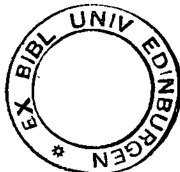
THE EXPERIMENTAL INDUCTION OF HETEROPLOIDY IN THE MOUSE.

R. G. Edwards.

Submitted to the University of Edinburgh as a Thesis for the degree of Doctor of Philosophy.

Institute of Animal Genetics,
University of Edinburgh,

August, 1955.



PREFACE.

It is only recently that the mammalian egg has been used as an experimental material. Pincus and his collaborators laid the foundation of much of the modern work; later experimenters have been Beatty, Chang, Fischberg, Häggqvist & Bane, Thibault, among others. The principal object of the following thesis has been to study the development of haploid and heteroploid mammalian eggs. The stimulus for experiment was given primarily by the work of Beatty and of Fischberg. The rationale for experiment comes largely from the study of haploid and heteroploid amphibian development by Dalcq, Fankhauser, the Hertwigs, etc., and from the effects of colchicine on mammalian development reported by Pincus & Waddington and by Häggqvist & Bane. The following experiments extend the results of many previous workers; future developments may be of application to animal breeding and genetics.

CONTENTS

	Page
Preface	
Chapter I. Natural mating, artificial insemination, and pre-implantation development in the mouse.	2
Appendix. Selective fertilisation following the use of sperm mixtures in the mouse.	18
Chapter II. Colchicine-induced heteroploidy in the mouse, *Mus musculus*.	21
1. The induction of triploidy by treatment of the gametes.	21
2. Heteroploidy induced after previous colchicine injections.	62
3. The induction of tetraploidy.	72
Chapter III. The experimental induction of gynogenesis in the mouse.	81
1. Ultra-violet irradiation of the sperm.	86
2. X-irradiation of the sperm.	119
3. Treatment of the sperm by trypaflavine.	146
4. The combined effect of trypaflavine and ultra-violet on the sperm.	165
5. Treatment of the sperm by toluidine blue.	169
6. The artificial insemination of sperm of other species into the mouse.	179
Chapter IV. The cell and cleavage number of haploid, diploid and polyploid mouse embryos at 3½ days development.	181
Chapter V. Aspects of fertilisation and of the cleavage of diploid and heteroploid eggs in the mouse.	202
Summary.	215
Acknowledgements.	

References, Tables, Text-figures, and Figures are appended to each chapter or sub-chapter.

CHAPTER I

NATURAL MATING, ARTIFICIAL INSEMINATION, AND PRE-IMPLANTATION DEVELOPMENT IN THE MOUSE.

In experiments described in subsequent chapters of this thesis, sperm and eggs of the mouse *Mus musculus* are treated by various agents. Some of these agents are added directly to the spermatozoa *in vitro*, and the mixture artificially inseminated into oestrous females. Other agents are injected into the uterus of oestrous females which are then paired with a male for natural mating. In assessing the effects of these agents on the gametes and on embryonic development, a comparison with control normal mating and development is necessary. The purpose of this chapter is to give the results obtained under normal conditions.

Three controls are needed. For experiments involving injections into the uterus prior to natural mating, the necessary control will be natural mating itself. For those experiments in which sperm is artificially inseminated after treatment *in vitro*, the necessary control will be the artificial insemination of untreated sperm. The experimental injections into the uterus were of solutions of various chemicals in 0.75% sodium chloride solution. The injection of the same volume of 0.75% saline, i.e. 0.1 ml., into each uterine horn, followed by natural mating, will be the third control. Details of the third control will not be given in this chapter, instead they will be given with their related experimental details.

Certain terms which are used throughout the thesis must be defined. An ovum is ovulated at oestrous. After fertilisation, the ovum commences cleavage and is then an embryo. After the first cleavage, the embryo is a group of cells, the morula; and at 3½ days gestation the morula commences to differentiate into a blastocyst. The early blastocyst is still approximately the same size as an ovum; the term 'egg' is used to cover all stages of growth from the ovulated ovum to the early blastocyst. Eggs can therefore be fertilised or unfertilised ova, morulae, or blastocysts. A blastocyst is usually considered to possess 16 cells or more.

The following analysis therefore describes the normal development of the mouse egg and the fertility of the inseminated females after natural mating, or after the artificial insemination of untreated sperm. For ease of presentation the analysis has been divided into three major parts. First, the females are analysed for their fertility. The numbers of eggs per female, and the percentage of the eggs which are embryos, are calculated from results obtained at 3½ days gestation. Secondly, the 3½ day embryos are analysed in three ways; their morphological stage of development as judged by examination alive, and their number of nuclei and chromosome complement as observed in squash preparations. Thirdly, cytological observations are made on live and sectioned material of the stages of fertilisation, pronucleus development, and the first cleavage, and of the degeneration of unfertilised eggs. Before giving the results, the techniques of artificial insemination, egg recovery and

squashing at $3\frac{1}{2}$ days gestation, and the examination alive or the sectioning of eggs at fertilisation or the first cleavage will be described.

Artificial insemination of the mouse.

The artificial insemination technique was based on the work of Long and Mark (1911), Merton (1939) and Snell, Hummel and Abelmann (1944).

Oestrous females were selected by the vaginal smear technique, using the pipette method (Snell, 1941). When the oestrous smear contained a high proportion of epithelial cells, leakage from the cervix was least, and the probability of mating was greatest. When paired overnight with a male, about 70% of the selected females mated, as judged by the presence of a vaginal plug.

Sperm were stripped from the excised vasa deferentia of mature males by gentle massage with forceps. They emerged as a white ribbon into the 0.75% sodium chloride solution used as diluent. This diluent (Long and Mark 1911) gave better fertility than others tested. Being a simple saline, it was less likely than complex diluents to interact chemically with the various agents added to treat the sperm. It is slightly hypotonic for mammalian tissues, but this had the advantage of compensating for the evaporation attendant on some treatments.

Most of the spermatozoa in the sperm ribbon could be dispersed into the diluent by gentle stirring. Usually, the sperm suspension was inseminated at once into oestrous females. But some of the experimental procedures demanded treatment of the sperm for up to an hour before insemination could be performed. In

controls for this type of experiment, insemination of untreated sperm was delayed for the same period.

Females to be inseminated were held in a pad of cotton wool, and the tail raised. Anaesthetic was not required. A speculum was inserted into the vagina, and the cervix illuminated with a head mirror. The sperm suspension was held in a hypodermic syringe bearing a blunted needle; the needle point was inserted through the cervical opening first into the left and then into the right horn of the uterus. 0.1 ml. of suspension was injected into each horn. After insemination, females were paired with vasectomized males for mating. The equipment was similar to that described by Snell, Hummel and Abelmann (1944).

A vaginal plug may not be necessary for fertilisation; Merton (1939) indicated that fertilisation occurred in unmated females. But, as many of the experimental results were obtained at 3½ days gestation or later, the sexual cycle induced by mating was necessary for embryonic development.

For vasectomy, the scrotal sacs were drawn through small incisions lateral to the area between the penis and the anus. The scrotal sac was opened, and the vas deferens gently exposed, ligatured, and sectioned about 1 cm. from the cauda. The organs were replaced, and the body wall sewn. From two weeks after the operation until their death, the males were paired with females to test their capacity for mating without inducing pregnancy. All the males gave a vaginal plugs; no litters were ever observed.

Inseminations were carried out about two hours after dark. Some females were checked for the presence of a vaginal plug two hours after injection; all of them were tested on the following

morning. Only females with a vaginal plug were examined for embryos. In the first series of inseminations, each suspension contained the sperm of a single male in approximately 0.5 ml. saline. It soon became evident, however, that larger samples were required for the experimental work. The sperm of several males was therefore mixed in one suspension of 1.25 ml., sperm being added until the density was considered satisfactory. Usually, the number of males contributing to one sample was between four and seven. The sperm was examined under the microscope for density and activity before and after the inseminations.

<u>The recovery and squashing of 3½ day old embryos.</u>

At 3½ days development, the embryos are free in the uterine lumen. After removal of the entire uterus from the female, the embryos were washed from the lumen by a gentle current of Pannet Compton saline. At this age, the great majority of the unfertilised eggs can also be collected from the uterus. The embryos were examined morphologically for their stage of differentiation, then fixed in aceto-carmine for three hours. They were made into squash preparations and stained with basic fuchsin according to the method of Beatty and Fischberg (1951).

The number of nuclei and chromosomes were counted in the squashed embryos. Chromosome counts were made under oil immersion lenses (x 1140). A second opinion (R. A. Beatty) was taken on every non-diploid mitosis seen, and it was included only if both opinions agreed. Counts were made with an approximate error of ± 5%, less accurate estimates being only provisional. This

method of classification probably gave a lower value to the number of heteroploid embryos than actually occurred.

<u>Live examination and sectioning of pronucleate and first cleavage eggs.</u>

Live examination of the eggs was made with the phase-contrast microscope according to the method of Austin and Smiles (1948). They were obtained by puncturing the fallopian tube in 0.85% saline. After transfer to a slide, a cover-slip with vaselined edges was inverted over them. Gentle pressure on the cover slip slightly compressed the eggs and improved observation. Only eggs from females killed 15 hours after mating were examined by this method.

The whole fallopian tube of the mouse is of a convenient size to handle for fixing, sectioning, and staining procedures. Females were killed between two and forty-two hours after mating, depending on the required stage of development of their eggs, and both fallopian tubes were removed <u>in toto</u>. The tubes were fixed overnight in Bouin, dehydrated in 70% alcohol for several hours and in 95% alcohol for one hour, and cleared in terpineol. Wax, M.P. 54°, was used for embedding. Sections of the whole tube were cut at 10 μ, stained by Delafield's haematoxylin, and mounted in balsam. The eggs could be clearly seen in the lumen of the fallopian tube. Orientation of the egg was not possible by this method.

The live eggs were examined for the presence or absence of a sperm-tail, and for their pronuclear content. Sectioned eggs were examined for the fate of the second meiotic spindle, the

sperm-head, the pronuclei, and for the morphology of the blastomere nuclei in the two-celled stage.

RESULTS.

Analysis of the fertility of the females and of embryonic development was made at 3½ days gestation. Details of the results after artificial insemination and after natural mating are given in Table 1.

Analysis of the fertility of the inseminated females.

47 females were artificially inseminated, 33 or 70.2% of these possessed embryos at 3½ days gestation. Of 31 females naturally mated, 27 or 87.1% had embryos. Fischberg and Beatty (1952) examined 99 females 3½ days after natural mating: 83 or 83.8% of these had embryos.

The mean number of eggs (fertilised and unfertilised) per female was analysed in two ways: over all females, and within females with at least one embryo. After artificial insemination, both values were 8.4 per female; after natural mating the value was 8.8 over all females, and 9.0 within females with embryos. The similarity of the two figures suggested that the degeneration and loss of unfertilised eggs was not excessive. The percentage of the total number of eggs which were embryos was therefore used as one of the estimates of fertility; but due to the degeneration of some unfertilised eggs the percentage will be somewhat over-estimated. After artificial insemination, 46.2%, and after natural mating 77.7% of the total eggs were embryos. Analysis within females with embryos gave 65.5% after artificial insemination and 87.6% after natural mating.

Seven artificially inseminated females were allowed to go to full term. Five had litters, the mean litter size being 6.0. The sex ratio was 19 females to 11 males.

Analysis of embryonic development at 3½ days gestation.

Embryos were examined alive for their stage of differentiation, and as squashes for their number of nuclei and chromosome complement. At 3½ days, the embryos are differentiating from advanced morulae into blastocysts. The percentage of the total embryos which were blastocysts was 51.8% after artificial insemination, and 63.2% after natural mating.

The mean number of nuclei per embryo was 41.2 ± 15.7 after artificial insemination, and 40.6 ± 13.6 after natural mating. The range in the former was between 11 and 80, and between 12 and 72 in the latter. Most of the embryos possessed between 30 and 60 nuclei (Fig. 8), i.e. they were in or near their sixth cleavage. Increase in the number of nuclei is geometric (2, 4, 8, 16, etc.) and is the product of successive cleavages. The time interval between cleavages declines slightly with successive cleavages; the following table is modified from Beatty (unpublished) who combined the results of several workers:-

Event	Time in hours after mating	Mean time in hours after mating	Difference between means
Sperm entry	7½	7½	
			17
1st. cleavage	21-28	24½	
			16
2nd. cleavage	38-43	40½	
			10
3rd. cleavage	50	50	
			12
4th. cleavage	60-64	62	
			3*
5th. cleavage	60-70	65	
			<15
6th cleavage	?-80	<80	

*This interval appears to be underestimated

increase in the cleavage number will therefore be approximately arithmetical. Conversion of the number of nuclei to the cleavage number is obtained from the formula

$$x = \frac{\log N}{\log 2}$$

where x is the number of cleavages, and N is the number of nuclei.

After both artificial insemination and natural mating, the cleavage number of the embryos was 5.4 The respective ranges were between 3.4 and 6.3, and between 3.6 and 6.2.

The diploid chromosome number of Mus musculus is 40 (Matthey, 1949). The majority of the squashed embryos either had no mitoses or their chromosomes could not be counted with accuracy. After artificial insemination, 56 embryos were certainly diploid, 15 could not be definitely classified but were probably diploid, and two were triploid. In other experiments using artificial insemination, 15 embryos were diploid; these have not been included in Table 1. After natural mating, 54 eggs were diploid (Fig. 9), 15 probably diploid, and one was tetraploid. Fischberg and Beatty (1952) obtained 222 diploids and one triploid in their natural mating controls.

Cytological observations on pronucleate, once-cleaved, and unfertilised eggs.

Fertilised eggs came mostly from natural matings; unfertilised eggs were obtained by mating females to vasectomised males. Females were killed between 2 and 42 hours after mating for the study; normal development will be described first, the degeneration of unfertilised eggs second.

Six females were killed between 2 and 5 hours after natural mating; one of them had not ovulated 3 hours after mating. Two

were killed 2 hours after mating. One of them had eight eggs; each was penetrated by sperm and was at anaphase or telophase of the second meiosis (Figs. 3 and 4). The second female had three eggs out of 15 penetrated, others having sperm in the perivitelline space. The three other females were killed between 4½ and 5 hours after mating, and all possessed pronucleate eggs. 26 of the eggs had two normal pronuclei and first and second polar bodies (Fig. 5), one egg had only one pronucleus and a normal second polar body. It could not be decided whether the single pronucleus was male or female.

Four females were killed six hours after mating. Two of these had been artificially inseminated, the other two being natural matings. After artificial insemination, one female had no eggs penetrated by sperm; the other had five eggs with two pronuclei each, five with swelling sperm-heads in the vitellus, and six others unpenetrated. After natural mating, one female had not ovulated, the other possessed eggs all of which were pronucleate. Seven of these eggs had two normal pronuclei, the other three were unusual. The latter resembled the fertilised 'immediate cleavage' eggs described by Braden and Austin (1954); instead of extruding a small second polar body, the eggs had divided into two equal halves and resembled two-celled eggs. One half of the egg possessed two normal pronuclei and was presumably the fertilised half of the egg, the other half had only one pronucleus and was presumably the second polar body (Fig. 6). The female which gave these eggs was an inbred A-strain animal.

Three females, one artificially inseminated, the others

normally mated, were killed 12 hours after mating. The artificially inseminated female possessed no penetrated eggs. One female had one recently penetrated egg and several unpenetrated. The third female had two pronucleate eggs and two with a sperm-head sinking into the vitellus. Penetration of the eggs by sperm could be therefore delayed for twelve hours after mating. Of the 13 sperm heads seen penetrating into the vitellus, 12 were entering sideways (Fig. 3) and the other was probably entering head-first.

Two females killed 18 hours after mating (one artificially inseminated, the other naturally mated) gave 12 pronucleate eggs. Two females, one killed 20 hours, the other 24 hours after normal mating, yielded six and nine eggs respectively. The former female possessed one egg in the two-celled stage, another one in syngamy, and four others with two pronuclei each. The latter female had nine eggs with two pronuclei and two eggs undergoing syngamy. These mice illustrate the variation between eggs in the time of the first cleavage.

Two mice were killed 36 hours after normal mating. Between them, they yielded 23 two-celled eggs.

To summarise, of the eggs seen in the pronuclear stages, 65 had two pronuclei, one had a single pronucleus, and three were "immediate cleavage" eggs. Immediate cleavage may have been due to the inbred mother; these eggs might have developed as normal diploids despite the loss of a large second polar body. All of the 24 two-celled eggs observed were normal; no sub-nuclei were seen in their blastomeres (Fig. 7).

Unfertilised eggs were studied by killing females between 6

and 42 hours after mating with a vasectomised male. Altogether, 79 eggs were examined. In every case, the chromosomes were still together in a metaphase-like group on the spindle. Soon after mating, the spindle and the chromosomes were clear and distinct and were located at the periphery of the egg (Fig. 1). The first polar body often contained a metaphase (Fig. 1). Between 36 and 42 hours after mating, degenerative changes began to appear in the spindle and the chromosomes; the former getting more ill-defined and indistinct, the latter losing their outline and merging together or moving slightly along the spindle (Fig. 2). The unfertilised egg of the mouse therefore retains its organisation for at least 42 hours after mating, and probably for longer periods (Charlton, 1917).

Seven females were killed 3½ days after mating to a vasectomised male. Their unfertilised eggs often give the appearance of possessing cell-like inclusions. Most of the unfertilised eggs disintegrated rapidly when fixed in aceto-carmine. No chromosomes or nuclei were seen in squashes of the remaining eggs.

DISCUSSION.

The primary object of this chapter is to investigate the fertility of naturally mated or artificially inseminated females, the development of their embryos at 3½ days gestation, and the early events of fertilisation and cleavage for comparison with the experimental results given later.

As might be expected, the fertility of females after artificial insemination was lower than after natural mating. In one respect, however, the artificial insemination technique had a

unique advantage over normal mating; this was due to the mixing of the sperm of several males in a single sperm suspension for artificial insemination. Infertility in the females due to factors in the male should be reduced in such mixtures. Further, the sperm of a particular male may fertilise proportionately more eggs than expected from its relative density in the mixed suspension. An investigation to test this possibility will be reported later (p. 18).

The primary comparison between artificial insemination and natural mating is in the percentage of the total number of eggs which were embryos at 3½ days gestation. After artificial insemination, 46.2% were embryos, after normal mating 77.7%. From these figures, artificial insemination was 59.5% as efficient as normal mating; this decreased efficiency was traceable partly to a lower percentage of females possessing embryos, and partly to these females having a lower mean number of embryos than after natural mating. The mean number of young born after artificial insemination, at 6.0 , was near to the mean number of embryos per female at 3½ days gestation (viz. 5.5). The mean number of offspring per female was slightly lower than that reported by Snell, Hummel and Abelmann (1944), but the number of females with embryos at 3½ days gestation, or with offspring at term, was slightly higher than reported by these workers. Snell, Hummel and Abelmann showed that artificial insemination was more successful if mating with the vasectomised male occurred soon after injection than if it was delayed; the present results included both early and late matings. The slight increase in fertility in the present work may have been due to the mixture of

sperm of different males in the same suspension.

The stage of development of the embryos produced by natural mating and by artificial insemination was the same at 3½ days gestation, i.e. a mean of 5.4 cleavages. All the classifiable embryos were diploid except for two triploids found after artificial insemination, and one tetraploid after natural mating. The triploids may be due to the technique; but, if so, the low numbers found showed that the effect was only slight. The number of heteroploids found in this work were slightly higher than, but did not differ significantly from, the numbers found by Fischberg and Beatty (1952).

The numbers of mice examined during the stages of sperm penetration, pronucleus formation, and first cleavage of the eggs were not large. Some interesting results emerge however. The females killed two hours after mating had many of their eggs already penetrated by sperm. These females were paired with males just before darkness and mating occurred immediately. The females could have been in a delayed oestrous from the previous evening or they could have ovulated early on the evening that they mated. Recent work by Braden (personal communication) suggests the latter; though ovulation usually occurs between five and six hours after darkness (Snell <u>et al.</u>, 1940; Snell, Hummel and Abelmann, 1944; Braden and Austin, 1954). A similar argument applies to the females killed up to six hours after mating, all of which possessed pronucleate eggs.

The time of sperm entry into the eggs also showed considerable variation. In the two females with penetrated eggs two hours after mating, and in the mice possessing pronucleate eggs four

hours after mating, penetration must have occurred very soon after mating. In contrast, however, two females killed 12 hours after mating also possessed eggs which had but recently been penetrated. The variation in time of penetration after mating is obviously fairly large; these results are similar to those of Braden and Austin (1954) who discussed the time of ovulation, maturation of the ovulated egg, and sperm penetration in the mouse. Evidence from the single female with penetrated eggs six hours after artificial insemination suggested that no delay occurred in sperm entry due to the technique, but much more evidence is needed to confirm this.

The majority of the pronucleate eggs contained two pronuclei. The two kinds of spontaneous abnormality which were seen have been previously described: fertilised 'immediate cleavage' eggs by Braden and Austin (1954), though their observations came from heat-treated eggs; and the presence of a single pronucleus in a fertilised egg, reported by Austin and Braden (1954). The times of syngamy and the first cleavage in the eggs of two females killed 20 and 24 hours after mating were, in general, similar to those reported by Lewis and Wright (1935) and Gresson (1941).

SUMMARY.

1. Embryonic development of the mouse, Mus musculus, after artificial insemination or after natural mating was compared at 3½ days gestation. The mean number of cleavages of the embryos produced by either type of semination was identical. Two triploid embryos out of 73 classified were found after artificial insemination, one tetraploid out of 70 after natural mating. Judged by the percentage of the eggs which were fertilised, artificial insemination was 59.5% as efficient as natural mating.
2. Sperm penetration and pronucleus formation in the eggs was probably undelayed by artificial insemination. Sperm penetration can occur between less than two hours and up to 12 hours after natural mating. Syngamy began at approximately 20 hours after mating.
3. Four eggs with abnormal pronuclear content are described.
4. The mean litter size after artificial insemination was 6.0.

APPENDIX. SELECTIVE FERTILISATION FOLLOWING THE USE OF SPERM MIXTURES IN THE MOUSE.

Artificial insemination has been carried out in the mouse using mixtures of sperm from a number of inbred lines each containing a suitable genetic marker. The lines are REB (containing the dominant hair structure gene rex, ReRe), G (coat colour and pattern tan, a^ta^t), and C_3H (coat colour agouti, AA); all possible combinations of these genes are phenotypically distinct in the offspring. The first two lines are derived from inbred lines A and CBA respectively. Sperm is obtained from the vas deferens of killed males and the density of each sample estimated by haemocytometer counts. Sperm mixtures were made with equal numbers of spermatozoa from each donor; the donor males for each mixture were of approximately the same age. Four types of sperm mixture were made, namely, a triple mixture of all three types and three double mixtures containing the three possible pairs of the types. These mixtures were used for inseminating oestrous females of the same three lines. Since fertility was rather low in the inbred lines, the same sperm mixtures have also been used on other females taken from highly fertile non-inbred strains. These are referred to as X in the tables, which give the results obtained up to the present time.

Certain facts are noteworthy in these results. First, the mixture of REB and C_3H sperm produced a significant excess of eggs fertilized by C_3H sperm. This is not due to the death and resorption of the homozygous REB animals, because the same result occurs where C_3H are the homozygotes, and also in the non-

inbred females. The result has been rather consistent throughout the series of nine such sperm mixtures which have been employed (Table 3). Secondly, in the other two double sperm mixtures there is less evidence of unequal efficiency of, or interaction between, sperm types; though the mixture C3H and G, and the small series from the triple mixture, again suggest that C3H is slightly more effective. From the triple mixture one tri-paternal litter has been produced, an REB female having given birth to a litter of five, three derived from the C3H sperm and one each from G and REB sperm.

These results clearly show that in sperm mixtures the chance of fertilization by a particular type may not be the same for all components of the mixture. At the present stage in the work, several different interpretations of this fact are possible. It may be that the C3H sperm is always more efficient than that of the other two lines involved. It may be, however, that the C3H sperm exerts a specifically depressant effect on REB sperm. The experiment is so designed that it should be possible to decide between these two possibilities as more numbers accumulate. One should, further, be able to discover whether there is any tendency for a reaction of one sperm type either with one particular egg type, or with a particular uterine environment; a possibility which should be borne in mind, though there is as yet little sign of it.

REFERENCES

AUSTIN, C.R. & SMILES, J. 1948. Phase-contrast microscopy in the study of fertilisation and early development of the rat egg. J. Roy. Micr. Soc. 68, 13-19.

AUSTIN, C.R. & BRADEN, A.W.H. 1954. Anomalies in rat, mouse, and rabbit eggs. Aust. J. Biol. Sci. 7, 537-542.

BEATTY, R.A. & FISCHBERG, M. 1951. Heteroploidy in mammals: I. Spontaneous heteroploidy in pre-implantation mouse eggs. J. Genet. 50, 345-359.

BRADEN, A.W.H. & AUSTIN, C.R. 1954. Fertilization of the mouse egg and the effect of delayed coitus and of hot-shock treatment. Aust. J. Biol. Sci. 7, 552-565.

CHARLTON, H. 1917. The fate of unfertilized eggs in the white mouse. Biol. Bull. 33, 21-29.

FISCHBERG, M. & BEATTY, R.A. 1952. Heteroploidy in mammals: III. Induction of triploidy in pre-implantation mouse eggs. J. Genet. 50, 455-470.

GRESSON, R.A.R. 1941. A study of the cytoplasmic inclusions during maturation, fertilization, and the first cleavage division of the egg of the mouse. Quart. J. Micr. Sci. 83, 35-60.

LEWIS, W.H. & WRIGHT, E.S. 1935. On the early development of the mouse egg. Pubbl. Carnegie Inst., No. 459; Contrib. Embryol. No. 148, 25, 113-143.

LONG, J.A. & MARK, E.L. 1911. The maturation of the egg of the mouse. Publ. Carnegie Inst., No. 142, 1-72.

MATTHEY, R. 1949. Les Chromosomes des Vertébrés. Univ. Lib. F. Rouge, Lausanne.

MERTON, H. 1939. Reproduction in the albino mouse: III Duration of life of sperm in the female reproductive tract. Proc. Roy. Soc. Edin. 59, 207-218.

SNELL, D.G. 1941. Biology of the Laboratory Mouse. Blakiston Co., Philadelphia, U.S.A. Chap. 2.

SNELL, D.G., FEKETE, E., HUMMEL, K.P. & LAW, L.W. 1940. Relation of mating, ovulation, and the oestrous smear in the house mouse to the time of day. Anat. Rec. 76, 39-54.

SNELL, D.G., HUMMEL, K.P. & ABELMANN, W.H. 1944. A technique for the artificial insemination of the mouse. Anat. Rec. 90, 243-253.

TABLE 1. DETAILS OF THE INSEMINATED FEMALES AND THEIR EMBRYOS AT 3½ DAYS GESTATION.

	FEMALES			EGGS AND EMBRYOS							CHROMOSOME COUNTS OF EMBRYOS									NUCLEAR NUMBERS OF EMBRYOS	
	No. of ovulated ♀♀ used	No. of ♀♀ with embryos	% total ♀♀ with embryos	Total no. of eggs found	No. of eggs from ♀♀ with embryos	Total no. of embryos found	% of total eggs which were embryos	% embryos of total eggs from ♀♀ with embryos	Mean no. of embryos from ♀♀ with embryos	% embryos which were blastocysts	Lost or unclassi-able	Haploid	Between haploid and diploid	Diploid	Probably diploid	Hyper-diploid	Triploid	Tetraploid	Others	Mean cell no. of all embryos	Mean cell no. of diploid embryos
Artificial Insemina-tion	47	33	70.2	394	278	182	46.2	65.5	5.5	51.8	109	-	-	56	15	-	2	-	-	41.2±15.7	44.1±15.4
Natural Mating	31	27	87.1	273	242	212	77.7	87.6	7.9	63.2	142	-	-	54	15	-	-	1	-	40.6±13.6	47.8±13.3

TABLE 2.

Total number of offspring born from mixed-sperm inseminations.

Females \ Males	Sperm mixtures: C_3H	REB	G	C_3H	REB	C_3H	G	REB	G
C_3H				24	4	1	2	5	16
REB	10	1	1	22	4				
G	2	0	0			2	4		
X				23	3	13	4	11	10
Total	12	1	1	69	11	16	10	16	26

TABLE 3.

Litter analysis of C_3H + REB sperm mixtures.

Sperm mixture No.	Female	Litters: Fertilizing sperm C_3H	REB
1	C_3H	4	3
2	C_3H	3	0
3	REB	8	2
4	C_3H	2	0
5	REB	7	1
	C_3H	5	0
	C_3H	4	0
6	REB	5	1
7	REB	2	0
8	C_3H	6	1
	X	1	0
	X	0	1
9	X	12	2
	X	10	0
Total		69	11

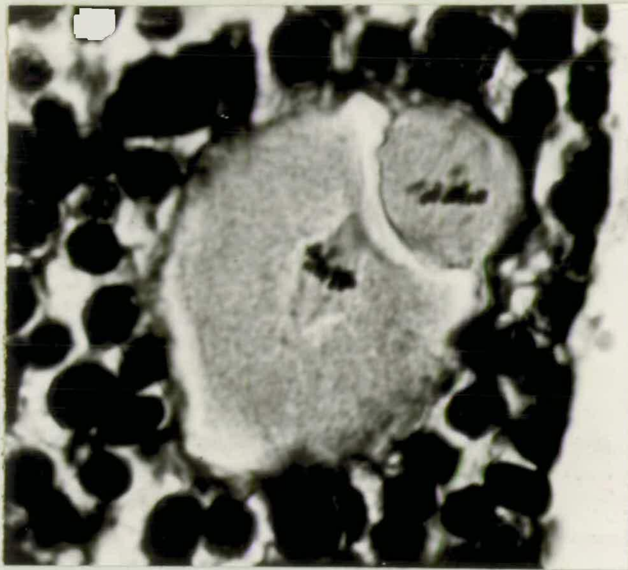
FIGURES

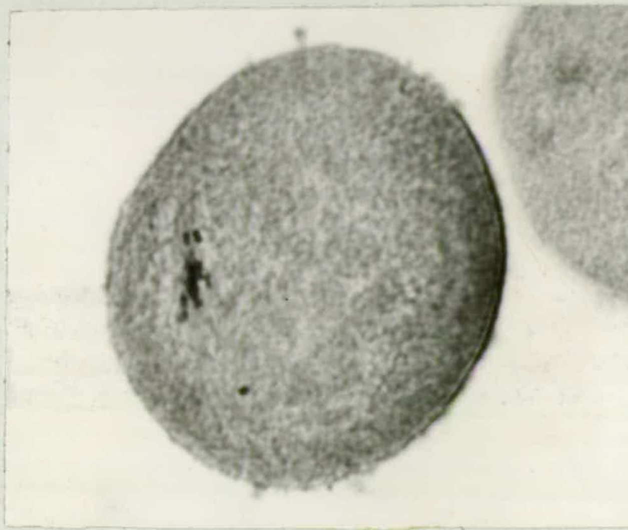
The negative of Fig. 8 was retouched around the periphery of the embryo. Figs. 1-7 inclusive are taken from sectioned eggs, Figs. 8 and 9 from squashed 3½ day old embryos.

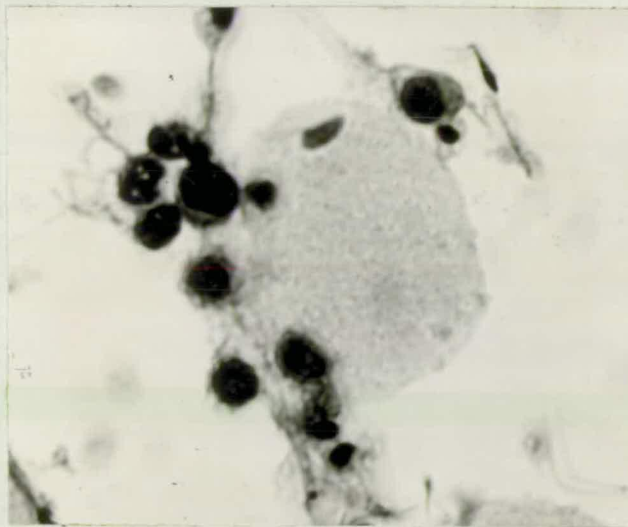
1. The metaphase of the second maturation division of an unfertilised mouse egg. The mother was killed six hours after mating to a vasectomised male. Note also the metaphase in the first polar body. x 1000.

2. The metaphase of an unfertilised egg taken from a mother killed 42 hours after mating to a vasectomised male. The spindle has degenerated, and the dyads are tending to fuse together or show slight scatter along the spindle. x 1000.

3. Sperm entry into the vitellus; the sperm-head is entering sideways. x 1000.



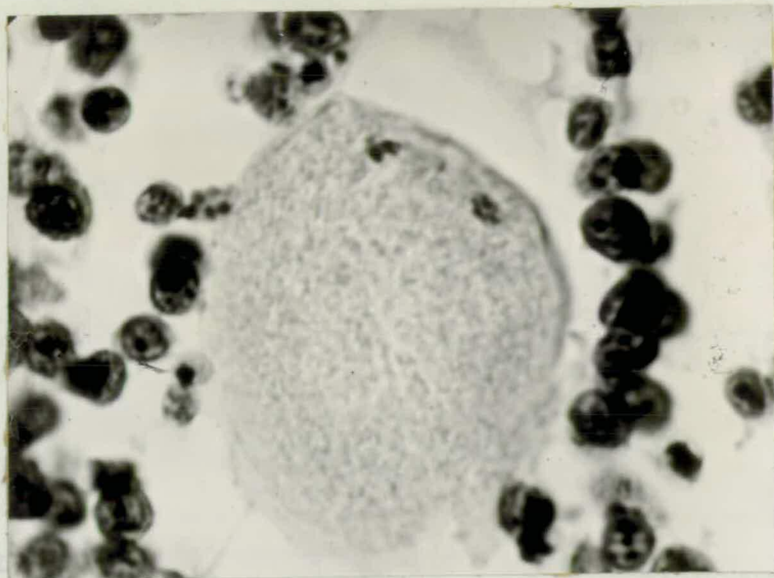


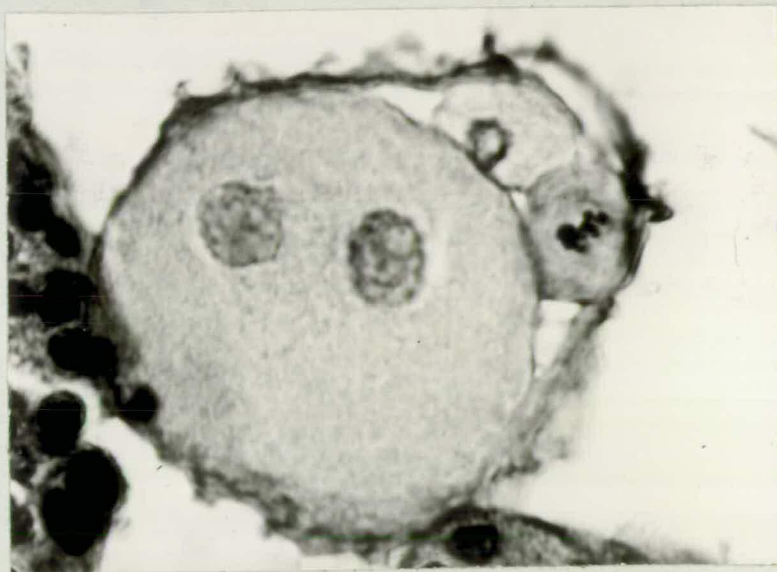


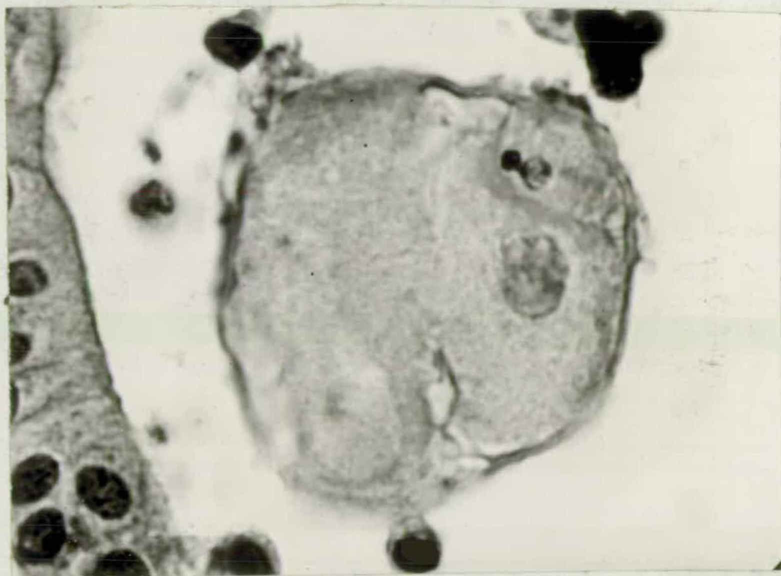
4. Anaphase of the second maturation division. x 1000.

5. Pronuclei in the mouse egg. The first and second polar bodies are also shown, the first polar body containing chromosomes. x 1000.

6. A fertilised "immediate cleavage" egg. The single pronucleus lies in the second polar body half of the egg; the other half possesses two pronuclei in other sections. The two halves were almost identical in size. The first polar body can also be seen in the top, right hand portion of the egg. x 1000.



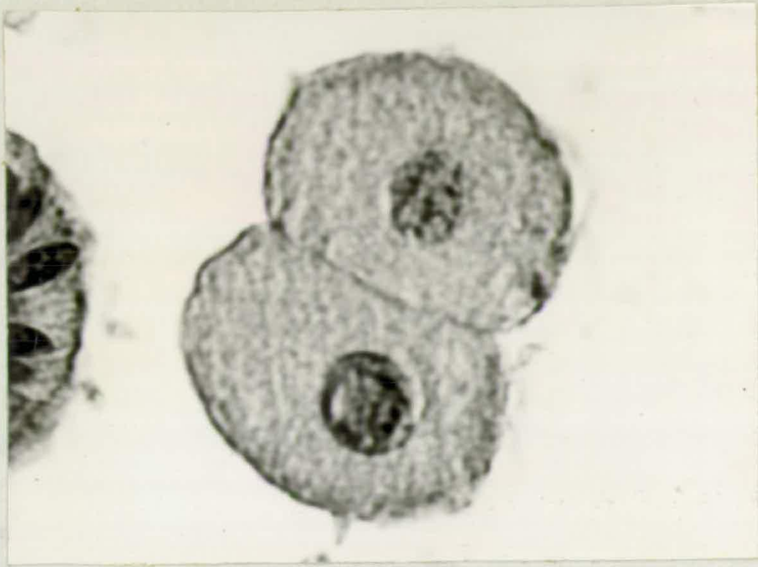


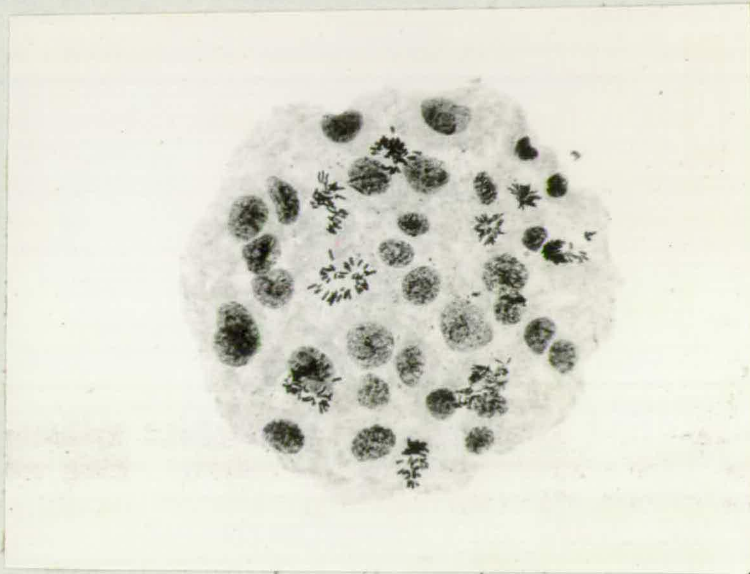


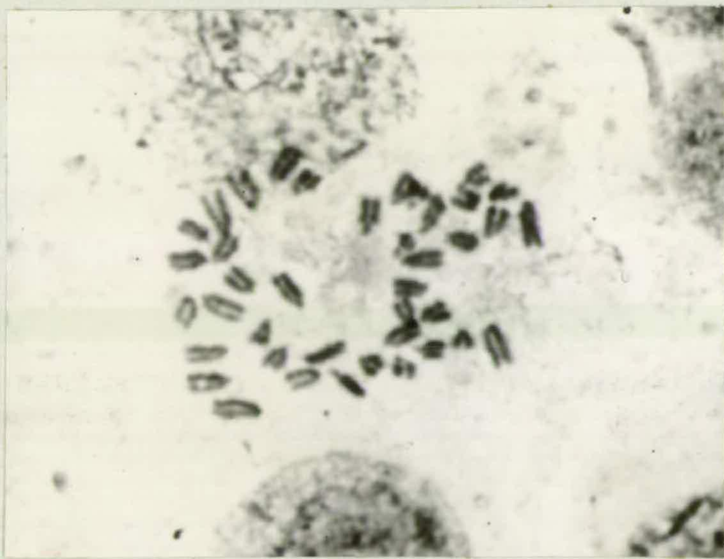
7. A two-celled mouse egg. x 1000.

8. Squash preparation of a $3\frac{1}{2}$ day old diploid blastocyst taken from an artificially-inseminated mother. This embryo possesses many mitoses. x 350.

9. Diploid mitosis in a $3\frac{1}{2}$ day old embryo taken from a naturally-mated mother. The forty chromosomes can be counted accurately. x 2000.







CHAPTER II

COLCHICINE--INDUCED HETEROPLOIDY IN THE MOUSE, MUS MUSCULUS.

1. THE INDUCTION OF TRIPLOIDY BY TREATMENT OF THE GAMETES.

Colchicine is the classical polyploidogenic agent in plants. Since its original use by Blakeslee (1937), the number of plant species in which polyploidy has been induced has steadily increased. In animals, however, colchicine has so far found remarkably little use, and the success variously reported had often been equivocal.

In the mammals, Pincus and Waddington (1939) originated the application of the chemical by culturing fertilised rabbit eggs in a dilute colchicine solutions; they observed one-celled tetraploid eggs after the treatment. Chang (1944) added colchicine to rabbit sperm suspensions and artificially inseminated the mixture. Häggqvist and Bane (1950 a & b, 1951) repeated Chang's experiment in the rabbit and extended it to the pig. If the fertilising sperm carries colchicine to the site of fertilisation, the chemical may affect the spindle of the second maturation division of the egg. Interference with the spindle mechanism may cause the retention of all the maternal chromosomes in the egg at fertilisation; the resultant embryos may then develop as triploids with two maternal and one paternal set of chromosomes. Chang obtained some abnormal young after the treatment, but did not examine them chromosomally. Häggqvist and Bane claimed that two of their rabbit offspring were triploid and that one of the pig offspring was heteroploid. Melander (1950, 1951) examined the chromosomes of one of the rabbits and the pig, and considered them

both to have been triploids. But the work of Häggqvist and Bane and of Melander has been severely criticised (for example Beatty and Fischberg, 1950), and attempts to repeat their results in the rabbit and cow have not succeeded (Beatty and Rowson, 1954).

Two other methods of treating mammalian gametes with colchicine are reported. Austin and Braden (1954) injected colchicine into the peritoneum of rat females and noted that second polar body extrusion was affected. Kliesch and Schmidtke (1954) injected colchicine into the testis of male rabbits, and noted polyploid meioses and mitoses, and an increased size of the sperm-heads which they attributed to polyploidy.

In the following work, colchicine has been used to induce heteroploid development in the mouse, Mus musculus. The technique of Chang and of Häggqvist and Bane was applied to the mouse but then abandoned in favour of alternatives. Treatment with colchicine was either at fertilisation, in order to retain the second polar body chromosomes within the egg by destroying the second maturation spindle; or at the first cleavage, in order to suppress the separation of the two diploid chromosome sets on the cleavage spindle. Treatment at fertilisation was designed to induce triploidy; suppression of the first cleavage division was intended to induce tetraploidy. The present section gives the results of the triploidy experiment, the induction of tetraploidy will be described later. Some of the results in this work have already been published in outline (Edwards, 1954).

MATERIALS AND METHODS.

Heteroploid development of the mouse egg can occur spontaneously in at least three ways. Random matings give about 0.4% of heteroploid embryos at 3½ days gestation (Fischberg and Beatty 1952a). The incidence of heteroploidy is significantly increased to 3.4% if mice of widely different strains are crossed (Fischberg and Beatty 1952b). If mice homozygous for the 'silver' factor are crossed with non-'silver' mice, 9.3% of the 3½ day old embryos are heteroploid (Beatty and Fischberg 1951a). Care was therefore taken to exclude factors causing spontaneous heteroploidy. A wide selection of males were used in the experiment; records of the performance of each of them ensured that induced heteroploidy due to male factors could be easily traced.

Methods for inducing triploidy.

In a similar experiment to that of Chang (1944) on the rabbit, and of Häggqvist and Bane (1950 a & b, 1951) on the rabbit and pig, colchicine was added to sperm suspensions of the mouse and the mixture artificially inseminated into oestrous females. Details of sperm collection, and of the artificial insemination of the mouse have been given previously (p. 4). Colchicine was dissolved in 0.75% sodium chloride to ten times the required concentration; addition of this solution to the sperm suspension in the ratio 1:9 achieved the desired strength. The concentrations of colchicine used in the suspensions were between 1/5,000 and 1/100,000 by weight.

During this experiment, a simplified method of treatment was adopted. Approximately 0.1 ml. of colchicine solution dissolved

in 0.75% saline was injected through the cervix into each uterine horn. The colchicine concentrations were between 1/2,500 and 1/500,000 by weight; except for the absence of sperm in the injected solution, the technique was the same as for artificial insemination. Immediately after the injection, the females were paired with males for natural mating.

Adoption of the injection method probably reduced the toxic effects of the chemical on the sperm. It is not possible to obtain timed ovulations in mature mice; the best indication of ovulation is probably the time of mating. If natural mating follows colchicine injection, the spermatozoa are subjected to the toxic action of the colchicine only for the time between mating and fertilisation. If colchicine is added to the sperm *in vitro*, the inseminated female must mate with a vasectomised male for the completion of the artificial insemination technique. The spermatozoa will be subjected to the colchicine for the time taken for copulation to be achieved. Error in timing oestrous by the vaginal smear method, and delay before copulation with the vasectomised male is complete may cause prolonged exposure of the sperm to the chemical.

The injection technique was therefore adopted to avoid excessive toxicity. Disadvantages were associated with the method however. If the injected colchicine solution leaked from the cervix, or was absorbed or neutralised by the uterine tissues and fluids before mating occurred, the gametes would be less efficiently treated than intended, or even completely untreated. Further reference will be made to this point.

Thirty-one females were injected with 0.1 ml. of 0.75% saline into each uterine horn before natural mating to serve as controls of the injection method.

Method of chromosomal and cytological examination of embryos.

Most embryos were recovered from their treated mothers at 3½ days gestation. Typically, the embryo at this age is a blastocyst lying freely in the uterine lumen. Details of the recovery, squashing, staining, and examination of these embryos have already been given (p. 6).

Based on the results obtained at 3½ days gestation, the experiment was extended to earlier and later stages of development. Unfertilised, pronucleate, and once-cleaved eggs were examined alive under the phase-contrast microscope or as stained sections of 10µ thickness. Details of the recovery, sectioning, staining, and examination of these eggs have been given previously (p. 7).

Later developmental stages were investigated by counting the chromosome complement of adult animals born to colchicine-treated mothers. Conjunctiva tissue of the eye was cultured in hypotonic solution by the method of Beatty (unpublished), then squashed and stained. After this treatment, many clear mitoses could be observed in the tissue, and exact chromosome counts were possible. Except where much of the conjunctiva tissue was lost from the slide during squashing and staining, at least 10 of the clearest mitoses per animal were counted.

RESULTS.

Results will be given in the order that they were obtained.

i.e. from the 3½ day old embryos first, details of fertilisation and the first cleavage next, and the chromosome counts on adults last.

A. 3½ Days Gestation.

Details of the fertility of the injected females and of the development of their embryos are given in Table 4. Most of the experiment was carried out with concentrations of colchicine between 1/9,000 and 1/20,000 by weight; the higher and lower concentrations were used to investigate the limits of effect of the chemical. Results obtained after the artificial insemination of colchicine-treated sperm will be given separately.

The toxic effect of colchicine.

