype of a conceptus on its survivability (Gropp, 1982). Whereas some trisomies may still be viable at the end of the first trimester and may occasionally survive to term, others with the same additional chromosome may die at or very shortly after implantation. For these reasons, any attempt to extrapolate from first trimester abortus findings to conclusions
regarding the situation at conception in man is likely to be fraught with
difficulties. Similarly, while meiotic (I and II) and first cleavage
observations in the mouse are likely to indicate, in the most general terms,
what occurs in man (see discussion in Bond & Chandley, 1983), it must be
appreciated that information regarding the latter species can only be obtained
from an analysis of appropriate human material. While many examples already
exist where the response to a single teratogen is particularly variable even
between strains within a single species (Fraser, 1977), it is unclear whether
this also applies to the wide range of agents that are known to be capable
of interfering with normal meiotic and mitotic spindle function (Mazia, 1961;
Sato, 1975; Bond & Chandley, 1983).
Acknowledgements

We would like to thank Mrs Lesley Cooke for expert technical assistance, and Dr Muriel Harris for analysing the post-implantation material in the experimental series. The work was supported by the National Fund for Research into Crippling Diseases (M.H.K.).
Literature cited


Table 1  Chromosome constitution of first cleavage mitoses and morulae* following ethanol administration at various times after HCG injection for inducing superovulation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time of ethanol administration after HCG injection(h)</th>
<th>Embryonic stage at time of analysis</th>
<th>Total embryos examined</th>
<th>Preparations not analysable(%)</th>
<th>Chromosome number&lt;sup&gt;xx&lt;/sup&gt;</th>
<th>Aneuploid (excluding triploids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>4</td>
<td>First cleavage</td>
<td>52</td>
<td>1 (1.9)</td>
<td>18:20 19:20 20:20 21:20 22:20</td>
<td>19.6</td>
</tr>
<tr>
<td>b</td>
<td>13.5</td>
<td>&quot;</td>
<td>85</td>
<td>5 (5.9)</td>
<td>2 5 61 11 -</td>
<td>22.8</td>
</tr>
<tr>
<td>c</td>
<td>17</td>
<td>&quot;</td>
<td>67</td>
<td>6 (9.0)</td>
<td>4 50 3 2 2</td>
<td>15.3</td>
</tr>
<tr>
<td>d</td>
<td>13.5 (control)</td>
<td>&quot;</td>
<td>34</td>
<td>6 (17.6)</td>
<td>- 28 - - -</td>
<td>-</td>
</tr>
<tr>
<td>2a</td>
<td>4</td>
<td>Morula</td>
<td>66</td>
<td>13 (19.7)</td>
<td>&lt;39 39 40 41 41+ Triploids</td>
<td>15.4</td>
</tr>
<tr>
<td>b</td>
<td>13.5</td>
<td>&quot;</td>
<td>155</td>
<td>27 (17.4)</td>
<td>4 3 107 8 3 3</td>
<td>14.4</td>
</tr>
<tr>
<td>c</td>
<td>17</td>
<td>&quot;</td>
<td>76</td>
<td>10 (13.2)</td>
<td>1 3 56 3 - 1</td>
<td>11.1</td>
</tr>
<tr>
<td>d</td>
<td>13.5 (control)</td>
<td>&quot;</td>
<td>92</td>
<td>16 (17.4)</td>
<td>- 73 1 - 2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

<sup>*</sup> Analysed at midday on 3rd day of gestation.

<sup>xx</sup> The incidence of aneuploidy at the morula stage was not significantly different from that observed at the first cleavage mitosis.

<sup>+</sup> The results from groups 1a-d have been published elsewhere (see Kaufman and Bain, 1984).
Table 2 Analysis of implantation sites on the 10th/11th day of gestation following ethanol administration at 13.5 hours after HCG injection for inducing superovulation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total implants examined</th>
<th>Total normal embryos (%)</th>
<th>Total resorptions (%)</th>
<th>Total abnormal/retarded embryos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ethanol exposed</td>
<td>380</td>
<td>342 (90)</td>
<td>22 (5.8)</td>
<td>16 (4.2)</td>
</tr>
<tr>
<td>2. control</td>
<td>146</td>
<td>129 (88.4)</td>
<td>6 (4.1)</td>
<td>11* (7.5)</td>
</tr>
</tbody>
</table>

* these embryos were all morphologically normal but retarded in development by about 12h when compared with their littermates.
Table 3  Relationship between morphology in abnormal/retarded embryos isolated on 10th/11th day of gestation and chromosome constitution.

<table>
<thead>
<tr>
<th>Group</th>
<th>Embryonic morphology</th>
<th>Chromosome constitution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diploid</td>
</tr>
<tr>
<td>1. ethanol exposed</td>
<td>a  Retarded but morphologically normal</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>b  Abnormal head region - usually exencephaly, otherwise normal</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>c  Grossly abnormal</td>
<td>2</td>
</tr>
<tr>
<td>2. control</td>
<td>a  Retarded but morphologically normal</td>
<td>10</td>
</tr>
</tbody>
</table>
Statement

a). The material presented here has not been submitted for any other degree or diploma of this or any other University

b). The research reported in these publications was entirely that of the candidate or, in those publications in which he was a member of a research group, the candidate made a substantial contribution to the work

Signed:

Dr. M.H. Kaufman