At concentrations 1/5,000 and above, no embryos were obtained from six females. This result, obtained with a small number of mice, was later reinforced by the injection of 1/5,000 colchicine for a slightly different purpose. In the later work, of 27 mice injected, only one gave birth to a litter, which contained two young. The prevention of fertilisation, development, or both was therefore definite at this concentration. Fertility increased with lower concentrations, and by 1/10,000 the number of females with embryos was as high as in natural mating (Text-fig. 1).

The toxic effect of colchicine on fertility was also estimated by comparing the numbers of embryos and unfertilised eggs at 3½ days gestation. Some of the unfertilised eggs will have degenerated (see p. 8) and the comparison will be biassed in favour of the embryos. The number of embryos as a percentage

of the combined number of embryos and unfertilised eggs was calculated for each concentration in two ways: over all females, and within females with embryos. The mean number of embryos taken from females with embryos was also calculated: this method overcame the bias due to degenerated unfertilised eggs. Each analysis showed that after a fairly sharp threshold at conc. 1/9,000, the toxic effects had largely disappeared at 1/10,000 and below (Table 4, Text-fig. 1).

The toxic effect of colchicine on the development of the embryos was reflected in their cell number. The four embryos found at conc. 1/7,500 were retarded, but at lower concentrations the colchicine had little effect on the mean cell number of the embryos (Table 4). At concs. 1/9,000 and 1/10,000, however, a few embryos had low cell numbers and were probably degenerate (Fig. 10). Embryos with low cell numbers caused the higher standard error of the mean cell number at conc. 1/9,000. But the mean and standard error for all embryos included embryos which were heteroploid, and which usually had fewer cells than their diploid sibs. Some of the variability in cell number was therefore due to the chromosome complement of the embryos.

Allowance for the effect of heteroploidy on cell number can be made by taking the diploid cell numbers only; delay in their development should be due to toxicity. The mean cell numbers of the diploids at concentrations of 1/9,000 and below were similar to each other and to controls (Table 5). But the diploids are a selected sample of embryos which may be expected to show normal development. The test was made more strict by comparing

two sets of diploids: those taken from females which produced one or more heteroploid embryos with those from females which produced only diploids. The former group must have developed under the influence of the chemical as attested by the presence of heteroploids, the latter group may have been largely free from the chemical. The test was not fully reliable, for females in the latter category had many unclassifiable embryos some of which were undoubtedly heteroploid. The results are given in Table 5. Except at concs. 1/10,000 and 1/30,000 the mean cell number of the diploids from females with heteroploids was higher than that from females without heteroploids. All means were comparable with controls. The toxic effect of colchicine was therefore slight, if present at all, at and below conc. 1/10,000.

A further indication of the effect of the treatment on embryonic development was to note the percentage of the total embryos which were blastocysts at each concentration. There was great variability in the results (Table 4), but they were not incompatible with the previous indications that the toxicity of colchicine was slight at conc. 1/10,000 and below.

The heteroploid-inducing effect of colchicine.

Heteroploid embryos occurred at all the concentrations which had no adverse effects on fertilisation or development. The percentage of heteroploids declined with decreasing colchicine concentration (Text-fig. 2). The single heteroploids found at each of the two lowest concentrations were slightly doubtful; but it was obvious that at these concentrations (1/200,000 and 1/500,000) the effect of the colchicine was slight if present at all. At the

high concentrations, the percentage of heteroploids reached 19% of the total embryos classified.

The numbers of heteroploid and diploid embryos found at each concentration were tested for significant differences against numbers found at adjacent concentrations. Tests were made on 2 x 2 contingency tables with Yates' correction for small numbers. The numbers of triploids found at each concentration were tested similarly. Only one significant difference was found: between 1/9,000 and 1/10,000 for heteroploid embryos where χ^2 was 4.17. But the wide range of effective concentrations of the colchicine made a significant difference between these two slightly differing concentrations unlikely. Further, the χ^2 on the number of triploids between them was very low (0.11), and indicated no difference. The significant difference in the number of heteroploids found between these concentrations was therefore ignored, and the data grouped into larger classes. Adjacent pairs of concentrations between 1/9,000 and 1/50,000 were grouped, e.g. concs. 1/9,000 and 1/10,000 were combined as 1/10,000, etc. The grouped concentrations, now labelled as 1/10,000, 1/20,000, and 1/40,000, have been used in the statistical tests given later and also in Text-fig. 2.

The intention of the treatment was to induce triploidy, and this was the heteroploid type produced in the largest numbers (Table 4, Figs. 16 & 17). The other chromosomal types produced varied from haploid to tetraploid, with many intermediate gradations (Figs. 11, 12, 13, 14, 15, 18). Several mosaics occurred, the most notable being two haploid-diploid mosaics (Fig. 19) and one diploid-tetraploid mosaic. Some of the mosaics

were highly abnormal: three of them had a group of chromosomes of less than the haploid number (7, 10, 13 respectively), and another was a multiform mosaic with three different chromosome counts of 46, 40, and 30.

The distribution of the triploid embryos is shown in Text-fig. 2. Three embryos with 55 ± 3 chromosomes, and which were therefore probably hypo-triploid, are included in the graph. The highest concentrations of colchicine were the most successful in inducing triploids; none were observed below conc. 1/40,000.

Five tetraploid embryos (Fig. 18) occurred at the highest concentrations, and presumably resulted after the suppression of the first cleavage division of a diploid. The origin of the diploid-tetraploid mosaic may have been due to a similar effect of the colchicine on one blastomere of a later cleavage division. Enough colchicine must have occasionally remained in the egg or the reproductive tract of the female to affect these later cleavages.

Four haploid embryos also resulted after the treatment (Figs. 11 & 13). Three occurred at concentrations 1/20,000, and the other at 1/100,000. Probably related to these were the haploid-diploid mosaics, which may have arisen by the action of the chemical on the second or a later cleavage of a haploid embryo. The fact that certain cells of haploid embryos may revert to diploid by this method implied that certain of the diploid embryos may have commenced development as haploids.

Many embryos had c-mitoses which were of the initial type (Levan, 1954) (Figs. 12, 15, 17). In some embryos this may have

led to an accumulation of metaphases in an embryo (Fig. 17 shows a triploid embryo). C-mitoses were present occasionally in all chromosomal types of embryo, including diploids, except for the haploids.

The classification of the control embryos is given in Table 4. After the injection of 0.75% sodium chloride before mating, no heteroploid embryos were found out of 105 classified. After natural mating only, one tetraploid embryo was observed among 69 diploids. In the tests of significance given later, both of these figures have been used as controls. Fischberg and Beatty (1952a) noted one triploid embryo and 222 diploids in their controls.

Tests of significance of the difference in proportion of heteroploid and of triploid embryos were made between the grouped concentrations (1.10,000, 1/20,000, 1/40,000), concentration 1/100,000, and the two controls. Tests were made by the 2 x 2 contingency table with the Yates correction. Significance levels are at 5% except where a stricter level is mentioned.

The numbers of heteroploids found after the two highest concentrations showed similar trends; no difference was found between them or from conc. 1/40,000, though both were significantly different from conc. 1/100,000 and the two controls. At conc. 1/40,000 the number of heteroploids was significantly different from the injected controls only. Tests on the numbers of triploids showed that conc. 1/10,000 was significant against conc. 1/100,000 and the controls. Conc. 1/20,000 was significant against the injected controls only.

To estimate the total effect of the colchicine, the two highest concentrations were amalgamated and their combined

incidence of all heteroploids and of triploids were tested against those of both control types. The results were:

Combined incidence of heteroploids tested

a. Against injected controls $\chi^2 = 21.76$ $P < .001$

b. Against natural mating $\chi^2 = 7.58$ $P < .01$

Combined incidence of triploids tested

a. Against injected controls $\chi^2 = 8.56$ $P < .01$

b. Against natural mating $\chi^2 = 5.42$ $P < .02$

To summarise, the tests of significance confirmed that colchicine induced heteroploidy in many embryos especially between concs. 1/10,000 and 1/40,000. Only the higher concentrations were effective in inducing triploidy.

One reservation was necessary in assessing the number of heteroploid embryos induced by the treatment. At all concentrations except 1/30,000, the cell number of the heteroploids was lower than the diploids. The chance of observing a mitosis in the diploids, and therefore of classifying them chromosomally, was consequently greater than in the heteroploids. A correction factor based on the mean cell number of the diploids and heteroploids was therefore applied to the observed number of heteroploids.

Calculation of the corrected number of heteroploids was made as follows:-

Let H be the number of embryos which commenced division as heteroploids, D the number starting as diploids, T the sum of these. A is the number of nuclei in the identifiable heteroploids, B in the identifiable diploids. h is the number of heteroploids observed chromosomally at 3½ days development, d the number of

diploids. k is a constant; based on the assumption that the chance of identifying the embryos is in direct proportion to their number of nuclei. It is identical for diploids and heteroploids.

Then

$$H + D = T \qquad kHA = h \qquad kDB = d$$

Hence

$$H = \frac{T}{1 + \frac{dA}{hB}}$$

Substituting the values found for each concentration from 1/10,000 to 1/30,000, the corrected percentages are (with the observed percentages in brackets):-

1/9,000: 38.5 (30.8)   1/10,000: 25.0 (15.2)

1/15,000: 20.0 (14.5)   1/20,000: 45.5 (29.3)

1/30,000: 25.6 (18.2)

If the assumptions made in the calculation are correct, the numbers of heteroploids found by observation are a low estimate. Despite this discrepancy, it is probably more appropriate to use the experimental results; further references to the number of heteroploid embryos will therefore be to the observed percentages and not to the corrected ones.

<u>Analysis within females of the numbers of heteroploid embryos.</u>

Early in the experiment, it became evident that the heteroploid embryos were not randomly distributed over all females; instead, there was a tendency for the heteroploids to be found within certain females. Two methods were used to clarify this phenomenon. The first was the statistical investigation of heterogeneity within females producing classifiable embryos; the

second was to relate the interval between colchicine injection and time of mating with the level of induced heteroploidy.

Analysis for heterogeneity in the distribution of heteroploid embryos was made between all females producing classifiable embryos within each of the concentrations that were combined previously. Significant heterogeneity between females within concentrations was present at 1/10,000 only ($P < .001$). No heterogeneity was found between concentrations. When the concentrations were grouped to test for heterogeneity between females over all concentrations, or over 1/10,000 plus 1/20,000, the significant heterogeneity was lost though χ^2 was still high.

The significant heterogeneity found at conc. 1/10,000 suggested that the differences between females were real. In view of this difference, analysis of the percentage of heteroploid embryos at this concentration, and, for comparative purposes, at the two lower concentrations, was made within females with at least one heteroploid embryo. Within these females more than 50% of the resulting embryos were heteroploid at conc. 1/10,000, the percentage at 1/40,000 being 30%. The numbers of triploids in the same females declined more steeply from 31% to 5% between the same concentrations.

The experimental method of analysis supported the statistical method. In the later stages of the experiment females were examined between 1½-2 hours after the colchicine injection for the presence or absence of a vaginal plug. Those which possessed vaginal plugs were labelled the 'evening plug' class; those which acquired plugs between the evening examination and examination on the following morning were called the 'morning plug' class.

These classes are not distinguished in Table 4. Numbers of embryos were rather small, but it was possible by combination of concentrations to use the χ^2 test between the 'evening and the 'morning' classes.

The number of heteroploids found after combination of concs. 1/10,000 and 1/20,000 was far higher in the 'evening' females, the numbers being significantly different ($P < .05$). Combination of concs. 1/10,000 to 1/50,000 also showed a significant difference. The cause of the greater yield of heteroploids and triploids in the 'evening' females may have been due to a less effective treatment of the gametes in the 'morning' females. In the latter, the colchicine may have leaked from the cervix or been neutralised by the uterine tissues; loss by either method could have been accentuated by delayed mating.

Both methods of analysis, experimental and statistical, indicated heterogeneity between females in the yield of heteroploid embryos. Under more controlled experimental conditions, a higher incidence of heteroploidy and triploidy may have resulted.

Cell numbers in haploid and heteroploid embryos.

The number of nuclei counted in haploid and the various types of heteroploid embryos, and a comparison with numbers in diploids, will be given subsequently (p.181).

In vitro treatment of sperm with colchicine.

The artificial insemination of sperm suspended in colchicine solution was the method used by previous workers on the rabbit and pig (Chang, 1944; Häggqvist and Bane, 1950a & b, 1951). A similar experiment was carried out on the mouse. The techniques

have been described previously (p. 23). The resulting embryos were examined at 3½ days gestation as squash preparations.

Detailed results are given in Table 6. Colchicine concentrations in the sperm suspensions were between 1/5,000 and 1/100,000. The same criteria of fertility were calculated as in the injection method. The toxic effect of the chemical closely resembled that found in the injection method; conc. 1/10,000 was near the upper threshold for fertilisation and development, 1/5,000 was toxic. Below conc. 1/10,000 the toxic effect had largely disappeared.

All classifiable embryos were diploid. But the low cell numbers found in two of the embryos at conc. 1/10,000 indicated that the chemical had an effect though this was not reflected in the ploidy of the embryos. The mean cell number of the embryos and of the diploids at each concentration was normal; the large standard error at concs. 1/10,000 and 1/50,000 was due to the retarded embryos.

B. The Stages of Fertilisation and the First Cleavage.

Two methods were used for the examination of the early embryonic stages. Living eggs were examined by phase-contrast microscopy according to the method of Austin and Smiles (1948). Other eggs were fixed, sectioned at 10μ and stained histologically by haematoxylin. Details of the techniques involved for the study of living eggs and for the sectioning and staining of fixed eggs have been given previously (p. 7).

Five females were killed between 5½ and 6½ hours after mating, one having been injected with concentration 1/7,500, two with 1/9,000, and two with 1/10,000. Their eggs were examined as

stained sections to investigate sperm entry and early pronucleus formation.

Nineteen mice were killed about 14 hours after mating and their eggs examined by phase-contrast. Eleven of these females had been injected with concentration 1/10,000, five with 1/20,000. Three had been injected with saline only to serve as controls. The pronuclei of their eggs were studied by phase-contrast microscopy.

One mouse, receiving concentration 1/7,500, was killed 17½ hours after mating to examine the first cleavage. Two females, one injected with 1/7,500 the other with 1/10,000, were killed between 27 and 36 hours after mating to observe events after the first cleavage. The embryos of these three females were sectioned and stained.

<u>Six hours after mating.</u>

None of the five mice killed six hours after mating had fertilised eggs. But the effect of the colchicine on the unfertilised eggs was very definite in two of these mice. Damage to the egg varied between that shown in Fig. 20, where the second maturation spindle was disappearing (compare the spindle of the untreated egg shown in Fig. 1), and that shown in Fig. 21, where the chromosomes had scattered in small groups round the periphery of the egg and the spindle had disappeared. The chemical had apparently destroyed the maturation spindle, and the dyads, freed from their attachment to the spindle, at first exhibited a false segregation along the remains of the spindle as shown in Fig. 20, and then moved freely through the egg. In the two mice, every

egg was affected. Of the other three mice, one had not ovulated, one showed no effect of the chemical, and the third had two eggs out of five affected.

Despite careful scrutiny with the ordinary microscope and with phase-contrast, no spermatozoa or pronuclei were seen in these eggs. Sometimes it is difficult to observe the spermheads in sectioned eggs, but it could hardly have been overlooked in all. The chemical which affected the spindles of most of these eggs must therefore have entered the egg by absorption, and not directly at sperm entry. Pretreatment of the egg _in vitro_ before fertilisation may induce the same effects.

The absence of fertilised eggs in these mice contrasted with the results from the control normal matings, where six of the eight females examined possessed penetrated eggs six hours after mating. Lack of fertilised eggs may have been due to several causes. The mechanical stresses created in the uterus following the injection of 0.1 ml. of solution into each uterine horn may have delayed the ascent of the sperm through the tubes and resulted in delayed fertilisation; for in uninjected mice, fertilisation can be delayed for 12 hours (p. 12). Alternatively, the colchicine may have had toxic effects on the sperm, especially in the female injected with concentration 1/7,500; or it may have affected the egg, rendering it impenetrable to sperm. The two latter possibilities were doubtful in view of the results obtained at 3½ days gestation, which showed no difference in the fertility of the injected females and controls (Table 4). Delayed penetration may therefore have been the cause of the lack of fertilised eggs.

Fourteen hours after mating.

One of the three females injected with saline had three unpenetrated eggs only. The other two together yielded 24 eggs, each of which had two normal pronuclei.

Three of the sixteen colchicine-injected females possessed unpenetrated eggs only. The other thirteen had one or more fertilised eggs. Many of their eggs were abnormal. Five were seen which contained a sperm-tail and enlarged mid-piece, but no sperm-head or pronucleus could be detected. Seventeen eggs possessed one large pronucleus, a sperm tail, and evidence of second polar body formation (Fig. 22a & b shows one of them sectioned and stained). Fifty eggs possessed two normal pronuclei, and seventy-three were unfertilised. The fourteen remaining eggs had more than one pronucleus of which one appeared to be normal and the others small in size (Fig. 23). Three of these eggs contained one small pronucleus in addition to the normal-sized one, five eggs had three pronuclei, two had four, and four had five pronuclei (Fig. 23). All eggs, except the unfertilised, had a sperm-tail.

The eggs containing several pronuclei presumably arose by the fertilisation of a colchicine-treated egg containing scattered groups of chromosomes. They may have possessed both sets of maternal chromosomes in addition to the sperm chromosomes, and thereby have been the precursors of triploid embryos. But, in the female killed at 17 hours after mating, histological examination revealed that eggs with several pronuclei often extruded polar bodies in addition to the first. Some of the maternal chromosomes could therefore have entered the polar bodies and others been retained in the egg. If only a few maternal

chromosomes were lost and the majority retained, the embryos would be hyper-diploid. If the majority of the maternal chromosomes were lost, the embryo would be a hypo-diploid. Eggs which contained one normal and one small pronucleus were presumably hypo-diploid, eggs with several pronuclei were hyper-diploid or triploid depending on the number of chromosomes lost into the polar bodies. Eggs with two pronuclei were probably, though not necessarily, diploid. Blandau (1952) has reported the presence of several sub-pro-nuclei in rat eggs in which fertilisation had been delayed by up to 12 hours. The greater the delay, the larger the number affected. The author did not consider the possibilities of hyper-diploidy in these eggs, but the work of Thibault (1949) shows that the spindle of the unfertilised rat egg starts to degenerate between 6 and 8 hours after ovulation. Delayed fertilisation of the rat egg may have induced the same changes as colchicine treatment of the mouse egg.

The last type of fertilised egg to be considered contained a single large pronucleus and a sperm-tail. Such eggs would almost certainly develop as haploids. Some of these ova were examined under phase-contrast to detect the fate of the second meiotic spindle. In none of them was it seen; hence the single pronucleus could have been either male or female. But histological examination of the eggs of the females killed at 17 or between 27 and 36 hours after mating indicated that the single pronucleus was derived from the sperm. The single uni-pronucleate egg observed histologically had extruded two small abnormal second polar bodies (Fig. 22b). Another egg was observed to have extruded similar polar bodies yet to have neither pronucleus nor sperm-head in the

egg itself. In the latter, all the maternal chromosomes had left the egg, leaving it anucleate; had fertilisation of this egg occurred it would have resembled the former egg in possessing a single large pronucleus. The inference was that eggs with a single large pronucleus were the precursors of androgenetic haploid embryos.

The effect of colchicine was therefore to destroy the meiotic spindle of the egg and liberate the chromosomes from their spindle attachment. The freed chromosomes scattered through the cytoplasm, became arranged at the periphery of the egg, and probably left the egg. Depending on the number of chromosomes lost this way into the abnormal second polar bodies, the egg developed with a chromosome complement anywhere between haploid and diploid. Such embryos were actually observed chromosomally at 3½ days gestation (Table 4).

On the assumption that the pronuclear content of the egg was an indication of its chromosome content, the following calculations have been made. Injection with concentration 1/10,000 yielded 58.7% diploid (i.e. two normal pronuclei), 20.6% haploid (one normal pronucleus), 3.1% hypo-diploid (one large and one small pronucleus), and 14.3% hyper-diploid or triploid embryos (several pronuclei). Injection with concentration 1/20,000 yielded 56.5% diploid, 17.4% haploid, 4.3% hypo-diploid, and 8.7% hyper-diploid or triploid. Eggs with sperm-tails and no pronuclei have not been included in any of these categories. The numbers of haploids and heteroploids induced were very similar for both concentrations. Unlike the results on 3½ day old embryos (p. 34), the 'morning plug' females had more eggs affected than had the 'evening plug' females.

The ratio of the different types of pronucleate eggs can be compared with the ratio of haploid, diploid, and heteroploid embryos identified by chromosome counts at 3½ days gestation. The number and kind of the pronucleate eggs have been given above; details of the 3½ day embryos have been abstracted from Table 4 for all concentrations of 1/20,000 and above:

	Haploid	Hypo-Diploid	Diploid	Hyper-Diploid or Triploid
Pronucleate eggs	17	3	50	10
3½ day old embryos	3	3	180	27

Multiplying the pronucleate eggs by a factor 180/50 to equalise the number of diploids, the comparison becomes:

	Haploid	Hypo-Diploid	Diploid	Hyper-Diploid or Triploid
Pronucleate eggs	61	11	180	36
3½ day old embryos	3	3	180	27

The small decline in the ratio of hyper-diploids or triploids at 3½ days gestation could be due to the lower cell numbers in these embryos than in the diploids causing the latter to be slightly overestimated (see p. 32). But a similar argument fails to explain the considerable decline in the number of haploids, for more than 95% of them failed to be traced at the 3½ day embryonic stage.

17½ hours after mating.

The single female killed at this stage had been injected with concentration 1/7,500; all of her eggs were fertilised. At 17½ hours, the eggs should be about to cleave (Lewis and Wright, 1935;

Gresson, 1941; and see p.12). Two types of egg were seen in this female. The first type (five examples) was already in the two-celled stage and apart from a slightly precocious cleavage, appeared to be quite normal. The second type was still in the pronuclear stage, and all four examples were abnormal. Three of the four possessed more than two pronuclei: two eggs possessed three, the third possessed four. In addition, one of the eggs with three pronuclei had no second polar body, the other had two small ones. The egg with four pronuclei possessed a rather small second polar body. In each case, one of the pronuclei was larger than the others, and, as previously indicated, resembled the male pronucleus. The fourth egg contained a single large pronucleus and two unusual, small second polar bodies (Figs. 22a & b). These abnormalities were similar to those found in the eggs of females killed 14 hours after mating, and again illustrate the possible mode of origin of triploid hyper-diploid and androgenetic haploid embryos.

It is notable that the four eggs which were still in the pronuclear stages were all abnormal, yet, with one exception, the five eggs in the two-cell stage appeared to be developing normally. The one exception had two nuclei in the second polar body. The only criterion of the normality of the five two-celled embryos was the appearance of the second polar body, for there was no certain method of ascertaining whether they were other than diploid. In view of the contrast between the second polar body of the two-celled embryos and that of the pronucleate eggs, it was quite possible that the former embryos were diploid. In effect, this meant that the abnormal embryos, which presumably

give rise to heteroploid development, were delayed in the pronuclear stages when compared to their normal sibs. The comparison fails, however, if the abnormal embryos were pathological.

27 to 36 hours after mating.

Two mice were killed for the histological examination of their eggs. One had received concentration 1/7,500, the other 1/10,000. The former mouse had only two eggs; one was in the two-celled stage and looked normal, while the other was unfertilised and was abnormal. The latter possessed no pronuclei or chromosomes in the egg proper, but had one first and two rather small second polar bodies. Previous comment has been made on this egg.

The other mouse yielded five normal-looking two-celled eggs, and seven unfertilised eggs. Six of the unfertilised eggs showed the same type of chromosome scatter as that noted previously (Fig. 21).

C. Heteroploid Development After 3½ Days Gestation.

Search for the later growth of heteroploid embryos was carried out by counting the chromosome complement of mature off-spring from colchicine-treated mothers. Chromosome counts were made on the conjunctive which had been previously cultured in hypotonic saline by the method of Beatty (unpublished). Females were injected with colchicine concentration 1/10,000 except in two cases where 1/7,500 was used. A constant stock of about fifty females was maintained for this part of the experiment; many of them received more than one injection of colchicine. The genetic combinations of some parents might have recombined in the offspring to show dosage effects of genes present in other than the diploid

complement; no dosage effects were noted in any of the offspring, however.

Altogether 37 matings after injection were recorded; 15 females (45.4%) gave birth to litters. Of these, one litter obtained by autopsy was dead, and three other litters were eaten by their mothers at birth. Another litter was lost when the mother died suddenly twelve days after giving birth. Details of the remaining ten litters are:-

Total number of offspring 65. Average litter size 6.5

Details of offspring:-

Dead at birth 3

Died later 13 (of which 7 died accidentally)

Total sexed 54

Sex ratio 30:24.

Of the 49 animals examined chromosomally, 46 were diploid, one was probably diploid (one mitosis appearing slightly above diploid, the others being certainly diploid), and two were unclassifiable due to loss of the conjunctiva tissue during the staining technique. In many mitoses every chromosome could be seen, and in every case of this kind exactly forty chromosomes were counted. With five exceptions, ten mitoses were counted in each animal. In the exceptions, only 5, 4, 2, 2, 1 mitoses respectively could be found. Apart from the doubtful case mentioned, no heteroploid mitoses were seen.

DISCUSSION.

At fertilisation the egg is in the first loop of the fallopian tube (Lewis and Wright, 1935). There are probably two barriers preventing the access of the injected colchicine solution to the egg. One is the tubo-uterine junction which is closed to ascending fluids except for a period during mating, and which at other times requires great pressure to open (see Alden, 1943 in the rat). The second barrier is the narrow, winding passage of the fallopian tube itself. Both of these barriers would be overcome by a successful mating and the fertilisation of the ovulated eggs. The opening of the tube-uterine junction at mating, and the ascent of the spermatozoa up the fallopian tube, would probably carry colchicine to the site of fertilisation. In addition, the pressure of the colchicine solution and the seminal fluids in the uterus may also cause a flow of the colchicine up the fallopian tube.

After the colchicine has been injected, there is no vaginal plug to retain the solution in the uterus until mating has taken place. Leakage of the colchicine from the uterus through the cervix would be assisted by the pressure of the injected solution on the uterine walls. A balance may therefore be created during mating in which loss of the injected colchicine occurred until the vaginal plug checked the leakage. This may have been the cause of the heterogeneity between females in the production of heteroploid embryos; females with no such embryos had probably lost most of the colchicine before mating occurred.

When the colchicine was retained in the uterus, it would reach

the site of fertilisation at the same time as, or maybe before, the arrival of the spermatozoa. To affect the meiotic spindle, the chemical must be taken into the egg before the extrusion of the second polar body. The time available for the colchicine to act will depend on the inter-relationships of several factors: the efficiency of the vaginal smear technique in tracing oestrous, the time taken for mating to occur, ovulation, and sperm entry into the eggs. The available time will vary between and within females; it may be less than one hour or up to several hours (Braden and Austin, 1954; Snell, Fekete, Hummel and Law, 1940; and see p. 15). Any delayed fertilisation induced by the treatment (p. 38) will extend this period. The experimental evidence shows that the unfertilised mouse egg can be severely affected by the colchicine; this suggests that sufficient time may exist before fertilisation for the colchicine to be absorbed on the surface of the egg. The results obtained from females which were not in oestrous when injected with colchicine (see later), and the effects of intraperitoneal injections of colchicine on the fertilised egg reported by Austin and Braden (1954), show that the carriage of colchicine to the egg may not necessarily occur via the fallopian tube; absorption of the chemical by various tissues, and transport in the blood to the ovary may be responsible.

Once in the egg, the colchicine destroys the second maturation spindle. The damage to the spindle leads to the false segregation and scattering of the dyads which, in many cases, come to lie at the periphery of the egg. Movements in the cyto-plasm of the unfertilised egg may cause this scatter; the

histological evidence shows that the chromosomes can even be completely extruded from the unfertilised egg. The disappearance of the spindle and the absence of any evidence of the parthenogenetic stimulation of the egg indicate that the extrusion is not due to a centriole-spindle mechanism. It could be due to the continuation of the movements of the egg cytoplasm causing the formation of small, nucleated cytoplasmic fragments separated from the egg proper; for the appearance of the extruded bodies (Fig. 22b) is often unlike the normal second polar body. At fertilisation, the contraction of the vitellus may assist in the expulsion of the chromosomes. It is clear from the pronuclear content of many eggs and from the chromosome counts on the 3½ day old embryos that all gradations between the complete loss and the complete retention of the female chromosomes are possible.

Pronucleus formation following colchicine treatment of the egg.

Eggs which have been affected by the colchicine and which show the false segregation or scattering of their chromosomes apparently retain the capacity of being penetrated by sperm. The detection of several small pronuclei in many fertilised eggs indicates that each small group of scattered maternal chromosomes can form a pronucleus at sperm entry. An alternative explanation of the origin of the sub-pronuclei based on the fragmentation of the female pronucleus after fertilisation appears doubtful in the face of the histological evidence. The mouse killed at the time of the first cleavage possessed five two-celled eggs which appeared to be normal, and four eggs in the pronuclear stages which were abnormal. Division of the five eggs to the two-celled

stage must have just occurred, the maximum time between fertilisation and fixing the embryos being $17\frac{3}{4}$ hours. It seems possible from this evidence that eggs which contain several pronuclei are slightly delayed in their pronuclear growth, syngamy, and first cleavage in comparison with normal eggs.

One of the four abnormal embryos possessed no second polar body or extruded fragment, and had three pronuclei of which one was presumably male and two female. All the female chromosomes had probably been retained in this egg, making its complement triploid. Two of the other three eggs had several pronuclei, and also one or more extruded fragments. It seems possible that these eggs possessed some, but not all, of the female chromosomes; they were probably between haploid and triploid. The last egg possessed one pronucleus only which resembled the male pronucleus, and had two extruded fragments. This was probably an androgenetic haploid. Many of the eggs examined by phase-contrast microscopy had similar types of abnormal pronuclear complements. The evidence suggests that, as the action of the colchicine is on the egg spindle only, the male gamete is not affected by the treatment and is normal. As no parthenogenetic stimulation of the eggs by the colchicine was observed, the chromosomes complement of the embryos developing after the treatment was probably composed of 20 male plus any number between 0 and 40 female. Proof of these origins requires the identification of the sex chromosome in the $3\frac{1}{2}$ day old embryos: half of the haploids possessing the Y chromosome, and all the triploids possessing at least two X chromosomes. Unfortunately the sex chromosomes of *Mus musculus* could not be identified in the embryos. Alternatively, radio-

active or vital dye markers in the sperm may show that all the uni-pronucleate ova possess a marked pronucleus which is therefore male, and that the multi-pronucleate eggs always contain only one male pronucleus.

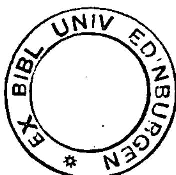
The reason for the delayed syngamy in eggs containing an abnormal pronuclear content is conjectural. The haploid may have been pathological, for the great majority of them failed to appear at 3½ days development. Fertilisation of the colchicine-treated eggs may have been delayed in relation to the diploids. Pincus and Waddington (1939) noted that colchicine applied to a pronucleate rabbit egg _in vitro_ arrested the inward movements of the pronuclei. The ratio of pronuclear to cytoplasmic volume is undoubtedly different in haploid, diploid, and triploid eggs. Excessive competition by the pronuclei for a cytoplasmic substrate in the last-mentioned, and an inability of the haploid to synthesise material as quickly as the diploid, may both lead to delay in pronuclear growth and syngamy. The pronuclear competition in hyper- and hypo-diploid eggs may be intermediate between the euploid types. The single pronucleus in the haploid eggs appeared very large in comparison with the pronuclei of normal diploids; the larger size could have been due to it synthesising more material than either of the two pronuclei in a diploid egg. Austin and Braden (1955) have also reported the enlarged growth of a pronucleus when by itself in an egg, and have postulated competition between pronuclei for a cytoplasmic substrate from their observations on these and other abnormal eggs.

In eggs containing four or five pronuclei, one pronucleus was probably the male and the others female. One or more of the

female pronuclei must have possessed a hypo-haploid chromosome complement because there can be a maximum of only two haploid pronuclei from the second maturation division of the egg. Further, one or more of these pronuclei must possess no centriole as there are presumably only two in the egg. Neither a haploid chromosome complement nor a centriole are therefore necessary for the formation of a pronucleus. The relative numbers of multi-pronucleate eggs and of hyper-diploid and triploid embryos at 3½ days gestation were very similar; the sub-pronuclei can apparently take part in syngamy. Failure of one or more of the numerous pronuclei to undergo syngamy in any particular egg may result in that pronucleus undergoing an unusual syngamy with one of the blastomere nuclei of the two-celled egg. This may have been the method of origin of some of the 3½ day old chromosome mosaics.

Heteroploid development at 3½ days gestation.

Many of the heteroploid embryos were capable of developing to 3½ days gestation. But the c-mitoses observed in some embryos probably indicated an excessive treatment by colchicine, and may have been due to the retention of free colchicine solution in the reproductive tract of the mother. Ideally, the treatment required that all trace of the chemical should be removed after affecting the maturation spindle; this is an undoubted advantage of the heat-treatment of eggs to induce triploidy (Fischberg and Beatty, 1952a). Besides this disadvantage, colchicine treatment *in vivo* also suffered from the difficulty of access of the solution to the eggs. The heterogeneity in the distribution of heteroploid embryos between females, and the effect of colchicine on the second maturation spindle of certain eggs, both


EX BIBL UNIV EDINBURGEN

indicated the considerable possibilities of the method when access was achieved.

Analysis of the cell number of the embryos showed that the toxic effects of the chemical were not profound. At conc. 1/10,000 lack of toxicity was combined with the capacity to affect the spindle of the egg. Addition of colchicine to the sperm *in vitro* before insemination also showed that conc. 1/10,000 was near the toxic threshold. Though no heteroploids were found after the *in vitro* treatment of sperm, some were presumably induced but were unrecognised due to the lack of classifiable mitoses.

The numbers of heteroploid embryos induced by the injection of colchicine (19% at the higher concentrations) is comparable with the number induced by heat-treatment of eggs in the fallopian tube (Beatty and Fischberg, 1949; Fischberg and Beatty, 1952a) which gave 15% heteroploid. The temperature treatment was more successful in inducing triploids as opposed to other types of heteroploid, for 19 out of the 26 heteroploids were triploid (compare 20 out of 43 between injected concs. 1/9,000 and 1/20,000). Spontaneous heteroploids taken from 'silver' strain mice contained at least 26 triploids out of 41 heteroploids (Beatty and Fischberg, 1951). Colchicine injection therefore gave less control than heat-treatment over the type of resultant heteroploidy.

The stage of development reached by the triploid and tetraploid embryos showed some variation, especially the tetraploids. The majority of the triploids, two of the tetraploids, and many other heteroploids were blastocysts when judged by cell number. Direct observation of the morphological stage of development of each embryo was not made; instead the number of blastocysts and

morulae taken from each female was noted. If all the embryos of a particular female were blastocysts, any polyploid from that female must have reached that stage of differentiation. Judged by this method, at least four of the triploids and one tetraploid were blastocysts. The mouse egg therefore resembles the amphibian egg in its capacity to differentiate to this level before chromosome unbalance interferes with its development.

Haploid development at the 3½ day embryonic stage.

Whereas the great majority of the induced hyper-diploid and triploid eggs succeeded in developing to morulae or blastocysts, 95% of the induced haploids failed to reach these morphological stages. The other 5%, however, were developing at almost the same rate as the diploid controls. The defection of the mass of the haploids poses the questions of their disability and their fate. The number of few-celled unclassifiable embryos observed in the experiment was so low that it cannot possibly have represented all of the missing haploids. The death of the haploids must have occurred at a very early stage of development. The explanation for their absence at 3½ days gestation is probably that these embryos failed to cleave at all, or cleaved but once or twice. Recent study has shown that many of the eggs which possess a single pronucleus fail to undergo their first cleavage; instead, considerable fragmentation occurs in their cytoplasm. The cause of the fragmentation and death of the haploids can scarcely be due to their chromosome complement, because those that do develop are quite normal. Death due to poisoning by colchicine is possible but it would hardly seem to explain the

remarkable all-or-none effect on the development of the haploid eggs only. Some other cause is presumably responsible for their decay.

The cause of the death of the majority of the haploids may be due to the possession of an abnormal cytoplasmic or centriolar complement. In the following discussion reference to the centriole will not exclude other cytoplasmic constituents. After the colchicine had dissolved the spindle of the egg, the chromosomes became arranged at the periphery of the egg due, probably, to movements in the cytoplasm of the unfertilised egg. By the same process, the egg centrioles can be scattered similarly. Both centrioles and chromosomes can therefore be lost from or retained in the egg. But the induction of haploid eggs is a more extreme effect on the egg than the induction of triploids: in the former the chromosomes are completely lost from the egg whereas they are merely scattered in the latter. Similarly, loss of the centrioles also occurs in most haploids. Dissolution of the spindle can result, therefore, in the loss of the chromosomes, the centriole, or both from the egg.

Based upon these assumptions, a theoretical scheme of fertilisation and development of colchicine-treated eggs can be postulated. Eggs in which all the egg chromosomes are retained will probably also retain their centriolar complement. At fertilisation, these eggs will possess a triploid chromosome complement, and both egg and sperm centrioles. Apart from delay due, for example, to pronuclear growth in triploids being slower than in diploids, these eggs will cleave at the same rate as diploids. On the other hand, eggs which have lost all their

chromosomes will almost certainly have lost their centrioles. At fertilisation these eggs will possess only the sperm centrioles, which are probably not sufficient to organise cleavage. Consequently, at the first cleavage these eggs will fail to divide normally, and, either by the formation of monocentric spindles or due to some other failure, will fragment and degenerate. If an egg which has lost its chromosomes has retained its centriole, however, cleavage and early development will be exactly the same as diploids. To complete the picture, if an egg which retained all its chromosomes but lost its centrioles was fertilised, it too would fail to cleave; there is some evidence for this in the lower number of triploids found in the 3½ day squashes than would be expected from the study of pronucleate eggs.

Adoption of this theory removes the responsibility for the success or failure of the early development of the mouse egg from the chromosomes to the centrioles. An all-or-none effect, depending on the retention or loss of the egg centriole, has been postulated. As it stands, the theory must be slightly modified because there are undoubtedly two centrioles in the egg, and only one of them, plus the sperm centriole, is necessary for normal development (see p.210). The theory is in opposition to results on amphibians, where the sperm contains the centrioles necessary for the developing embryo; and it also fails to explain why tripolar mitoses are not found in the triploid embryos. Considerable support for the theory is obtained from the study of the developmental rate of 3½ day old haploid, diploid, and polyploid mouse embryos (p.181), for these may all be cleaving at an equal rate. Further support comes from the total absence of 95% of

the haploids at 3½ days development, from the observations on gynogenetic haploid embryos (p. 209), and from the parthenogenetic stimulation of the rabbit egg (Thibault, 1949). This evidence will be considered in detail later.

Chromosome examination of mature animals.

Judged by the percentage of triploids developing at 3½ days development, 6.0 of the animals born to injected mothers, should have been triploid, but no triploid offspring were found. The sex ratio was not different from a 1:1 ratio; the slight excess of females was probably due to chance. All of the animals which died, either at or after birth, appeared normal. Unless the triploids died between birth and maturity, the colchicine treatments have so far failed to produce any triploid offspring.

Unfortunately, the heteroploid embryos will, in almost every case, be present alongside diploid embryos in the uterus. Unless triploid embryos keep pace with diploid sibs in developmental rate, their fate will quite possibly be death and resorption *in utero*. Some of the causes which probably bring about delay in triploid development, viz. delayed pronuclear growth, slower mitotic rate, multipolar spindles, and the toxic effect of the colchicine have been discussed. Some of the triploids can, despite these drawbacks, still show an equal or greater cell number at 3½ days development than diploid sibs; the competition that such triploids have to face probably comes later in development. Factors causing retarded triploid development in later embryonic stages may be physiological (Fischberg and Beatty, 1951), morphological, or mechanical. It seems that, unless such

diploid competition be almost or entirely eliminated from triploid development, the number of adult triploids to be found will be extremely small. Considerably improved techniques yielding very high percentages of triploids appear to be necessary; the *in vitro* cultivation of freshly ovulated eggs in colchicine before fertilisation may be the answer.

SUMMARY.

1. After the cervical injection of colchicine solution of various concentrations into the uterus of females immediately before natural mating, 19% of the resultant $3\frac{1}{2}$ day old embryos were heteroploids, many of them triploids.
2. 95% of the haploid eggs induced by this treatment failed to develop to the $3\frac{1}{2}$ day embryonic stage.
3. The toxic effect of colchicine on embryonic development was very marked above injected concentration 1/9,000 by weight. The heteroploid-inducing effect was greatest at concentration 1/9,000 and decreased in intensity down to 1/100,000. Lower concentrations probably had no effect.
4. Tetraploids, triploids, and, judged by cell number alone, haploids and various other chromosomal types, had all reached the blastocyst stage of development at $3\frac{1}{2}$ days.
5. By destroying the maturation spindle of the egg, colchicine induced heteroploid embryonic development with chromosome complement ranging from androgenetic haploid to triploid. Tetraploid and some mosaic development probably occurred by the action of the chemical on the first or one of the subsequent cleavages.

6. 107 mature offspring from females which had been treated by colchicine were examined chromosomally. All were diploid. Judged by the $3\frac{1}{2}$ day embryos, 6.0 of these offspring should have been triploid.

REFERENCES.

ALDEN, K.H. 1943. The utero-tubal function in the albino rat. Anat. Rec. 85, 290-291.

AUSTIN, C.R. & BRADEN, A.W.H. 1954. Induction and inhibition of the second polar division in the rat egg and subsequent fertilisation. Aust. J. Biol. Sci. 7, 195-210.

____________________ 1955. Observations on nuclear size and form in living rat and mouse eggs. Exp. Cell Res. 8, 163-172.

AUSTIN, C.R. & SMILES, J. 1948. Phase-contrast microscopy in the study of fertilisation and early development of the rat egg. J. Roy. Micr. Soc. 68, 13-19.

BEATTY, R.A. & FISCHBERG, M. 1949. Spontaneous and induced triploidy in pre-implantation mouse eggs. Nature 164, 807-808.

____________________ 1950. Polyploidy in rabbits. Nature 166, 238-239.

____________________ 1951a. Heteroploidy in mammals. I. Spontaneous heteroploidy in pre-implantation mouse eggs. J. Genet. 50, 345-359.

BEATTY, R.A. & ROWSON, L.E.A. 1954. Note on motility and fertility of colchicine-treated bull and rabbit sperm. J. Agric. Sci. 45, 254-256.

BLAKESLEE, A.F. 1937. Cytologie vegetale. Dedoublement du nombre de chromosomes chez les plantes par traitement chimique. C. R. Acad. Sci., Paris 205, 476-479.

BLANDAU, R.J. 1952. The female factor in fertility and infertility. I. Effects of delayed fertilization in the development of the pronuclei in rat ova. Fert. & Ster. 3, 349-365.

BRADEN, A.W.H. & AUSTIN, C.R. 1954. Fertilization of the mouse egg and the effect of delayed coitus and of hot-shock treatment. Aust. J. Biol. Sci. 7, 552-565.

CHANG, M.C. 1944. Artificial production of monstrosities in the rabbit. Nature 154, 150.

EDWARDS, R.G. 1954. Colchicine-induced heteroploidy in early mouse embryos. Nature 174, 276-277.

FISCHBERG, M. & BEATTY, R.A. 1951. Spontaneous embryos in mouse embryos up to mid term. J. exp. Zool. 118, 321-336.

________________ 1952a. Heteroploidy in mammals. II. Induction of triploidy in pre-implantation mouse eggs. J. Genet. 50, 455-470.

________________ 1952b. Heteroploidy in mouse embryos due to crossing of inbred strains. Evol. 6, 316-324.

GRESSON, R.A.R. 1941. A study of the cytoplasmic inclusions during maturation, fertilization and the first cleavage division of the egg of the mouse. Quart. J. Micro. Sci. 83, 35-60.

HAGGQVIST, G. & BANE, A. 1950a. Polyploidy in rabbits induced by colchicine. Nature 165, 841-843.

________________ 1950b. Chemical induction of polyploid breeds of mammals. Kungl. Svenska Vetens. Handl. 1, No. 10.

________________ 1951. Kolchizininduzierte Heteroploidie bein Schwein. Kungl. Svenska Vetens. Handl. 3, No. 2.

KLIESCH, J. & SCHMIDTKE, C. 1954. Untersuchungen über die Wirkung von Colchicin auf die Spermatogenese bein Kaninchen unter besonderen Berücksichtung den Polyploidie. Zeit. f. Tierz. und Zücht. 63, 127-154.

LEVAN, A. 1954. Colchicine-induced c-mitosis in two mouse ascites tumours. Hereditas 40, 1-64.

LEWIS, W.H. & WRIGHT, E.S. 1935. On the early development of the mouse egg. Publ. Carnegie Inst., No. 459, Contrib. Embryol. No. 148, 25, 113-143.

MELANDER, Y. 1950. Chromosome behaviour of a triploid adult rabbit as produced by Häggqvist and Bane after colchicine treatment. Hereditas 36, 335-341.

________________ 1951. Polyploidy after colchicine treatment of pigs. Hereditas 37, 288-289.

PINCUS, G. & WADDINGTON, C.H. 1939. Comparative behaviour of mammalian eggs in vitro and in vivo. V The effects of mitosis-inhibiting treatments on normally fertilized pre-cleavage rabbit eggs. J. Hered. 30, 515-518.

SNELL, D.G., FEKETE, E., HUMMEL, K.P. & LAW, L.W. 1940. Relation of mating, ovulation and the oestrous smear in the house mouse to time of day. Anat. Rec. 74, 39-54.

THIBAULT, C. 1949. L'oeuf des mammifères, son développement parthénogénétique. Ann. Sc. nat., Zoologie, 11, 136-219.

TABLE 4. DETAILS OF THE FERTILITY OF THE INJECTED FEMALES AND OF THEIR EMBRYOS AT $3\frac{1}{2}$ DAYS GESTATION.

	MALES	FEMALES			EMBRYOS:-							CHROMOSOME COUNTS											CELL NOS.
Injected concentration (x 1000)	No. of different ♂♂ used	No. of ovulated ♀♀ used	No. ♀♀ with embryos	% of total ♀♀ with embryos	Total no. of eggs found	No. of eggs from ♀♀ with embryos	Total no. of embryos	% of total eggs which were embryos	% embryos of total eggs from ♀♀ with embryos	Mean no. embryos per ♀ with embryos	% embryos which were blastocysts	Lost or unclassified	Haploid	Between haploid and diploid	Diploid	Probably diploid	Hyper-diploid	Triploid	Tetraploid	Others	% triploid of total classified	% not diploid of total classified	Mean cell no. of all embryos
1/5 & above	5	6	0	.0	30	0	0	0	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-
1/7	8	8	2	25.0	49	8	4	8.2	50.0	2.0	0	3	-	-	1	-	-	-	-	-	0	0	19.0±6.1
1/9	17	17	11	64.7	155	120	73	47.1	60.8	6.6	53.4	47	-	2 (34) (35)	17	1	3 (42) (46) (48)	2	-	1	11.4±3.3	19.0±4.5	35.4±19.4
1/10	24	28	25	89.3	270	266	204	75.6	76.7	8.0	71.1	125	-	1(37)	64	3	-	10	1	-			40.5±17.6
1/15	20	26	18	69.2	228	173	155	68.0	89.6	8.6	75.5	79	3	-	59	6	1(47)	3	1	3	6.8±2.3	19.7±4.7	42.6±15.0
1/20	19	24	18	75.0	192	154	129	67.2	83.8	7.0	55.8	88	-	-	25	4	3 (45) (50) (53)	5	3	1			40.8±17.8
1/30	6	6	5	83.3	66	51	51	77.3	100.0	10.2	49.0	29	-	-	18	-	-	-	-	4	2.0±2.0	12.0±4.6	37.9±16.2
1/50	6	6	5	83.3	64	53	51	79.7	96.2	10.2	76.5	23	-	-	23	3	-	1	-	1			43.9±12.3
1/100	4	4	4	100.0	40	40	33	82.5	82.5	8.3	90.9	21	1	-	11	-	-	-	-	-	0	8.3	41.2±13.0
1/200	9	9	8	88.9	72	65	54	75.0	83.1	6.8	66.7	37	-	-	16	-	-	-	-	1?	0	5.9	46.1±16.5
1/500	7	9	9	100.0	79	79	69	87.3	87.3	7.7	71.0	48	-	-	30	-	-	-	-	1?	0	3.2	43.8±14.4
saline only	28	43	38	88.4	371	361	298	80.3	82.5	7.8	66.1	193	-	-	103	2	-	-	-	-	0	0	45.2±18.1
natural mating	17	31	27	87.1	273	242	212	77.7	87.6	7.9	63.2	142	-	-	54	15	-	-	1	-	0	1.4±1.5	40.6±13.6

TABLE 5. ANALYSIS OF THE CELL NUMBERS OF DIPLOID EMBRYOS.

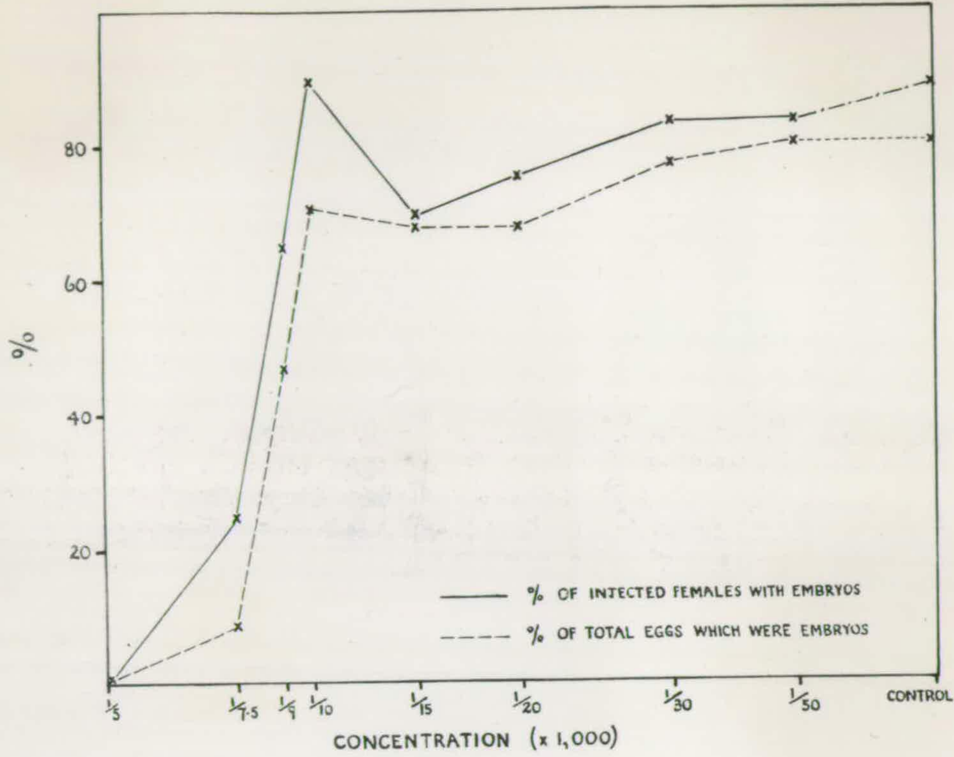
	All Diploids		The mean cell number of: Diploids taken from females with heteroploids		Diploids taken from females without heteroploids	
Colchicine Conc.	No. of embryos	Mean	No. of embryos	Mean	No. of embryos	Mean
1/100	11	44.5	2	53.0	9	42.7
1/50	21	48.7	4	46.3	17	49.2
1/30	15	43.7	5	35.2	10	47.9
1/20	23	47.5	11	52.0	12	43.3
1/15	60	46.0	31	46.7	29	45.1
1/10	64	49.2	11	37.1	53	51.7
1/9	16	48.6	6	50.7	10	47.4
Saline only	71	51.7				
Natural mating	46	47.6				

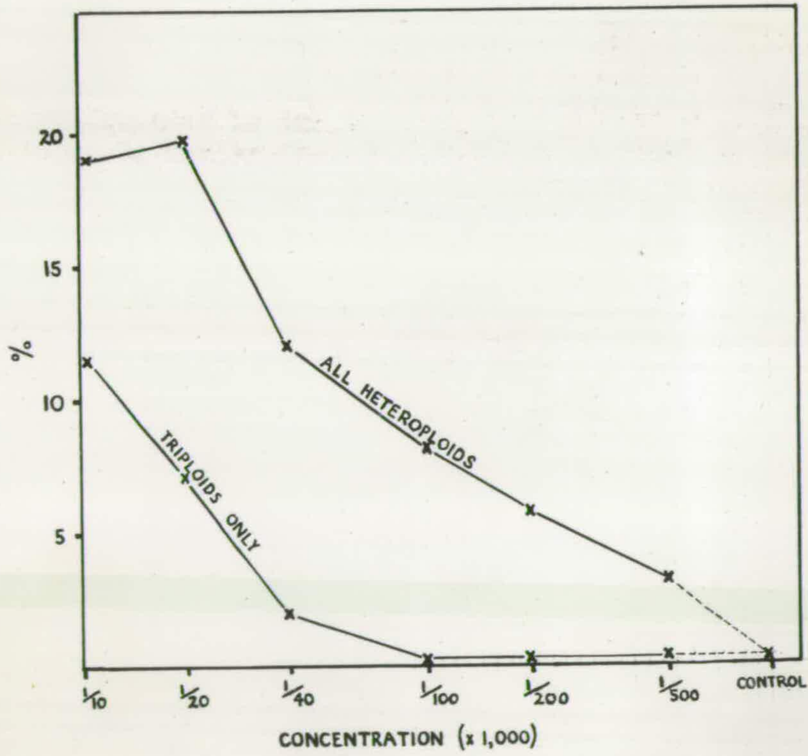
TABLE 6. DETAILS OF THE FERTILITY OF THE INSEMINATED FEMALES AND OF THEIR EMBRYOS AT 3½ DAYS GESTATION.

	SPERM	FEMALES			EMBRYOS:—							CHROMOSOME COUNTS					CELL NOS.	
Injected concentration (x 1000)	No. of sperm samples	No. of ovulated ♀♀ used	No. of ♀♀ with embryos	% of total ♀♀ with embryos	Total no. of eggs found	No. of eggs from ♀♀ with embryos	Total no. of embryos	% of total eggs which were embryos	% embryos of total eggs from ♀♀ with embryos	Mean no. of embryos from ♀♀ with embryos	% embryos which were blastocysts	Lost or unclassified	Below diploid	Diploid	Probably diploid	Above diploid	Mean cell no. of all embryos	Mean cell no. of diploids
1/5	5	4	–	0	21	–	–	0		0		–	–	–	–	–		
1/10	18	10	3	30.0	88	28	13	14.8	46.4	4.3	76.9	4	–	6	3	–	57.2±25.7	63.3±18.1
1/20	11	6	3	50.0	50	36	12	24.0	33.3	4.0	91.7	5	–	7	–	–	50.8±15.3	54.6±14.4
1/30	5	4	2	50.0	42	24	10	23.8	41.7	5.0	80.0	7	–	3	–	–	38.6±9.6	45.7±14.8
1/50	5	5	3	60.0	45	29	11	24.4	37.9	3.7	90.9	6	–	5	–	–	54.0±23.8	58.0±3.3
1/100	4	2	2	100.0	20	20	12	60.0	60.0	6.0	91.7	6	–	5	1	–	51.8±18.0	54.5±15.6

Text-fig. 1. Percentage of females with embryos, and the percentage of the total eggs which were embryos at 3½ days gestation after the injection of various colchicine concentrations at fertilisation. Data taken from Table 4.

Text-fig. 2. Percentage of triploid and of heteroploid embryos induced by various concentrations of colchicine.


%
80
60
40
20
% OF INJECTED FEMALES WITH EMBRYOS
% OF TOTAL EGGS WHICH WERE EMBRYOS
1/5
1/7.5
1/9
1/10
1/15
1/20
1/30
1/50
CONTROL
CONCENTRATION (x 1,000)


%
20
15
10
5
ALL HETEROPLOIDS
TRIPLOIDS ONLY
1/10
1/20
1/40
1/100
1/200
1/500
CONTROL
CONCENTRATION (x 1,000)

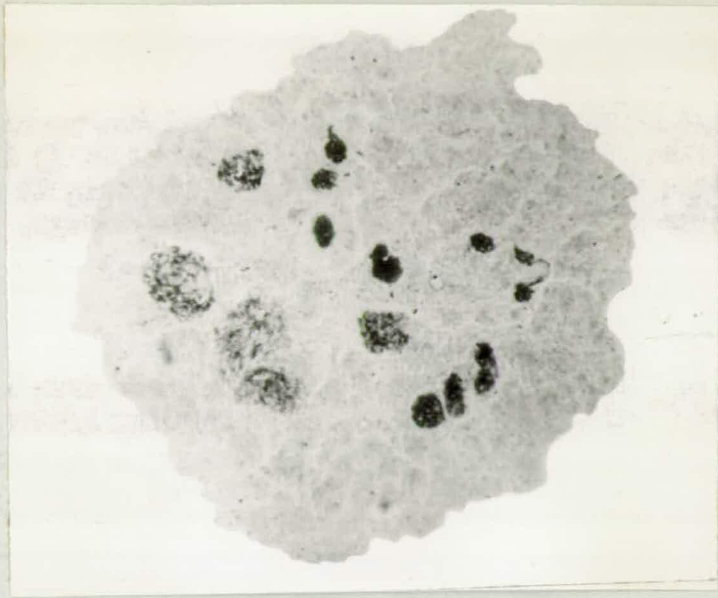
FIGURES

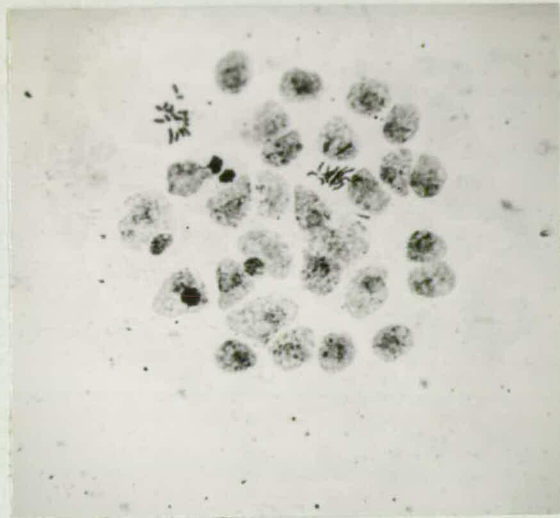
The negative of Fig. 10 was retouched around the periphery of the embryo. Figs. 10-19 inclusive are from squashed $3\frac{1}{2}$ day old embryos, Figs. 20-22 are from sectioned eggs, Fig. 23 is a living egg photographed under the phase-contrast microscope.

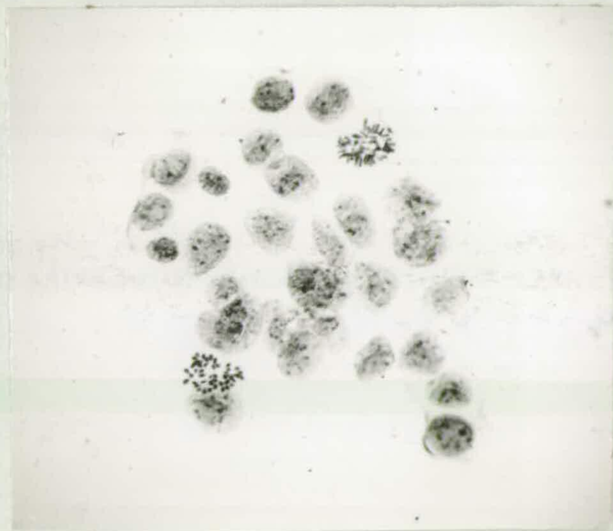
10. A highly retarded $3\frac{1}{2}$ day old embryo taken from a mother injected with colchicine of concentration 1/9000. x 350.

11. A $3\frac{1}{2}$ day old haploid blastocyst. The mother was injected with colchicine concentration 1/15,000. x 350.

12. An embryo with a complement of between 46 and 48 chromosomes. There is some contraction in the chromatids of the lower mitosis. x 350.



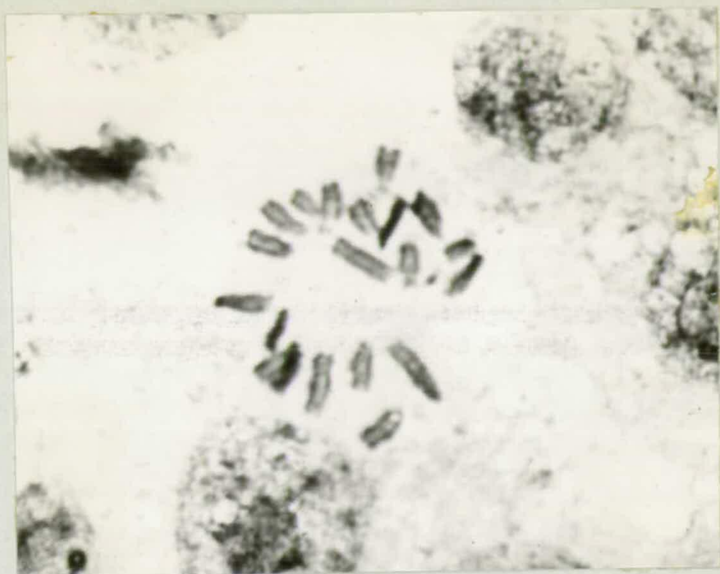


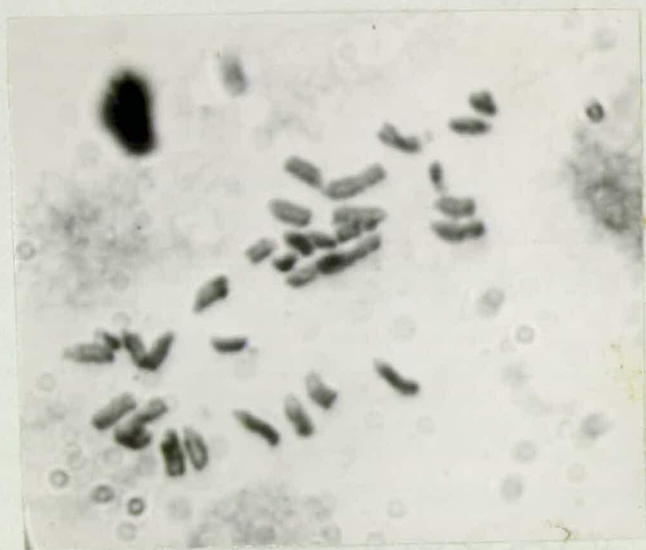


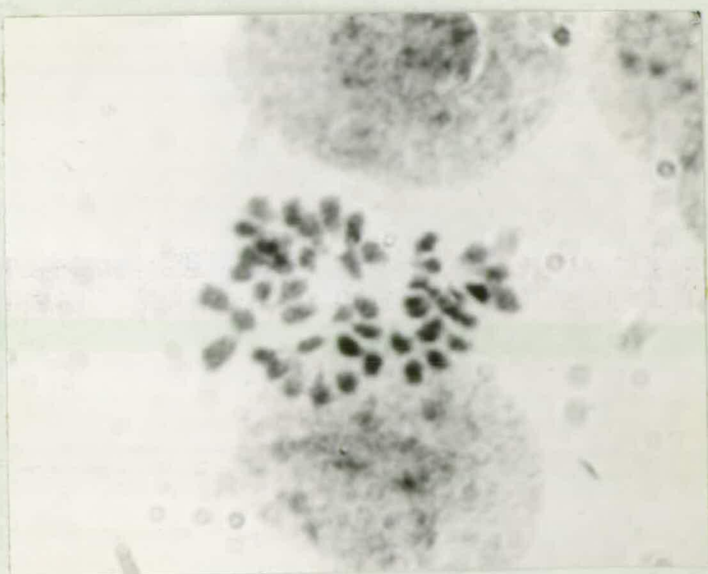
13. Haploid chromosome complement in a squashed blastocyst taken from a female injected with concentration 1/100,000. x 2000.

14. A mitosis of 35 ± 1 chromosomes. x 2000.

15. A mitosis of 46 ± 2 chromosomes. The contracted chromatids are difficult to focus for photography. x 2000.



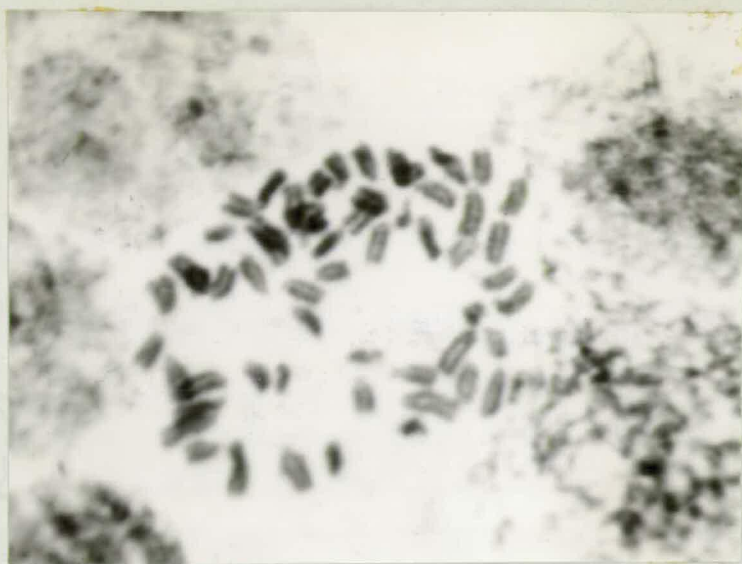


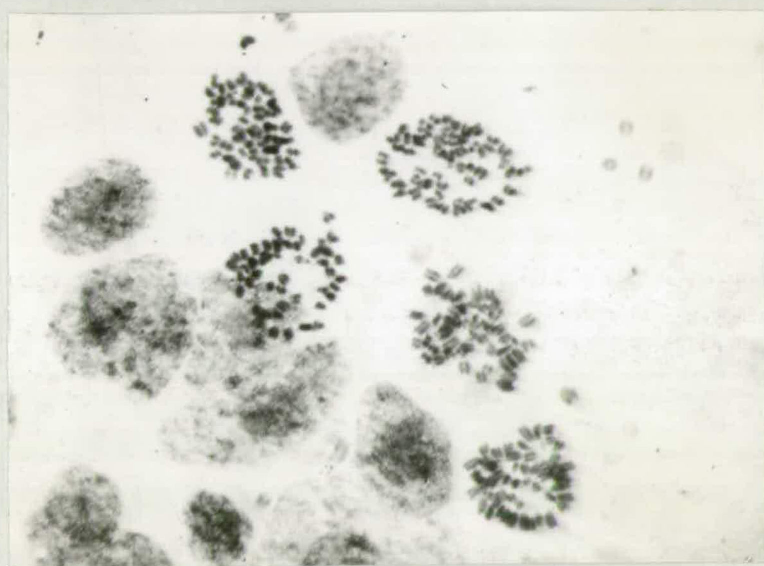


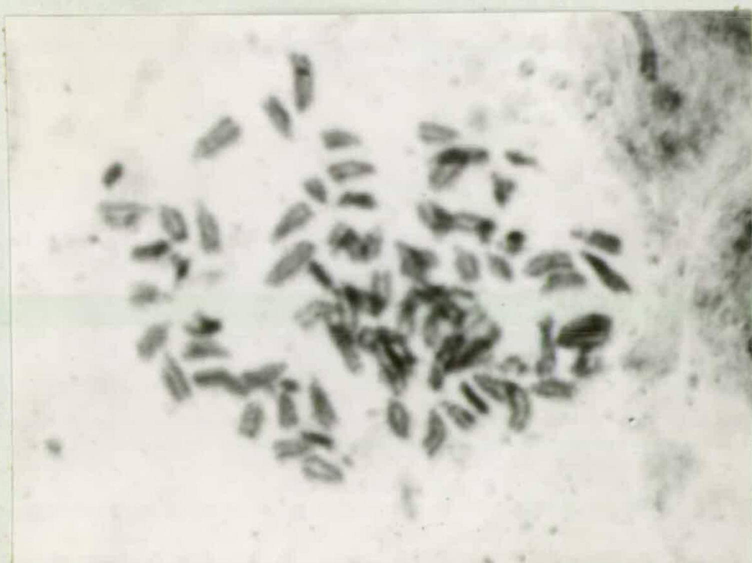
16. A triploid mitosis. x 2000.

17. A group of five triploid mitoses in a squashed embryo; some contraction is evident in two mitoses. The mother was injected with concentration 1/10,000. x 800.

18. A tetraploid mitosis. x 2000.



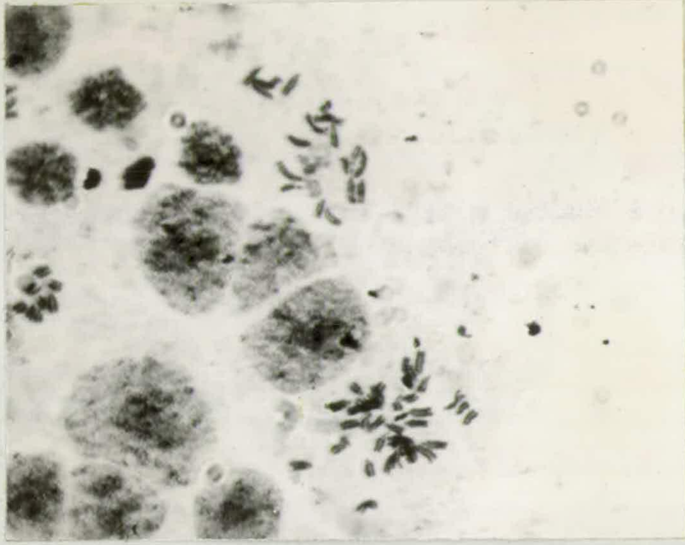


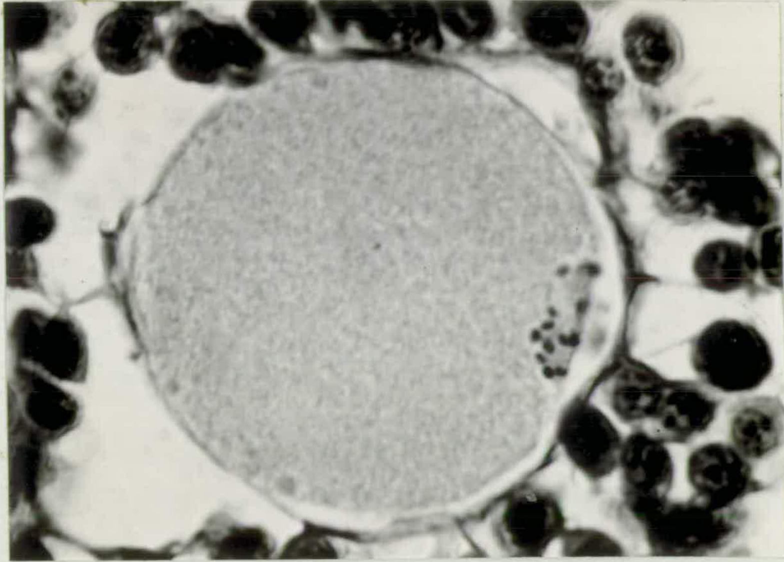


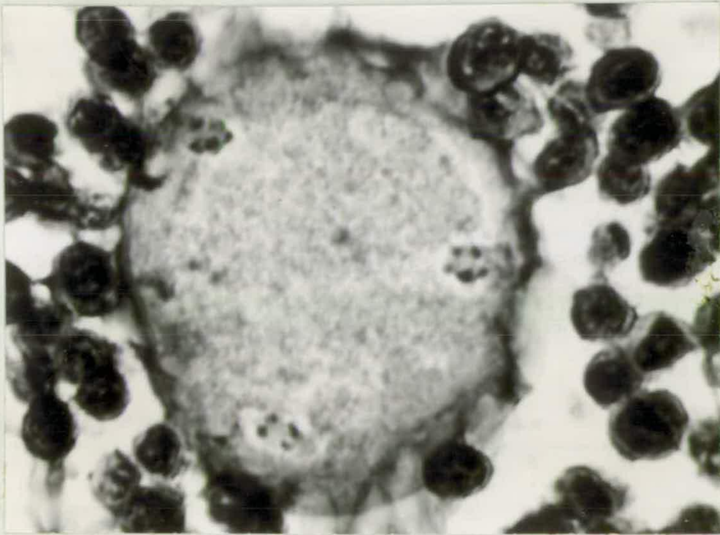
19. A haploid/diploid mosaic taken from a female injected with concentration 1/30,000. x 800.

20. Destruction of the spindle of the second maturation division after treatment by colchicine of concentration 1/7500. The dyads are moving along the remnants of the spindle. x 1000.

21. Groups of chromosomes scattered round the periphery of the unfertilised egg after injection of colchicine concentration 1/10,000 into the mother. x 1000.

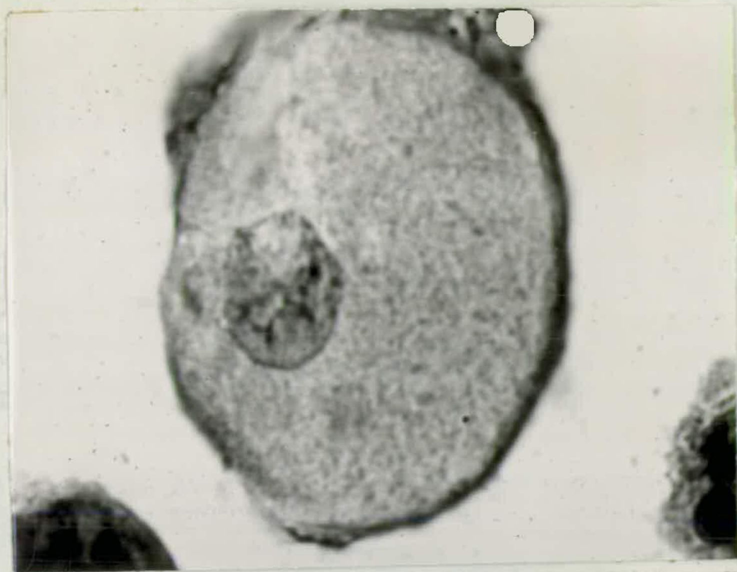


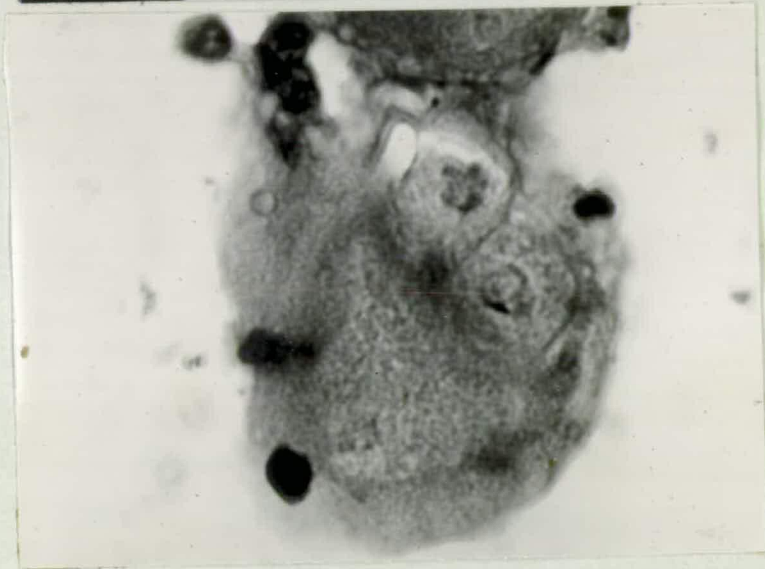


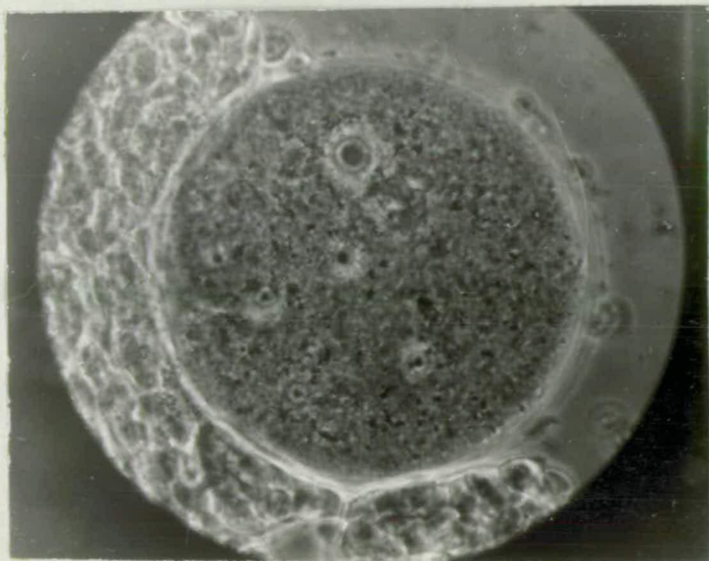


22a and b. A single large pronucleus in an egg treated with colchicine concentration 1/7500. Two small, abnormal second polar bodies have been abstricted from the egg in Fig. 22b (lower plate). x 1000.

23. Five pronuclei ina living egg treated with colchicine concentration 1/20,000. One pronucleus is normal-sized and is probably male; the other four are very small and are probably female. Examined by phase-contrast. x 600.







2. HETEROPLOIDY INDUCED AFTER PREVIOUS COLCHICINE INJECTIONS.

The method generally used to select oestrous females is the vaginal smear technique. This is not a perfect tool; judged by the numbers of selected females which mated, it gave about 70% success throughout previously-described experiments in which colchicine was injected into the uterus before mating (p. 21). Necessarily, therefore, certain of the injected females failed to mate, and numbers of these mice accumulated. Some were injected again (see later), and, in turn, a residue of these failed to mate after subsequent injections. To discover whether any effect of the injected colchicine remained in these mice, they were placed full-time with normal males and allowed to mate at will. At mating, therefore, these mice had been previously injected either once or more with various colchicine concentrations, though none were injected at the time of mating itself. Three and a half days after mating, the females were killed and their embryos made into squash preparations for chromosome counts; details of this technique have been given on p. 6.

RESULTS.

Twenty-four females were killed; details of their fertility and of the embryos recovered from them are given in the first row of Table 7. Nineteen mice gave fertilised eggs. The numbers of eggs fertilised, and embryonic development after fertilisation, were similar to those found after natural mating. But seven of the 72 embryos examined chromosomally were heteroploid; they comprised three triploids, one hyper-diploid with 50 chromosomes,

one probable haploid, and two mosaics. At least one of the triploids, the hyper-diploid, and a diploid-tetraploid mosaic were blastocysts. The number of heteroploid embryos was not significantly different from the number obtained after natural mating, but the test was rather strict because of the high estimate of spontaneous heteroploids in the controls. If, in fact, the heteroploidy was spontaneous, though this is unlikely, it is a remarkably high yield for such heterogeneous mice. For example, Fischberg and Beatty (1952) discovered 0.4% of heteroploid embryos in control matings; and in the controls injected with sodium chloride (p. 31), no heteroploids were found out of 105 classifiable embryos. For convenience, mice which had been previously injected with colchicine without having mated will be termed 'residual' mice.

Efforts were made to induce the residual effect in other females by controlled means. Certain females were injected with various concentrations of colchicine (1/5,000 to 1/15,000 by weight) when they were definitely not in oestrous, and mated later. The classification of their embryos is shown in Table 7, row 2. All eleven mice had fertilised eggs, and two embryos were heteroploid out of 29 classified.

The second method used to induce the residual effect was to inject through the cervix into the uterus a colchicine colution known to be toxic to the gametes. A concentration of 1/5,000 by weight was selected. Oestrous females were injected and then paired with a male for natural mating. It was intended that the uterine contractions during mating, the opening of the tubo-uterine junction, and the ascent of the sperm up the fallopian

tube would facilitate the carriage of the colchicine solution to the ovary. Contamination of the ovary may affect the maturation spindle of the eggs while still in follicle, resulting in the ovulation of diploid or tetraploid eggs on a later oestrus. This actually amounted to <u>in vivo</u> treatment of the ovary while the female was in pseudopregnancy following mating. At the end of pseudopregnancy, the female was mated without further injection, and her embryos were examined at 3½ days gestation as squash preparations. Of the 27 females injected, one gave birth to a litter which contained two young. Details of the others and of their embryos are given in Table 7, row 3. No heteroploid embryos were found out of 35 classified. The only notable result was the measure of infertility induced in some of the females.

The third method used to induce the residual effect was, by operation, to inject colchicine solution beneath the ovarian capsule on the ovary. A wide range of concentrations was used. No heteroploids were found after the subsequent mating of these females; instead a measure of infertility was induced if mating closely followed the operation.

Despite the comparative failure to induce the residual effect by other means, residual mice were used for three other purposes. Some of them were given further injections just before mating, others were injected after mating; both of these types will be dealt with later. The third purpose was to allow some of these females, which had various genetic markers, to rear their offspring to maturity. The chromosome complement of the offspring was counted in conjunctiva tissue pre-cultured in hypotonic

saline by the method of Beatty (unpublished).

Twelve litters were born. One of them was obtained by autopsy, but all the young were dead. Two other litters were eaten by their mother at birth; and one litter was lost by accident. From the remaining eight litters, 49 offspring were obtained of which six were dead when first seen. The remaining 43 were raised to maturity, the sex ratio being 25:18. This unequal ratio was due to one particular litter which consisted of nine females and no males, though two other members of the same litter were eaten before being sexed. In the chromosome counts, one animal was unclassifiable because the conjunctiva tissue was lost from the slide during staining, another mouse died before examination; all of the 41 others were diploid. No heteroploid mitoses were seen. Except for three of these mice where 5, 4, and 1 mitosis respectively were counted, ten clear mitoses were used for each classification. No abnormal gene dosage effects were seen.

Repeated injections of colchicine into the uterus.

When they came into oestrus, colchicine was injected into the uterus of certain residual females and they were paired with males for mating. The intention of the treatment was twofold. First, to combine the 'residual' effect with the action of colchicine on the gametes (p. 21) and increase the yield of heteroploidy; second to test whether the combination of the two methods induced more complex heteroploidy, for example hexaploidy.

Injected concentrations were between 1/9,000 and 1/15,000. The results found in 3½ day old squashed embryos are given in

Table 8. The number of females with embryos, and the number of embryos per female, were lower than that of residual females or of once-injected females (p. 26). Forty-five embryos were classifiable, of which five were heteroploid. No triploids were found, and apart from one embryo which possessed a mitosis of 69 ± 2 chromosomes, no heteroploids occurred which were qualitatively different from those produced by either the injection method or the residual method alone.

The mean cell number of all embryos and of the diploid embryos are given for each concentration in Table 8. The means were similar to those of control embryos, but the high standard error at conc. 1/10,000 was due largely to four embryos each of which possessed 10 nuclei or less. Apart from these, embryonic development appeared to be normal. The number of cells in the various types of heteroploid, and a comparison with diploid cell numbers, will be given separately.

Examination of offspring from colchicine-treated 'silver' mice.

Seven females from a line of mice selected for a high incidence of spontaneous heteroploidy in 3½ day old embryos (Beatty and Fischberg, 1951; Beatty, 1954) were used for colchicine injections. These mice contained the 'silver' factor which is responsible for or related to the origin of spontaneous heteroploidy (Beatty & Fischberg, 1951). Combination of the effect of the 'silver' factor with colchicine injection was intended to raise the incidence of triploidy. The chromosome complement of the mature offspring of these mothers was counted in conjunctiva tissue which had been cultured in hypotonic saline by

the method of Beatty (unpublished).

It was intended to inject colchicine into the uterus of oestrous 'silver' females just before mating. Difficulties were encountered in tracing the oestrous cycle of these females by the vaginal smear technique, but despite this eight matings following injection were obtained. Only two of these matings produced litters, one of four and the other of two young. Subsequently the 'silver' females were placed full-time with a male to make use of any residual effect induced by the previous injections. Five litters were obtained in this manner. Details of the females and of their offspring were:-

	Injected colchicine	Residual
Number of females injected	8	
Number of litters	2	5
Litters lost through miscarriage		1
Total number of offspring	6	18
Sex ratio	4:2	10:8
Chromosome classification of offspring:		
Diploid	5	14
No mitoses	1	2
Material lost from slide		2

After the examination of their offspring, the seven 'silver' females were remated and killed $3\frac{1}{2}$ days later. Squash preparations were made of their embryos. Results are given in Table 7, row 4. One of the females had not ovulated, three others possessed only unfertilised eggs. The other three females gave 13 embryos, 12 of which were morulae. Nine squashed embryos had no mitoses, three were diploid, one was a tetraploid. Whether the tetraploid was spontaneous or residual is conjectural.

DISCUSSION.

The small percentage of heteroploidy induced in residual females occurred at periods of up to 3 weeks after the previous colchicine injection. It seems unlikely that colchicine remained in the fallopian tube or beneath the ovarian capsule for this length of time; the chemical may have been absorbed in the blood or in the tissues of the female and affected the ovary or the egg via the blood. Austin and Braden (1954) reported second polar body suppression after intraperitoneal injections of colchicine into female rats; but, as these workers injected colchicine immediately after mating, the injected chemical could have affected the eggs directly or via the bloodstream.

Of the heteroploids, triploids occurred in the largest numbers. This evidence, together with the induction of a haploid embryo, suggests that the second maturation spindle of the egg may have been damaged in a similar manner to that caused by the injection of colchicine into the uterus before mating (p. 21). The time available to affect the second maturation spindle of the egg is limited to between the completion of the first maturation division and the extrusion of the second polar body. If sufficient colchicine remained in the females to affect the second maturation spindle, it could also affect the first maturation spindle of the egg, or both. Destruction of both spindles could result in the ovulation of tetraploid eggs; destruction of the first spindle only could result in diploid eggs. At fertilisation, the embryos would then be pentaploid or triploid. Suppression of the first maturation division would result in

triploids which were genetically distinct from those caused by suppressing the second maturation division. In the triploids arising by the former method, the complete heterozygosity of the mother would be retained in the secondary oocyte; the only loss of heterozygosity would be by the chance segregation of similar cross over segments into the second polar body. In contrast to this, suppression of the second maturation would retain only that heterozygosity caused by odd-numbered cross overs from the centromere in the first maturation division. Residual triploids may therefore be of a different origin genetically than those produced by treatment of the gametes at fertilization by heat-treatment (Fischberg and Beatty, 1952) or by colchicine (p. 21).

Judged by the number of embryos which were triploid at 3½ days gestation, 4 of the 96 mature offspring of 'silver' or non-'silver' mothers should have been triploid. The absence of mature triploid offspring may have been due to chance or to their death in uterus or after birth. The possibility of uterine competition between diploid and triploid embryos, resulting in the death of the latter has been discussed previously (p. 56). Though chromosome counts are the primary evidence for assessing ploidy, observation of a disturbed sex ratio in the offspring of treated mothers may be of considerable importance in heteroploidy-inducing experiments. After attempts to cause polar body suppression in the egg, an excess of female offspring is very suggestive; any induced triploids would possess two X chromosomes from the egg, and may be biassed towards femaleness whether they receive an X or a Y chromosome from the sperm. One female gave a

litter of nine females and no males; her subsequent litters had a 1:1 sex ratio. The femaleness of the first litter may have been due to an influence of residual colchicine, the influence having been lost by the time of conception of subsequent litters. But the diploidy of the offspring suggests that it was merely a chance result.

SUMMARY.

1. Females which were mated several days after receiving an intra-uterine injection of colchicine yielded some heteroploid embryos at 3½ days gestation. About 10% of the embryos were heteroploid, triploids being the most numerous.
2. Attempts to produce the same phenomenon by other experimental procedures did not succeed.
3. Chromosome counts on the mature offspring of these females showed that no heteroploids had developed to maturity.

REFERENCES.

AUSTIN, C.R. & BRADEN, A.W.H. 1954. Induction and inhibition of the second polar division in the rat egg and subsequent fertilisation. Aust. J. Biol. Sci. 7, 195-210.

BEATTY, R.A. 1954. Haploid rodent eggs. Proc. 9th Inter. Congr. Genet., Bellagio. Caryologia, 6, Suppl.

BEATTY, R.A. & FISCHBERG, M. 1951. Heteroploidy in mammals: I. Spontaneous heteroploidy in pre-implantation mouse eggs. J. Genet. 50, 345-359.

FISCHBERG, M. & BEATTY, R.A. 1952. Heteroploidy in mammals: II. Induction of triploidy in pre-implantation mouse eggs. J. Genet. 50, 455-470.

TABLE 7. DETAILS OF THE FERTILITY OF 'RESIDUAL' FEMALES AND OF THEIR EMBRYOS AT 3½ DAYS GESTATION.

Treatment	MALES: No. of different ♂♂ used	FEMALES: No. of ovulated ♀♀ used	No. of ♀♀ with embryos	% of total ♀♀ with embryos	EMBRYOS:— Total no. of eggs found	No. of eggs from ♀♀ with embryos	Total no. of embryos	% of total eggs which were embryos	% embryos of total eggs from ♀♀ with embryos	Mean no. of embryos from ♀♀ with embryos	% embryos which were blastocysts	CHROMOSOME COUNTS: Lost or unclassified	Haploid	Between haploid and diploid	Diploid	Probably diploid	Hyper-diploid	Triploid	Tetraploid	Others	% not diploid of total classified	CELL NOS.: Mean cell no. of all embryos	Mean cell no. of diploids
I	15	24	19	79.2	191	184	161	84.3	87.5	8.5	68.9	89	1?(26)	-	56	9	1(50)	3	-	2	9.7±3.5	45.9±16.8	49.0±15.
II	6	11	11	100.0	95	95	76	80.0	80.0	6.9	60.5	47	-	1?(29)	23	4	-	-	-	1	6.9±4.7	45.5±17.5	46.3±14.
III	26	26	19	73.1	207	184	166	80.2	90.2	8.7	75.3	131	-	-	22	13	-	-	-	-	0	45.4±15.9	55.3±11.
IV	6	6	3	50.0	22	18	13	59.1	72.2	4.3	7.7	9	-	-	2	1	-	-	1	-	25.0±21.6	16.6± 8.6	19.0± 9.
[N]at. Mat. [C]ontrols	17	31	27	87.1	273	242	212	77.7	87.6	7.9	63.2	142	-	-	54	15	-	-	1	-	1.4±1.4	40.6±13.6	47.9±13.

I Injected (probably more than once) when on a previous oestrous, now naturally mated.
II Injected when not on oestrous, now naturally mated.
III Injected once, with 1/5000 or 1/7500 colchicine when on oestrous, now naturally mated.
IV 'Silver' mice injected (probably more than once) when on oestrous, now naturally mated.

TABLE 8. DETAILS OF THE FERTILITY AND EMBRYOS AT $3\frac{1}{2}$ DAYS GESTATION OF 'RESIDUAL' FEMALES RE-INJECTED AT OESTROUS

	MALES	FEMALES			EMBRYOS:-							CHROMOSOME COUNTS								CELL NOS.	
Injected concentration (x 1000)	No. of different ♂♂ used	No. ovulated ♀♀ used	No. ♀♀ with embryos	% of total ♀♀ with embryos	Total no. of eggs found	No. of eggs from ♀♀ with embryos	Total no. of embryos	% of total eggs which were embryos	% embryos of total eggs from ♀♀ with embryos	Mean no. of embryos from ♀♀ with embryos	% embryos which were blastocysts	Lost or unclassified	Between haploid and diploid	Diploid	Probably diploid	Hyper-diploid	Tetraploid	Others	% not diploid of total classified	Mean cell no. of embryos	Mean cell no. of diploids
1/9	9	13	6	46.2	101	57	30	29.7	52.6	5.0	67.7	18	-	11	-	-	1	-	8.3±8.0	39.4±12.5	43.7±6.7
1/10	6	6	4	66.7	57	41	25	43.9	61.0	6.3	68.0	13	1(34)	10	1	-	-	-	8.3±8.0	41.6±20.1	52.8±9.8
1/15	9	10	6	60.0	90	68	45	50.0	66.2	7.5	82.2	25	-	17	1	1(43)	2	1(68)	14.3±7.6	41.3±14.0	39.6±13.9

3. THE INDUCTION OF TETRAPLOIDY

The destruction of the spindle of the second maturation division of the egg by colchicine may cause all the maternal chromosomes to be retained in the egg at fertilisation. Such an egg may then develop as a triploid (p. 49). If the spindle of the first cleavage division of the egg can be destroyed in a similar manner, the first cleavage may be suppressed. Retention in one cell of the two diploid chromosome sets on this spindle may cause further development of the egg to be tetraploid.

Pincus and Waddington (1939) subjected pronucleate rabbit eggs to supranormal temperatures, or to dilute solutions of alcohol, ether, or colchicine _in vitro_. Ether and hyperthermia resulted in 2- or 4-celled tetraploid embryos; after colchicine treatment, many one-celled tetraploids were discovered. Beatty and Fischberg (1952) subjected pronucleate mouse eggs to heat _in vivo_ and examined the treated eggs at $3\frac{1}{2}$ days gestation. Of twelve heteroploid embryos discovered by these authors, at least seven were tetraploid. In the following work, the induction of tetraploidy in the mouse, _Mus musculus_, has been attempted by subjecting pronucleate eggs to colchicine _in vivo_. A short preliminary report of the work has been published (Edwards, 1954).

MATERIAL AND METHODS.

At the time of the first cleavage, the mouse egg is in the second loop of the fallopian tube (Lewis and Wright, 1935). The narrowness of this tube hinders access of a solution to the eggs. Two methods of approach wefe used to reach the fallopian tubes.

One was via the cervix into the uterus; the technique was similar to that described for the injection of colchicine into the uterus before mating (p. 23). The second approach was by the injection of colchicine solution beneath the ovarian capsule. Injections were carried out about 15 hours after mating, when the eggs were still in the pronuclear stage.

The vaginal plug persists for various periods after mating, and injection via the cervix often necessitated its removal before treatment. Leakage of the injected solution from the uterus was often pronounced; in several cases all the injected solution was immediately ejected. If a very small vaginal plug remained over the cervix only, colchicine was injected through the plug, and the solution was retained more successfully. Care was necessary when injecting through the plug because of damage to the female, and no attempt was made to inject through large plugs. The pressure of the injected solution in the uterus was intended to cause a flow of colchicine up the fallopian tube to the eggs.

Injection beneath the ovarian capsule was intended to cause a flow of colchicine down the fallopian tube to the eggs. Females were anaesthetised with ether and small incisions made in their dorsal body wall. The ovaries and capsules were drawn through the incisions, and enough colchicine was injected to cause a slight distension of the capsule. Leakage sometimes occurred from the capsule. Replacement of the ovaries and capsules, and sewing the body wall, completed the operation.

Concentrations of colchicine (by weight) used in the two experiments were: via the cervix, 1/2,000 to 1/9,000; via the

ovarian capsule, 1/1,000 to 1/4,000. Injections via the cervix were carried out on normal and residual females (see p. 62), only normal females were injected via the ovarian capsule. Three females were injected cervically with 0.75% sodium chloride solution to serve as controls.

RESULTS.

1. Injection via the cervix.

Results are given in detail in Table 9, figures for normal and residual mice being given separately. The three normal mice which were injected with 0.75% saline showed no effects of the treatment. After the injection of higher concentrations of colchicine, occasional females suffered from the treatment; six mice, not included in the Table, had to be killed a few hours after the injection.

The fertility of the injected mice was compared with that of normal and residual mice after natural mating. Both types of injected mice showed decreased fertility; the number of females with embryos, and the numbers of embryos obtained, were both lower than in controls, especially at the higher concentrations. Analysis of the numbers of embryos within females with embryos indicated, however, that the normal mice were similar to their controls, while residual mice were less fertile than their controls. This may represent a greater sensitivity to colchicine of mice previously treated with it.

The mean cell number of the embryos showed that the treatment retarded embryonic development; many embryos had very few cells at the higher concentrations. But the variable success of the

technique led to many mice being relatively untreated; their embryos had similar cell numbers to controls and influenced the mean at each concentration. The percentage of the total embryos which were blastocysts was lower than in controls, but was an uncertain guide to the effect of the treatment on development.

A few heteroploids were induced; these were mainly tetraploids (Fig. 24) or diploid/tetraploid mosaics. Presumably the latter arose by an effect of the chemical on a blastomere in a later cleavage than the first. From the residual mice, one triploid and one haploid/diploid mosaic were recovered. The triploid was almost certainly residual; the mosaic was probably a residual mosaic which became partially doubled to diploid on a second or a later cleavage. Two hypo-diploids were also found. Though the number of heteroploids was not significantly different from the number in controls, the treatment was presumably responsible for the heteroploidy. The c-mitoses found in both diploids and tetraploids (Fig. 25) indicated that colchicine was still present in many embryos.

2. Injection beneath the ovarian capsule.

Detailed results of the experiment are given in Table 10. Three colchicine concentrations, 1/1,000, 1/2,000, and 1/4,000 were used, eight females being injected at each concentration. One mouse, not included in the Table, had to be killed shortly after the operation. The number of females with embryos, and the number of embryos per female, declined with increasing colchicine concentration. At the highest concentration, for example, only half of the operated females had embryos, and the numbers of embryos in these females was only one-half of those in

control females.

The mean number of nuclei in the embryos declined with increasing colchicine concentration (Table 10); no blastocysts were recovered at the highest. Some embryos had escaped the complete treatment, however, and had fairly high cell numbers; others were restricted to the second or third cleavage by the treatment.

Two tetraploid embryos were found (Fig. 26); both were taken from the same mother treated with conc. 1/2,000. At the same concentration, 11 other classifiable embryos were diploid. In addition, the nine embryos classifiable at conc. 1/1,000, and the 14 at 1/4,000 were diploid. C-mitoses were often seen, and accumulation of mitoses had occurred in both tetraploids (Fig. 26).

The cell number of the heteroploids resulting from both methods of injection, and a comparison with diploids, will be given later.

DISCUSSION.

Embryos with tetraploid mitoses occurred after both treatments. Some of these embryos also had diploid mitoses, and were certainly mosaic; others had large numbers of tetraploid mitoses only, and were probably uniform. Tetraploid cells presumably arose by the suppression of a diploid cell division. Embryos which were wholly tetraploid must have been affected at a very early cleavage, and in most the first cleavage was probably suppressed. The mosaics must have been affected in the second cleavage at the earliest: the affected blastomere giving rise to the tetraploid lineage, the unaffected blastomere producing the diploid lineage.

Sufficient colchicine must either have been absorbed by these embryos, or enough chemical must have remained in the confine of the fallopian tube, to cause this effect on the later cleavages. The presence of c-mitoses in 3½ day old embryos which are undergoing their sixth cleavage also indicated the presence of excess colchicine.

The stage of differentiation of each embryo was not noted; instead, the number of blastocysts and morulae taken from each female was recorded. The embryos of one female were all blastocysts, and as they included one diploid/tetraploid mosaic, the mosaic complement had not upset differentiation to this stage. The stage of differentiation of the other heteroploids can only be judged from their cell numbers; with the exception of three of the tetraploids and the hypo-diploid, all of them would be blastocysts. In addition to the number of chromosomes, the number of centrioles may have also been doubled at the first cleavage. But, except in the hypo-diploid and in the multiform mosaic, no non-euploid mitoses were found. Centromere function was therefore apparently undisturbed by the chromosome doubling; if the number of centromeres was doubled, a regulating mechanism must have operated on them to prevent the formation of multipolar spindles.

Heat treatment of eggs at the time of the first cleavage (Beatty and Fischberg, 1952) yielded approximately the same amount of heteroploidy as did the higher concentrations of colchicine in the present experiment. Of the twelve heteroploids found by Beatty and Fischberg, seven were tetraploid and three near-tetraploid. After colchicine treatment, five of the eleven

heteroploids were tetraploid, and four were euploid mosaics; induced heteroploidy by hyperthermia was therefore more consistently tetraploid than that induced by colchine. The difficulty common to both methods is that the first cleavage in different eggs occurs over a wide period (Lewis and Wright, 1935; Gresson, 1941; and see p. 12). Beatty and Fischberg heated the egg for five minutes; the treatment would presumably affect all the eggs which were susceptible at the time of heating. In contrast, colchicine would be less likely to reach the egg in the fallopian tube, but once there it would probably exert its influence over a long period. The euploid mosaics, for example, must have been affected by the chemical at the second or a later cleavage. By its better access to, and easier removal from the eggs, heat treatment therefore gave a more consistent result than did colchicine.

By its effect on the second maturation division, colchicine induced all gradation of heteroploids between haploid and triploid (see p. 41). The action of colchicine on the eggs of residual mice was probably similar (p. 68). If the chemical also suppressed the first cleavage of these eggs, the heteroploids would now range from diploid to hexaploid. The diploids would be unidentifiable from normal untreated eggs except, perhaps, for the possession of a low number of cells. If the second action of colchicine was delayed until the second or a later cleavage, the range of mosaicism would be from haploid/diploid to triploid/hexaploid. The former was induced deliberately in the present experiment by injecting residual mice with colchicine after mating, and it has also occurred by chance following colchicine

injection before mating (p. 29). More complex mosaics have not been seen, but the possibility of observing two mitoses in such ponderous mosaics must be very slight indeed.

SUMMARY.

1. Various concentrations of colchicine dissolved in 0.75% saline were injected either into the uterus via the cervix or beneath the ovarian capsule about 15 hours after mating. The colchicine was intended to reach the fallopian tube and suppress the first cleavage of the egg.

2. Tetraploids, diploid/tetraploid mosaics, and other heteroploids were found in low numbers at 3½ days gestation. High concentrations of colchicine adversely affected embryonic development.

3. Some colchicine was probably retained within the confines of the fallopian tube or in the embryos, and affected later cleavages in addition to the first.

4. Judged by their number of cells, some tetraploids were blastocysts.

REFERENCES.

BEATTY, R.A. & FISCHBERG, M. 1952. Heteroploidy in mammals: III. Induction of tetraploidy in pre-implantation mouse eggs. J. Genet. 50, 471-479.

EDWARDS, R.G. 1954. Colchicine-induced heteroploidy in early mouse embryos. Nature 174, 276-277.

GRESSON, R.A.R. 1941. A study of the cytoplasmic inclusions during maturation, fertilisation and the first cleavage division of the egg of the mouse. Quart. J. Micr. Sci. 83, 35-60.

LEWIS, W.H. & WRIGHT, E.S. 1935. On the early development of the mouse. Publ. Carnegie Inst., No. 459, Contr. Embryol. No. 148, 25, 113-143.

PINCUS, G. & WADDINGTON, C.H. 1939. Comparative behaviour of mammalian eggs *in vitro* and *in vivo*: V. The effects of mitosis-inhibiting treatments on normally fertilised pre-cleavage rabbit eggs. J. Hered. 30, 515-518.

TABLE 9. DETAILS OF THE FERTILITY AT 3½ DAYS GESTATION AND OF THE EMBRYOS OF FEMALES INJECTED CERVICALLY WITH COLCHICINE ABOUT 15 HOURS AFTER MATING.

	MALES	FEMALES			EMBRYOS:—							CHROMOSOME COUNTS											CELL NOS.	
Injected concentration (x 1000)	No. of different ♂♂ used	No. of ovulated ♀♀ used	No. of ♀♀ with embryos	% total ♀♀ with embryos	Total no. of eggs found	No. of eggs from ♀♀ with embryos	Total no. of embryos found	% of total eggs which were embryos	% embryos of total eggs from ♀♀ with embryos	Mean no. of embryos from ♀♀ with embryos	% embryos which were blastocysts	Lost or unclassified	Haploid	Between haploid and diploid	Diploid	Probably diploid	Hyper-diploid	Triploid	Tetraploid	Others	% tetraploids of total classified	% not diploid of total classified	Mean cell no. of all embryos	Mean cell no. of diploids
1. Into uterus of normal females																								
1/2	2	2	1	50.0	16	2	2	12.5	100	2.0	0	2	-	-	-	-	-	-	-	-	-	-	15.0 ± 1.4	
1/3	13	19	16	84.2	173	147	125	72.3	85.0	7.8	43.2	89	-	1(31)	32	-	-	-	2	1(4n/2n)	5.6	11.1	34.5 ± 16.2	42.6 ± 14.4
1/5	3	3	1	33.3	13	10	10	76.9	100	10.0	20.0	9	-	-	1	-	-	-	-	-	0	0	24.1 ± 1.1	24
1/9	2	2	2	100	26	26	24	92.3	92.3	12.0	100	7	-	-	16	1	-	-	-	-	0	0	56.6 ± 10.0	55.9 ± 9.5
saline after matg.	3	3	3	100	24	24	24	100	100	8.0	79.2	16	-	-	7	1	-	-	-	-	0	0		
2. Into uterus - mice previously used for colchicine, i.e. 'Residual' mice.																								
1/2	5	6	4	66.7	43	42	16	37.2	38.1	4.0	68.8	10	-	-	5	-	-	-	-	1?	0	20.0	30.8 ± 27.1	63
1/3	7	23	14	60.9	144	109	87	60.4	79.8	6.2	40.2	60	-	-	23	-	-	1	1	2 (1n/2n) (4n/2n)	3.7	14.8	35.6 ± 15.1	38.2 ± 15.1
1/5	4	4	3	75.0	35	29	15	42.9	51.7	5.0	66.7	6	-	-	8	-	-	-	-	1(37/35/29/)	0	11.1	40.0 ± 15.9	44.7 ± 10.1
'Residual' controls	15	24	19	79.2	191	184	161	84.3	87.5	7.5	68.9	89	1?	-	56	9	1	3	-	2	0	9.7	45.9 ± 16.8	49.0 ± 15.5

TABLE 10. DETAILS OF THE FERTILITY AT $3\frac{1}{2}$ DAYS GESTATION AND OF THE EMBRYOS OF FEMALES INJECTED BENEATH THE OVARIAN CAPSULE WITH COLCHICINE 15 HOURS AFTER MATING.

	MALES	FEMALES			EMBRYOS:—							CHROMOSOME COUNTS						CELL NOS.	
Injected concentration (x 1000)	No. of different ♂♂ used	No. of ovulated ♀♀ used	No. of ♀♀ with embryos	% total ♀♀ with embryos	Total no. of eggs found	No. of eggs from ♀♀ with embryos	Total no. of embryos found	% of total eggs which were embryos	% embryos of total eggs from ♀♀ with embryos	Mean no. of embryos from ♀♀ with embryos	% embryos which were blastocysts	Lost or unclassified	Diploid	Probably diploid	Tetraploid	% tetraploids of total classified	% not diploid of total classified	Mean cell no. of all embryos	Mean cell no. of diploids
1/1	4	8	4	50.0	39	25	16	41.0	64.0	4.0	0	7	7	2	-	0	0	17.9±11.4	20.9±8.0
1/2	4	8	6	75.0	61	57	43	70.5	75.4	7.2	23.3	31	8	3	2	15.4	15.4	29.8±18.1	42.4±23.7
1/4	6	8	7	87.5	69	62	44	63.8	71.0	7.7	52.3	30	9	5	-	0	0	35.1±22.9	42.7±23.2
Natural mating	17	31	27	87.1	273	242	212	77.7	87.6	7.9	63.2	142	54	15	1	1.4	1.4	40.6±13.6	47.9±13.3

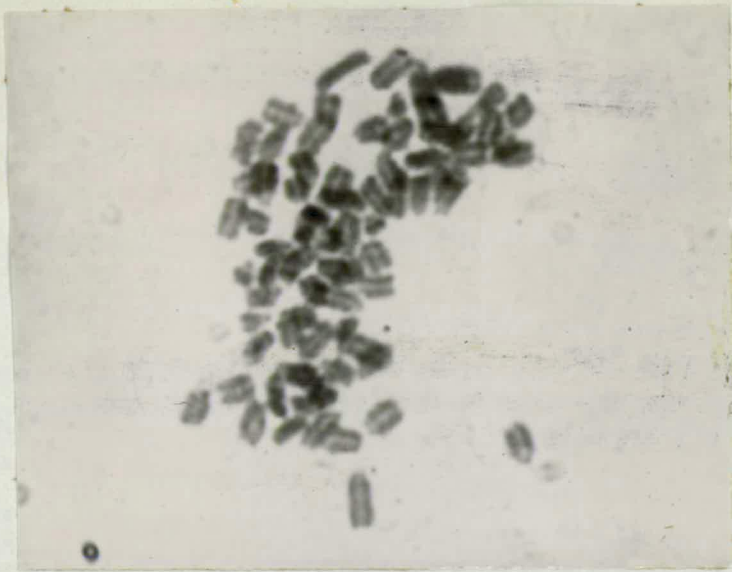
FIGURES

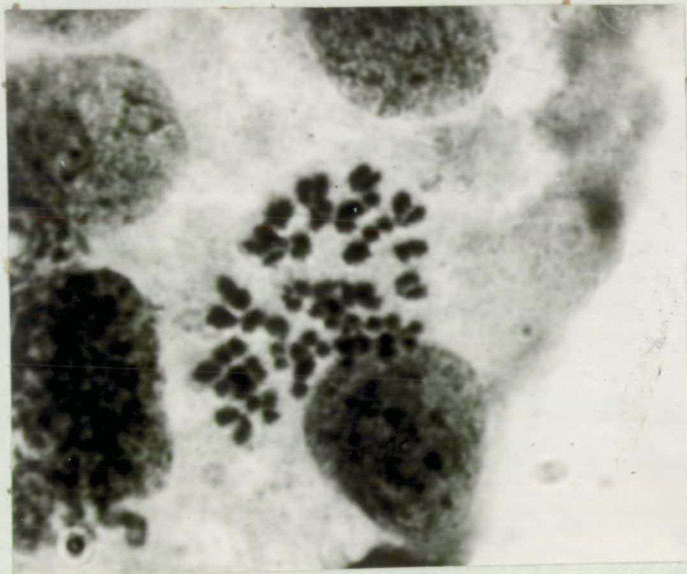
The negative of Fig. 26 was slightly retouched around the periphery of the embryo.

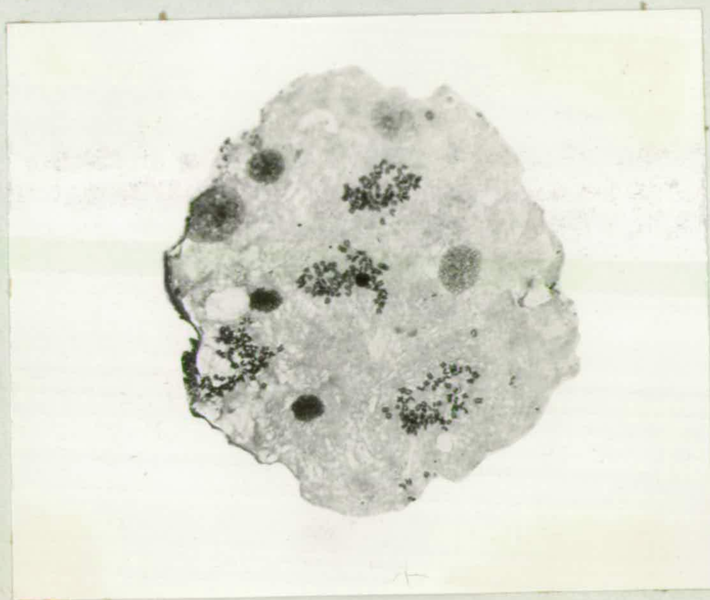
24. A tetraploid mitosis in an embryo taken from a mother injected via the cervix with colchicine concentration 1/3,500. Squash preparation x 2000.

25. A contracted diploid mitosis in an embryo taken from a mother injected with concentration 1/9000. Squash preparation x 2000.

26. A tetraploid embryo taken from a female injected beneath the ovarian capsule with concentration 1/2000. Squash preparation x 350.







CHAPTER III.

THE EXPERIMENTAL INDUCTION OF GYNOGENESIS IN THE MOUSE

GENERAL INTRODUCTION.

Parthenogenetic embryos develop from only one of the two gametes, usually the egg. The impetus given to the egg to commence development may occur naturally, as in many insect species, or it may be experimentally applied. The experimental stimuli can be classified into two groups. The first group includes those given directly to the egg; they initiate development without the presence of a spermatozoon. This type of development can be considered as 'true' parthenogenesis. In the second group, the stimulus to the egg is by fertilisation with a pre-treated sperm, the treatment of the sperm preventing the male chromatin from partaking in embryonic development. This type of development is gynogenesis. The induction of true parthenogenesis therefore necessitates treatment of the egg, the induction of gynogenesis requires the pre-fertilisation treatment of the sperm.

The present study is devoted to the induction of gynogenesis in a mammal, viz. the mouse, Mus musculus. In mammals, knowledge of the treatments which induce parthenogenesis has considerably increased in recent years (Pincus, 1939; Thibault, 1949; Chang, 1954; Austin & Braden, 1954). Less work has been reported on the induction of gynogenesis in mammals. In the amphibians, however, considerable information is available on experimentally-induced gynogenesis (see the reviews by Fankhauser, 1945; Briggs, 1952; Drebinger, 1951). Several of the sperm treatments used by these writers have been adapted for the present investigation.

The original experiments in this field were reported in 1911 by O. Hertwig. He and his collaborators noted that, after moderate dosages of radium irradiation on the sperm, embryonic development was very abortive. If the dosage was increased, embryonic development improved until, at high dosages, it was almost normal. The return to normal development after high dosages he termed the 'paradoxical effect'. To explain this effect, Hertwig suggested that the moderate dosages had injured the sperm chromatin, whereas the high dosages had completely inactivated it. The injured chromatin contributed to the embryo but disorganised development. The inactivated chromatin took no part in development, however, and the embryos contained only the haploid set of maternal chromosomes. Haploid development, inheriting no encumbrance from the treated sperm, was similar to normal development until advanced larval stages.

This hypothesis has since been substantially confirmed. In terms of modern genetics, the explanation of the 'paradoxical effect', or as it is now termed the Hertwig phenomenon, is that low dosages of irradiation induce lethal mutations or structural alterations in the sperm chromosomes. The induced genetic damage then adversely affects embryonic development. High dosages completely inactivate the sperm chromosomes as suggested by Hertwig, and there is no lethal effect of the sperm chromatin on the resulting haploid gynogenetic development. Since its discovery, subsequent investigators using other treatments on the sperm have also reported finding a Hertwig phenomenon.

Other treatments used to inactivate the chromatin of amphibian sperm before fertilisation have included the use of X-rays (Dalcq

& Simon, 1931; Rugh, 1939) which showed a clear Hertwig phenomenon (Rugh, 1939); trypaflavine (Hertwig, 1924); ultra-violet (Dale and Simon, 1931) which showed a clear Hertwig phenomenon; toluidine blue (Briggs, 1952); and nitrogen-mustard (Drebinger, 1951). The haploid gynogenones induced were able to reach advanced stages of morphogenesis (Fankhauser, 1945).

In the mammals there is no evidence of gynogenetic development beyond early cleavage. Irradiation of rabbit sperm _in vitro_ by ultra-violet affected the pronuclear stages and the first cleavage (Pincus and Enzmann, 1936). In a similar experiment, Beatty and Selman (unpublished) detected no haploids in 5½ day old rabbit embryos. X-irradiation of rabbit sperm _in vitro_ over a range of dosages up to 100,000r by Amoroso and Parkes (1947) induced increasingly severe abnormality at syngamy and early cleavage; though gynogenetic development of the egg was not induced by the treatment. Pincus (1939) induced the gynogenetic development of a rabbit egg to the 8-celled stage after fertilisation with rat sperm. Thibault (1949) inseminated sheep with trypaflavine-treated spermatozoa, but though observing spindle rotation in the egg, induced neither pronucleus formation nor gynogenetic development.

A number of haploid mouse embryos have been reported from other experiments. They occurred as advanced morulae or as blastocysts from three sources: from a line of mice selected for a a high incidence of spontaneous heteroploid embryos (Beatty and Fischberg, 1951a)(Beatty, 1954); after heat-treatment of newly fertilised eggs in the fallopian tubes (Fischberg and Beatty 1952a); and after colchicine treatment of the gametes at fertilisation

(see pp.21 & 62). In all cases the haploid embryos were produced in association with triploids.

Four of the sperm treatments which induce gynogenesis in the amphibian egg have been used on the mouse, *Mus musculus*. They include two irradiations, ultra-violet and X-rays; and two chemicals, trypaflavine and toluidine blue. In addition, trypaflavine and ultra-violet were combined in one experiment. Lastly, rat or rabbit sperm was inseminated into mouse females; this was a variation of the experiments of Pincus (1939). A brief report of the experiments has been published (Edwards, 1954).

THE PRESENTATION OF THE RESULTS

The method of presentation of the results obtained after the various sperm treatments will be standardised. This will simplify perusal of the different criteria, and permit direct comparison between the effects of the different treatments. The basis of the work was observation at 3½ days gestation after the insemination of the treated sperm. Earlier embryos were examined at fertilisation and the first cleavage. Other embryos were allowed to develop beyond 3½ days gestation; these were examined after implantation or at and after birth, often with the aid of marker genes. The programme of investigation was carried out in entirely after most, but not all of the sperm treatments. The results will be presented in the order that they were experimentally obtained, i.e. in the order just described.

Presentation will be under the following headings:-

1. The effect of the treatment on sperm motility.
2. Results at 3½ days gestation:

a) Analysis of the fertility of the artificially inseminated females.

b) Analysis of the numbers of embryos and unfertilised eggs obtained

i. from all females

ii. from females which produced one or more embryos.

c) Analysis of the morphological stage and number of nuclei of the embryos.

d) Chromosome counts of the embryos.

e) Cytological observations on the embryos.

3. Observations at fertilisation and the first cleavage:

a) Sperm entry and pronucleus formation.

b) Syngamy and the first cleavage.

4. Observations at implantation, birth and maturity:

a) The number of occupied placentae.

b) The number of offspring.

c) Observations on the genetic markers, and other genetic observations.

I. ULTRA-VIOLET IRRADIATION OF THE SPERM.

INTRODUCTION.

The effect of the ultra-violet irradiation of rabbit sperm on fertilisation and the first cleavage was reported by Pincus and Enzmann (1936). They used a lamp giving an output of 37 ergs/sec/ cm^2. After irradiation times of one and of five minutes, they noted degeneration of the sperm chromatin, and irregular and delayed first cleavage. Beatty and Selman (unpublished) noted the retarded development of some 5½ day old embryos after ultra-violet irradiation of rabbit sperm, but found no haploid or heteroploid embryos.

In the experimental results which follow, the effects on embryonic development following ultra-violet irradiation *in vitro* of the sperm of the mouse, *Mus musculus* have been investigated. Treatment of mouse sperm *in vitro* by ultra-violet necessitated the artificial insemination of the treated sperm; details of this technique have been given on p. 4 .

MATERIAL AND METHODS.

A precaution was necessary in selecting the mouse strains to be used in the experiment. The mating of mice carrying the factor 'silver', or the crossing of mice of widely different strains, are known to give an incidence of spontaneously heteroploid embryos at 3½ days gestation (Beatty and Fischberg, 1951a; Fischberg and Beatty, 1952b). Care was taken to avoid the incidence of spontaneous heteroploidy.

Ultra-violet irradiation of the sperm.

Details of the artificial insemination technique have been given on p. 4. Sperm were excised from the vas deferens into 1.25 ml. of 0.75% saline; usually between four and seven bucks provided a sufficiently dense sperm sample. The depth of the sample was approximately 2 mm.; it was placed in the centre of the area illuminated by ultra-violet for the required time, then inseminated into oestrous females. A sperm sample so obtained and treated is referred to as one sperm treatment in the tables of results; several such treatments were made at each dosage level (Table 11). The sperm was examined under the microscope (x 80) before and after the irradiation, and after the last female had been inseminated. At each examination, the activity and density were estimated by eye.

The source of the ultra-violet irradiation was an Osram 125 watt mercury vapour lamp type MB/V. It was housed in a container next to an aluminised mirror. The mirror reflected the light through a fused quartz lens which concentrated the beam on a limited area. Little loss of light of wavelength 2600A occurred by this construction. The illuminated area received radiant energy at a rate of 8.7×10^3 ergs/cm^2/second between wavelengths 2100A and 3200A. The spectral distribution of the lamp is shown in Text-fig. 3; the maximum output coincided very closely with the absorption maximum for nucleic acids.

Treatment of the sperm was for various times between 15 seconds and 30 minutes. 30 minutes irradiation corresponded to 1.6 Joules per sq. cm. on the surface of the sperm suspension in the tables and figures given later, dosage amounts will be given

in terms of exposure time.

Examination of the embryos resulting from the treated sperm.

Embryos were examined at various ages after fertilisation. The methods of examination, in experimental and not developmental order, were:

i. The 3½ day embryonic stage. At this stage of development, the embryos can be easily obtained by gently passing a current of saline through each uterine horn. Details of the recovery, squashing, staining, and examination of these embryos have been given previously (see p. 6).

ii. The stage of fertilisation and the first cleavage. The entire fallopian tube of the mouse is of a convenient size to handle for sectioning. It was removed containing eggs of the required age and sectioned at 10u thickness. Details of the sectioning, staining, and examination of these eggs have been given previously (see p. 7).

iii. Implantation, birth, and maturity. Examination for implantation sites was at first planned to coincide with the deposition of eye pigment in the embryos, which is first observable at about 12 days gestation. To allow for any delayed development in abnormal embryos, examination was delayed to 13 days. But as results accumulated, it became clear that very few of the embryos would reach this stage of development. Accordingly, examination for placentae was made at earlier times, some examinations being made at 10 days gestation, others at six or seven days when the placentae is merely knot-like. In four cases, examination was made at 4½ days to make certain of development beyond the 3½ day

stage. Apart from these, two other mice were examined, at 13 and 16 days respectively. These females had been intended to carry their embryos to term, but they were obviously not pregnant when killed. Where possible, females which had been inseminated with treated sperm and which had been allowed to carry their embryos to term were killed when it was apparent that no young would be born. These females were checked for the presence or absence of placentae.

Examination for births was made as carefully as possible near the expected time of arrival. Genetic markers were present in many females and in the sperm samples to ascertain the origin of the offspring. The markers were planned so that the inseminated female was homozygous for a gene for pink-coloured eyes (e.g. any one of the genes 'albino', 'pink-eye', 'pallid'); the male was homozygous for the dominant normal eye-coloured allele. If the sperm chromatin had been inactivated by the treatment, the pink eye of the female's recessive gene would appear in the offspring. If the sperm chromatin had not been inactivated, and had therefore contributed to development of the embryo, the eye colour would be normal black. Various other recessives (e.g. 'waved-2', 'dilute', 'brown', etc.) were also homozygous in some of these females, and provided a similar test. To ensure that the sperm samples were homozygous for the dominant markers, males of inbred lines CBA and C3H were used as sperm donors.

RESULTS.

1. The effect of ultra-violet on sperm motility.

Low dosages had little apparent effect on sperm motility; but effects were noted after two minutes exposure and above. The

activity declined, and occasionally the sperm exhibited a shaking or shuddering movement instead of the normal action. These effects became more pronounced with increased dosage; at the high dosages some of the sperm was observed to be motionless. At the two highest dosages (20 and 30 minutes exposure), the activity of the sperm was greatly reduced in four of the five samples, the fifth being less affected. Where only a proportion of the sperm in a sample was affected, the affected sperm may have been that which had been swimming near the surface of the suspension. Due to the low penetrating power of ultra-violet light, sperm in the lower layers of the salinex may not have been so heavily treated and so retained their activity.

2. Results at 3½ days gestation.

Detailed results of the females used, the embryos obtained, and the chromosome counts and mean cell numbers of the embryos are given in Table 11. Exposure times were between ½ and 30 minutes; several samples of sperm were treated at each dosage.

Analysis of the fertility of the inseminated females.

The controls of the artificial insemination technique yielded 33 out of 47 females possessing one or more embryos at 3½ days gestation, or 70.2% of the females inseminated. After ultra-violet irradiation of the sperm, the percentage of females with embryos was lower than the control figure, especially after the longest exposure. Lower exposures did not excessively depress fertility. Because embryonic development was often very retarded after irradiation, some embryos may easily have been overlooked at 3½ days gestation; the percentage of inseminated

females which possessed embryos must therefore be considered as the minimum.

Analysis of the numbers of embryos and unfertilised eggs.

At $3\frac{1}{2}$ days gestation, normal untreated embryos are advanced morulae or early blastocysts. Unfertilised eggs, which are the same size as the embryos, usually burst open and disintegrate when fixed in aceto-carmine. Unfortunately, not all unfertilised eggs can be seen $3\frac{1}{2}$ days after mating because some have previously cytolised. Consequently the comparison between the numbers of embryos and unfertilised eggs is biassed in favour of the embryos. The percentage of the total eggs which were embryos was calculated. Results are given in Table 11, the analysis having been made first on all eggs, and second only on the eggs taken from females with embryos. The percentage was consistently lower than that in controls; but within the experiment the percentage was maintained at a fairly constant level except at the two highest exposures. Despite the numbers of immotile sperm seen at the higher dosages, the percentage was not greatly depressed; either the physiological damage to the sperm did not affect their fertilising power, or sperm suspensions were so dense that numbers of sperm escaped the complete treatment.

Calculation of the mean number of embryos obtained from females with embryos overcame the difficulty due to degeneration of unfertilised eggs. The results are given in Table 11. The mean was consistently just below the control mean except at exposure time of one minute, where it was slightly higher, and at the two highest exposures, where it dropped

steeply. The pronounced effects on sperm motility may have been the cause of the lack of embryos at the highest exposures.

Examination of eggs at fertilisation (see later) showed that the three criteria used above actually compared the number of fertilised eggs which cleaved with the number unfertilised plus those which were fertilised but failed to cleave. Two factors were therefore simultaneously measured: the effect of ultra-violet on the fertilising power of the sperm, and the ability of the fertilised egg to undergo cleavage. A study of eggs in the pronuclear stages would give a stricter measure of the fertilising capacity of the sperm after ultra-violet irradiation.

Analysis of the morphological stage and number of nuclei of the embryos.

The effect of irradiation of the sperm on embryonic development was measured by the analysis of the stage of morphological differentiation and the number of nuclei of the embryos. In controls, 51.8% of the embryos were blastocysts, the remainder were morulae. After low exposures to ultra-violet, the percentage of embryos which were blastocysts was far less than in controls. Increased exposure further reduced the percentage, and no blastocysts at all were found after exposure for 10 minutes or more(Table 11). After the two highest dosages, most of the embryos were in the one- or two-celled stage, and were probably degenerate.

The restriction of embryonic development to the two-cell stage was not a phenomenon restricted to the highest dosages of ultra-violet. Even after exposure for 30 seconds, some 3½ day old

embryos were still two-celled; these occurred in the same mother that also produced blastocysts. With increasing exposure, the proportion of retarded embryos increased, and the number of embryos which had reached the more advanced cleavage stages declined. In effect, an approximation to an all-or-none effect was apparent, embryonic development at 3½ days gestation being almost unaffected or highly retarded by the treatment.

In assessing the number of nuclei per embryo, some difficulty occurred due to the appearance of a number of small sub-nuclei (Fig. 27). Where recognised, the sub-nuclei were excluded from the nuclear counts. In addition, some apparently one-celled eggs contained a number of nuclei (this will be described later). The nuclear counts given in the following data do not therefore necessarily correspond to the cell number of the embryos. The all-or-none response shown in the morphological stage of development was also observed in the nuclear counts. At all dosages up to 15 minutes exposure, one female would often yield some embryos which possessed as many nuclei as controls, and others which were still in their first or second cleavage. Above 15 minutes exposure, all the embryos observed had only one or two nuclei, with the exception of two embryos which each had three nuclei.

For each exposure time, the mean number of nuclei per embryo, and its standard error was calculated. The results are given in Table 11 and plotted in Text-fig. 4. The mean declined with dosage, the highest dosage restricting development to two-neculei per embryo. But the all-or-none tendency in embryonic development gave large values to the standard errors; sometimes the standard error was greater than the mean.

Chromosome counts of the embryos.

The diploid number in Mus musculus is 40 (Matthey, 1949). Due to the low numbers of nuclei after long exposures, very few embryos possessed classifiable mitoses at these exposures. Details of chromosome counts of the 3½ day old embryos are given in Table 11. Counts were made to an accuracy of ±5% of the number of chromosomes in the mitosis. Where an exact or nearly exact count could not be made on embryos which appeared to be diploid, they have been classified as 'probably diploid'; where the count on non-diploid embryos is only approximate, a question-mark has been appended to the particular embryos in Table 11. Four chromosomal classifications other than diploid were made. They were: haploid, between haploid and diploid, hyper-diploid, and mosaic. With the exception of the haploids, the number of chromosomes counted in these embryos has been inserted in brackets for each embryo in Table 11. The hyper-diploids consisted of two tetraploids or hypo-tetraploids (Fig. 30), and two embryos possessing 43 and 50 chromosomes respectively. Five mosaics were found; one of them was a haploid/diploid.

The embryos which were between haploid and diploid fell into two fairly sharply defined groups. One group possessed from 35 to 39 chromosomes and will be referred to as hypo-diploids (Fig. 31). The other group possessed a few chromosomes more than the haploid number (Fig. 32), and these will be termed hyper-haploids. The first haploids occurred after a sperm exposure of 2 minutes (0.1 Joules per sq. cm.), and were present in small numbers up to 15 minutes exposure (Figs. 28, 29, 33). The total number of

haploids was seven, and four hyper-haploids were found. Diploids and hypo-diploids occurred at all the exposures except the highest. The proportion of non-diploid embryos increased with higher dosage; the percentage of the embryos which were not diploid or which were haploid or near-haploid is given in the Table and plotted in Text-fig. 5.

Besides the counts given, three other chromosome observations were made which cannot properly appear in the Table. First, several embryos appeared to have one or two chromosomes less than the diploid number, but their number could not be established with certainty. These have been included, among others, in the 'probably diploid' column. Second, six embryos had counts of approximately the haploid number but the chromosomes were abnormal: four appearing extremely pycnotic, the others very small. These have not been included in the Table.

The third type of abnormality was often observed. It consisted of one-celled eggs which contained exactly 20 chromosomes; sometimes the chromosomes were meiotic (Fig. 34), in other eggs they appeared to be mitotic. In some cases, the egg possessed sub-nuclei in addition to the chromosomes. Such eggs could have been unfertilised, but in the embryos of several females mated to vasectomised males, no similar eggs were observed. If not unfertilised, the eggs may have been penetrated by the sperm without having been activated to maturation (see later, p.101). These would be expected to contain merely meiotic chromosomes and/or sub-nuclei if sperm entry allowed them to retain their organisation to 3½ days. Thirdly, the presence of twenty mitotic chromosomes could be due to the failure of a haploid

embryo to achieve the first cleavage division. Several of the eggs appeared to contain mitotic chromosomes in exactly the haploid number, and could have been haploid embryos. All of these embryos have been classified as 'unfertilised'; the subjectivity involved in the assessment of the chromosomes as meiotic or mitotic condemned the one-celled eggs as doubtful experimental value. The exclusion of the one-celled eggs from the results necessarily means that in subsequent analysis of the cell and cleavage numbers, the smallest possible number of cells in the haploid embryos is two. Due to this, the mean values for the haploids may be higher than actually occurred.

<u>The number of nuclei in haploid, diploid, and heteroploid embryos.</u>

The number of nuclei in the seven haploid embryos was 11, 9, 6, 3 (from exposure time of two minutes); 2 (exposure 5 mins.); 3 (exposure 10 minutes); 14 (exposure 15 minutes). The nuclear numbers in the hyper-haploids i.e. those with a few chromosomes more than haploid, was 2, 15, 11, 8. The mean of the eleven values is 7.6. For comparison, the mean nuclear number of the diploid embryos has been calculated for each exposure time, and this was 29.5 ± 9.9 after 15 secs. exposure, rising to 40.0 ± 20.0 after 2½ mins. exposure, and falling to 19.7 ± 11.2 after 10 mins. and above. But this comparison, which suggested that diploids were uniformly superior to haploids, was not strictly true. Several retarded diploids were seen, for example two were two-celled and others had between four and ten nuclei. In controls, the mean nuclear number of the diploid embryos was 44.1 ± 15.4.

The number of cleavages which must have occurred in the

embryos to produce the number of nuclei observed can be found from the formula:

$$2^x = N$$

where x is the number of cleavages, and N the number of nuclei observed. Because of the possibility of sub-nuclei being present in a cell in addition to the ordinary nucleus, the number of nuclei may be greater than the number of cells; the calculated cleavage number may therefore be too high. From the formula, the number of cleavages is given by

$$x = \frac{\log N}{\log 2}$$

Conversion of the previously given cell numbers to cleavage numbers gave the values:

Diploids: Controls 5.5; 15 secs. exposure 4.8; 2 mins. exposure 5.3; 10 mins. and above 4.3

Haploids: 3.5, 3.2, 2.6, 1.6, 1.0, 1.6, 3.8.

Hyper-haploids: 1.0, 3.9, 3.5, 3.0.

Mean of haploids and hyper-haploids: 2.6.

Ten of the hypo-haploid embryos had countable nuclei, the mean of them being 25.4 ± 10.9. This is the equivalent of 4.7 cleavages. They were only slightly retarded in comparison with the diploids, several of them possessing more nuclei than some of their diploid sibs. The hyper-diploids were retarded, the tetraploids having 10 and 3 nuclei respectively, the 43 and 50 chromosome embryos having 5 and 15 nuclei respectively. The haploid/diploid mosaic had 27 nuclei, the other mosaics between 3 and 17.

Cytological observations on the embryos.

The $3\frac{1}{2}$ day embryos were examined as squash preparations. This method, which is ideal for chromosome counts, is not a good method for the observation of cytological detail *in situ*. Further, artefacts may be caused by the pressure exerted in squashing. Considerable support is given to the following observations, however, by two facts. First, the control embryos showed very few or none of these abnormalities. Second, observations on pronucleate and once-cleaved ova and the chromosome counts of the $3\frac{1}{2}$ day old embryos, fully supported the cytological irregularities seen in the squashes.

The most common abnormality was the occurrence of sub-nuclei in many embryos (Fig. 27); some embryos contained only one or two, others possessed many. They occurred in the embryos after all ultra-violet dosages. As will be shown later, sub-nuclei were observed *in situ* when sections of 2-celled embryos were examined just after the first cleavage.

Many chromosomal abnormalities were seen. Several embryos possessed isolated or lagging chromatids (Fig. 35). Occasionally, fragmented chromatids occurred (Fig. 35). Certain chromosomes had a wavy appearance (Fig. 32), and were probably dying. In the two tetraploids, the whole group of chromosomes appeared sticky and bunched together (Fig. 30). Contracted mitoses occurred infrequently. The presence of pycnotic chromosomes, and of one-celled eggs containing meiotic or mitotic chromosomes (Fig. 34), has been mentioned previously. Some embryos were probably undergoing phagocytosis.

In the subsequent discussion of these results, the cytological abnormalities are related to the chromosome counts. The suggested pattern is that mitotic irregularities, i.e. sticky, lagging, and fragmented chromatids, cause the loss of a few chromosomes from the diploid blastomeres, and so induce the hypo-diploid embryos observed.

3. Observations at fertilisation and the first cleavage.

Sperm suspensions were exposed to ultra-violet for periods between 15 seconds and 10 minutes, the same dosage levels being used as in the recovery of 3½ day old embryos. The response to dosages of more than 10 minutes was not examined; it appeared from the results at 3½ days gestation that all the effects of irradiation were present after 10 minutes exposure.

Three phenomena were investigated. Sperm entry was studied in the eggs of females killed six hours after mating with the vasectomised male. Pronuclear growth in the eggs was followed by killing females between 12 and 18 hours after mating. Lastly, syngamy and the first cleavage were examined in the eggs of females killed between 36 and 42 hours after mating. Syngamy was not observed directly, but the nuclear content of the blastomeres or the fate of the pronuclei could be observed in the eggs of these females.

For each irradiation dosage, six or more females were killed. The embryos of four of these were examined for sperm entry and pronucleus formation, the embryos of the other two for syngamy and the first cleavage. Only the fertilised eggs were analysed. Full details are given in Table 12.

Sperm entry and pronucleus formation.

None of the ten mice killed six hours after mating had any eggs penetrated by sperm. Judged by the fertility of females killed at 3½ days gestation, about half of these mice would eventually have contained fertilised eggs. The lack of penetrated eggs in these mice suggested that fertilisation was delayed after ultra-violet irradiation of the sperm. More evidence of this came from a female which was inseminated with sperm irradiated for 10 minutes. She was killed 12 hours after mating, and possessed 13 eggs. One of her eggs was unpenetrated, another was in the pro-nuclear stages, the remaining eleven were in the telophase of the second meiosis. Sperm entry must have but recently occurred in the 11 eggs. For comparison, one of the two females artificially inseminated with untreated sperm, and six out of eight naturally-mated females possessed fertilised eggs six hours after mating (p. 11). Caution is necessary in postulating delayed fertilisation after ultra-violet irradiation of the sperm, however, for in normally-mated controls penetration can be delayed for 12 hours (see p. 12).

After natural mating, sperm entry into the vitellus was observed in 13 cases, and in twelve of these the sperm-head was entering sideways. Nine cases of sperm entry were seen after ultra-violet irradiation; they were all found in the eggs of a single female inseminated with sperm irradiated for 10 minutes. Eight of the sperm-heads were entering normally (Fig. 36); the other sperm-head was curiously bent and anvil-shaped (Fig. 37). Possible abnormalities were seen in the eggs of other females. In three cases the sperm-head was not seen though the eggs had

been activated; but this may not be unusual, for the sperm-head can often be overlooked in controls. In five other eggs, the sperm-head appeared to be either on or just in the vitellus of an unactivated egg (Fig. 38). If the sperm was in the vitellus, activation of the second maturation division should have occurred; penetration without activation may have resulted because of the irradiation.

Apart from the possible exceptions just mentioned, sperm penetration always stimulated the extrusion of the second polar body. This was shown by observation of twelve telophase spindles, one pole leading to a polar body (Fig. 36), and also by the observation of two pronuclei in 37 eggs, the second polar body being observable in these eggs. The two pronuclei were presumably one male and one female. In only one egg were more than two pronuclei seen. This egg was fertilised by sperm which had been illuminated for two minutes; it contained one normal pronucleus (presumably female), and two abnormally swollen male pronuclei which still had the outline of huge sperm-heads (Fig. 39a & b). No evidence was found of chromosome doubling at the second maturation division.

Apart from the polyspermic egg which contained two male and one female pronuclei, all the pronuclei appeared to be normal.

Syngamy and the first cleavage.

Direct observations were not made on syngamy or the first cleavage. Instead, eggs were examined between 36 and 42 hours after mating, when the egg should be about to divide from the two-celled to the four-celled stage. The presence of abnormalities

in the blastomeres or in the pronuclei of undivided eggs could be demonstrated, and inferences made about syngamy and the first cleavage.

Two types of egg were observed. The first type had undergone cleavage and was in the two-cell stage; the other type was still pronucleate and had not cleaved. Each type could be subdivided into two main classes. The two-cell stage embryos were either normal in appearance, with well-defined nuclei, or they possessed sub-nuclei in one or both of their cells (Fig. 40). In one two-celled embryo resulting after 10 minutes irradiation of the sperm, it was impossible to decide which were nuclei and which were sub-nuclei, for each blastomere contained two nuclei of exactly the same size (Fig. 41). Though numbers were rather low, it can be seen from the results in Table 12 that, after two minutes irradiation and less, approximately ¼ of the two-celled embryos had sub-nuclei; after 4 minutes irradiation and above approximately one half possessed sub-nuclei. In one of the two-celled embryos, the sub-nuclei, together with a small amount of cytoplasm, appeared to have been extruded from the embryo. In two two-celled embryos resulting after irradiation for 10 minutes, a body was observed in the cytoplasm which resembled an abnormally-developed sperm pro-nucleus. In one of these embryos, this stained body was attached to the blastomere nucleus (Fig. 42), in the other it was lying freely in the cytoplasm. Reference to these two embryos will be made subsequently.

The second type of egg was still in the pronuclear stages and was therefore very retarded. Most of these eggs still possessed

two unchanged pronuclei (Fig. 43), others being more abnormal. In one of them, one of the pronuclei appeared to be condensing into a group of pycnotic chromosomes (Fig. 44). In another the pronuclei appeared to be undersized and there were small extruded cytoplasmic bodies on the egg. These occurred after sperm irradiation of 30 secs. and 1 min. respectively. After 10 mins. irradiation, two eggs in the pronuclear stages possessed obviously abnormal male pronuclei. In one, the male pronucleus was still shaped like a swollen sperm-head, and the female pronucleus was enlarged and lying at the periphery of the egg (Fig. 45). In the other egg, one pronucleus was small and degenerate and again incompletely transformed from a sperm-head (Fig. 46). In the eggs which possessed two apparently normal pronuclei, one or both of the pronuclei sometimes appeared to be swollen or slightly pycnotic (Fig. 43). Clearly the ultra-violet had pronounced effects on the growth of the male pronucleus and on syngamy.

Two other embryos were observed which were probably degenerating. Both were in the one-cell stage, but possessed several nuclei. In one, what appeared to be an abnormally developed male pronucleus lay at the periphery of the egg and two other nuclei were lying close together in the centre of the eggs (Fig. 47). The female pronucleus may have divided without cytoplasmic division, or the nuclei may have been degeneration products. It could have also been a polyspermic egg with two male and the female pronucleus. The other egg was more extreme, possessing four nuclei. Identification was not so obvious in this egg, but one of the nuclei was larger than the others, and

in addition, appeared slightly pycnotic and separate from the others (Fig. 48 a & b). This may have been the male pronucleus; the smaller nuclei could have resulted from division of the female pronucleus. In this, the postulate of trispermy seems less probable than division or fragmentation of the female pronucleus. The evidence of both of these eggs indicated, therefore, that when the ultra-violet interfered with pronuclear growth and syngamy, the female pronucleus could divide or fragment independently of cytoplasmic division.

The last two embryos described illustrated a method by which the haploid female complement may double to diploid. Division of the haploid female pronucleus without the participation of the male pronucleus or cytoplasmic division would result in a one-celled egg containing a diploid set of maternal chromosomes. Whether such an egg could develop further is conjectural.

The distribution of the various anomalous embryos showed that the higher dosages induced the greater numbers (Table 12). In particular, the observations on the embryos after 10 minutes irradiation of the sperm illustrated the numbers and type of effect. If syngamy was not prevented by the irradiation, some of the resultant two-celled embryos were normal, while others possessed sub-nuclei in addition to normal blastomere nuclei. The sub-nuclei may have been due to the exclusion of degenerate male chromatin during the first cleavage, or to loss of chromatids through stickiness, etc., or due to the partial breakdown of the blastomere nucleus. If the effect of the ultra-violet was pronounced, syngamy was prevented, probably by an effect on the male pronucleus. Failure of syngamy caused delay or decay in the

pronuclear stages; cleavage of the egg with the maternal nucleus only, the male chromatin remaining uncondensed in one of the two blastomeres; or division of the female pronucleus without cytoplasmic division. From these observations and analyses, the haploid embryos observed at $3\frac{1}{2}$ days development were probably only a small sample of the gynogenone induced, most haploids failing to cleave to the two-celled and later stages.

4. Observations at implantation, birth and maturity.

Details of the mice killed, the placentae and embryos found, and the young born, are given in Table 13. Sperm samples were irradiated for one of the following periods: $\frac{1}{4}$, $\frac{1}{2}$, 1, and 2 minutes.

The number of placentae occupied.

The females were examined for numbers of placentae and embryos at either 6-7 days, 9-10 days, or 13-16 days gestation. After irradiation of the sperm for two minutes, no placentae were observed in two mice at 6-7 days, or in ten mice at 13 days. To ensure that development beyond the $3\frac{1}{2}$ day stage was possible after this dosage, three mice were killed at $4\frac{1}{2}$ days after mating. One of these mice yielded three embryos, two of which were $4\frac{1}{2}$ day blastocysts, and one a $3\frac{1}{2}$ day blastocyst.

Irradiation of the sperm for 60 seconds did not inhibit implantation, for one female had two very small, unoccupied placentae at 9 days gestation. No development beyond 9 days was observed in eight females.

At the two lower dosages, 30 and 15 seconds irradiation, all stages up to birth were observed. One female inseminated with sperm irradiated for 30 seconds had 13 placentae at 7 days gesta-

tion, of which 8 appeared normal and 5 were very small. The latter were probably already degenerate. Foetal death was confirmed by examination at 10 days gestation of four other females. Three of these females had received sperm irradiated for 30 seconds, and only one of them had placentae: she possessed two, neither of which had embryos. The fourth female received sperm irradiated for 15 seconds, and she had five placentae, only one of which possessed an embryo. Two females, killed at 13 and 16 days respectively, had neither embryos nor placentae; these females were examined at this age only because they appeared to be not pregnant by external inspection. Ultra-violet irradiation of the sperm for 30 or 15 seconds was therefore sufficient to cause the death of a large number of the embryos after implantation.

The number of offspring born, and the genetic observations on them.

As shown by the analysis of the number of implantations, no young could be expected after the sperm had received 60 seconds or more irradiation. Four females which received sperm irradiated for 60 seconds gave no offspring.

Seven females which received sperm irradiated for 30 seconds were allowed to term: only one produced a litter, which contained one young. This offspring was very abnormal, its hinder parts being curiously rotated in relation to its head and shoulders. Such an abnormality is rare in this laboratory (J. Isaacson, personal communication). It could conceivably have been due to a dominant mutation induced in the sperm chromosomes by the ultra-violet. Unfortunately it was eaten by its mother shortly after birth.

Twelve females inseminated with sperm irradiated for 15 seconds were allowed to term. Three of them gave litters, size 5, 3, and 1 respectively. The single offspring was eaten by its mother within twelve hours. One of the offspring in the litter of five was born dead. The sex ratio in the offspring classified was three females to five males. Eight of the offspring had genetic markers of the type described (p. 88); the markers showed that the sperm chromosomes had contributed to development in all eight offspring.

DISCUSSION.

In experiments designed to inactivate the sperm chromatin prior to fertilisation of the egg, the primary evidence of success should be the production of embryos carrying the haploid complement of chromosomes. The discovery of such embryos after the ultra-violet irradiation of the sperm is therefore a positive indication of the success of the method. But if haploids had not been observed, it would not necessarily have meant the failure of the technique. If, for example, the induced haploids failed to cleave, or if they regulated to diploid at either the second maturation division of the egg or at the first cleavage, the chromosome count alone would fail as a criterion and other methods of identification would be necessary. In such an eventuality, the most direct method would be to observe the cytological events at fertilisation and cleavage in order to trace the fate of the sperm chromosomes. Alternatively, the use of dominant gene markers in the sperm, and their recessive alleles in the egg, may show, by the presence or absence of the dominants in the resulting

embryos, whether the sperm chromosomes had or had not contributed to embryonic development.

The chromosome counts of the 3½ day old embryos and the cytological evidence at fertilisation both indicated an all-or-none effect of the ultra-violet on the sperm. The most pronounced result of the treatment was on the growth of the male pronucleus and on syngamy, though delayed fertilisation, abnormal sperm entry into the vitellus, and polyspermy also occurred. The male pronucleus sometimes failed to be fully differentiated and remained as a greatly swollen sperm-head. The largest number of embryos were affected at syngamy, however, the pronuclei either failing to condense into chromosomes or condensing only after a considerable delay. Certain eggs therefore contained enlarged pronuclei when their sibs were late two-celled embryos. The eventual fate of these pronucleate eggs varied. Some may have successfully undergone a delayed syngamy, though this was doubtful, judging from their cytological appearance. In others, degeneration of the embryo had apparently begun. Degeneration occurred either by the division of the female pronucleus without cytoplasmic cleavage, or by the fragmentation of the cytoplasm with or without nuclear division. In some embryos, however, the female pronucleus had apparently been capable of dividing to produce a two-celled embryo, for undifferentiated male chromatin was found in one of the two blastomeres. In these embryos, the male chromatin was probably inert. This last type of egg probably developed as a true haploid; the others probably degenerated. Elimination of the male chromatin as an inert mass at the first cleavage closely resembles the method of action of ultra-violet on the male

chromatin of Rana fusca described by Dalcq and Simon (1931).

After treatment of the sperm by ultra-violet, two types of embryo were probably represented in the two-celled stage. They were, first, the true haploids just described, and second, those eggs in which the male chromosomes had successfully undergone syngamy with the maternal chromosomes. But this clear duality became modified almost immediately, for, even in two-celled eggs, the presence of sub-nuclei in addition to the normal blastomere nucleus could be detected. The sub-nuclei were clearly not male pronuclei, for they were far too small. They must have represented therefore, small amounts of chromatin lost from the normal nuclei. The loss of chromatin may have been due to lagging or fragmented chromatids being excluded at anaphase from the restitution nuclei of the first cleavage; the hypo-diploid embryos found at 3½ days gestation may have been the product of this mechanism. The mitotic irregulatities were probably caused in turn by ultra-violet-induced damage to the chromosomes or centriole of the sperm. Further expressions of this damage may have been chromatid stickiness at anaphase, or the failure of the chromosomes to separate after metaphase, resulting respectively in the hyper-diploid and tetraploid mitoses seen in 3½ day old embryos (see for example the tetraploid mitosis in Fig. 30 in which the chromosomes appear to be adhering to one another).

In the chromosome counts of the 3½ day old embryos, the all-or-none effect of the ultra-violet become obscured due to the interplay of the above factors. Two general types of embryo were still present, however, either diploid and near-diploid, or haploid and hyper-haploid. The hyper-haploids did not rest wholly

comfortably in this classification, for, if they commenced development with haploid maternal chromosomes only, their only damaged constituents were the irradiated male centriole and cytoplasm. If the damage to male centriole caused the increase to a hyper-haploid chromosome complement of some mitoses due, for example, to sticky chromatids, then the compensating presence of hypo-haploid chromosome complement may be expected in other mitoses. No hypo-haploid mitoses were, in fact, observed. The lack of these mitoses may have been due to chance, to the failure of such mitoses to divide, or it may be that the hyper-haploids originated by a different process. They could have arisen in a similar manner to the hypo-diploids, by a more excessive loss of male chromatin in successive cleavages; but the absence of mitoses with chromosome complements intermediate between the hypo-diploids and the hyper-haploids militates against this suggestion.

Some indirect evidence can be calculated on the stage of development at which the hypo-diploids lost their missing chromosomes. If the total loss occurred at the first cleavage, the embryo concerned would be a uniform hypo-diploid. If the loss occurred in later cleavages, the rate of loss may vary between different mitoses, and the resulting embryo would be a chromosome mosaic. Comparison of the numbers of uniform and mosaic hypo-diploids will therefore give an indication of the time of loss of chromatin. To trace the uniformity of mosaicism of the $3\frac{1}{2}$ day embryos, the presence of at least two mitoses is necessary in each of them. Only four of the hypo-diploids were classifiable on two or more mitoses; three of them were uniform, and one was a mosaic. The missing chromosomes therefore appear to be lost

early in development in many cases. The presence of sub-nuclei in many of the two-celled embryos supports this observation, though further loss may occur in later cleavages.

All of the haploids and near-haploids were retarded in their development at 3½ days gestation. The most advanced haploid had 14 nuclei, or, alternatively, had undergone 3.8 cleavages, and was more than one complete cleavage behind the diploid controls. The other haploids were even more retarded, some having cleaved but once. In comparison, many of the hyper-diploids were advanced morulae or blastocysts though some of these were also highly retarded. Clearly there was no Hertwig phenomenon (Hertwig, 1911). Some developmental abnormality was consistently present in the haploids, and present also in some of the non-haploids. The cleavage of these haploids was in sharp contrast to that of other haploids and heteroploids occurring either spontaneously after normal mating (Beatty, 1954; Beatty and Fischberg, 1949a, 1951a); or experimentally after heat treatment at fertilisation (Beatty and Fischberg, 1949, 1951b, 1952; Fischberg and Beatty, 1952) ; of colchicine treatment of the gametes (see p. 21). The spontaneous, heat-, and colchicine-induced haploids and heteroploids often developed to blastocysts and beyond. Delayed fertilisation would not induce a similar developmental result to that caused by ultra-violet (Gates and Beatty, 1954). The cause of the abnormal embryonic development following ultra-violet irradiation of the sperm must therefore be due to something other than the abnormal chromosome complement of the embryos.

Despite this conclusion, however, embryonic development was nevertheless correlated with the amount of chromosome abnormality.

Thus the most retarded embryos were the haploids, hyper-diploids, and mosaics; the hypo-diploids were intermediate; diploids were the most advanced. The chromosome content of these embryos, while not the primary cause of retarded development, reflected developmental abnormalities. The primary cause must have been some agent, cytoplasmic or nuclear, which directly concerned the chromosomes; it could possibly be traced to the centriole of the irradiated sperm.

Adoption of the theory of irradiation damage to the sperm centriole or to a cytoplasmic factor in the sperm can explain the non-chromosomal abnormalities of embryonic development. In the subsequent discussion, reference to the sperm centriole is meant to include also the cytoplasmic factors of the sperm which enter the egg at fertilisation. At the commencement of embryonic development, the embryos can therefore possess two treated entities from the sperm: the chromosomes and the centriole. The first of these entities, the chromosomes, are either completely inactivated and play no part in development; or they are slightly affected if at all, and contribute to embryonic development. The second entity, the centriole, is also more or less damaged by the treatment, but damage to it may be quite independent of the all-or-none response of the chromatin. If the chromosomes have received sufficient energy to cause their complete inactivation, the centriole of the same sperm will also have received a high dosage. The two types of damage are positively correlated. Embryos receiving inactivated sperm chromatin will usually also receive a heavily damaged centriole; embryos receiving active sperm chromosomes will usually receive a lightly-treated centriole. The

former type, i.e. the presumptive haploids, will therefore show effects related to abnormal cleavage; and this is what was actually found. The haploids failed either at the first or in early cleavage. None of them had completed their fourth cleavage at 3½ days. The number of cleavages undergone by each particular haploid was therefore probably a measure of the damage inflicted on the sperm centriole by the ultra-violet.

The second type of embryo received from the sperm a lightly-damaged chromosome complement and, generally, a slightly treated centriole. If centriolar damage was independent of chromosome inactivation, some spermatozoa may contribute a severely affected centriole with a normal chromosome set. The embryos receiving this particular combination would be diploid yet show the same kind of developmental effects as the haploids. The numbers of diploids so affected should be fewer than in the haploids, however, because of the lower amount of irradiation received by such sperm. This effect was observed in the results. Two diploids were still two-celled at 3½ days gestation, others were delayed in their third and fourth cleavage. But the retarded diploid or near-diploid was exceptional, for most of them were similar to controls in completing or having completed their fifth cleavage. In general, therefore, when the sperm chromosome contributed to embryonic development, cleavage was fairly normal; only when the centriole was heavily affected was the development of these embryos arrested.

It remains formally to establish the existence of the mammalian centriole. The presence of centrioles in mouse spermatozoa has been indicated by Gresson (1941). Amoroso and

Parkes (1947) X-irradiated rabbit sperm before fertilisation and observed centrosomes in some of the accessory sperm pronuclei of uncleaved ova. Thibault (1949) inferred the existence of two egg centrosomes from his experiments on parthenogenetically stimulated rabbit, rat, and sheep ova. No centrioles have been seen in the sperm or eggs in the present work. But the indirect evidence for the existence of such bodies given by Thibault, and the theories given above for the failure of radiation-induced haploid gynogenones to cleave normally, and on p. 53 to explain the abnormalities of androgenetic haploid development, give considerable grounds for postulating the existence of centrioles in the sperm and eggs of mammals. Also, many of the cytological anomalies reported in the present work could be due to abnormal centriolar function in cleavage.

The developmental capacity of the haploids was almost completely exhausted at 3½ days gestation, any further development being probably slight. This retarded gynogenetic development of the mouse is in contrast to similar type of development in amphibians where gynogenones develop to metamorphosis (Hertwig, 1911; Fankhauser, 1945; etc.). It appears that an evolutionary difference exists between the mammals and the amphibians in the response of embryonic development to irradiation of the sperm by ultra-violet. In the Amphibia, the sperm may be more resistant to damage, or the egg more capable of independent development, than in the Mammalia. In addition to the abortive development of the haploids, the other mouse embryos produced after irradiation of the sperm for one minute or longer were also abnormal. For, with the exception of two very small placentae in one female,

irradiation for one minute or more completely suppressed implantation.

Irradiation for 15 or 30 seconds did not prevent the implantation of large numbers of the resulting embryos. But the majority of these died immediately after implanting. Judged from the $3\frac{1}{2}$ day chromosome counts, all these embryos were diploid; the cause of death may have been due to genetic changes in the sperm chromatin induced by the ultra-violet. The small size of the resultant litters, and the presence of a deformed offspring at birth, are probably further indications of mutagenic effects of the treatment. The ultra-violet apparently had three progressively increasing effects on the sperm chromatin with longer exposures: mutations, physical damage causing the loss of individual chromosomes, and the total elimination of the sperm chromatin from development.

The reduction in the activity of the sperm after the irradiation may partly account for the reduction in fertility noted especially after the high dosages. Delayed fertilisation of the eggs may have resulted from the same cause. The discovery of one case of abnormal sperm penetration into the vitellus out of nine observed is probably indicative of an effect of the ultra-violet. The presence of more than one sperm or male pronucleus in only one egg out of 37 examined suggests that polyspermy is not an important product of the treatment.

SUMMARY.

1. Sperm of the mouse, Mus musculus, was irradiated by ultra-violet in vitro with dosages of up to 1.6 Joules per sq. cm., then artificially inseminated into oestrous females.
2. At 3½ days gestation, many haploid, hyper-haploid, hypo-diploid, hyper-diploid, and mosaic embryos were found in addition to diploids. The haploids, hyper-haploids, and hyper-diploids were very retarded in development, the hypo-diploids and diploids were almost normal.
3. Ultra-violet had an all-or-none effect on the participation of the male chromosomes at syngamy, and occasionally suppressed the growth and differentiation of the male pronucleus. Many presumptive gyngenetic haploids degenerated without cleavage.
4. A considerable number of cytological abnormalities occurred in 3½ day old embryos.
5. Other effects of the ultra-violet were a reduction in the activity of the sperm, and abnormal sperm penetration into the vitellus. Delayed fertilisation may also have been induced. Polyspermy, if induced, was very rare.
6. At the lowest dosages considerable embryonic mortality occurred in diploid embryos at implantation, and litter size was very small. One of the few offspring was deformed. Slight increase in dosage suppressed implantation completely. These effects may have been due to induced mutations in the sperm chromosomes.
7. A theory of irradiation damage to the centriole or the cyto-plasm of the sperm in addition to inactivation of its chromatin is postulated to account for the retarded development of the haploids and other embryos.

REFERENCES.

AMOROSO, E.C. & PARKES, A.S. 1947. Effects on embryonic development of X-irradiation of rabbit sperm in vitro. Proc. Roy. Soc., B, 134, 57-78.

AUSTIN, C.R. & BRADEN, A.W.H. 1954. Induction and inhibition of the second polar division in the rat egg, and subsequent fertilization. Aust. J. Biol. Sci. 7, 195-210.

BEATTY, R.A. 1954. Haploid rodent eggs. Proc. 9th Inter. Congr. Genetics, Bellagio.

BEATTY, R.A. & FISCHBERG, M. 1949. Spontaneous and induced triploidy in pre-implantation mouse eggs. Nature 163, 807.

__________ 1951a. Heteroploidy in mammals: I Spontaneous heteroploidy in pre-implantation mouse eggs. J. Genet. 50, 345-359.

__________ 1951b. Cell number in haploid, diploid, and polyploid mouse embryos. J. Exp. Biol. 28, 541-552.

__________ 1952. Heteroploidy in mammals: III Induction of tetraploidy in pre-implantation mouse eggs. J. Genet. 50, 471-479.

BRIGGS, R. 1952. Analysis of the inactivation of the frog sperm nucleus by toluidine blue. J. Gen. Physiol. 35, 761-780.

CHANG, M.C. 1954. Development of parthenogenetic rabbit blastocysts induced by low temperature storage of unfertilised ova. J. Exp. Zool. 125, 127-150.

DALCQ, A. 1931. Contribution à l'analyse des fonctions nucléaires dans l'ontogénèse de la grenouille. I. Etude cytologique des oeufs fécondés par du sperme trypaflaviné. Arch. Biol. 41, 143-220.

DALCQ, A. & SIMON, S. 1931. Contribution à l'analyse des fonctions nucléaires dans l'ontogénèse de la grenouille. III. Etude statistique et cytologique des effets de l'irradiation d'un des gamètes sur la gastrulation chez Rana fusca. Arch. Biol. 42, 107-165.

DREBINGER, K. 1951. Kerngifte und Lichtstrahlung. Eine Studie an Froschspermien zur Wirkungsanalyse der Kerngifte. Arch. EntwMech. Org. 145, 174-204.

EDWARDS, R.G. 1954. The experimental induction of pseudogamy in early mouse embryos. Experientia 10, 499

FANKHAUSER, G. 1945. The effects of changes in chromosome number on Amphibian development. Quart. Rev. Biol. 20, 20-78.

FISCHBERG, M. & BEATTY, R.A. 1952a. Heteroploidy in mammals: II Induction of triploidy in pre-implantation mouse eggs. J. Genet. 50, 455-470.

________________ 1952b. Heteroploidy in mouse embryos due to crossing of inbred strains. Evolution 6, 316-324.

GATES, A.H. & BEATTY, R.A. 1954. Independence of delayed fertilisation and spontaneous triploidy in mouse embryos. Nature 174, 356-357.

GRESSON, R.A.R. 1941. A study of the cytoplasmic inclusions during maturation, fertilization, and the first cleavage division of the egg of the mouse. Quart. J. Micr. Sci. 83, 35-60.

HERTWIG, G. 1924. Trypaflavin als Radiumersatz zur Gewinnung haploidkerniger Froschlarven. Verh. der Anat. Ges. 58, 223-227.

HERTWIG, O. 1911. Die Radiumkrankheit tierischer Keimzellen. Arch. mikr. Anat. 77, 97-164.

MATTHEY, R. 1949. Les Chromosomes des Vertébrés. F. Rouge, Lib. Univ., Lausanne.

PINCUS, G. 1939. Comparative behaviour of mammalian eggs in vivo and in vitro. IV Development of fertilised and artificially activated rabbit eggs. J. exp. Zool. 82, 85-129.

PINCUS, G. & ENZMANN, E.V. 1936. The comparative behaviour of mammalian eggs in vivo and in vitro. II The activation of tubal eggs of the rabbit. J. exp. Zool. 73, 195-208.

RUGH, R. 1939. Developmental effects resulting from exposure to X-rays. 1. Effect on the embryo of irradiation of frog sperm. Proc. Amer. Phil. Soc. 81, 447-472.

THIBAULT, C. 1949. L'oeuf des mammifères, son développement parthénogénétique. Ann. Sci. nat., Zool. 11, 136-219.

TABLE 11

DETAILS OF THE FERTILITY OF THE INSEMINATED FEMALES AND OF THEIR EMBRYOS AT 3½ DAYS GESTATION.
In the Table, 'eggs' refers to embryos and unfertilised eggs. 30 mins. exposure = 1.6 Joules per cm^2 on the surface of the sperm suspension.

SPERM		FEMALES			EGGS & EMBRYOS							EMBRYO CHROMOSOME COUNTS (2n = 40)									EMBRYO NUCLEAR NOS.	
Dosage (mins. exposure	No. of sperm treatments	No. of ovulated ♀♀ used	No. of ♀♀ with embryos	% of total ♀♀ possessing embryos	Total no. of eggs found	No. eggs from ♀♀ with embryos	Total no. of embryos found	% embryos of total eggs found	% embryos of total eggs found from ♀♀ with embryos	Mean no. embryos per ♀ with embryos	% of embryos which were blastocysts	Lost or unclassifiable	Haploid	Between haploid and diploid	Diploid	Probably diploid	Hyper-ploid	Mosaic	% haploid or near-haploid embryos of total classified	% not diploid of total embryos classified	Mean no. of nuclei of all embryos	Mean no. of nuclei of the diploid embryos
Control	-	47	33	70.2	394	278	182	46.2	65.6	5.5	51.8	109	-	-	56	15	2(3n)	-	0	2.7	41.2±15.7	44.1±15.4
0.25	6	13	7	53.8	81	49	27	33.3	55.1	3.9	29.6	20	-	-	6	1	-	-	0	0.0	26.4+ 9.6	29.5+ 9.9
0.50	6	12	8	66.7	83	69	36	43.4	52.2	4.5	25.0	21	-	-	12	2	-	1(40/70)	0	6.7	26.2+15.9	31.6+13.8
1.0	8	22	14	63.6	217	150	84	38.7	56.0	6.0	20.2	41	-	1(23) 2(37) 1(25) 2(38) 1(39)	23	10	1(43)	1(35/40) 1(20/40)	4.7	23.3	25.4±16.6	35.6±16.9
2.5	14	40	18	45.0	308	179	84	27.3	46.9	4.7	22.6	56	3+1?	1(25) 1?(35) 3(37) 1(38)	9	7	1(4n)	1?(32/40)	17.9	42.9	20.0±18.2	40.0±20.0
5	9	28	12	42.9	202	104	53	26.2	51.0	4.4	7.5	46	1	1(25) 1?	3	2	-	-	25.0	37.5	9.2+10.1	17.8+11.5
10	7	24	12	50.0	182	102	46	25.3	45.1	3.8	0.0	41	1	-	1	1	1(4n) 1(50)	-	20.0	60.0	5.3± 6.4	
15	3	9	5	55.6	74	42	23	31.1	54.8	4.6	0.0	17	1	1(36)	2	1	-	1(20/50)	16.7	50.0	7.1+ 8.5	19.7+11.2
20	3	9	5	55.6	48	38	10	20.8	26.3	2.0	0.0	9	-	-	1	-	-	-	-	-	1.6+ 0.7	
30	2	9	2	22.2	41	14	2	4.9	14.3	1.0	0.0	2	-	-	-	-	-	-	-	-	2.0+ 1.4	

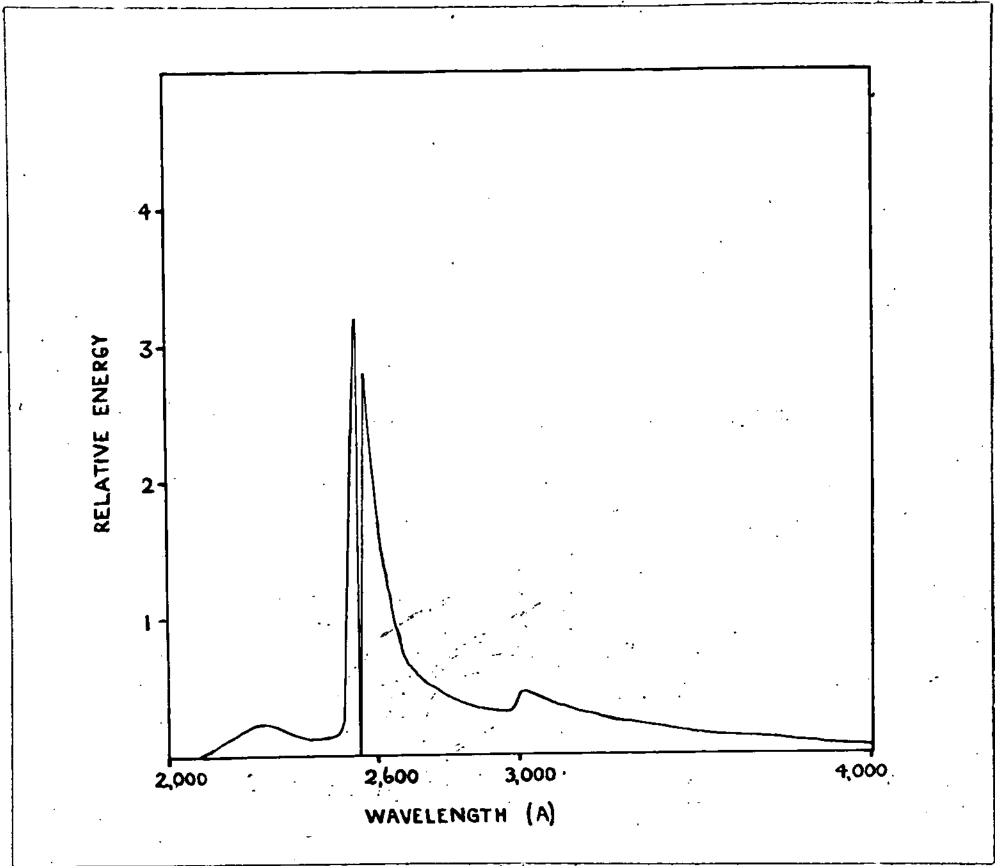
TABLE 12. DETAILS OF THE FERTILISED EGGS EXAMINED UP TO 42 HOURS AFTER MATING

10 minutes exposure corresponds to 0.5 Joules per cm^2 on the surface of the sperm suspension.

Stage of development of controls at similar times of examination.	Type of abnormality	Minutes exposure of the sperm to ultra-violet					
		0.25	0.50	1.0	2.5	5	10
Sperm entry	Normal						8
	Sperm head not seen						3
	Abnormal sperm head						1
	Sperm head in vitellus, egg at metaphase					2?	3?
Pronucleate	Normal	5	3		27		2
	Polyspermic				1		
Two-celled	Normal	8	3	14	4	1	2
	Sub-nuclei in blastomeres	2	1	4	1		3
	Inactivated male chromatin in one blastomere						2
	Still pronucleate				1	3	
	Still pronucleate, pronuclei abnormal		1				2
	Fragmenting nucleus or cytoplasm			1			2

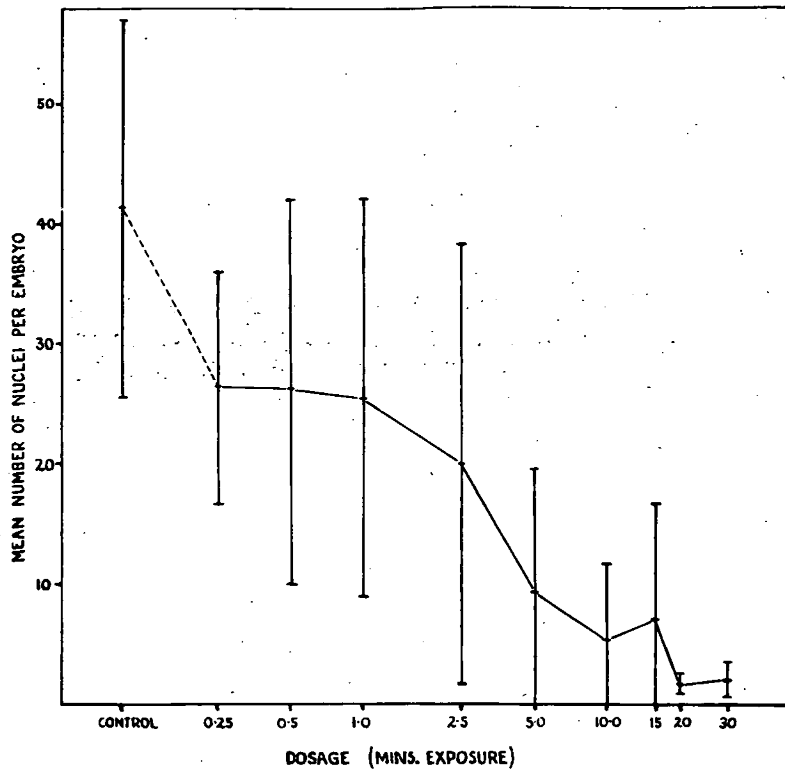
TABLE 13. DETAILS OF THE FERTILITY OF THE INSEMINATED FEMALES AND OF THE DEVELOPMENT OF THEIR EMBRYOS TO BIRTH.

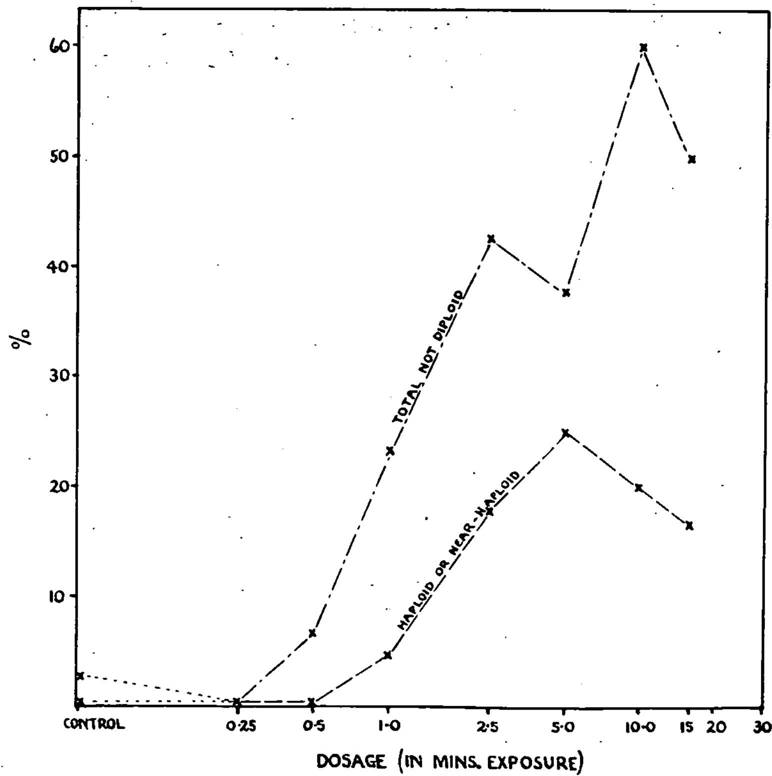
Minutes exposure of the sperm to ultra-violet.	4½ days			6 or 7 days			9 or 10 days			Between 13 & 16 days			Birth		
	No. of ♀♀ examined	No. ♀♀ with embryos	Morphological age of embryos (in days)	No. of ♀♀ examined	No. ♀♀ with placentae	Fraction of no. of placentae occupied by embryos	No. of ♀♀ examined	No. of ♀♀ with placentae	Fraction of no. of placentae occupied by embryos	No. of ♀♀ examined	No. ♀♀ with placentae	Fraction of no. of placentae occupied by embryos	No. of ♀♀ examined	No. ♀♀ with litters	Total no. offspring
2.0	3	1	2(4½) 1(3½)	2	-	-	10	-	-						
1.0				2	-	-	1	1	0/2 (very small placentae)	4	-	-	4	-	-
0.5				1	1	8/13	3	1	0/2	1	-	-	7	1	1
0.25							1	1	1/5	1	-	-	12	3	9
0 (controls)													7	5	30


RELATIVE ENERGY
4
3
2
1
2,000
2,600
3,000
4,000
WAVELENGTH (A)

Text-fig. 4. Relationship between dosage of ultra-violet on the sperm and the number of nuclei in $3\frac{1}{2}$ day old embryos
I = one standard error above and below the mean.

Text-fig. 5. Relationship between dosage of ultra-violet on the sperm and the percentage of the total classified embryos which were not diploid, or which were haploid or near-haploid at $3\frac{1}{2}$ days gestation.


MEAN NUMBER OF NUCLEI PER EMBRYO
50
40
30
20
10
CONTROL
0·25
0·5
1·0
2·5
5·0
10·0
15
20
30
DOSAGE (MINS. EXPOSURE)


%
60
50
40
30
20
10
TOTAL NOT DIPLOID
HAPLOID OR NEAR-HAPLOID
CONTROL
0·25
0·5
1·0
2·5
5·0
10·0
15
20
30
DOSAGE (IN MINS. EXPOSURE)

FIGURES

Figs. 27-35 inclusive are taken from squash preparations of whole $3\frac{1}{2}$ day embryos. Figs. 36-48 inclusive are from sections of eggs taken from mothers killed between 6 and 42 hours after mating.

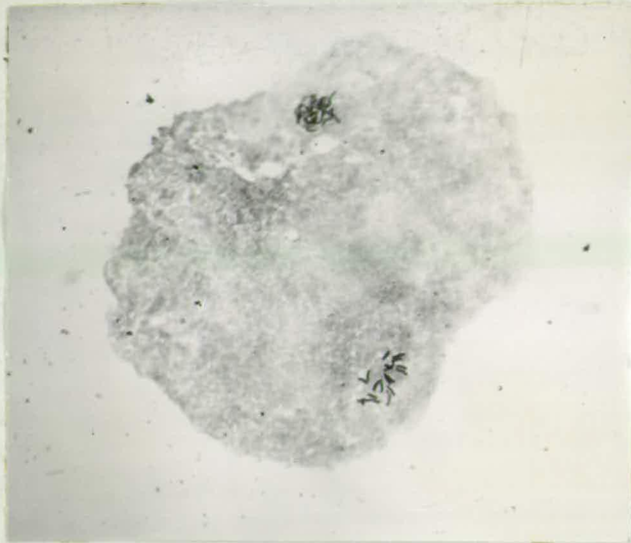
The irradiation dosage on the sperm for the various figures was: Fig. 40, $\frac{1}{4}$ minute; Fig. 44, $\frac{1}{2}$ min.; Figs. 31, 32, 35, 1 min.; Figs. 30, 39, 43, $2\frac{1}{2}$ mins.; Figs. 29 & 33, 5 mins.; Figs. 34, 36-38, 41-42, 45-48, 10 mins.; Figs. 27 & 28, 15 mins. 10 mins. illumination is equivalent to 0.5 Joules per sq. cm. on the surface of the sperm suspension.

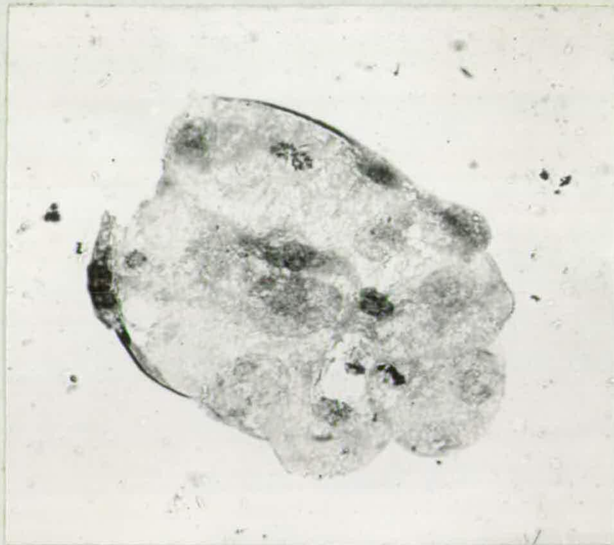
None of the negatives were retouched.

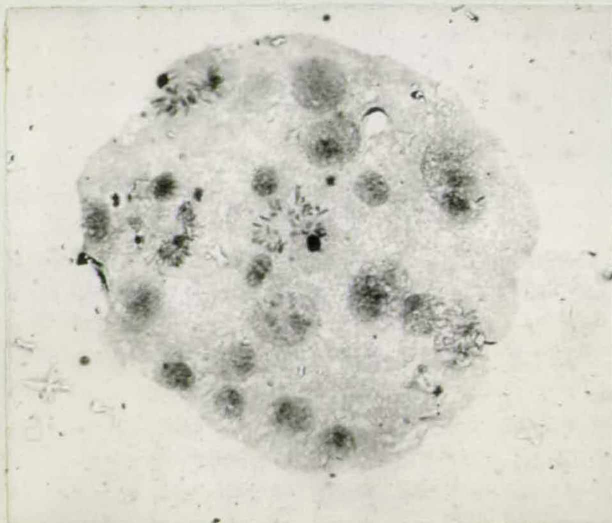
27. A whole $3\frac{1}{2}$ day old embryo containing a mitosis of 36 ± 1 chromosomes. Several sub-nuclei can be seen in addition to the normal nuclei. x 350.

28. The most advanced haploid embryo found in the experiment. The embryo which was damaged during re-staining, contained 14 nuclei. x 350.

29. A haploid embryo found at $3\frac{1}{2}$ days gestation. It possesses only two nuclei, both being in division; one of these mitoses is shown enlarged in Fig. 33. x 350.



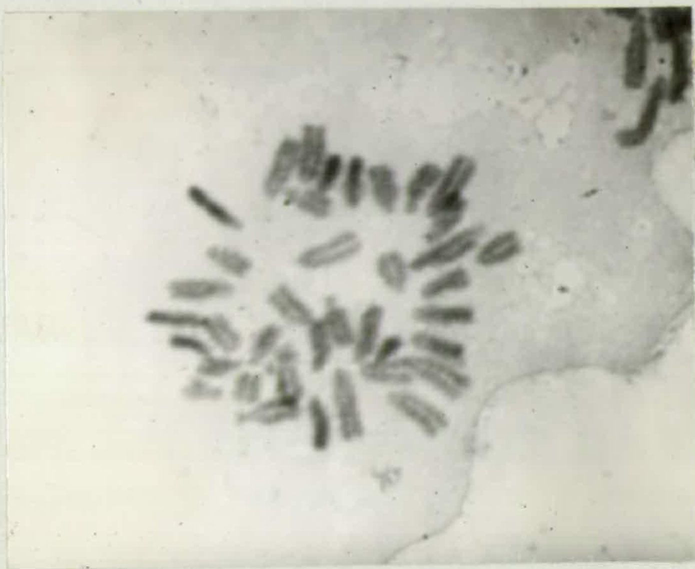


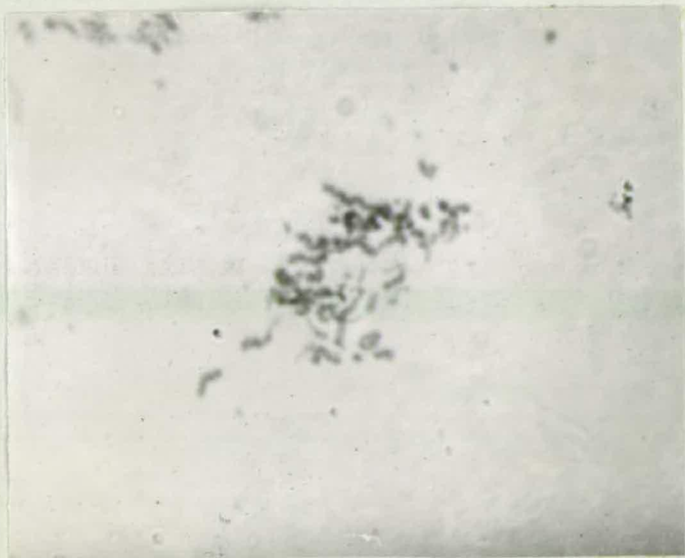


30. A tetraploid or near-tetraploid mitosis. The chromosomes appear to be sticky and adhering to one another. x 2000.

31. A mitosis of 39 chromosomes (2n = 40). x 2000.

32. A mitosis of approximately 50 abnormal chromatids. The chromatids are small and often wavy in appearance. x 2000.

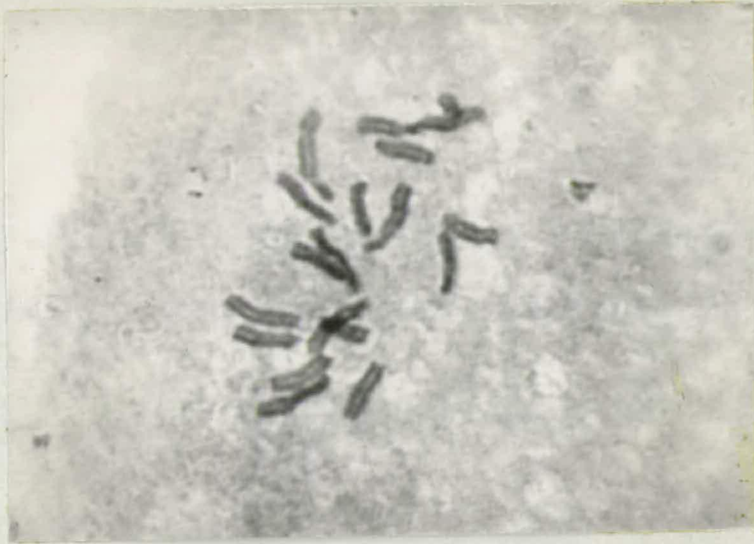


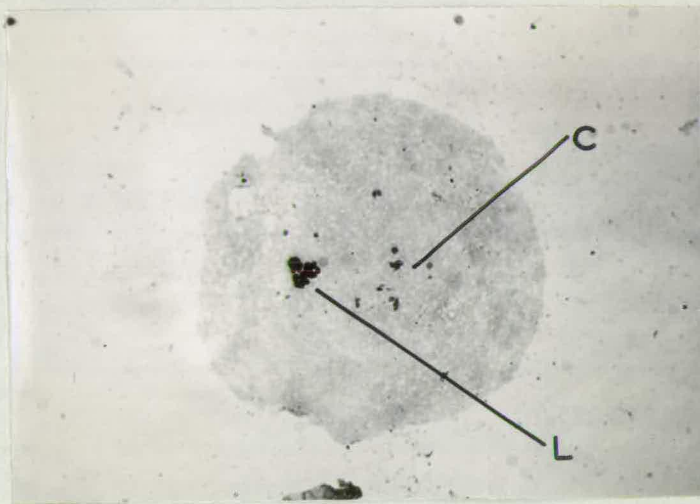


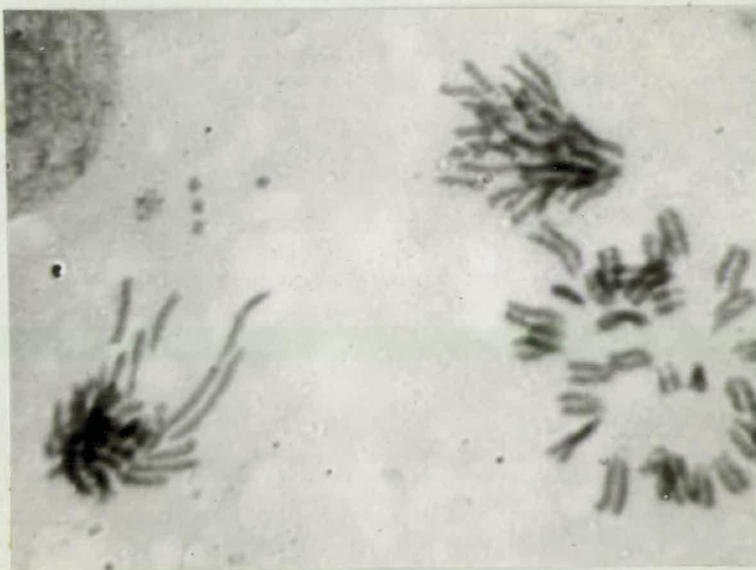
33. A haploid mitosis of 20 ± 1 chromosomes. x 2000.

34. A $3\frac{1}{2}$ day old egg possessing the remains of a haploid set of chromosomes(C) and no nuclei. The egg is being phagocytosed, the leucocytes can be seen in the cytoplasm(L). x 350.

35. Anaphase showing lagging and fragmented chromatids. Four small fragments can be seen above the left half of the anaphase. x 2000.



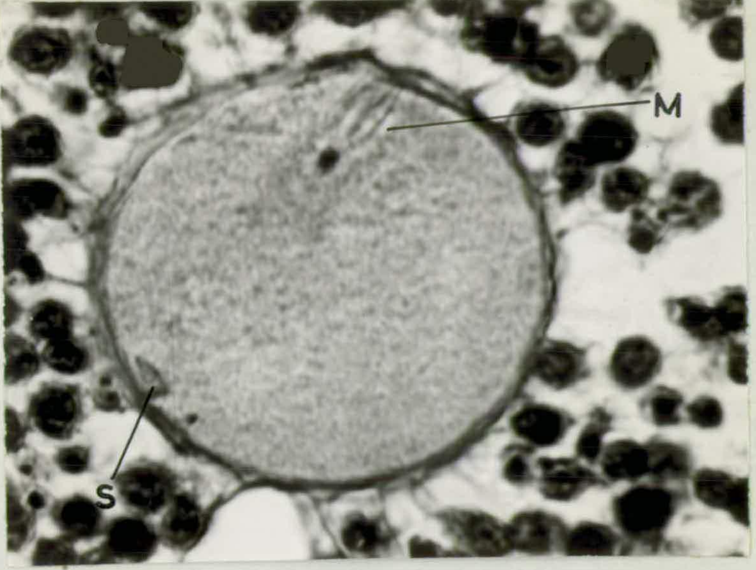

C
L

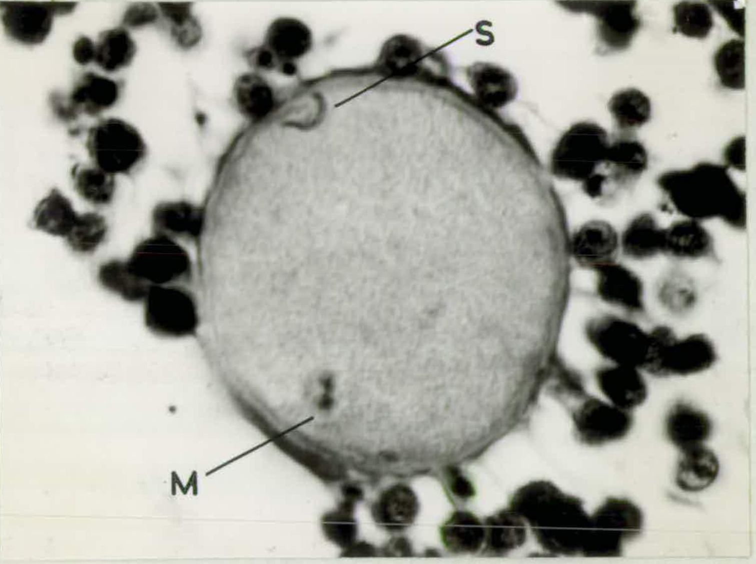


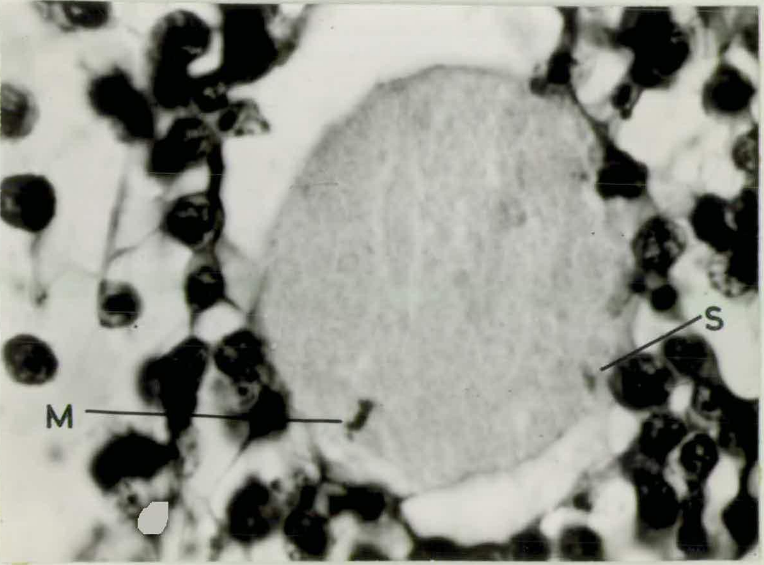
36. Normal sperm penetration and activation of the egg after the sperm was irradiated for 10 min. The second maturation division is at telophase; the egg chromosomes, part of the spindle(M), and the penetrating sperm-head can be seen in the egg(S). The second polar body chromosomes cannot be seen in this section. x 1000.

37. Abnormal sperm penetration into the vitellus; the sperm-head has become bent and sickle-shaped(S). Telophase chromosomes of the second maturation division can be seen indistinctly in the egg(M). x 1000.

38. An egg containing an unstimulated second maturation division at metaphase(M) and a sperm-head(S) which is on the surface of or just inside the vitellus. x 1000.

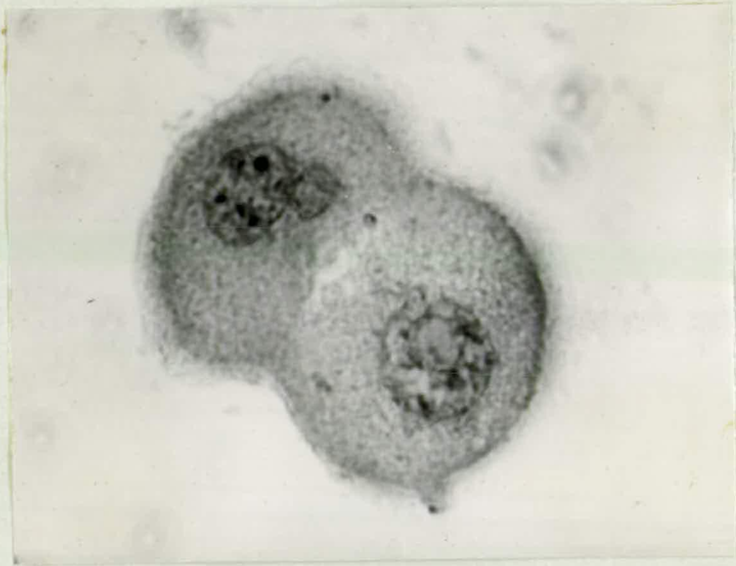

M
S

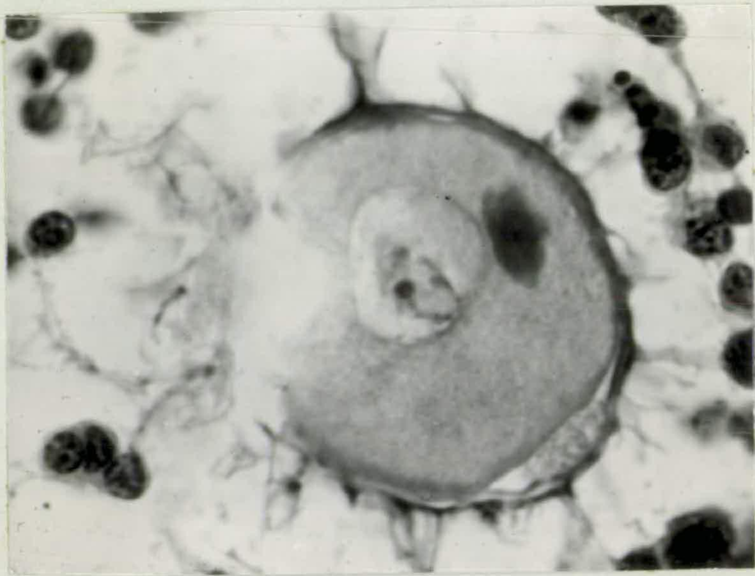

S
M

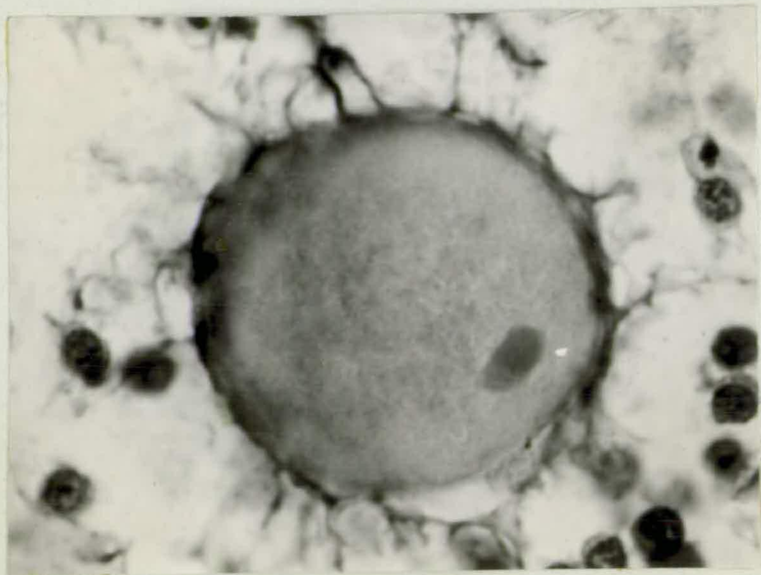

S
M

39a and b. Two sections of an egg containing two very swollen sperm-heads, and a very large, presumably female, pronucleus. x 1000.

40. A two-celled embryo with a sub-nucleus in the blastomere. x 1000.



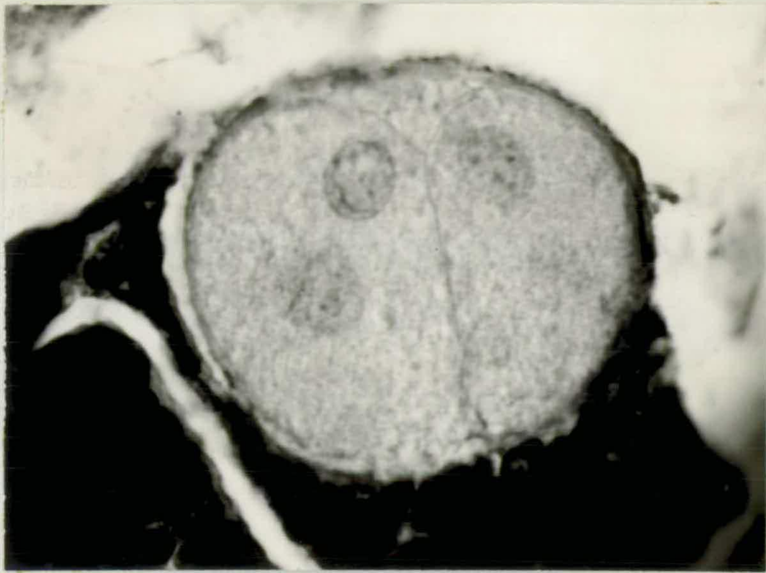


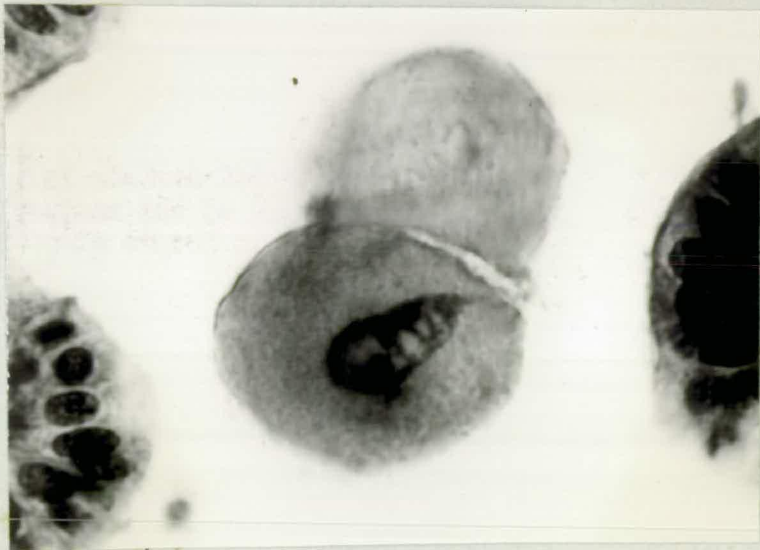


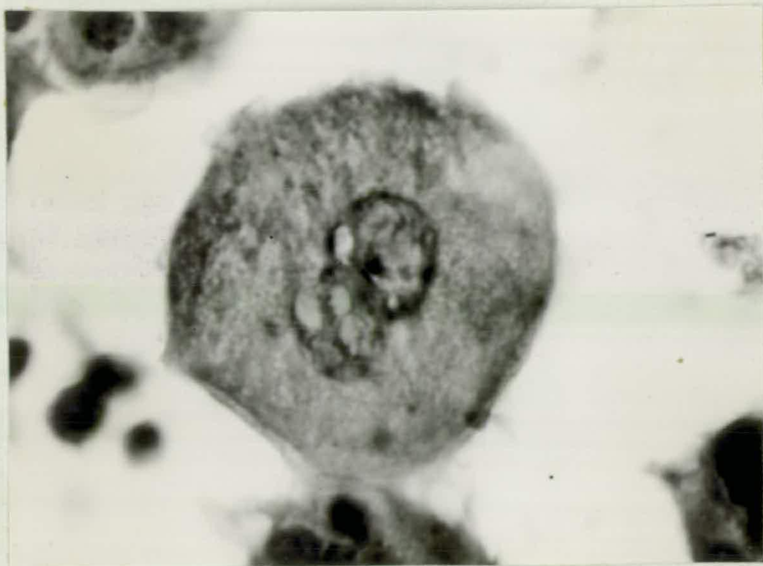
41. A two-celled embryo with two nuclei in one blastomere. The other blastomere also possessed two equal-sized nuclei, only one of which can be seen in this section. One of the nuclei in each blastomere could be a sub-nucleus; alternatively, nuclear division may have occurred without cleavage. x 1000.

42. A two-celled embryo with an elongated nucleus in one of its blastomeres. The elongated 'tail' of the nucleus may be inactivated male chromatin which has become attached to a haploid blastomere nucleus. x 1000.

43. Two swollen, vacuolated pronuclei in an egg taken from a female killed 36 hours after mating. Syngamy is very delayed in this egg: the pronuclei are abnormal. x 1000.



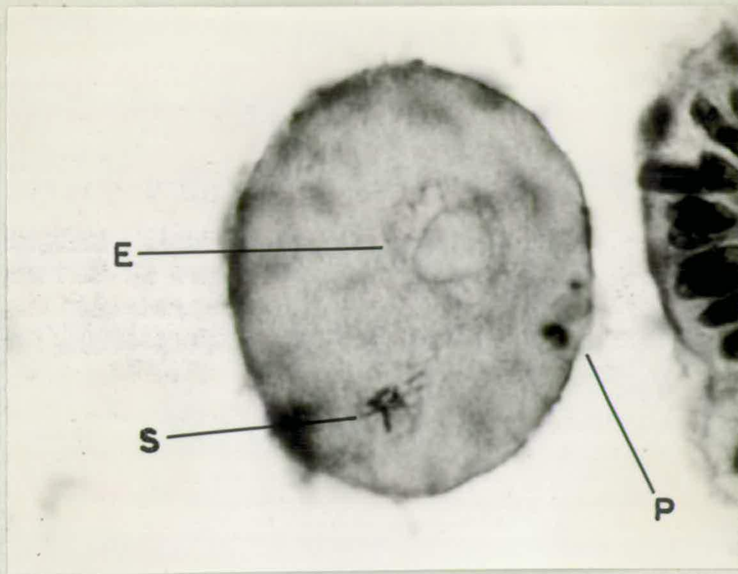


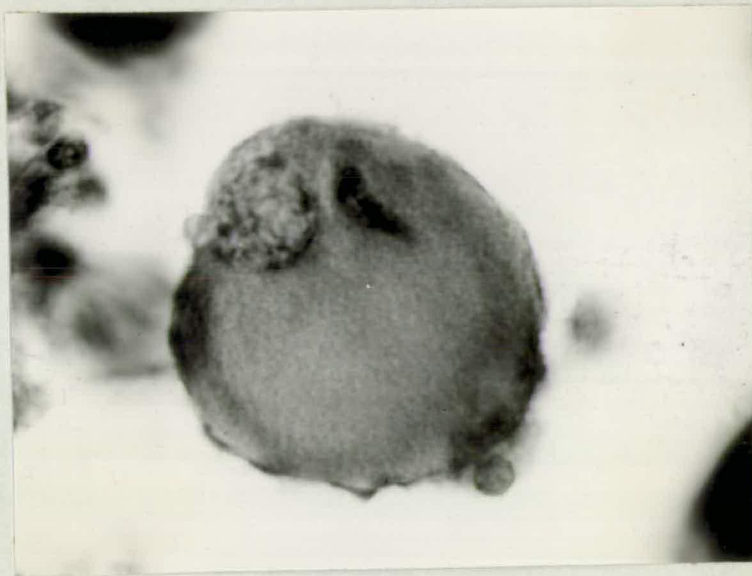


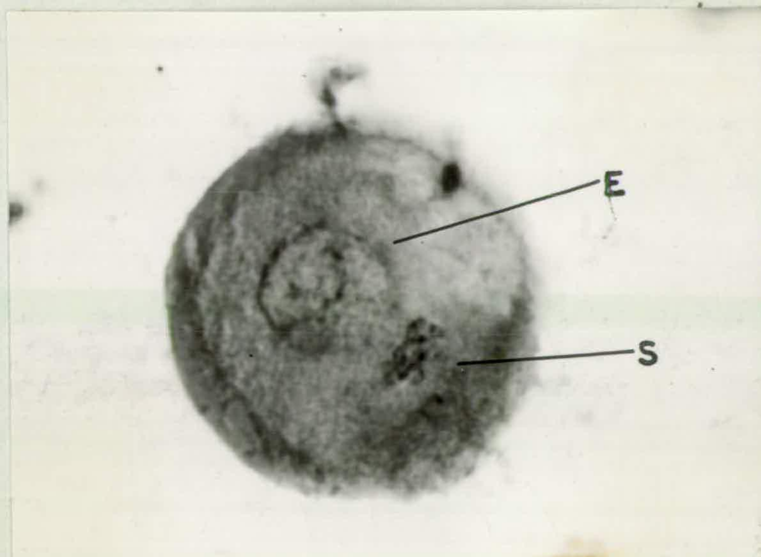
44. Two pronuclei in an egg taken from a female killed 42 hours after mating. Syngamy is delayed; one of the pronuclei, probably the male, is condensing into pycnotic chromosomes (S), the other pronucleus being greatly enlarged(E). A polar body can be seen in the section(P). x 1000.

45. Pronuclei in an egg taken from a female killed 42 hours after mating. The male pronucleus has failed to differentiate and resembles an enlarged sperm-head; the female pronucleus is greatly swollen and situated at the periphery of the egg. Cleavage is delayed. Irradiation time 10 mins. x 1000.

46. Another egg from the same female as in Fig. 45. The male pronucleus(S) has failed to differentiate normally, and is small and pycnotic. The female pronucleus(E) is swollen. x 1000.

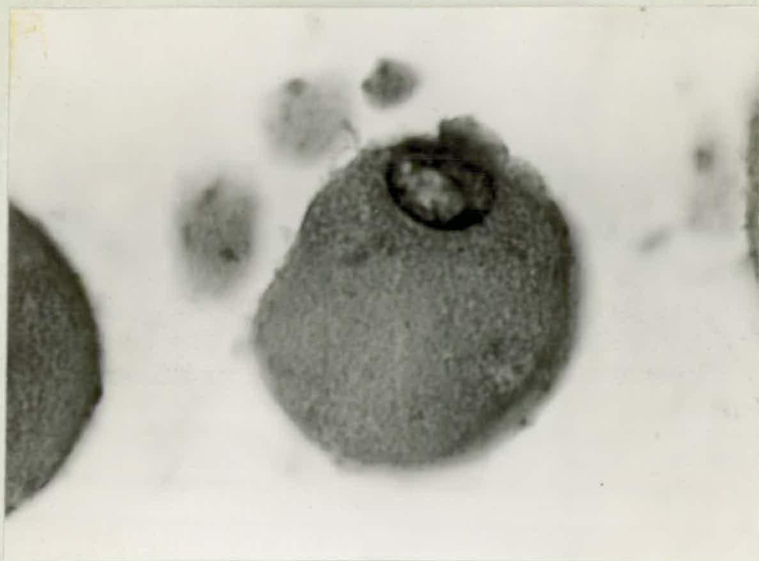

E
S
P

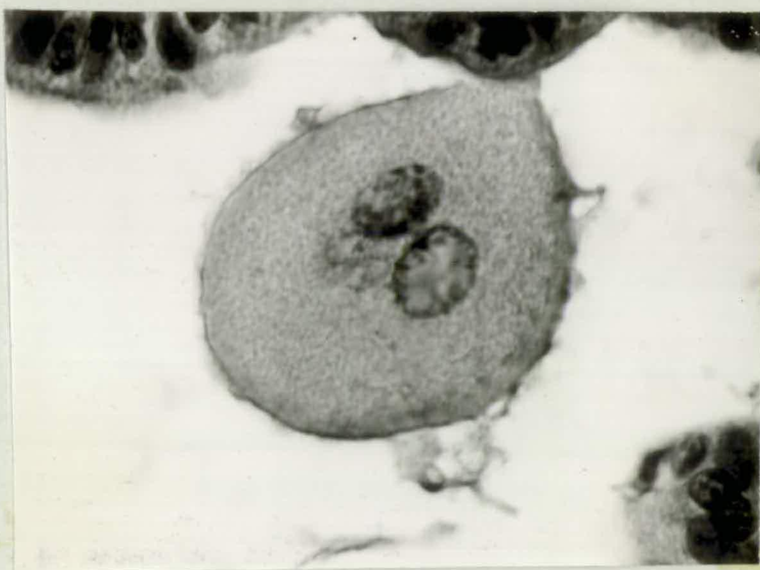


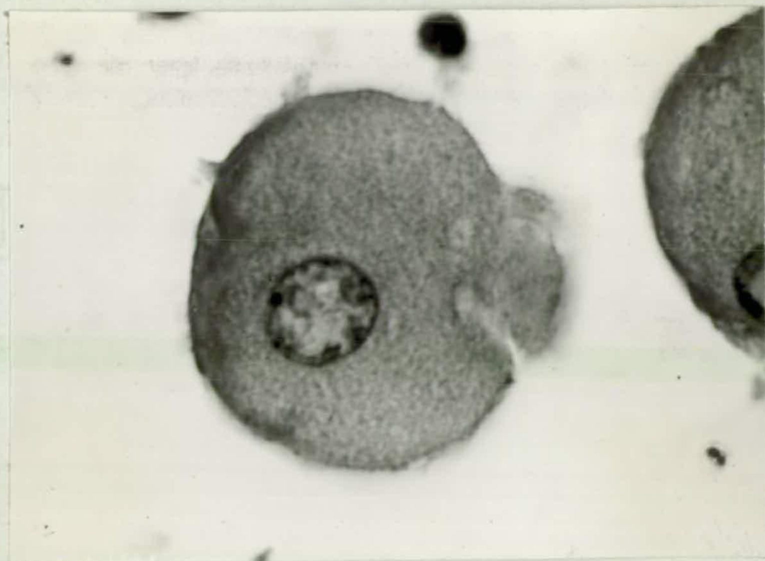

E
S

47. Another egg from the same female as in Fig. 45, showing a swollen pronucleus, probably male, on the periphery of the egg. Two other nuclei are in other sections of this egg. x 1000.

48a and b. Several nuclei in a one-celled egg taken from a female killed 42 hours after mating. The single nucleus in Fig. 48b (lower photo.) may be the male pronucleus; the other pronuclei may be the products of division of the female pronucleus. x 1000.







2. X-IRRADIATION OF THE SPERM.

INTRODUCTION

A considerable amount of work on the effects of low X-irradiation dosages on the testes of mice has been reported. Irradiations of up to 800r. induced testicular atrophy and male sterility (Snell, 1933), translocations in the male chromosomes (Snell, 1933; 1935; and 1946; Koller and Auerbach, 1941), and embryonic mortality (Plagens, 1933; Snell, Bodemann and Hollender, 1934; Snell and Picken, 1935; Hertwig, 1938, 1940).

Higher dosages have been used by Brenneke (1937), who irradiated the testes of rats and mice with dosages of between 800 and 1800r. and between 800 and 2,200r. respectively. She observed sub-nuclei, lagging chromosomes, asynchronous division of blastomeres, and enucleated cells in the early cleavage stages of the resulting embryos. In later embryonic stages, she also reported considerable embryonic mortality in implants.

Extremely high dosages of X-rays were applied <u>in vitro</u> to rabbit sperm by Amoroso and Parkes (1947). They used dosages between 50 and 100,000r., and artificially inseminated the irradiated sperm. The number of offspring declined up to 500r., where most of the offspring were sterile. Above 2,500r., all embryonic development was retarded, sperm penetration of the egg was delayed, and polyspermy was not uncommon. After the high dosages, many abnormalities were observed: the male pronucleus failed to develop normally, syngamy was delayed and irregular, and many activated ova failed to divide. The arrest of

segmentation of the embryos was associated with irregular nuclei, and anucleate or multinucleate cells. There was evidence of slight activation without syngamy, but none of continued gyngenetic development.

In the experimental results which follow, details are given of the effects on embryonic development of the X-irradiation <u>in vitro</u> of the sperm of the mouse, <u>Mus musculus</u>. The primary intention of the treatment was to induce gynogenetic development. Dosages were between 100 and 50,000r; the experiment was similar to that of Rugh (1939) on the frog, and Amoroso and Parkes (1947) on the rabbit. Treatment of mouse sperm <u>in vitro</u> necessitated the artificial insemination of the treated sperm; details of the technique have been given on p. 4.

MATERIAL AND METHODS

Details of the stocks used, the artificial insemination technique, and the examination of the embryos resulting from the treated sperm, are exactly similar to those given for the ultra-violet irradiation of the sperm (p. 86). Sperm suspensions were about 2 mm. deep; between 4 and 7 bucks usually provided a sufficiently dense sperm sample.

The X-ray machine.

Irradiation of the suspension was made at two distances from the X-ray source: at 8" and at 2½". At 8" distance, the output of the machine was 180r/min. Dosages of 100r and 500r were given at this distance. For dosages between 1,000r and 50,000r, irradiation was made at 2½". Output at the shorter distance was

calculated from the 8" output by the relationship between intensity and the square of the inversed distance. Even at $2\frac{1}{2}$" distance, the time of exposure of the sperm for 50,000r to be given was approximately one hour, including time for the machine to cool. For dosages between 20,000 and 50,000r, it was necessary to cover the sperm suspension with thin plastic to prevent evaporation. The plastic offered no detectable barrier to the X-rays. Some sperm samples were given the same treatment without irradiation to ensure that no loss of fertility resulted from this delay.

The constants of the machine were: tube voltage 70 kV., current 7 mA., filtration 0.5mm. Al, HVL 0.8mm. Al, dose-rate 180r/min. at 8" from the source.

RESULTS.

1. The effect of X-rays on sperm motility.

No pronounced effects on sperm motility were observed except, possibly, at the highest dosages, where activity appeared to have declined in several samples. Unlike the effect of the ultra-violet, the whole sperm sample appeared to be evenly affected; all the sperm in any one suspension had the same activity. The control samples which were exposed for one hour without irradiations showed no diminution of activity.

2. Results at the $3\frac{1}{2}$ days gestation

Details of the fertility of the inseminated females, of the number of embryos and unfertilised eggs, and of the chromosome counts and mean cell numbers of the embryos are given in Table 14. Dosages were between 100 and 50,000r; the number of sperm

samples treated at each dosage are given in the Table.

Analysis of the fertility of the inseminated females.

Controls of the artificial insemination technique yielded 33 out of 47 females possessing embryos at 3½ days gestation, or 70.2% of the females inseminated. In controls, and also in the experimental animals, care was taken to exclude females which had not ovulated. After X-irradiation of the sperm, some variation occurred between dosages in the percentage of females with one or more embryos. Some of the variation was accidental, for at dosages 100r., 500r., and 2,000r., one of the samples used at each dosage was probably chilled slightly before insemination. None of the females inseminated with either of these three sperm samples possessed embryos. If the low figure at these three dosages is excluded for this reason, the percentage of females with embryos was similar to controls at all dosages up to 20,000r. Above this, the percentage declined. The cause of this decline may have been due to effects on the fertilising power of the sperm, or due to the difficulty of observing at 3½ days gestation the embryos which had failed to cleave (see later). After the highest dosages, the numbers of females with embryos must therefore be considered as a minimum figure.

Analysis of the numbers of embryos and unfertilised eggs.

The comparison between the numbers of fertilised eggs (represented by the embryos), and numbers of unfertilised eggs is biassed due to the degeneration of some of the unfertilised eggs before 3½ days gestation. The comparison has nevertheless been made first over all females, and secondly on the eggs of females

which possessed one or more embryos. The results are given in Table 14. Analysis over all females included those in which the lack of embryos was probably due to accidental cold-treatment of the sperm, and this was presumably responsible for the low values at dosages 100, 500, and 2,000r. Analysis within females with embryos overcame this difficulty. Both analyses gave a consistently lower percentage after X-irradiation of the sperm than in untreated controls. The percentage declined steadily with increasing dosage; the decline could have been actual or due to the difficulty of identifying highly retarded embryos at 3½ days gestation (see later).

Observational difficulties may have also affected the calculation of the mean number of embryos taken from females with embryos. This analysis overcame the obstacle of the defenerating unfertilised eggs. The mean was the same as that in controls at 100r. (i.e. 5.5 embryos per female), lowered slightly to 4.5 at 10,000r., and declined steeply to 2.7 at 50,000r. (Table 14).

Examination of fertilised ova (see later) showed that the three criteria used above actually compared the number of fertilised eggs which cleaved with those unfertilised plus those which were fertilised but failed to cleave. Two factors were therefore simultaneously measured: the effect of X-rays on the fertilising power of the sperm, and the ability of the egg to undergo cleavage. The study of numbers of eggs in the pronuclear stages would give a stricter measure of the fertilising capacity of the sperm after X-irradiation.

<u>Analysis of the morphological stage and number of nuclei of the developing embryos.</u>

The effect of X-irradiation of the sperm on embryonic development at 3½ days gestation was measured in two ways: first, the stage of differentiation of the embryos; second, their number of nuclei. In controls, 51.8% of the embryos were early or medium blastocysts, the rest being advanced morulae. After X-irradiation of the sperm, the proportion of blastocysts declined from a value identical with controls (57.6%) after 100r., to 0% after 2,000r. and above. The decline in this percentage was very marked between 500 and 1,000r. (Table 14). After 30,000 and 50,000r. all of the embryos appeared to be in the 2, 3, or 4 cell stage when viewed alive under the microscope. Unlike the all-or-none tendency induced by ultra-violet irradiation, all the embryos were affected more or less equally by the X-rays, especially after 2,000r. and above. Below this dosage, some variation occurred, for example at 100r., one embryo had not developed beyond the two-celled stage while the remainder were either blastocysts or advanced morulae.

Some reservations must be made concerning the number of nuclei counted in embryos produced from X-irradiated sperm because sub-nuclei were often present in addition to the normal-sized nuclei (Figs. 50, 51). Where they could be distinguished, the sub-nuclei were excluded from the count. The numbers of nuclei given in the following data may therefore not necessarily represent the number of cells.

For each irradiation dosage, the mean number of nuclei per

embryo and its standard error was calculated. Results are given in Table 14 and plotted in Text-fig. 6. The mean declined with dosage, especially after 500r. (see Figs. 49 to 52). The number of cleavages which must have occurred to give the mean nuclear number can be calculated from the formula

$$x = \frac{\log N}{\log 2}$$ where x is the number of cleavages and N is the number of nuclei.

After 100 and 500r., cleavage numbers at 5.3 and 5.6 respectively were identical to controls (i.e. 5.4). 1,000r. caused a slowing down equivalent to about one cleavage, to 4.2 cleavages. After 5,000r. and above, very few embryos had completed their third cleavage. After 30,000 and 50,000r., the mean cell number was lower than 2.0; many embryos had probably failed to undergo their first cleavage. The standard error of the mean nuclear number was fairly low for the medium and high dosages; response to X-irradiation was evenly distributed over all embryos, unlike the all-or-none response to ultra-violet irradiation of the sperm (p. 92).

Analysis of the chromosome counts of the embryos.

The diploid number in Mus musculus is 40 (Matthey, 1949). Details of the chromosome counts of the $3\frac{1}{2}$ day old embryos are given in Table 14. Few embryos could be classified at the high dosages; due to their low number of nuclei, very few of them xxxx possessed mitoses. Counts were made with an accuracy of $\pm 5\%$ of the number of chromosomes in the mitosis. If this accuracy was not possible on embryos which were approximately diploid, they were classified as 'probably diploid'. Where the

count on non-diploids was only approximate, a question-mark has been appended behind the particular embryos in the Table. If an embryo was neither haploid or diploid, its chromosome complement has been bracketed in the Table.

The outstanding trend in the chromosome counts was the gradual decline from the diploid to the haploid number with increasing dosage (see Text-fig. 7; figs. 49-54). Even at 100r., the lowest dosage used, some of the embryos were hypo-diploid; at this dosage one hyper-diploid and one mosaic were recovered. The proportion of hypo-diploids increased rapidly up to 1,000r., and above 2,000r. no diploid embryos were found. At the higher dosages, the number of mitoses declined considerably. Sufficient were observed, however, to show that the mean chromosome count of the embryos was very close to haploid at these dosages. Four embryos were provisionally classified as haploid, two of which had only approximate counts. The other two were highly retarded. One of them possessed three cells, of which one cell was in division with 19 contracted chromosomes; the other had two cells, of which one was in division with approximately 20 small chromosomes. The last-mentioned embryo was probably being phagocytosed (Fig. 52). Above 20,000r., no mitoses were observed in any of 45 embryos.

More detailed study of certain mitoses revealed an unusual feature in them. After the chromosome classification under the microscope, some of the hypo-diploid mitoses were projected on a screen at magnification x 2,000. At this magnification, what had previously been classified as complete chromosomes were often found to be small fragments of chromosomes. The mitoses

consisted of 20 normal-sized chromosomes and several small pieces (Fig. 54). Some of the hyper-haploid chromosome counts were therefore nearer to the haploid number than estimated; the small pieces of chromatin were probably the remains of the treated sperm chromosomes. Much of the sperm chromatin had apparently been lost in previous mitoses. The normal chromosomes were presumably the haploid maternal complement, the embryos being in the process of reversion to haploid.

In addition to the definite chromosome counts, other observations on chromosomes were made which could not be included in the Table. They were essentially similar to those described following ultra-violet irradiation of the sperm. Many embryos possessed pycnotic, fragmented, elongated, or contracted chromatids or chromosomes (Figs. 49, 53). The majority of these were unclassifiable. The largest group excluded from the chromosome counts was a number of one-celled embryos containing exactly twenty chromosomes. Sometimes the chromosomes appeared to be meiotic, others were probably mitotic. The possible origin of similar embryos has been discusses previously in the ultra-violet series; meiotic chromosomes may have represented unfertilised eggs (though none have been seen in controls), or eggs which had been penetrated by sperm without being activated. Embryos with mitotic chromosomes may be haploids arrested at the first cleavage. Due to the subjectivity necessary in deciding the type of chromosomes, all one-celled eggs have been excluded from the chromosome counts given, and they have been classified as unfertilised eggs. This type of abnormality occurred mostly at the higher dosages; some experimental evidence for their

being inactivated, fertilised eggs will be given presently.

The exclusion of the one-celled haploids from the results necessarily means that in subsequent analysis the smallest possible number of cells in the haploids is two. Their number of nuclei and cleavage number may therefore be higher than actually occurred.

The number of nuclei in haploid, diploid, and heteroploid embryos.

As previously, shown, the mean number of nuclei of all embryos decreased with increased irradiation, the highest dosages restricting development to the one- or two-celled stage. In Table 14, the mean nuclear number of all embryos of the diploid, and of the hypo-diploid embryos are given. For this purpose, all the hypo-diploid embryos have been regarded as a group, regardless of their chromosome complement. Included in the group are the four haploid embryos which had cell numbers of 11 (the haploid occurring after 5,000r.); 2, 10, 2 (those occurring at 10,000r.).

The number of nuclei in the diploid embryos declined from approximately 45 at dosages 100 and 500r., to 23 at 1,000r., and 8.5 at 2,000r. The diploids therefore also conformed to the pattern of declining nuclear number with increasing dosage; they must also have inherited some developmental abnormality from the irradiated sperm. The hypo-diploids had, in general, lower nuclei numbers than the diploids, and also conformed to the pattern of decreased capability of development with increased dosage of X-rays. At the higher dosages (2,000 to 20,000r.), the hypo-diploids consistently possessed nuclear numbers higher than the mean of all embryos. But this result might be expected,

because the embryos which possessed mitoses were a selected sample giving proof of continued development. The mean nuclear number of the hypo-diploids at 20,000r. was 5.17, the mean of all the embryos at this dosage being 3.75. For comparison, the mean nuclear number of the diploid embryos in control artificial inseminations without treatment of the sperm was 44.1±15.4.

If the mean nuclear number of the hypo-diploid embryos is transformed to the cleavage number, the values for dosages 5,000, 10,000 and 20,000r. were 3.28, 2.65, and 2.38 respectively. At these dosages, the male chromatin was probably being excluded from development with successive mitoses, and the embryos were becoming gynogenetic in the sense. The cleavage number of the four haploids found was: 3.47, 1.00, 3.33, 1.00 respectively. No comparisons with diploids were possible within each dosage because no diploids occurred at these dosages. It was clear, however, that the dosage of X-rays necessary to inactivate the sperm chromatin also suppressed normal embryonic development.

The hyper-diploid with 45 chromosomes, and the mosaic with mitoses of 31 and 40 divisions, possessed 32 and 29 nuclei respectively.

Cytological observations on the embryos.

The precautions necessary in the interpretation of cytological abnormalities in 3½ day old squashed embryos have been discussed on p. 98. The absence of similar abnormalities in controls, observations on eggs at fertilisation and the first cleavage which will be given later, and chromosome counts on 3½ day old embryos, all indirectly confirmed the following observations.

The most common abnormality seen in the $3\frac{1}{2}$ day squashes was the presence of numbers of sub-nuclei in addition to normal sized nuclei (Figs. 50, 51). Sub-nuclei accurred after all dosages used, some embryos containing few, others possessing many. The chromosomal anomalies were lagging and fragmented chromatids (Figs. 52, 55), and several contracted mitoses (Fig. 49). The presence of pycnotic chromosomes, and of one-celled eggs containing meiotic or mitotic chromosomes, has been mentioned previously. An interpretation of these cytological abnormalities will be given later.

Some of the embryos at $3\frac{1}{2}$ days gestation were probably dead and undergoing phagocytosis (Fig. 52).

3. Observations at fertilisation and the first cleavage.

Dosages of 100, 1,000, 10,000, 20,000, or 30,000r. were applied to the sperm. Few eggs were fertilised after the two highest dosages; results from these dosages have been grouped. Sperm entry, pronuclear growth, and syngamy and the first cleavage were investigated. Eggs of females killed between 6 and 8 hours, 12 and 18 hours, and 36 and 42 hours after mating were examined for these purposes respectively. Five mice or more were killed at each dosage; the eggs of one of them were studied for sperm entry, two for pronuclear growth, and two for syngamy and the first cleavage. Syngamy was not observed directly, but inferences about it could be made from the appearance of the two-celled eggs. Only the fertilised eggs have been analysed; details are given in Table 15.

Sperm entry and pronucleus formation.

Only one of the females killed between six and eight hours after mating possessed penetrated eggs. She had been inseminated with sperm receiving 20,000r. Seven of her eggs were in telophase, and in five of these the sperm was swelling normally (Fig. 56). In the other two eggs, no sperm head could be seen, but, as this occasionally happens in controls, it may have been due to difficulties in observation. None of the four other females killed between six and eight hours after mating had penetrated eggs. The absence of sperm penetration into these eggs may have been due to delayed fertilisation after X-irradiation of the sperm. For comparison, after artificial insemination of untreated sperm, one female of two inseminated had penetrated eggs six hours after mating. In natural matings, six out of eight females possessed penetrated eggs at this time. More data is needed to ascertain whether delayed fertilisation occurs after X-irradiation of the sperm.

Two kinds of anomaly were observed during pronuclear growth. In both cases, eggs possessed only one pronucleus. The only means of deciding whether this pronucleus was male or female was to examine the egg for the presence or absence of the maturation spindle. After 30,000r., three eggs possessed vacuous pronuclei together with an unchanged second meiotic spindle (Fig. 57a & b). One of these eggs possessed two of these unusual pronuclei, the other two eggs possessed one each. These highly abnormal pronuclei were presumably male; sperm entry into the egg had apparently failed to stimulate the second maturation division of

the egg. Four other eggs possessed only one pronucleus; in these eggs the pronucleus was normal in appearance, and the eggs had no meiotic spindle but did possess a second polar body. In one of these eggs, dark striations appeared in the cytoplasm. The presence of a second polar body indicated that meiosis was complete, and hence the single pronucleus in these eggs could have been either male or female. Judged from its position close to the second polar body, the single pronucleus could have been female; the dark striations in the cytoplasm may have been the remains of an abortive sperm-head. At 30,000r., six eggs appeared to have two normal pronuclei, three eggs possessed the vacuous pronuclei and the second maturation spindle, and four eggs possessed a single normal pronucleus and no spindle. After 10,000r., 10 eggs had two normal pronuclei, one had three pronuclei and one egg had one normal pronucleus.

Polyspermy was observed in two eggs. One of these contained two vacuous pronuclei in an unstimulated egg. The other is shown in Fig. 58 and occurred after 20,000r; a slightly swollen sperm-head lies next to one of the two normal pronuclei present in the egg.

Syngamy and the first cleavage.

Examination of the embryos between 36 and 42 hours after mating revealed only two types of egg: those which had cleaved to the two-cell stage, and those which had not been activated and were probably unfertilised. These was no evidence of a delay in the pronuclear stages similar to that occurring after ultra-violet irradiation (p. 101). Nor is any evidence seen of the

fate of those eggs which had possessed only one pronucleus. But the numbers examined were low (Table 15).

Many of the two-celled embryos possessed sub-nuclei (Fig. 59), the numbers of embryos affected being proportional to the amount of irradiation of the sperm. At 30,000r., each of the five two-celled embryos possessed at least one sub-nucleus in one of its blastomeres; three of these embryos had more than one (Fig. 60). At 1,000r., four embryos of eleven examined had sub-nuclei; at 100r., one embryo certainly had a sub-nucleus, two others probably had one and eight were normal.

The only evidence of a doubling of the maternal chromosome set was the egg possessing three pronuclei following irradiation of 10,000r. In appearance this egg suggested polar suppression rather than polyspermy. Polyspermy occurred in two eggs, but in these either one or both of the sperms appeared to be taking no part in development. Forty-four other eggs had either one or two pronuclei; these, together with direct observations on seven telophase spindles which appeared normal, showed that chromosome doubling at the second maturation was very rare. No evidence of chromosome division without cytoplasmic division at the first cleavage was seen (cf. the ultra-violet results, p. 103). To conclude, observations at fertilisation and the first cleavage supported the chromosome counts on $3\frac{1}{2}$ day embryos and confirmed that chromosome doubling did not occur by either second polar body suppression, polyspermy, or suppression of the first cleavage.

4. Observations at implantation, birth and maturity.

Details of the fertility of the inseminated females, of their placentae and embryos, and of their young, are given in Table 16. The irradiation dosages used were 100, 500, and 1,000r.

The number of placentae occupied.

Mice were killed at two stages of gestation: at 10 days, and at 15 to 16 days. After dosage of 1,000r., none of the four mice examined at these gestation periods possessed placentae. Six females which were killed at term when they were obviously not pregnant, were examined for placentae. One of them possessed two very small, degenerate placentae. This dosage was therefore not incompatible with implantation, but was lethal for embryonic development to term. Four females were examined after insemination with sperm irradiated with 500r. One female possessed 10 placentae at 16 days, but only six contained embryos. None of the other three possessed placentae. The six embryos were derived from a mother homozygous for the dominant allele of this gene. All of the embryos had black eyes; the father's genes had not been eliminated by the X-rays. After irradiation of 100r., one female was examined at 10 days gestation; she possessed two placentae, only one of which contained an embryo.

To summarise, 1,000r. almost completely inhibited implantation; 500 and 100r. caused considerable embryonic mortality after implantation.

The number of offspring born, and genetic observations on them.

After 1,000r., none out of six females had litters. Three

females out of seven inseminated produced litters from sperm irradiated by 500r., the litter sizes being 3, 3, 2. All of these offspring were marked by the presence of a black eye gene present in the male chromatin; the male chromosomes must have taken part in the development of these embryos. After 100r., two out of eight females gave litters, size eight and three respectively. The litter of eight showed the presence of the black eye gene derived from the father. The sex ratio of the offspring was: after 500r., 5 females, 3 males; after 100r., 5 females, four males (two others were lost by accident before sexing).

DISCUSSION.

The chromosome complement of 3½ day old embryos resulting from the fertilisation of normal eggs by irradiated sperm declined from diploid to haploid with increasing dosage on the sperm. Each of these embryos would contain a normal haploid set of chromosomes from the untreated egg. The inference is that the number of sperm chromosomes contributing to embryonic development at 3½ days gestation is inversely correlated to the applied dosage. After high dosages, the fate of the sperm chromatin could be seen in certain mitoses of 3½ day old embryos; it remained as fragments of chromosomes interspersed among twenty normal, presumably female, chromosomes. The presence of these fragments, and of sub-nuclei in the blastomeres of cleaving embryos, indicated the method by which chromosome loss occurred in the embryos. The X-rays either directly fragmented the chromosomes in the sperm-head, or so affected the chromosomes or

other sperm elements that fragmentation occurred during the cleavage mitoses of the embryo. The presence of chromosomes and/or fragments presumably derived from the sperm indicated that the X-rays did not prevent male pronucleus formation or syngamy, but that the loss of the male chromatin occurred in subsequent development.

Direct observation on the pronuclear stages largely supported this indication. The majority of the embryos possessed two normal pronuclei. Certain embryos were exceptional, however, in that they had abnormal pronuclei. Some had one pronucleus which was presumably female, others had one or two pronuclei which may have been derived from the sperm. Embryos with a single pronucleus were found after 10,000r and above, the high dosages seemingly affecting to some degree the normal processes of pronucleus formation. But, at all dosages, the majority of embryos possessed two apparently normal pronuclei.

The effect of the X-rays was therefore unlike that of ultra-violet in that no all-or-none inhibition of the sperm chromatin occurred; instead, the male chromosomes usually took part in syngamy, and cleavage was not delayed. The effects of the irradiation became apparent during development, when the fragmenting or lagging male chromosomes became lost from the dividing blastomere nuclei into small sub-nuclei. Similar mitotic irregularities were reported by Brenneke (1937) in cleaving rat and mouse ova after irradiation of the testis; Amoroso and Parkes (1947) observed sub-nuclei in cleaving rabbit ova after irradiation of sperm _in vitro_. The present work shows that after high dosages

almost all the male chromosomes were lost in the course of one, two, or three cleavages. Two causes may have determined the rate of loss of male chromatin during development: the dosage of X-rays, and the number of cleavages undergone by the embryo. With increasing dosages, the embryos cleaved progressively less, yet, despite this, their chromosome complement still declined. The primary agent causing chromosome loss therefore appeared to be the dosage of X-rays applied to the sperm. The loss of chromatin as sub-nuclei could be observed as early as the two-celled stage, the number and size of the sub-nuclei being positively correlated with dosage. In two-celled embryos resulting after high irradiations of the sperm, the sub-nuclei were almost as large as the blastomere nuclei. These large sub-nuclei, and the decline to near-haploid in the chromosome content of once- and twice-cleaved embryos, were undoubtedly closely related and induced by similar mitotic mechanism.

Of the embryos which possessed a single pronucleus, some contained a second maturation spindle still at metaphase; in others the spindle was not present and a second polar body had formed. The presence of a pronucleus and an unstimulated maturation spindle in the former may have been due to a heavily X-rayed sperm penetrating the egg without stimulating the completion of the second maturation division. In these eggs, the appearance of the pronuclei was clearly abnormal; this could have been due to the development of the male pronucleus in an unstimulated egg cytoplasm. This kind of development may be considered as androgenetic! In the second type of egg, the pronucleus appeared to be normal. In one of these eggs, the

detection of degenerate chromatin in addition to the pronucleus may have been due to the decay of the sperm-head after fertilisation. The decay of irradiated male chromatin was seen in rabbit ova by Amoroso and Parkes (1947). Such eggs may therefore have originated by the degeneration of the fertilising sperm after normal entry and stimulation of the egg. The eventual fate of eggs containing a single pronucleus is speculative. Those which contained an unchanged meiotic spindle probably quickly degenerated or may have been the precursors of the one-celled eggs containing merely meiotic chromosomes at 3½ days gestation. The unipronucleate eggs which had been stimulated may have cleaved; in this respect, it is interesting to note that the two-celled 3½ day old haploid embryo shown in Fig. 52 possessed twenty chromosomes. This is contrary to the normal appearance of many chromosome fragments in mitoses after the X-irradiation of the sperm. This particular embryo may have resulted from the cleavage of an egg containing a single pronucleus; such an embryo would be expected to possess an exact haploid complement. Judged by the number of nuclei in 3½ day old embryos, this type of gynogenetic development was very abortive and ended after one or two cleavages.

With increasing X-irradiation of the sperm, embryonic development became more retarded until, at the highest dosages, none of the embryos had completed their second cleavage at 3½ days gestation. No Hertwig phenomenon was found, which agrees with the findings of Amoroso and Parkes (1947) after X-irradiation of the rabbit sperm. The retardation of embryonic development

was very constant at each dosage level, and quite unlike the all-or-none effect of the ultra-violet (p. 92); the greater penetration power of the X-rays probably caused the higher uniformity of effect. The reduced chromosome complement of these embryos was probably not the cause of their retarded development, for haploid and many kinds of heteroploid mouse embryos can develop quite successfully to blastocysts (Beatty, 1954; Beatty and Fischberg, 1949, 1951a & b, 1952; Fischberg and Beatty, 1952 a & b; and see p. 53 of this thesis). Delayed fertilisation caused by the treatment was also unlikely to have produced this result (Gates and Beatty, 1954). A non-chromosomal factor in the sperm may have been affected to retard embryonic development in this manner. The factor may have been cytoplasmic, and because it appeared to be closely connected with cleavage, it could have been the centriole. Increasing irradiation of the sperm could therefore have had two effects: greater damage to the chromosomes and to the centriole. Dosages of 30,000 and 50,000r. so affected the centriole that all of the embryos resulting from the fertilisation of a normal egg by this sperm failed to cleave more than once. It seems unlikely that the high uniformity of retarded development after these dosages was due to genetic damage to the sperm chromosomes, especially as such embryos possessed an untreated haploid complement from the egg.

Certain of the phenomena induced in embryonic development were the same after either ultra-violet or X-irradiation of the sperm. After either of them, embryonic development was adversely affected, chromosomes were lost during development, and

cytological abnormalities included sub-nuclei in the blastomeres, chromosome fragmentation, and other mitotic disturbances. The clear difference between the two treatments lay in the inactivation of the male chromatin at syngamy by the ultra-violet, which had no counterpart after X-irradiation. An effect on the centriole and the chromosomes of the sperm has been suggested to explain the abnormalities resulting from both treatments. Neither treatment has induced a Hertwig phenomenon, all the haploids and near-haploids being excessively retarded.

A comparison between mammals and amphibians can be made on the effects of the X-irradiation of the sperm on embryonic development. Rugh (1939) reported a well-defined Hertwig phenomenon in the frog with dosages up to 50,000r. on the sperm _in vitro_. Haploids were induced by the higher dosages, and developed to advanced larval stages. The degeneration of mouse embryos, and also of rabbit embryos (Amoroso and Parkes, 1947), in or before the two-celled stage is in direct contrast with the Amphibian results. Either the dosages given to mammalian sperm have not been sufficiently high, or a difference exists between the two classes. The former theory is unlikely because the male chromatin appeared to be totally destroyed in certain mouse eggs. The difference between the two classes may therefore be due to the greater resistance of some part of the amphibian sperm to X-ray damage; or to a greater capacity of the amphibian egg for haploid development.

The death of mouse embryos before and just after implantation following relatively low dosages of X-rays on the sperm is

similar to that produced when the testes and ducts of live males are irradiated (Snell and Picken, 1935; Brenneke, 1937; etc.). One of the females receiving sperm irradiated by 1,000r. had placentae, and these were very small and ill-formed. This dosage was therefore near the lethal limit for implantation. Pre- and post-implantation death occurred at 500 and 100r., and was also reflected in the low litter size. The death of these embryos may have been caused by induced dominant lethals or chromosome re-arrangements (Snell, 1935; Hertwig, 1940; Koller and Auerbach, 1941; etc.). One other cause of death of these embryos was evident in the 3½ day chromosome counts, for many embryos possessed unbalanced non-diploid chromosome complements. After such a relatively low dose as 100r., one quarter of the embryos classified were no longer diploid, while after 500r., one-third were affected. Radiation damage of such magnitude following these dosages on mature sperm assumes considerable importance as a factor in male sterility and embryonic death, especially as earlier stages of spermatogenesis are more susceptible to X-rays (Hertwig, 1938).

The delayed fertilisation indicated by the observations on the eggs of females killed six hours after mating may have been due to reduced activity of the sperm after X-irradiation. Amoroso and Parkes (1947) also reported delayed fertilisation after X-irradiation of rabbit sperm. Judged by the observations at 3½ days gestation, the fertilising capacity of the sperm was not affected until very high dosages were given, and the lack of embryos at the high dosages may have been due to premature embryonic death rather/

rather than failure of fertilisation. Two polyspermic eggs, one of them unstimulated by sperm entry, out of 20 examined after 30,000r. indicated that polyspermy may be a product of the treatment as also noted by Amoroso and Parkes (1947). In the three pronucleat eggs which contained an unstimulated metaphase of the second meiosis, two had a single pronucleus and the third had two pronuclei. The occurrence of polyspermy in one out of three of these eggs suggested that sperm entry had also failed to stimulate fully the zona reaction against the occurrence of polyspermy (Braden, Austin, and David, 1954).

SUMMARY.

1. Sperm of the mouse, *Mus musculus*, was X-irradiated *in vitro* before fertilisation. Dosages were between 100 and 50,000r.
2. With increasing dosage, the chromosome complement of $3\frac{1}{2}$ day old embryos declined from diploid to haploid. Higher dosages increasingly retarded embryonic development, 30,000 and 50,000r. restricting development to the first cleavage. Many cytological abnormalities were found in the $3\frac{1}{2}$ day old embryos.
3. X-irradiation did not normally prevent male pronucleus formation or syngamy. Chromosome loss occurred in the embryos during development, probably by the loss of chromosome fragments into sub-nuclei.
4. After 30,000r., abnormal pronuclear relationships were found. In some eggs, sperm entry and the growth of the male pronucleus did not stimulate completion of the second maturation division. In other eggs, the single pronucleus

was probably female, the male pronucleus having degenerated. Polyspermy occurred at the same dosage, and may have been correlated with failure to stimulate the egg.

5. 1,000r. almost completely suppressed implantation. 500r. caused considerable embryonic mortality.

6. A theory of irradiation damage to the centriole or cytoplasm of the sperm in addition to destruction of its chromosomes is postulated to account for the retarded development of the embryos.

REFERENCES

AMOROSO, E.C. & PARKES, A.S. 1947. Effects on embryonic development of X-irradiation of rabbit spermatozoa _in vitro_. Proc. Roy. Soc., B, _134_, 57-78.

BEATTY, R.A. 1954. Haploid rodent eggs. Proc. 9th Intern. Congr. Genetics, Bellagio.

BEATTY, R.A. & FISCHBERG, M. 1949. Spontaneous and induced triploidy in pre-implantation mouse eggs. Nature _163_, 807-808.

________________ 1951a. Heteroploidy in mammals: I Spontaneous heteroploidy in pre-implantation mouse eggs. J. Genet. _50_, 345-359.

________________ 1951b. Cell number in haploid, diploid and polyploid mouse embryos. J. exp. Biol. _28_, 541-552.

________________ 1952. Heteroploidy in mammals: III Induction of tetraploidy in pre-implantation mouse eggs. J. Genet. _50_, 471-479.

BRADEN, A.W.H., AUSTIN, C.R. & DAVID, H.A. 1954. The reaction of the zona pellucida to sperm penetration. Aust. J. Biol. Sci. _7_, 391-409.

BRENNEKE, H. 1937. Strahlenschädigung von Mäusen und Rattensperma, beobachtet an der Frühentwicklung der Eier. Strahlentherapie _60_, 214-238.

FISCHBERG, M. & BEATTY, R.A. 1952a. Heteroploidy in mammals: II Induction of triploidy in pre-implantation mouse eggs. J. Genet. _50_, 455-470.

________________ 1952b. Heteroploidy in mouse embryos due to crossing of inbred strains. Evolution _6_, 316-324.

GATES, A.H. & BEATTY, R.A. 1954. Independence of delayed fertilisation and spontaneous triploidy in mouse embryos. Nature _174_, 356-357.

HERTWIG, P. 1938. Unterschiede in der Entwicklungsfähigkeit von F1 Mäusen nach Röntgen-Bestrahlung von Spermatogonien, fertigen und unfertigen Spermatozoen. Biol. Zentralbl. _58_, 273-301.

________ 1940. Vererbbare Semisterilität bei Mäusen nach Rontgenbestrahlung verursacht durch reziproke Chromosomentranslokationen. Z. indukt. Abstamm.-u. VererbLehre _79_, 1-27.

KOLLER, P.C. & AUERBACH, C. 1941. Chromosome breakage and sterility in the mouse. Nature 148, 501-502.

MATTHEY, R. 1949. Les Chromosomes des Vertébrés. F. Rouge, Lib. Univ., Lausanne.

PLAGENS, G.M. 1933. An embryological study of a special strain of deformed X-rayed mice. J. Morph. 55, 151-183.

RUGH, R. 1939. Developmental effects resulting from exposure to X-rays. 1. Effect on the embryo of irradiation of frog sperm. Proc. Amer. Phil. Soc. 81, 447-472.

SNELL, G.D. 1933. X-ray sterility in the male house mouse. J. exp. Zool. 65, 421-441.

________ 1935. The induction by X-rays of hereditary changes in mice. Genetics 20, 545-567.

________ 1946. An analysis of translocations in the mouse. Genetics 31, 157-180.

SNELL, G.D., BODEMANN, E. & HOLLANDER, W. 1934. A translocation in the mouse and its effect on development. J. exp. Zool. 67, 93-104.

SNELL, G.D. & PICKEN, D.I. 1935. Abnormal development in the mouse caused by chromosome unbalance. J. Genet. 31, 213-235.

TABLE 14. DETAILS OF THE FERTILITY OF THE INSEMINATED FEMALES AND OF THEIR EMBRYOS AT 3½ DAYS GESTATION.

SPERM		FEMALES			EMBRYOS							CHROMOSOME COUNTS OF EMBRYOS (2n=40)								EMBRYO NUCLEAR NOS.		
Dosage of X-rays (in 1000r units)	No. of sperm treatments	No. of ovulated ♀♀ used	No. of ♀♀ with embryos	% total ♀♀ with embryos	Total no. of eggs found	No. of eggs from ♀♀ with embryos	Total no. of embryos found	% of total eggs which were embryos	% embryos of total eggs from ♀♀ with embryos	Mean no. of embryos from ♀♀ with embryos	% embryos which were blastocysts	Lost or unclassified	Haploid	Between haploid and diploid		Diploid	Probably diploid	Above diploid	Mosaic	Mean no. of nuclei of all embryos	Mean no. of nuclei of diploid embryos	Mean no. of nuclei of hypo-diploid embryos
ontrols	-	47	33	70.2	394	278	182	46.2	65.5	5.5	51.8	109	-	-	-	56	15	2(3n)	-	41.2±15.7	44.1±15.4	-
0.1	3	12	6	50.0	110	58	33	30.0	56.9	5.5	57.6	14	-	-	1(37) 2(38)	6	8	1(45)	1(31/40)	37.8±15.3	45.1±16.3	28.7±10.5
0.5	3	11	6	54.5	85	42	21	24.7	50.0	3.5	42.9	8	-	1(26)	1(34) 1(37) 1(37)	5	4	-	-	47.2±15.9	45.9±17.7	36.0± 8.6
1	4	11	8	72.7	84	70	38	45.2	54.3	4.8	7.9	25	-	1(28) 1(30)	1(33) 1(35) 1(36) 1(37)	5	2	-	-	18.4±13.5	23.9±13.9	13.5± 4.2
2	5	12	5	41.6	113	52	26	23.0	50.0	5.2	0	23	-	-	1(34)	2	-	-	-	10.8± 7.4	8.5± 3.5	13
5	5	17	11	64.7	147	95	47	32.0	49.5	4.3	0	45	1?	1?(28)	1?(32)	-	-	-	-	5.6± 2.5		9.7± 5.1
10	3	11	8	72.7	86	77	36	41.9	46.8	4.5	0	32	3?	1(24)	-	-	-	-	-	4.8± 2.9		6.3± 4.4
20	3	11	8	72.7	95	73	23	24.2	31.5	2.9	0	16	-	1(22) 1(25) 1(26) 1(28)	2(29)	-	-	-	-	3.8± 2.0		5.2± 1.5
30	4	14	6	42.9	102	44	17	16.7	38.6	2.8	0	16	-	-	-	-	-	-	-	1.7± 0.7		
50	8	23	11	47.8	189	102	30	15.9	29.4	2.7	0	30	-	-	-	-	-	-	-	1.4± 0.8		

TABLE 15. DETAILS OF THE FERTILISED EGGS EXAMINED UP TO 42 HOURS AFTER MATING.

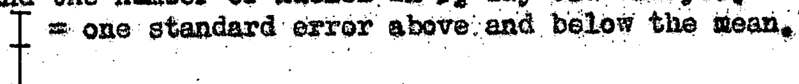
Stage of development of controls at similar times of examination.	Type of abnormality	Dosage of X-rays on the sperm			
		100r	1,000r	10,000r	20,000r & 30,000r.
Sperm entry	Normal				5
	Sperm-head not seen				2
	Sperm-head in vitellus, egg at metaphase			2?	
Pronucleate	Normal	5	17	10	6
	One pronucleus and an unchanged metaphase				3
	One pronucleus only			1?	4
	Three pronuclei			1	
	Polyspermic				2
Two-celled	Normal	8	7		
	With at least one sub-nucleus in the blastomeres	1+2?	4		5

TABLE 16. DETAILS OF THE FERTILITY OF THE INSEMINATED FEMALES AND OF THE DEVELOPMENT OF THEIR EMBRYOS TO BIRTH.

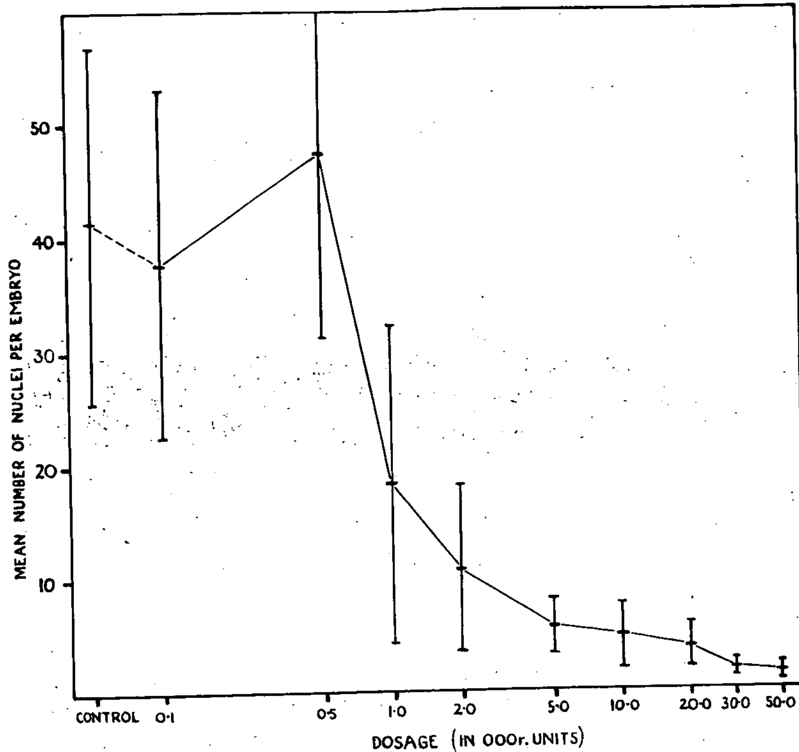
Dosage of X-rays	Time of gestation when examined: 10 days — No. of ♀♀ examined	10 days — No. of ♀♀ with embryos	10 days — Fraction of no. of placentae occupied by embryos	15-16 days — No. of ♀♀ examined	15-16 days — No. of ♀♀ with embryos	15-16 days — Fraction of no. of placentae occupied by embryos	Birth — No. of ♀♀ examined	Birth — No. of ♀♀ with embryos	Birth — Total no. of offspring
1000r.	2	-	-	2	-	-	6*	-	-
500r.	1	-	-	3	1	6/10	7	3	8
100r.	1	1	1/12	-	-	-	8	2	11
0 (Controls)	-	-	-	-	-	-	7	5	30

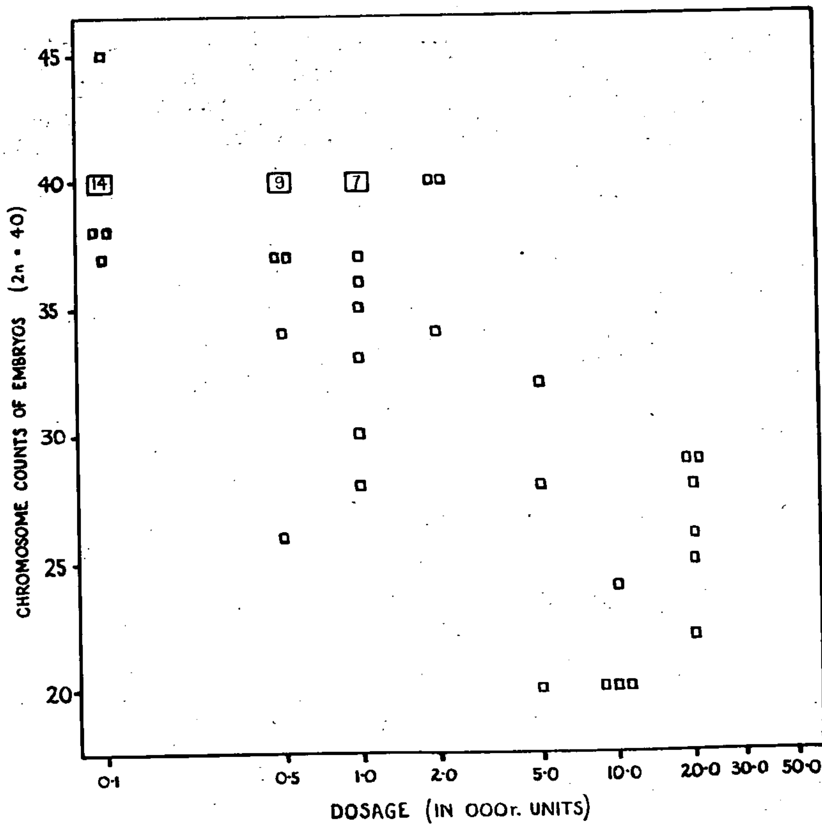
* One of the females possessed the remains of two placentae when examined at term.

Text-fig. 6. Relationship between dosage of X-rays on the sperm and the number of nuclei in $3\frac{1}{2}$ day old embryos.


= one standard error above and below the mean.

Text-fig. 7. The chromosome complement of $3\frac{1}{2}$ day old embryos after X-irradiation of the sperm before fertilisation. Each square represents one embryo, except where a different number is shown in the square.


MEAN NUMBER OF NUCLEI PER EMBRYO
50
40
30
20
10
CONTROL
0·1
0·5
1·0
2·0
5·0
10·0
20·0
30·0
50·0
DOSAGE (IN 000r. UNITS)


CHROMOSOME COUNTS OF EMBRYOS (2n = 40)
45
40
35
30
25
20
14
9
7
0·1
0·5
1·0
2·0
5·0
10·0
20·0
30·0
50·0
DOSAGE (IN 000r. UNITS)

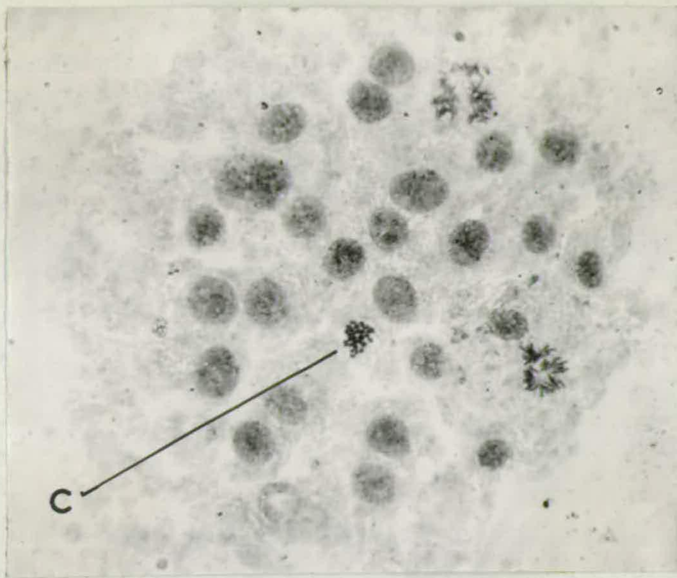
FIGURES

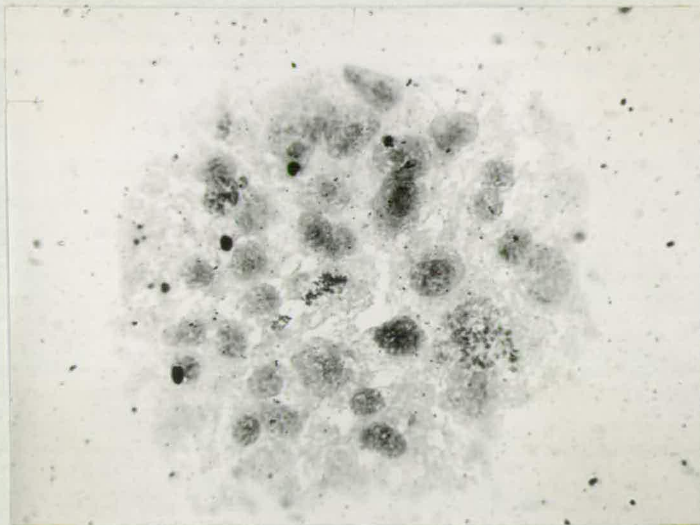
Figs. 49-55 inclusive are taken from squash preparation of whole $3\frac{1}{2}$ day old embryos. Figs. 56-60 inclusive are from sections of eggs taken from mothers killed between 6 and 42 hours after mating. The amount of irradiation given to the sperm is appended to the description of each figure. None of the negatives were retouched.

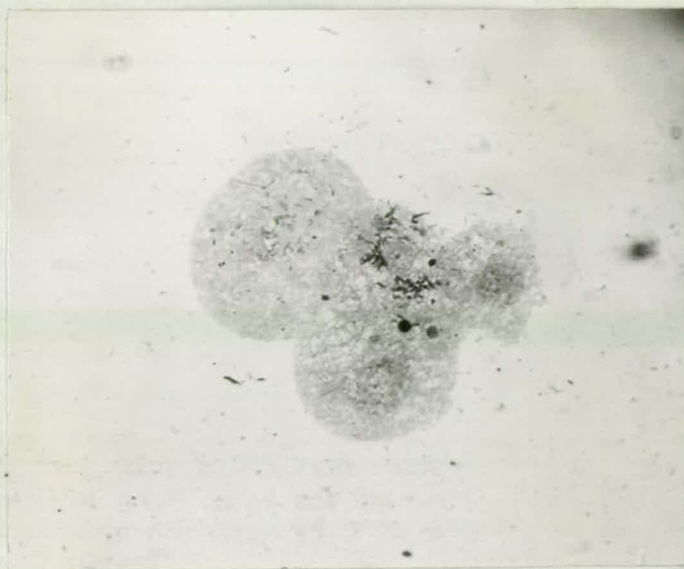
49. A whole $3\frac{1}{2}$ day old embryo containing two diploid mitoses and one contracted mitosis of 31 chromosomes(C). x 350, 100r.

50. A whole $3\frac{1}{2}$ day old embryo containing a mitosis of 34 ± 1 chromosomes. Several sub-nuclei can be seen in addition to the normal-sized nuclei. x 350, 500r.

51. A whole $3\frac{1}{2}$ day old embryo containing only 5 nuclei of which 3 are in mitosis. One of the metaphases possessed 29 ± 1 chromosomes, the other 29 ± 2; and the anaphase on the left of the embryo had approximately 46 chromatids. Sub-nuclei can be seen in the embryo. x 350, 20,000r.


C

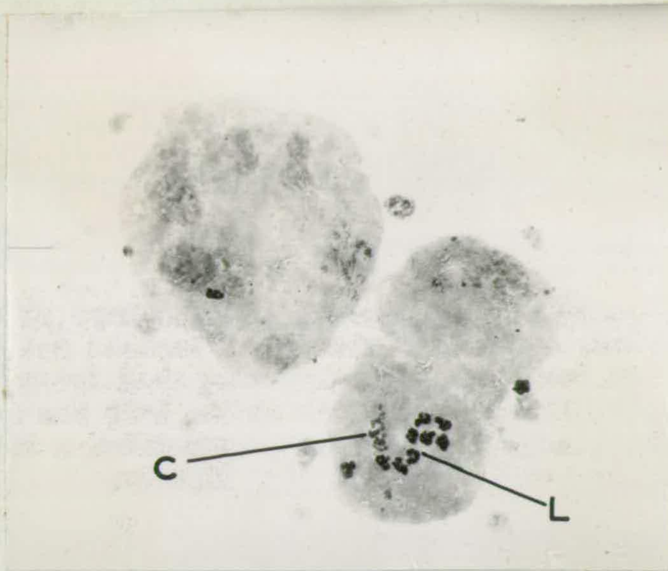




52. Two whole $3\frac{1}{2}$ day old embryos. The embryo on the right is two-celled, one cell containing a haploid set of chromosomes (C); the embryo is probably being phagocytosed by leucocytes(L). The embryos on the left has seven nuclei of which one is in an anaphase containing a large number of fragmented chromatids. x 350, 10,000r.

53. A mitosis of approximately 24 chromosomes. x 2000, 10,000r.

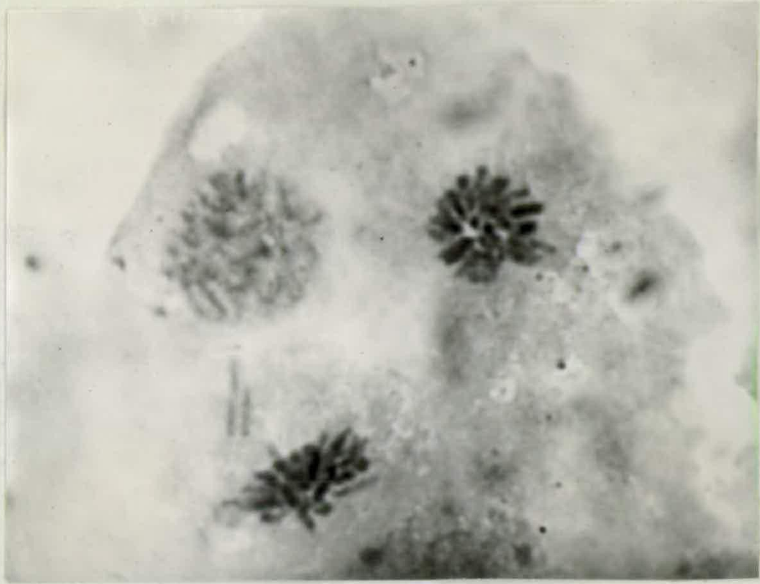
54. A mitosis containing a haploid set of normal, presumably maternal, chromosomes, and small chromosome fragments, which are presumably male, interspersed among them. x 2000, 20,000r.

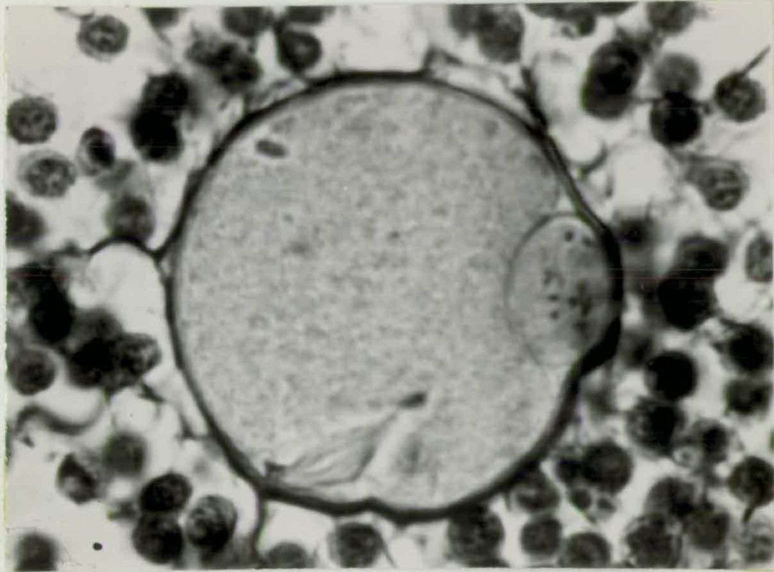

C
L

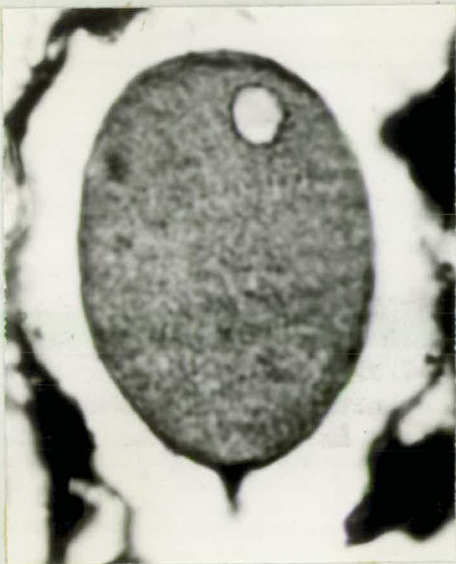
55. An anaphase with three lagging chromatids. x 2000, 500r.

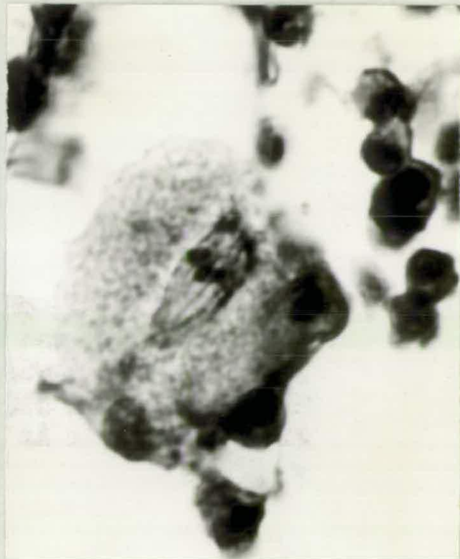
56. Normal sperm penetration into the vitellus and stimulation of the second maturation division after the sperm had been inactivated with 20,000r. The first polar body can be seen on the right of the egg. x 1000, 20,000r.

57a and b. Abnormal pronuclear growth and failure of normal stimulation of the second maturation of the egg after the sperm was irradiated with 30,000r. The maturation division is still at metaphase, the presumably male pronucleus is vacuous in appearance. x 1000, 30,000r.





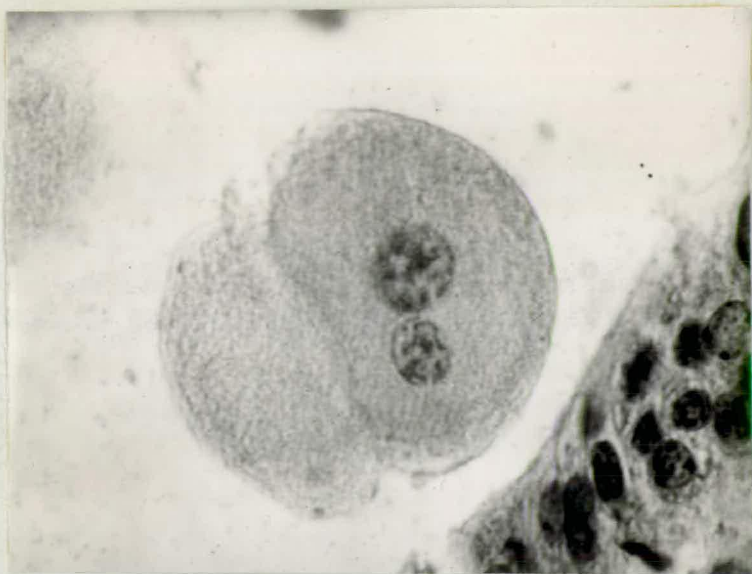


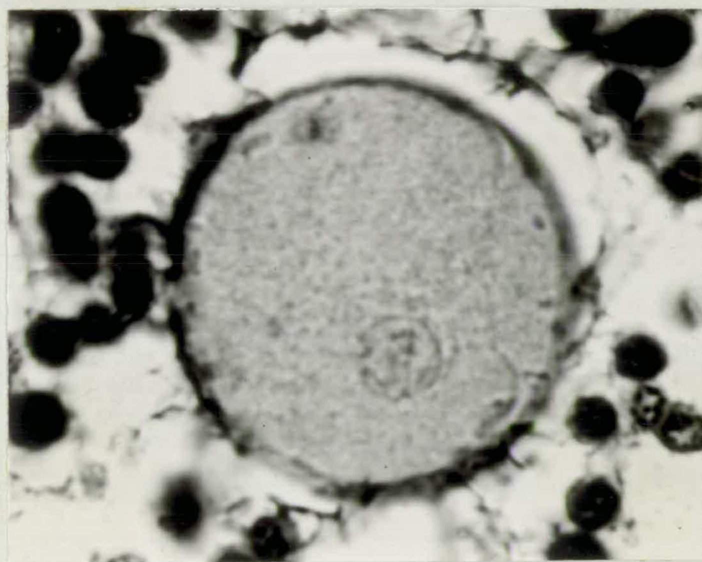


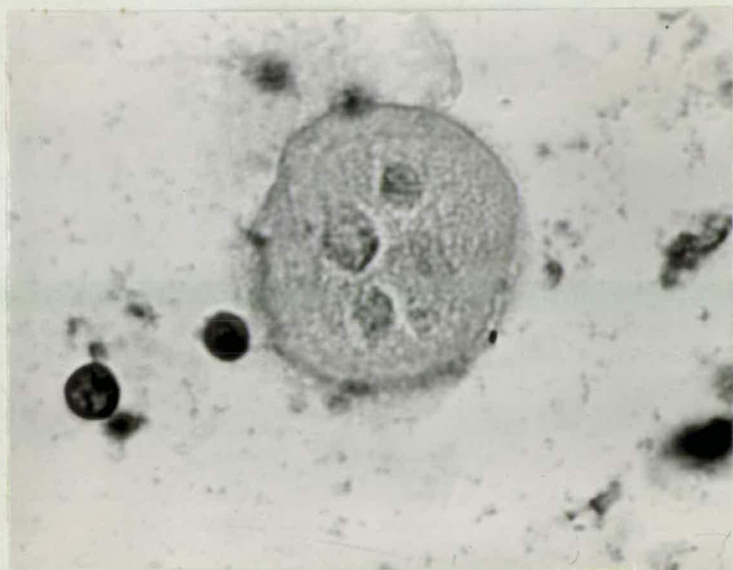
58. A large sub-nucleus present in addition to the normal blastomere nucleus in a two-celled embryo. x 1000, 30,000r.

59. Polyspermy occurring after the sperm was irradiated by 20,000r. The pronucleus in the figure is the male; the female pronucleus is in another section of the egg. Below the male pronucleus, an unchanged sperm-head can be seen in the vitellus. x 1000, 20,000r.

60. A blastomere of a two-celled egg. Three nuclei are present in the blastomere; the larger, central nucleus may be the blastomere nucleus, the others being sub-nuclei. x 1000, 30,000r.







3. TREATMENT OF THE SPERM BY TRYPAFLAVINE.

INTRODUCTION.

Since its original application by Hertwig (1924), trypaflavine has been one of the principal agents used to inactivate amphibian sperm chromosome before fertilisation. Similar treatment of mammalian sperm was carried out by Thibault (1949) on the sheep, and by Beatty (unpublished) on the rabbit. At fertilisation, Thibault observed spindle rotation and migration in the eggs, emission of the second polar body, and polyspermy; he obtained no evidence of pronuclear growth or of gynogenetic development. Beatty examined 5½ day rabbit blastocysts resulting from the treated sperm; all the embryos were diploid and apparently normal, though the numbers developing after treatment were low.

The following experiment gives the results of the trypaflavine treatment *in vitro* of the sperm of the mouse, *Mus musculus*, before fertilisation. The concentrations of the chemical on the sperm covered a range which included the concentrations used in the amphibians (Hertwig, 1924; Dalcq, 1931). Embryos were examined at fertilisation, the first cleavage, at the 3½ day embryonic stage, implantation, and birth. Treatment of the sperm by trypaflavine *in vitro* necessitated the artificial insemination of the treated sperm; details of this technique have been given on p. 4.

MATERIAL AND METHODS

With one exception, the mouse stocks were the same as those used for the ultra-violet irradiation of the sperm (p. 86). The exception was a single female which was probably heterozygous for

the 'silver' factor. The recovery, preparation and examination of the embryos resulting from the treatment were exactly similar to those given for the ultra-violet experiment (p. 88).

Treatment and artificial insemination of the sperm.

In the early stages of the experiment, the sperm of each mouse male was separately treated by trypaflavine. But progress by this method was painfully slow and hence the alternative method was adopted of mixing the sperm of several males. The size of the containers was arranged so that the depth of sperm suspensions at 2mm. was the same for the individual and the mixed sperm samples. The sperm suspension was 0.75% saline.

Trypaflavine has a photochemical action on the sperm (Drebinger, 1951). Immediately after the addition of the trypaflavine to the sperm, the resulting mixture was placed at 30 cm. distance beneath a 100W lamp for 10 minutes. The activity of the sperm was checked under the microscope before and after the illumination. The treated sperm was then artificially inseminated into oestrous females withminimum delay. After completion of the inseminations, the activity of the sperm was re-checked. A mixed sperm suspension usually inseminated three or four females; an individual sample averaged one inseminated female.

The trypaflavine, obtained from Messrs. Bayer Products Ltd., Dublin, was made into a solution of ten times the required concentration by weight in 0.75% saline immediately before use. Addition of this solution to the pure sperm suspension in the ratio 1:9 achieved the desired concentration on the sperm. The trypaflavine concentrations in the sperm suspensions were between

1/5,000 and 1/100,000 by weight.

RESULTS.

1. The effect of trypaflavine on sperm motility.

Only the two highest concentrations, 1/5,000 and 1/7,500, reduced sperm motility, though not all of the sperm samples were affected. For confirmation of this effect, sperm samples were illuminated for 20 minutes; reduced motility in these samples was pronounced at concentration 1/5,000, and noticeable at concentration 1/10,000. Sperm illuminated for 20 minutes was not inseminated into females.

2. Results at the 3½ day embryonic stage.

Examination of the 3½ day embryos taken from inseminated females at first indicated that development was normal, though the numbers developing were reduced at the higher concentrations. When the experiment was about half completed, various highly retarded embryos were noted for the first time; these must have been overlooked previously. In the results given in Table 17, analysis of the fertility of the inseminated females and of the numbers of embryos has been made using the normally-developing embryos only. No quantitative estimate of the retarded embryos was attempted.

Analysis of the fertility of the inseminated females.

The percentage of females with one or more normal embryos steadily increased with decreasing concentration from 0% at conc. 1/7,500 to 80% at conc. 1/1000,000 (Text-fig. 8). Large numbers of females established this effect with accuracy at the

semi-lethal concentrations. Controls, inseminated with untreated sperm, gave 70.2% of the females with one or more embryos.

Analysis of the numbers of embryos and unfertilised eggs.

The mean number of eggs, both fertilised and unfertilised, taken from females at the different concentrations rose from 5.2 per female at 1/5,000 to 8.6 at 1/100,000. The low figure at the high concentration was probably due to the degeneration of unfertilised eggs, the chemical probably assisting this process. The loss of more unfertilised eggs at the higher concentrations will bias the comparison in favour of the embryos.

The percentage of the total eggs which were normal embryos was analysed in two ways: over all females, and within those females which had one or more normal embryos. Both criteria, and especially the first, showed that the percentage steadily increased with decreasing trypaflavine concentration, though the figure was below controls even at the lowest concentration (Table 17, Text-fig. 8). The highest concentration compatible with normal embryonic development was 1/10,000.

The same character was measured by a different method: the calculation of the mean number of normal embryos within females which possessed one or more of them. This method overcame the difficulty due to the degeneration of the unfertilised eggs. The results are given in Table 17. The mean increased fairly steadily from 1.8 per female at concentration 1/10,000 to 4.5 at conc. 1/100,000; controls were 5.5 per female.

Analysis of the morphological stage, and of the number of nuclei in the embryos.

The effect of the treatment on embryonic development was measured in two ways: first, the examination of live embryos for their stage of differentiation; and second, the number of nuclei in squashed preparations.

In controls, 51.8% of the developing embryos were early or medium blastocysts, the remainder were advanced morulae. Two types of embryo appeared after trypaflavine treatment: one type was normal, the other was highly retarded. The great majority of the normal embryos were blastocysts (Table 17). The retarded embryos were occasionally two- or three-celled, but in most cases appeared to be one-celled or somewhat fragmented.

The mean nuclear number of the normal embryos is given for each concentration in the Table. Each of the means was above the control figure. Some embryos possessed low numbers of nuclei for 3½ day embryos however; for example one embryo had 15 nuclei, two had 11 nuclei, and one had only 5. These formed a link with the highly retarded embryos which were at first overlooked. In many cases it was doubtful if the latter had cleaved at all (Fig. 61), for the nuclei were often indistinct and observation of the embryos before squashing often failed to show evidence of cleavage. The number of nuclei in these retarded embryos was one, two, or three; they were found at all concentrations except the lowest (1/100,000). 3½ days gestation was obviously far too late to examine these embryos; evidence from the squashes must therefore be interpreted with some reservations. Further evidence of highly delayed and abnormal development will be given later.

The pH of the sperm media influences the inactivation of amphibian sperm chromosomes by trypaflavine (Hertwig, 1924). 0.75% saline consistently gave an acid reaction (pH between 5 and 6). To ensure that the retarded development was not due to acidity, the saline was buffered in several sperm samples. The buffers used were: Pannet-Compton saline (pH app. 7.0), Baker's glucose saline (pH.7), a saline-borate suspension (pH app. 9.0), and a saline-bicarbonate suspension. Nineteen females were inseminated with trypaflavine sperm in these suspensions, the concentration of the chemical being 1/10,000. Only unfertilised eggs or highly retarded embryos were taken from these females; no normal embryos were found. The results given in this paragraph have not been included in any of the Tables or Figures, and will not be considered further in the presentation of the results.

Analysis of the chromosome counts of the embryos.

The diploid complement of *Mus musculus* is 40 (Matthey 1949). Details of chromosome counts on the experimentally-produced embryos are given in Table 17. Only the normal embryos were classifiable; none of the retarded embryos had recognisable mitotic chromosomes. All the embryos occurring at or below concentration 1/15,000 were diploid. After conc. 1/10,000 one haploid (Fig. 62, 65), one triploid and seven diploid embryos were found. As the experiment was designed to induce haploid gynogenetic development, the discovery of the haploid was at first considered to indicate the success of the method. But the female which donated the haploid embryo was possibly heterozygous for the gene 'silver'; mice which are homozygous for this gene produce a number of spontaneous

haploid embryos after normal mating (Beatty, 1954; Beatty and Fischberg, 1951). The haploid could therefore have been of spontaneous origin and quite independent of the trypaflavine treatment. Also, despite the insemination of 100 other females with sperm receiving the same treatment, no other haploid was produced; this again suggested that the haploid was not due to the treatment. After the discovery of this haploid, females which were homozygous or heterozygous for the 'silver' factor were rigorously excluded from the experiment.

The single triploid embryo had a peculiar importance to the experiment. It was possible that the diploid embryos were gynogenetic haploid embryos which had doubled their chromosome complement at the second maturation division of the egg or at the first cleavage. This suggestion would be denied if the Y chromosomes of the male was recognisable in them or if implants and offspring could be genetically marked by dominant genes derived from the male. Unfortunately, the Y chromosome was not identifiable, and the embryos did not implant or come to term for the male marker gene to be recognised, (see later). Indirect denial of chromosome doubling was observed in two ways. First, the number of nuclei in the advanced embryos was the same as, or greater than normally-developing embryos (see back). Embryonic development had therefore not been checked at the first cleavage. Second, the presence of a triploid embryo is difficult to explain by any chromosome-doubling process; it would entail development starting with 30 chromosomes. The most reasonable origin of the triploid appeared to be either the normal fertilisation of an egg which failed to extrude the second polar body, or by polyspermy.

Either of these origins implies that the sperm chromosomes were not inactivated by the trypaflavine. The triploid therefore provides evidence for the failure of the method.

In addition to the retarded embryos containing one, two, or three nuclei, some one-celled eggs occurred which possessed chromosomes but no nuclei. The chromosome complement was always haploid, and in most eggs they appeared to be meiotic (Fig. 64). The one-celled eggs occurred at all concentrations used, often in large numbers. Their origin is conjectural; they may be the remains of unfertilised eggs, though none were seen in the eggs of females mated to vasectomised males. They may have been penetrated by sperm without being activated. Lastly, they could have been rudimentary parthenogenetic eggs which had been stimulated to some activity by the presence of sperm or of trypaflavine at ovulation. Some evidence for and against these origins will be given later.

The number of nuclei in haploid, diploid, and triploid embryos.

The mean number of nuclei in the diploid embryos at each trypaflavine concentration is given in Table 17. The means were between 47.5 and 64.4; the diploid mean of controls was 44.1. Converted into cleavage number (see p. 9) the values were between 5.6 and 6.0 following trypaflavine treatment, and 5.4 for the controls. The diploids obtained after trypaflavine treatment of the sperm were therefore dividing at the same rate as normal embryos.

The haploid embryo had 40 nuclei (Fig. 62); the triploid 27.

Cytology of the embryos.

The presence of highly retarded embryos possessing one or two nuclei, and one-celled eggs possessing chromosomes only has been previously mentioned. Some of the latter also possessed sub-nuclei (Fig. 64).

In the advanced embryos, the only abnormality was that some of the mitoses contained slightly contracted chromatids.

Controls of the photochemical action of trypaflavine.

To ensure that trypaflavine had no action on the sperm in the absence of light, a solution of trypaflavine of concentration 1/4,000 by weight in 0.75% saline was injected via the cervix into the uterus of seven oestrous females. The females were then paired with males for natural mating. The sperm would become mixed with the trypaflavine *in utero* before fertilisation; and any action of the chemical would occur in the absence of light. Six of the females were killed 3½ days after mating; they yielded 47 eggs of which 39 were blastocysts or advanced morulae and eight were unfertilised. No retarded embryos were seen. All the classifiable embryos were diploid except for one which was a diploid-tetraploid mosaic.

The other three injected females were killed 13 days after mating and examined for placentae. None were seen. There was evidence of damage to the uterus by the trypaflavine. The absence of placentae may have been due to this damage, or it may have been because of a toxic effect of the trypaflavine on the embryo after 3½ days gestation. The former explanation appeared the more likely.

III

*

Since its original application by Hertwig (1924), trypaflavine has been one of the principal agents used to inactivate the chromosomes in amphibian sperm before fertilisation. Thibault (1949) treated ram sperm with trypaflavine, but obtained no gynogenetic development of the egg. Beatty (unpublished) examined 3½ day rabbit blastocysts resulting from trypaflavine-treated sperm and found that the numbers of embryos recovered were low but all were diploid & apparently normal.

Recent work by Briggs (1952) has shown that the basic dye toluidine blue effectively inactivates the sperm chromosomes of the frog Rana pipiens before fertilisation, & that over 90% of the resultant embryos were haploid. Uehlinger (1951) showed that trypaflavine acted photochemically on the sperm, & Briggs confirmed the same method of action by toluidine blue. No report has appeared concerning the use of toluidine blue in mammals.

Several experimental agents which inactivate the chromatin of Amphibian sperm before fertilisation and so induce haploid gynogenetic development of the egg have been used on the sperm of the mouse, Mus musculus, for a similar purpose. Previous papers (Edwards, 195 a & b) have briefly reviewed earlier work on the induction of gynogenesis in mammals, & described the effects on embryonic development of the X-irradiation or ultraviolet irradiation of mouse sperm before fertilisation. This paper describes in detail an attempt to induce gynogenesis in the mouse by treating the sperm in vitro by trypaflavine or toluidine blue before fertilisation. Some of this work has been briefly reported (Edwards 1954 b).

The primary evidence for induced gynogenesis was considered to be the occurrence of haploid embryos at 3½ days gestation. Some eggs were then examined after fertilisation & the first cleavage, others allowed to develop beyond 3½ days.

The large numbers of normal embryos taken from the injected females at 3½ days after mating confirmed that, in the absence of light, the trypaflavine had little or no effect on the sperm.

3. Observations at fertilisation and the first cleavage.

Observations on sectioned eggs were made after treatment of the sperm with concentrations 1/10,000 and 1/15,000. Seven females were killed between six and ten hours after mating to study sperm entry and pronucleus formation in their eggs. Eleven females were killed between 36 and 42 hours after mating to observe the fate of the pronuclei and the results of syngamy and the first cleavage. Details of the observations on the eggs are given in Table 18.

Sperm entry and pronucleus formation.

Some of the females may have been killed before sperm entry into the eggs had commenced, for the majority of their eggs were unfertilised. After concentration 1/10,000, 45 eggs were examined, of which three had two pronuclei each. One egg, which was still in the metaphase of the second meiotic division, had a sperm-head beneath the zona pellucida. Two other eggs had chromosomes in the centre of the egg instead of at the periphery; no sperm-head could be seen in these eggs but the appearance of the chromosomes suggested that spindle rotation had occurred. These two eggs may therefore have been activated. After conc. 1/15,000, all of the 19 eggs were unfertilised. Delayed fertilisation may have been induced by the treatment.

Syngamy and the first cleavage.

Three types of egg were observed at this stage. One type had developed normally to the two- or four-celled stage, another type was undergoing the normal degeneration of unfertilised eggs. The third type had various abnormalities.

After concentration 1/10,000 a total of 41 eggs were examined; three were two- or four-celled, one of the four-celled eggs having a sub-nucleus in one of its blastomeres. Conc. 1/15,000 yielded two normal two-celled embryos out of a total of 39 examined. If numbers at the two concentrations are combined, the percentage of normally-developing embryos was higher than the percentage seen at 3½ days gestation.

The abnormal eggs had the same deformities after both concentrations. All of them were one-celled. Some eggs had their chromosomes scattered around the cytoplasm (Fig. 65), others had chromosomes which were in the form of a telophase (Fig. 66). Two eggs had sperm-heads in the vitellus yet the second meiosis had not been activated and was showing normal degeneration typical of unfertilised eggs (Fig. 67 a & b). Three other eggs had sperm-heads either in the vitellus or beneath the zona, these also had not been activated. The last two kinds of abnormality showed that sperm penetration of the vitellus was not necessarily synonymous with activation of the egg. Altogether, thirteen of the eggs examined were of one of the above types. In most of the eggs, the sperm-head was not directly observed; the abnormalities may have been due to induced parthenogenesis, to premature degeneration, or to fertilisation by an abnormal sperm which was overlooked in the examination. In the study of the normal degeneration of

unfertilised eggs, none of these occurrences were seen in 30 eggs taken from females killed between 36 and 42 hours after mating to a vasectomised male.

The remainder of the eggs had the appearance of normal unfertilised eggs, i.e. they possessed the remains of a degenerate spindle with the chromosomes still attached.

4. Observations at implantation, birth and maturity.

To follow embryonic development beyond 3½ days gestation, females which had been inseminated with trypaflavined sperm were either killed during pregnancy and examined for the presence of placentae or young, or allowed to go to full term for the birth of litters. Three concentrations of trypaflavine were tested: 1/10,000, 1/25,000, and 1/100,000. At the highest concentration, examination for placentae and embryos was made at 14 days gestation; at the two lower concentrations the females were examined for placentae when they were seen to be not pregnant.

Sperm treatments with concentrations 1/10,000 and 1/25,000 yielded neither placentae nor young. At the higher concentration 14 females were examined at 14 days gestation and three were allowed to go to full term; at the lower concentration five females were examined at 16 days gestation and 4 allowed to term.

At conc. 1/100,000, no placentae were seen in three females examined at 17 days gestation, though the remains of one placenta was seen in a female which gave no young at full term. Two litters were obtained from five other females. One, a litter of three or four, was immediately eaten by the mother. The other, a litter of seven, grew to maturity. The sire of this litter was

genetically homozygous for dominant black eye colour, the mother homozygous for recessive pink-eye. All the offspring had black eyes, so the male chromatin must have partaken in embryonic development. The sex ratio in this litter was five females to two males.

DISCUSSION.

With two exceptions, all the classifiable 3½ day embryos were shown to be diploid; the two exceptions were a haploid and a triploid, and both occurred at concentration 1/10,000. Chromosome counts were possible only in embryos which were developing at about the same rate as controls. The proportion of the total number of eggs with normal development steadily increased with decreasing concentration, from 2.2% at concentration 1/10,000 to 41.9% at concentration 1/100,000. The use of eye-colour genes in these embryos which developed to full term gave direct demonstration of their hybrid origin at the latter concentration. They were therefore not gynogenetic. This direct identification of parentage was not possible for the embryos produced at the higher concentration however, for none developed to the stage of eye pigment deposition. But three kinds of indirect evidence indicated that they too were normal hybrids. First, observations on newly fertilised eggs showed that the male and female pronuclei both formed normally and, judging by the absence of degenerate chromatin in two- and four-celled eggs, that both contributed to embryonic development. Second, the identification of a triploid embryo was evidence of the presence of sperm chromatin in it (see p.152). Thirdly, the gradual decline in number of normal

embryos with increasing concentration is symptomatic of a steadily increasing inhibition by the chemical of normal fertilisation and development.

The diploids and the triploid were therefore presumably hybrid. The effect of the treatment was to reduce the number of them at high concentrations of trypaflavine. The only embryo with normal development which could not be fitted into this classification was the haploid. This may have been a gynogenetic embryo induced by the treatment. But it was taken from a female possibly heterozygous for the gene 'silver', and may have been of spontaneous origin (Beatty, 1954). Against its spontaneous origin is the very low incidence of such haploids even after selection for spontaneous heteroploids over several generations (Beatty, 1954), whereas this particular haploid occurred as one of two embryos taken from the only 'silver' female inseminated in the experiment. Its origin may therefore have been due to a combination of the treatment and spontaneity. The trypaflavine induced one triploid embryo out of nine classified at the same concentration that induced the haploid, and, in the without-light controls, induced a diploid tetraploid mosaic. Seemingly, therefore, the chemical may have an effect on the spindle mechanism of the egg or of a dividing blastomere. This effect was also directly observed in some presumably unfertilised eggs examined a few hours after mating; no spindle was seen in these eggs and the meiotic chromosomes had scattered in the egg cytoplasm. Interference with the meiotic spindle of the egg may have led to all of the maternal chromosomes being retained in the egg, or to all of them entering the second polar body at fertilisation, or, by a

similar action to that after colchicine, (see p. 47) to them being expelled from the egg after scattering had occurred. Fertilisation of these affected eggs would produce either triploid or androgenetic haploid embryos; this might have been the method of origin of the triploid and haploid observed.

If the above deductions are correct, all of the normally-developing embryos found after treating the sperm with trypaflavine contained an unreduced sperm chromosome complement. None of them was gynogenetic. One class of embryo remains to be considered: those that were highly retarded at 3½ days gestation. A few of these had three nuclei, others had one or two. Some had no nuclei, but possessed only meiotic chromosomes with or without sub-nuclei. A few of these embryos had cleaved, others had apparently not. Examination of eggs at the normal time of pro-nucleus formation or the two-celled stage revealed that some embryos did commence an abortive development. Mention of the disappearance of the spindle in some of these eggs has already been made; other eggs had other kinds of spindle and chromosomal behaviour in that they formed anaphase- of telophase-like nuclei. Sperm-heads were not detectable in many of these embryos; the sperm may have degenerated after penetration, become unstainable because of the experimental treatment, or such eggs may have been parthenogenetic. Other eggs were penetrated, and these were of two types: normally stimulated eggs either containing a normal pronuclear content or possessing normal blastomeres, and eggs with an unchanged sperm-head in the vitellus and a maturation division still at metaphase. The proportion of pronucleate ova and cleaved eggs was higher than the proportion of embryos developing normally

at 3½ days gestation after similar sperm treatments. Some of the pronucleate ova or cleaving embryos may have therefore degenerated without further development, but the numbers examined were probably too low for statistical comparison.

The observation of penetrated eggs which had not been stimulated to complete their maturation division may explain the occurrence of the uncleaved eggs containing meiotic chromosomes at 3½ days gestation. In these eggs, the sperm-head had failed to differentiate into a pronucleus. Penetration without activation of the egg was also a feature of high dosages of X-rays on the sperm (p. 131), and probably occurred after the ultra-violet irradiation (p. 101). Two all-or-none responses of the egg must occur at fertilisation: the block to polyspermy achieved by the zona reaction (Braden, Austin and David, 1954), and the stimulation to complete the second meiotic division. The two events are probably related, for it was noticed that one of the three unstimulated eggs penetrated by X-irradiated sperm was polyspermic. Trypaflavine treatment of the sperm may also induce polyspermy in association with failure to stimulate completion of the second maturation division, for one of the unstimulated eggs had another sperm-head in either the vitellus or the perivitelline space. Unfortunately, sperm-heads can easily be overlooked in sectioned eggs: even in controls the sperm-head can often not be detected. However, Thibault (1949) reported that polyspermy occurred after trypaflavine treatment of sheep sperm, and he also noted that, apart from spindle rotation, some eggs failed to respond to sperm entry. Trypaflavine

apparently reduces the capacity of sperm to penetrate eggs and, of the eggs which are penetrated, some fail to acquire the block to polyspermy and to complete their second maturation. The loss of stimulating power of the sperm appeared traceable to the illumination of the sperm-chemical mixture, for in the no-light controls fertilisation and development occurred despite the use of higher trypaflavine concentrations. Drebinger (1951) showed that the inactivation of amphibian sperm chromatin by trypaflavine was essentially photodynamic, and that the cytoplasm of the sperm was affected by the treatment. His observations may also apply to mammalian sperm.

Two kinds of embryonic development occur after trypaflavine treatment of the sperm before fertilisation: one if similar to control, the other is highly retarded (see also Thibault, 1949). They occurred over a wide range of concentrations, and probably over wide pH range. Delayed fertilisation is unlikely to have induced such retarded development (Gates and Beatty, 1954). If the solitary haploid is excluded because of its doubtful origin, there was no definite evidence of gynogenetic development or of a Hertwig phenomenon (Hertwig, 1911), though the retarded embryos may have been gynogenetic or parthenogenetic. The failure to induce haploid gynogenesis in the mouse, sheep (Thibault, 1949), or rabbit (Beatty, unpublished) by this technique is in sharp contrast to the success of the same method in amphibians. Fertilisation with trypaflavine-treated sperm stimulates the amphibian egg to develop as a haploid to advanced larval stages without the participation of the sperm chromatin (Hertwig, 1924; Dalcq, 1931).

Embryos which were developing normally at 3½ days gestation came successfully to term after the lowest dye concentrations in the sperm suspensions, but failed to implant after higher concentrations. The death of these embryos may have been due to induced genetic effects in the sperm chromatin as has been suggested in amphibians (Hertwig, 1924). But the cause of death may have been entirely accidental, and due to the effect of the chemical on the uterine tissues of the mother.

SUMMARY.

1. Sperm of the mouse, Mus musculus, was treated with various concentrations of trypaflavine in vitro before fertilisation.
2. With increasing concentration of dye, the number of normal embryos at 3½ days gestation steadily decreased; none were found at concentration 1/7,500 by weight and above. In addition to the normal embryos, many highly retarded embryos occurred over a wide range of concentrations.
3. Chromosome counts were possible in the normal embryos only. Except for a single haploid which was possibly not gynogenetic, no certain evidence of the induction of gynogenesis was found. Histological examination of eggs up to 42 hours after mating indicated that some penetrated eggs were not activated, other eggs may have commenced an abortive parthenogenesis, and that trypaflavine sometimes destroyed the spindle of the second maturation metaphase of the egg.
4. Implantation was suppressed by concentrations 1/10,000 and 1/25,000. Damage to the uterine tissues rather than embryonic mortality was probably responsible.

REFERENCES.

BEATTY, R.A. 1954. Haploid rodent eggs. Proc. 9th Intern. Congr. Genetics, Bellagio.

BEATTY, R.A. & FISCHBERG, M. 1951. Heteroploidy in mammals: I Spontaneous heteroploidy in pre-implantation mouse eggs. J. Genet. 50, 345-359.

BRADEN, A.W.H., AUSTIN, C.R. & DAVID, H.A. 1954. The reaction of the zona pellucida to sperm penetration. Aust. J. Biol. Sci. 7, 391-409.

DALCQ, A. 1931. Contribution à l'analyse des fonctions nucléaires dans l'ontogénèse de la grenouille. I Etude cytologique des oeufs fécondés par du sperme trypaflaviné. Arch. Biol. 41, 143-220.

DREBINGER, K. 1951. Kerngifte und Lichtstrahlung. Eine Studie an Froschspermien zur Wirkungsanalyse der Kerngifte. Arch. EntwickMech. 145, 174-204.

GATES, A.H. & BEATTY, R.A. 1954. Independence of delayed fertilization and spontaneous triploidy in mouse embryos. Nature 174, 356-357.

HERTWIG, O. 1911. Die Radiumkrankheit tierischer Keimzellen. Arch. mikr. Anat. 77, 97-164.

HERTWIG, G. 1924. Trypaflavin als Radiumersatz zur Gewinnung haploid kerniger Kroschlarven. Verh. Anat. Ges. 58, 223-227.

MATTHEY, R. 1949. Les Chromosomes des Vertébrés. F. Rouge, Lib. Univ., Lausanne.

THIBAULT, C. 1949. L'oeuf des mammifères, son développement parthénogénétique. Ann. Sci. Nat., Zool. 11, 136-219.

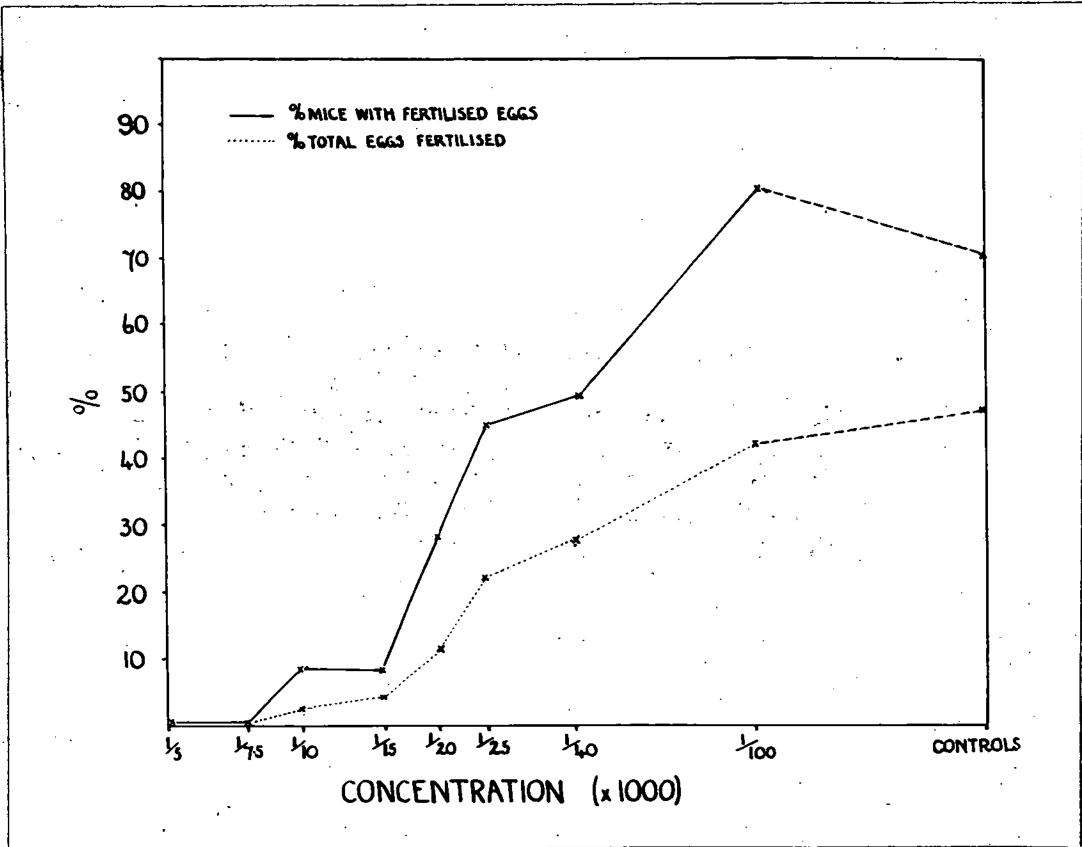
TABLE 17. DETAILS OF THE FERTILISATION OF THE INSEMINATED FEMALES AND OF THEIR EMBRYOS AT 3½ DAYS GESTATION.

	SPERM	FEMALES			EGGS AND EMBRYOS							CHROMOSOME COUNTS OF EMBRYOS (2n=40)					NUCLEAR NOS. OF EMBRYOS	
Dye concentration (x 1000)	No. of sperm treatments	No. of ovulated ♀♀ used	No. of ♀♀ with embryos	% total ♀♀ with embryos	Total no. of eggs found	No. of eggs from ♀♀ with embryos	Total no. of embryos found	% of total eggs which were embryos	% embryos of total eggs from ♀♀ with embryos	Mean no. of embryos from ♀♀ with embryos	% embryos which were blastocysts	Lost or unclassified	Haploid	Diploid	Probably diploid	Above diploid	Mean no. of nuclei of all normal embryos	Mean no. of nuclei in diploid embryos
1/5	2 +5 ind.	13	-	0	67	-	-	0	0	0	-	-	-	-	-	-		
1/7.5	4	17	-	0	100	-	-	0	0	0	-	-	-	-	-	-		
1/10	31 +5 ind.	101	9	8.9	727	70	16	2.2	22.9	1.8	62.5	7	1	4	3	1(3n)	46.9±14.4	51.1±15.2
1/15	13	48	4	8.3	287	34	12	4.2	35.3	3.0	100	7	-	2	3	-	55.9±18.4	63.6± 5.2
1/20	6	21	6	28.6	153	84	19	12.4	22.6	3.2	63.2	11	-	8	-	-	42.6±15.7	47.5±11.8
1/25	8	26	12	46.2	132	98	30	22.7	30.6	2.5	90.0	15	-	12	3	-	50.3±18.0	52.4±15.5
1/40	2	6	3	50.0	48	30	13	27.1	43.3	4.3	100	3		5	5	-	55.5±11.4	54.1±10.9
1/100	2	5	4	80.0	43	37	18	41.9	48.6	4.5	100	9		5	4		57.9±15.8	64.4±10.7
Controls	-	47	33	70.2	394	278	182	46.2	65.5	5.5	51.8	109	-	56	15	2(3n)	41.2±15.7	44.1±15.4

TABLE 18. DETAILS OF THE EGGS EXAMINED AT SYNGAMY AND THE FIRST CLEAVAGE.

Stage of development of controls at similar times of examination	Histological observation on the egg	Trypaflavine concentration on sperm	
		1/10,000	1/15,000
Pronucleate	No metaphase spindle, chromosomes in centre of egg	2	
	Sperm-head beneath zona	1	
	Two normal pronuclei	3	
	Unfertilised	39	19
2-celled or 4-celled	Normal 4-celled	1	
	Normal 2-celled	1	2
	4-celled with sub-nucleus in one blastomere	1	
	Unfertilised egg, chromosomes scattered through cytoplasm	1	1
	Sperm-head beneath zona, egg unstimulated	3	
	Egg possessing a group of chromosomes resembling a telophase	1	5
	Sperm-head in egg, second maturation at metaphase	1	1
	Unfertilised	32	30

Text-fig. 8. Percentage of mice with fertilised eggs, and the percentage of the total eggs which were fertilised after the addition of various concentrations of trypaflavine to the sperm before fertilisation. "Fertilised eggs" is restricted to normally-developing embryos only; the highly retarded embryos have been excluded.



% MICE WITH FERTILISED EGGS
% TOTAL EGGS FERTILISED
90
80
70
60
50
40
30
20
10
%
1/5
1/7.5
1/10
1/15
1/20
1/25
1/40
1/100
CONTROLS
CONCENTRATION (x 1000)

FIGURES

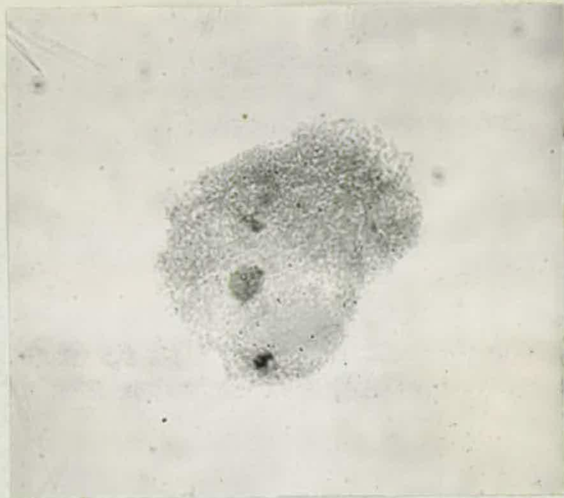
None of the negatives were retouched.

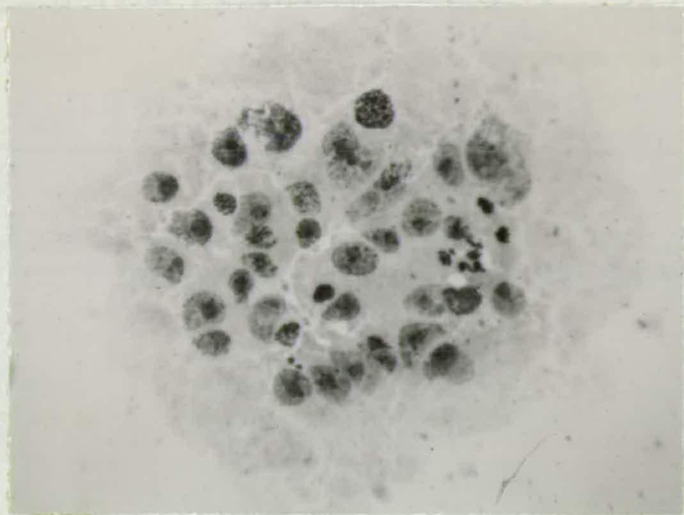
61. A squashed $3\frac{1}{2}$ day old embryo. It is highly retarded in its development, possessing two or three nuclei (cf. Fig. 8). x 350.

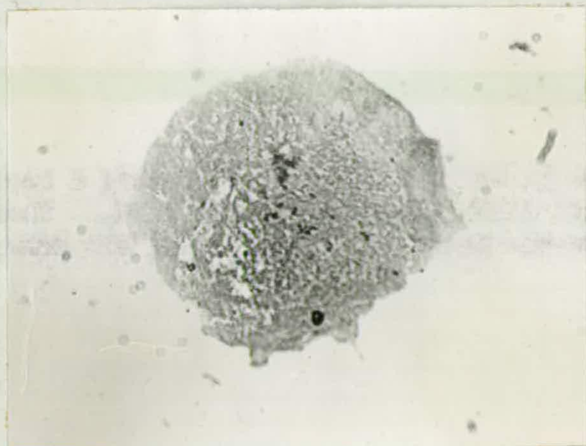
62. Squash preparation of the only haploid embryo found in the experiment. x 350.

63. The mitotic complement of the haploid embryo. The metaphase has 20 ± 1 chromosomes; the anaphase is small in comparison with diploid controls. x 2000.

64. A squashed $3\frac{1}{2}$ day old embryo containing a haploid set of chromosomes which appear to be meiotic. There is also a small pycnotic patch of chromatin in the embryo. x 350.



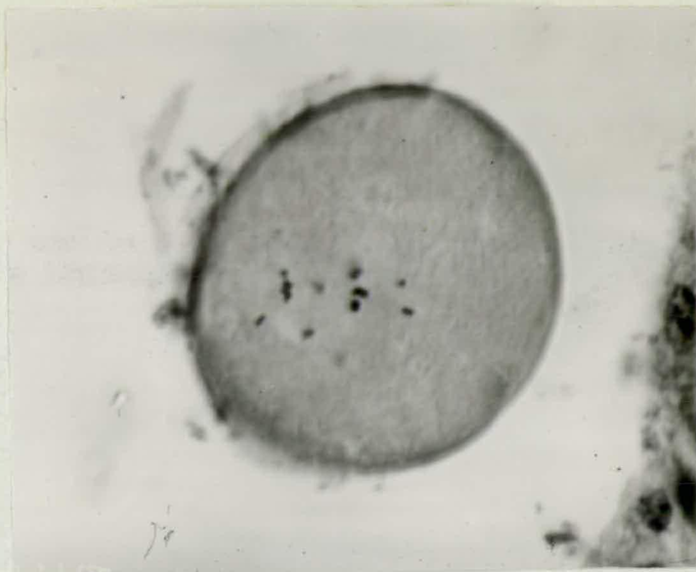


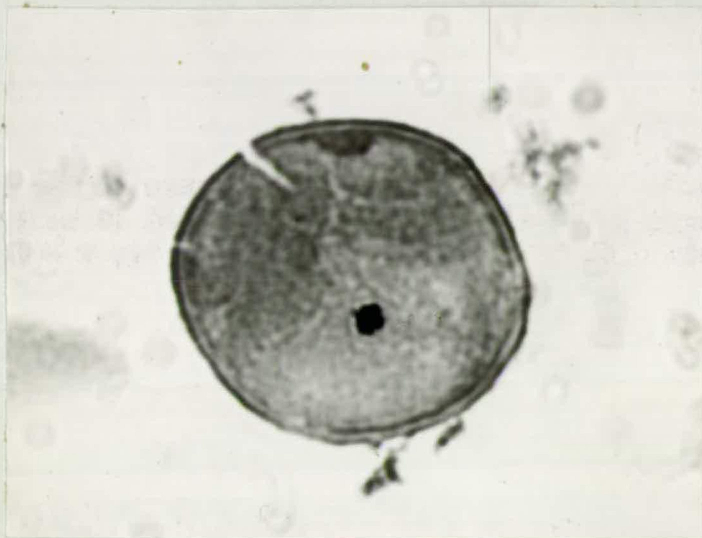


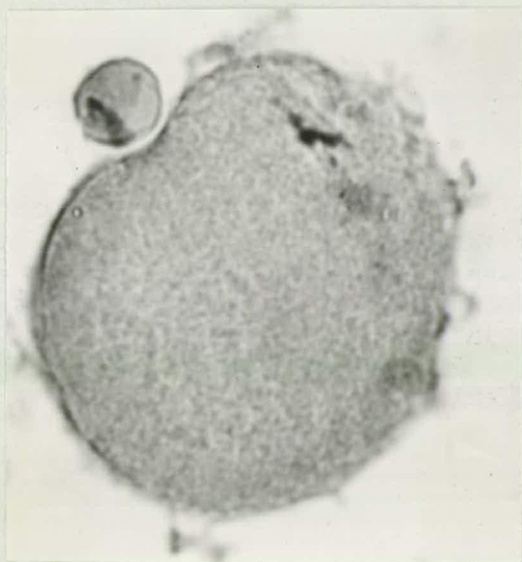
65. The chromatids of an egg scattered through the ooplasm 40 hours after the insemination of trypaflavined sperm into the mother. Sectioned egg x 1000.

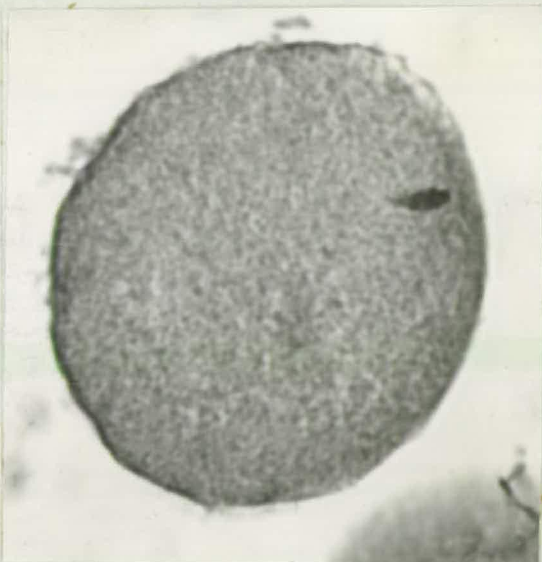
66. A one-celled egg containing chromosomes in the form of a telophase. The mother was inseminated 42 hours previously, with trypaflavined sperm. Sectioned egg x 1000.

67a and b. Two sections of an egg taken from a mother killed 42 hours after being inseminated with trypaflavined sperm. The metaphase of the second maturation division of the egg has not been stimulated by sperm entry and shows the degeneration typical of unfertilised eggs (cf. Fig. 2). The sperm-head lies unchanged in the vitellus. x 1000.









4. THE COMBINED EFFECT OF TRYPAFLAVINE AND ULTRA-VIOLET ON THE SPERM.

The following small experiment was carried out to determine whether a combination of two sperm treatments would inactivate the male chromatin more effectively than either by itself. The two treatments selected were trypaflavine and ultra-violet; the former probably has a fluorescent effect which might be increased by the use of the latter. The addition of trypaflavine to the sperm, and the ultra-violet irradiation of the mixture, were exact combinations of the separate techniques previously described, (p. 147 and 87).

The experiment consisted of two parts. The first was made with a standard illumination time of 30 seconds ultra-violet after treatment of the sperm with trypaflavine concentrations between 1/25,000 and 1/100,000. The second part utilised a standard trypaflavine conc. of 1/200,000 by weight and ultra-violet irradiation of either ½, 3, or 10 minutes. The resulting embryos were examined at 3½ days gestation as squashed; details of the squashing technique have been given previously (p. 6).

RESULTS.

Analysis of the fertility of the females and of the number of embryos.

A total of eight mice were inseminated with sperm treated with trypaflavine between concentrations 1/25,000 and 1/100,000 and 30 seconds ultra-violet. Only one female possessed embryos; one of them was a normal diploid blastocyst, the others were highly retarded and had very few nuclei. The trypaflavine

concentration was then reduced to 1/200,000 and the ultra-violet irradiation increased to ½,3, or 10 minutes. 17 females were inseminated with this sperm; ten of them possessed developing embryos at 3½ days gestation. Details of the experimental results are given in Table 19.

The number of embryos expressed as a percentage of the total number of eggs was analysed over all females and within females with embryos. The percentage declined with increasing ultra-violet illumination. The mean number of embryos taken from females with embryos also declined similarly.

Embryonic development was adversely affected by increased ultra-violet illumination. The mean number of nuclei in the embryos decreased from 33.6 for ½ minute illumination to 7.2 for 10 minutes. In terms of the number of cleavages, this decline was from 5.0 to 2.9. Comparable mean nuclear numbers following ultra-violet irradiation only were 26.2 after ½ minute illumination and 5.3 after 10 minutes. The two types of embryo - one apparently normal, the other retarded - which were observed after ultra-violet irradiation alone were also observed following the combined trypaflavine and ultra-violet treatment.

The percentage of embryos which were blastocysts declined with increased ultra-violet illumination; none were seen in the two females which received the most heavily-irradiated sperm. This result was also similar to ultra-violet irradiation alone (p. 92).

<u>Chromosome counts and cytological observations on the embryos.</u>

No haploid embryos were found in the experiment. Apart from

the diploids, which occurred at all dosages, one hypo-diploid of 37 chromosomes, one hyper-diploid of 47 chromosomes, and one tetraploid were found. They possessed 26, 12, and 11 nuclei respectively.

Many one-celled eggs contained chromosomes and no nuclei; the chromosomes appeared to be meiotic in all cases. Other cytological anomalies included sub-nuclei in many embryos, and, in a few embryos, the presence of pycnotic nuclei.

DISCUSSION.

The use of either trypaflavine or ultra-violet as a separate sperm treatment induced an all-or-none effect on subsequent embryonic development. After trypaflavine alone, embryos are either almost normal or excessively retarded. Ultra-violet alone induces an all-or-none immobilisation of the sperm chromatin, which results in haploid or diploid embryonic development; it also has deleterious effects on development. Combination of the two was intended to inactivate the sperm chromatin without inducing the abnormalities associated with haploid development. Unfortunately, no haploid embryos were observed after the treatment, but the numbers of embryos examined was low. The results show, however, that the combination of the two methods resulted in the same abnormalities induced by each treatment used separately. If the ultra-violet irradiation was held constant at 30 seconds and the trypaflavine concentration varied, embryonic development was either normal or highly retarded. Reduction to a very dilute dye concentration and variation in the length of the ultra-violet irradiation removed some of the pronounced effect

of trypaflavine, but superposed on the remainder the typical developmental effects caused by ultra-violet alone.

Embryonic development after the combined sperm treatment suggested that either the trypaflavine or the ultra-violet individually affected the sperm, depending upon which was present in the greater relative strength. Most of the highly retarded embryos, and the decrease on the fertility of the females, were presumably due to the dye; the less retarded embryos and the hypo-and hyper-diploid chromosome counts were probably induced by the radiation. As the respective actions of the two treatments do not appear to be complementary, the method is probably of little use for the induction of haploid gynogenesis in the mouse.

TABLE 19. DETAILS OF THE FERTILITY OF THE INSEMINATED FEMALES AND OF THEIR EMBRYOS AT 3½ DAYS GESTATION.
10 minutes exposure is equal to 0.5 Joules per sq. cm. on the surface of the sperm suspension.

TREATMENT		SPERM	FEMALES			EMBRYOS							CHROMOSOME COUNTS OF EMBRYOS (2n=40)						EMBRYO NUCLEAR NOS.
Dye concentration (x 1000)	U.V. exposure (mins.)	No. of sperm treatments	No. of ovulated ♀♀ used	No. of ♀♀ with embryos	% total of ♀♀ with embryos	Total no. of eggs found	No. of eggs from ♀♀ with embryos	Total no. of embryos found	% of total eggs which were embryos	% embryos of total eggs from ♀♀ with embryos	Mean no. of embryos from ♀♀ with embryos	% embryos which were blastocysts	Lost or unclassified	Haploid	Between haploid and diploid	Diploid	Probably diploid	Above diploid	Mean no. of nuclei of all embryos
1/25	½	1	3	-	0	20	-	-	0	0	0	-	-	-	-	-	-	-	
1/50	½	1	5	1	20.0	37	8	5	13.5	62.5	5	20.0	4	-	-	1	-	-	
1/100	½	1	1	-	0	5	-	-	0	0	0	-	-	-	-	-	-	-	
1/200	½	2	8	5	62.5	85	63	36	42.4	57.1	7.2	38.9	22	-	1(37)	10	1	1(47) 1(4n)	33.6±19.3
1/200	3	2	6	3	50.0	60	31	16	26.7	51.6	5.3	6.3	12	-	-	4	-	-	19.2±16.6
1/200	10	2	3	2	66.7	25	21	9	36.0	42.9	4.5	0	8	-	-	1	-	-	7.2± 4.3
Controls		-	47	33	70.2	394	278	182	46.2	65.5	5.5	51.8	109	-	-	56	15	2(3n)	41.2±15.7

5. TREATMENT OF THE SPERM BY TOLUIDINE BLUE.

INTRODUCTION.

Recent work by Briggs (1952) has shown that the basic dye toluidine blue is a most effectivo chemical agent for the inactivation of the sperm chromosomes before fertilisation of eggs of the frog *Rana pipiens*. Under certain conditions of dye concentration, over 90% of the resulting embryos were haploid.

No report has appeared concerning the use of toluidine blue in mammals. To investigate whether the result obtained by Briggs in the frog might be obtained in mammals, the sperm of the mouse, *Mus musculus*, has been treated by toluidine blue *in vitro* before fertilisation. The conditions of the experiment, and the concentrations of the dye-sperm mixutre, were the same as in the experiment described previously where trypaflavine was added to the sperm (p. 146). The resultant embryos were examined at 3½ and 12 days gestation. Treatment of the sperm by toluidine blue *in vitro* necessitated the artificial insemination of the treated sperm; details of this technique have been given on p. 4.

MATERIAL AND METHODS.

Details of the mice used, treatment of the sperm and artificial insemination, and examination of the resultant embryos at 3½ and 12 days gestation, were similar to those described for the treatment of mouse sperm by trypaflavine (p. 146). No 'individual' sperm samples were treated by toluidine blue; only mixed samples were used. Mothers carrying the factor 'silver' yield a low incidence of spontaneously haploid embryos at 3½ days gestation (Beatty, 1954; Beatty and Fischberg, 1951); these

females were excluded from the experiment.

The toluidine blue was the standard stain of the British Drug Houses, Ltd., London. Dye concentrations in the sperm suspensions were between 1/5,000 and 1/100,000 by weight.

RESULTS.

1. The effect of toluidine blue on sperm motility.

At all concentrations the activity of the sperm was considerably affected after illumination. In many samples the activity of the sperm was completely lost, and all the sperm were motionless. But, to observe the motility of the sperm, an ordinary light microscope was employed; and the necessary use of a fairly strong beam of light adversely affected sperm activity. By focussing the sperm with the reflecting mirror averted, and then adjusting the mirror so that light beams passed through the sperm to the eye piece, it was possible to observe the almost immediate cessation of sperm activity. A considerable photo-dynamic action was obviously exerted on the sperm. A small test to discover any recovery of motility after removal of the illumination gave no definite result; possibly a slight recovery occurred. Despite the effect on the motility of the sperm, its fertilising power was not completely lost as was shown by the discovery of embryos in females inseminated with it.

2. Results at the $3\frac{1}{2}$ day embryonic stage.

Examination of the $3\frac{1}{2}$ day old embryos taken from inseminated females at first suggested that development was normal, but the numbers of embryos found after the higher concentrations were low.

When the experiment was about half complete, various highly retarded embryos were noted for the first time; these must have been overlooked previously. In Table 20, analysis of the fertility of the females and of the numbers of embryos has been made, using the normal embryos only. No estimate of the numbers of retarded embryos was attempted.

Analysis of the fertility of the inseminated females.

The percentage of females with one or more normal embryos increased from 0% at conc. 1/7,500 to 50% at 1/100000, the rise commencing somewhat abruptly at conc. 1/10,000. The percentages were consistently lower than controls (Table 20). The increase was probably gradual, but obscured by sampling variations.

Analysis of the numbers of embryos and unfertilised eggs.

The mean number of eggs, both fertilised and unfertilised, taken from females at the different concentrations varied between 4.9 and 7.5. As this variation was not correlated with concentration, the chemical probably had little effect on the degeneration of the unfertilised eggs. The percentage of the total number of eggs which were developing normally was analysed in two ways: over all females, and within those females which had advanced embryos. Due to the degeneration of some unfertilised eggs, the percentage will be biassed in favour of the embryos. Both criteria indicated that the percentage steadily increased with decreasing toluidine blue concentration, except for a rise at concentration 1/10,000. This concentration was the highest at which normal 3½ day old embryos were obtained, but after conc. 1/7,500 normal development of an embryo to 13 days gestation occurred in one female.

The mean number of normal embryos within females with normal embryos was calculated for each concentration. Fertility was reduced at all concentrations, the mean being constantly lower than the controls.

Analysis of the morphological stage and number of nuclei of the developing embryos.

Two types of embryo resulted after toluidine blue treatment of the sperm. One type was normal, the embryos being either blastocysts or advanced morulae. The other type was highly retarded; occasionally they possessed two or three cells, but more often appeared to be one-celled or somewhat fragmented. A similar result occurred after trypaflavine treatment (p. 150).

The mean nuclear number of the normal embryos at each concentration is given in Table 20. Apart from two embryos which had 17 and 19 nuclei respectively, the number of nuclei in these embryos was always 25 or above. All the means were below controls, but only very slightly so, except at concs. 1/10,000 and 1/40,000. Converted to cleavage number, the means were all between 4.9 and 55 after toluidine blue, controls being 5.5. Clearly there was little difference between the experimental and control embryos.

The retarded embryos possessed one or two nuclei, occasionally with sub-nuclei. Most of them had probably failed to cleave. The $3\frac{1}{2}$ day embryonic stage was obviously far too late to make observations on these embryos, and any conclusions about them must be tentative.

A high pH of the sperm media can increase the amount of the inactivation of amphibian sperm chromosomes by toluidine blue

(Briggs, 1952). As 0.75% saline consistently gave an acid reaction, the saline was buffered in several smaples. The buffers and their pH were: Pannet-Compton, app. 7.0; Baker's glucose saline (7.7); a saline-borate (app. 9.0); and saline-bicarbonate. Thirteen females were inseminated with sperm treated with toluidine blue in these suspensions, the concentration of the chemical being 1/10,000. No embryos were recovered from these females. The results given in this paragraph have not been included in any of the Tables or Figures, and will not be considered further in the presentation of the results.

Analysis of the chromosome counts of the embryos.

The diploid number of *Mus musculus* is 40 (Matthey, 1949). Details of the chromosome counts in the embryos are given in the Table. Only the normal embryos were classifiable; none of the retarded embryos had definite mitotic figures. All the normal embryos which were classifiable were diploid.

In addition to the retarded embryos which had one or two nuclei, some one-celled eggs were seen which possessed merely a haploid set of chromosomes. The chromosomes were definitely meiotic in many of these eggs, but in other eggs they appeared to be mitotic (see Figs. 68 & 69). In appearance, the chromosomes varied from highly degenerate to clear and distinct, and several of the latter which appeared to possess mitotic-like chromosomes were provisionally classed as one-celled haploid embryos. But the subjectivity involved in deciding whether the chromosomes were meiotic or mitotic devalued their evidence. One-celled eggs with meiotic chromosomes may have been unfertilised,

and had retained their organisation to 3½ days gestation. The few embryos which possessed mitotic-like chromosomes may have been haploid embryos restricted to the first cleavage division. All eggs which contained only chromosomes have been classified as unfertilised in Table 20.

The number of nuclei in the diploid embryos.

With the exception of two possessing 17 and 19 nuclei respectively, all the diploids were undergoing their sixth cleavage (i.e. more than 32 cells).

Cytological observations on the embryos.

The highly retarded embryos possessing one or two nuclei, and the one-celled eggs possessing chromosomes only, have been previously mentioned. Some of these eggs and embryos also had sub-nuclei.

Controls of the photochemical action of toluidine blue.

To ascertain that treatment of the sperm by toluidine blue was photochemical, a solution of the dye, concentration 1/4,000 by weight, dissolved in 0.75% saline, was injected via the cervix into the uterus of eleven females. The females were then paired with males for natural mating. The sperm would become mixed with the toluidine blue *in utero* before fertilisation; the action of the chemical would occur there in the absence of light. All the females were killed 3½ days after mating; they yielded 87 embryos. Of those with mitoses, 44 were diploid, one was triploid, and one was haploid (Fig. 70). The triploid and the haploid were taken from the same female. The possible origin of the haploid

will be discussed later.

3. Observations at implantation.

To follow embryonic development beyond 3½ days gestation, females which had been inseminated with toluidine blue-treated sperm were killed at 12 days gestation and examined for implants. The sperm had been treated with concentrations 1/7,500 or 1/10,000. Four females were used at the higher concentration, five at the lower. In both cases, one female had one embryo *in utero*. All the females were genetically homozygous for a recessive gene for pink eye-colour, the sires being homozygous for dominant black eye colour. The two embryos both had black eyes, hence the paternal chromosomes must have taken part in embryonic development.

DISCUSSION.

Toluidine blue is reported by Briggs (1952) to be an effective agent for the inactivation of amphibian sperm chromatin, and may be more effective than trypaflavine, the chemical originally used for this purpose (Hertwig, 1924). Both substances induce a considerable proportion of the fertilised amphibian eggs to develop as haploids. In mammals, trypaflavine failed to induce any definite gynogenetic development of the mouse egg (p. 146), and of the eggs of the sheep (Thibault, 1949) and rabbit (Beatty, unpublished). Mouse embryos which may have been gynogenetic were highly abortive (p. 150). The present results show that in the mouse, toluidine blue has the same result as trypaflavine. After treatment with toluidine blue, two kinds of development are found: one being diploid and very similar to controls, the

other highly retarded. Some confirmation of the normal hybridity of the former is shown by the two embryos with male eye-marker genes present after high concentrations of toluidine blue. Probably all of the normal embryos are therefore hybrid.

After treatment of the sperm in the dark *in utero*, however, a haploid embryo was obtained. This may have been a gynogenetic embryo induced by the treatment. But these controls suggested, the haploid apart, that toluidine blue in high concentrations did not interfere excessively with fertilisation and development if light was not applied to the sperm-dye mixture. Briggs (1952) reported a photochemical action of the dye in his original amphibian studies. The haploid may therefore not have been an experimentally-induced gynogenone. Some indication of its origin comes from the discovery of a triploid embryo taken from the same mother. Haploids and triploids occurring after the same treatment suggest an effect on the second maturation division of the egg causing either the expulsion of all of the egg chromosomes into the second polar body, or their retention in the egg. Fertilisation of such eggs would result in androgenetic haploids being associated with triploids. This mechanism was also indicated after colchicine (p. 40) or trypaflavine (p. 156) treatment at fertilisation. On this theory, the haploid embryo was androgenetic.

Many embryos were highly retarded at 3½ days gestation. Some of these embryos had one, two, or three nuclei, others possessed merely chromosomes with or without sub-nuclei. Chromosome counts were only possible on the latter, and all possessed the haploid complement. The majority of these embryos appeared to possess meiotic chromosomes, though some may

have possessed mitotic chromosomes. Their origin may have been the same as similar embryos appearing after trypaflavine treatment (p. 156) i.e. abortive gynogenetic or parthenogenetic development induced by the treatment, or failure of the fertilising sperm to stimulate the development of the egg. Histological examination of these eggs just after mating may clarify their origin. The cause of the retarded development was directly related to the illumination of the dye-sperm mixture, for no comparable embryos came from the without-light controls.

It is evident that toluidine blue, used over a wide range of concentration and pH, failed to induce haploid development of the mouse egg except, possibly, to the first or second cleavage. The response of the mammalian sperm or egg to this treatment appears to be qualitatively different to that of amphibians where gynogenones develop to advanced larvae (Briggs, 1952). In the present experiments, neither trypaflavine nor toluidine blue were effective agents for the induction of the haploid gynogenetic development of the mouse egg.

SUMMARY.

1. Sperm of the mouse, Mus musculus, was treated with various concentrations of toluidine blue in vitro before fertilisation.
2. Increasing concentration of dye decreased the numbers of normal embryos at 3½ days gestation. Many highly retarded embryos occurred over a wide range of concentrations.
3. Chromosome counts were possible on the normal embryos only. Apart from the occurrence of a haploid of suspect origin, no certain evidence was found of the induction of gynogenesis.
4. Implantation was not suppressed by the highest concentrations.

REFERENCES.

BEATTY, R.A. 1954. Haploid rodent eggs. Proc. 9th Intern. Congr. Genetics, Bellagio.

BEATTY, R.A. & FISCHBERG, M. 1951. Heteroploidy in mammals: I Spontaneous heteroploidy in pre-implantation mouse eggs. J. Genet. 50, 345-359.

BRIGGS, R. 1952. Analysis of the inactivation of frog sperm nucleus by toluidine blue. J. Gen. Physiol. 35, 761-780.

HERTWIG, G. 1924. Trypaflavine als Radiumersatz zur Gewinnung haploidkerniger Roschlarven. Verh. Anat. Ges. 58, 223-227.

MATTHEY, R. 1949. Les Chromosomes des Vertébrés. F. Rouge, Lib. Univ., Lausanne.

THIBAULT, C. 1949. L'oeuf des mammifères, son développement parthénogénétique. Ann. Sci. Nat., Zool. 11, 136-219.

TABLE 20. DETAILS OF THE FERTILITY OF THE INSEMINATED FEMALES AND OF THEIR EMBRYOS AT 3½ DAYS GESTATION.

	SPERM	FEMALES			EGGS AND EMBRYOS							CHROMOSOME COUNTS OF EMBRYOS				NUCLEAR NOS. OF EMBRYOS	
Dye concentration (x 1000)	No. of sperm treatments	No. of ovulated ♀♀ used	No. of ♀♀ with embryos	% total ♀♀ with embryos	Total no. of eggs found	No. of eggs from ♀♀ with embryos	Total no. of embryos found	% of total eggs which were embryos	% embryos of total eggs from ♀♀ with embryos	Mean no. of embryos from ♀♀ with embryos	% embryos which were blastocysts	Lost or unclassified	Diploid	Probably diploid	Above diploid	Mean no. of nuclei of all embryos	Mean no. of nuclei in diploid embryos
1/5	1	2	-	0	15	-	-	0	0	0	0	-	-	-	-		
1/7.5	3	8?	-	0	42	-	-	0	0	0	0	-	-	-	-		
1/10	5	11	3	27.3	82	23	6	7.3	26.1	2.0	33.3	5	1	-	-	29.8±11.0	17
1/20	4	16?	2	12.5	79	14	3	3.8	21.4	1.5	66.7	1	2	-	-	43.7±13.2	51.0±12.7
1/40	8	25?	4	16.0	129	35	8	6.2	22.9	2.0	25.0	3	2	3	-	33.5± 9.1	35.8±11.1
1/100	7	18	9	50.0	134	68	28	21.0	41.2	3.1	53.6	18	6	4	-	40.9±10.6	43.4± 5.9
Controls	-	47	33	70.2	394	278	182	46.2	65.5	5.5	51.8	109	56	15	2(3n)	41.2±15.7	44.1±15.4

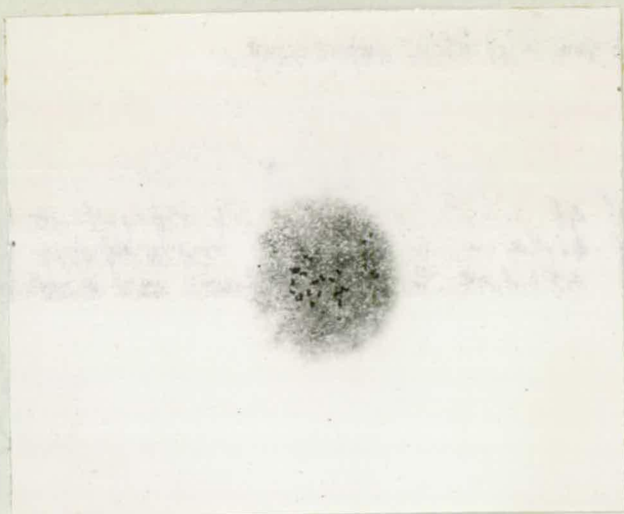
FIGURES

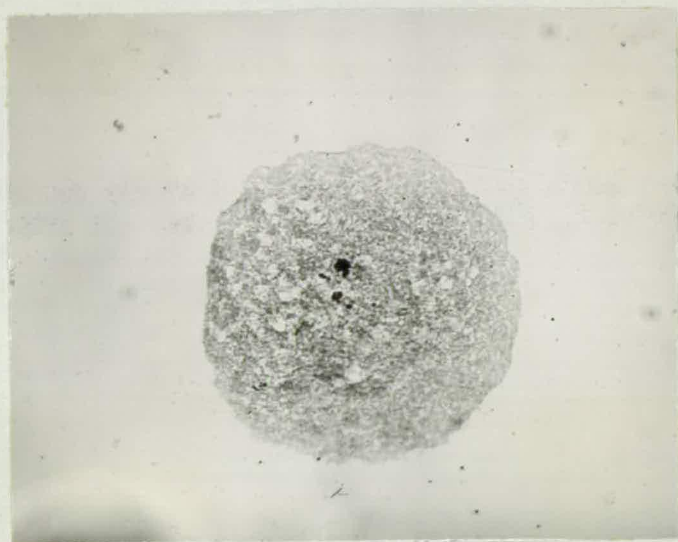
None of the negatives were retouched.

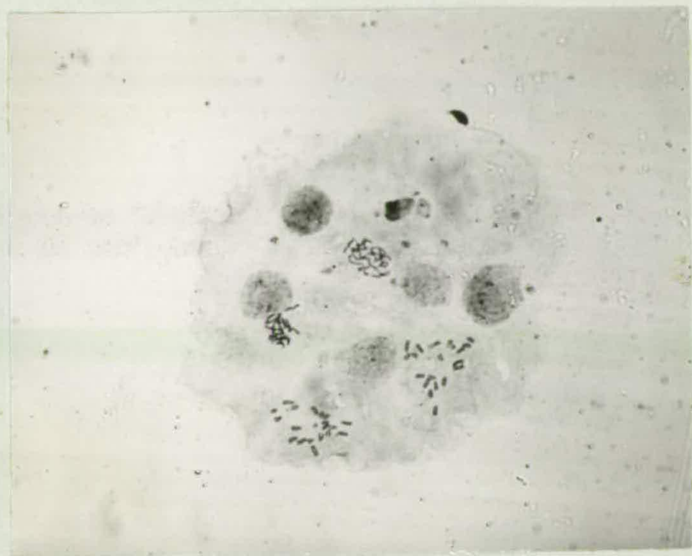
68. Squash preparation of a whole $3\frac{1}{2}$ day old embryo containing 20 chromosomes and no nuclei. This embryo was only slightly squashed, the chromosomes can clearly be seen. x 350.

69. Squash preparation of a $3\frac{1}{2}$ day old embryo containing chromatids in the form of a telophase. x 350.

70. Squash preparation of the single haploid embryo found in the experiment. Four mitoses can be seen, two of which are exactly haploid. x 350.







6. THE ARTIFICIAL INSEMINATION OF SPERM OF OTHER SPECIES INTO THE MOUSE

Several attempts to make interspecific crosses in mammals have been reported. Rabbit females have been artificially inseminated with sperm of the hare (Yamane and Egashira, 1924; Hammond and Walton, 1929), the rat (Pincus, 1939), or the cotton-tail rabbit (Chang and McDonough, 1955). No young have resulted from these crosses, but Pincus noted the activation of the egg by rat sperm, and Chang reported the survival of fertilised eggs up to the blastula stage at latest. In the following experiment, rat or rabbit sperm was inseminated by the usual technique (see p. 4) into mouse (Mus musculus) females.

The rat sperm was taken from the vas deferens of killed males, the rabbit sperm obtained with an artificial vagina. The mouse eggs were recovered 3½ days after insemination. Eight females, which gave 38 eggs, were inseminated with rat sperm; two females, which gave 4 eggs, were inseminated with rabbit sperm. None of these eggs was developing, and squash preparations of them failed to disclose the presence of nuclei or chromosomes. Examination of mouse eggs a few hours after the insemination to determine whether or not they are activated by the foreign sperm is at present being undertaken.

REFERENCES

CHANG, M.C. & McDONOUGH, J.J. 1955. An experiment to cross the cottontail and the domestic rabbit. J. Hered. 46, 41-44.

HAMMOND, J. & WALTON, A. 1929. An attempt to cross hare and rabbit. J. Genet. 20, 401-404.

PINCUS, G. 1939. Comparative behaviour of mammalian eggs *in vivo* and *in vitro*. IV Development of fertilised and artificially activated rabbit eggs. J. exp. Zool. 82, 85-129.

YAMANE, J. & EGASHIRA, T. Uber Kreuzungsversuche zwischen Kanninchen und Schneehasen durch natürliche Paarung und künstliche befruchtung. Zool. Mag. (Tokyo) 36, No. 340.

CHAPTER IV.

THE CELL AND CLEAVAGE NUMBER OF HAPLOID, DIPLOID, AND POLYPLOID MOUSE EMBRYOS AT 3½ DAYS DEVELOPMENT.

At 3½ days gestation the embryo of the mouse, *Mus musculus*, is an advanced morula or a blastocyst. It can be made into a squash preparation and, after staining, the number of nuclei in the embryo can be counted. One nucleus usually represents one cell, but, after certain treatments of the gametes designed to induce haploid or heteroploid development of the resultant embryo, some of the cells may contain one or more sub-nuclei in addition to their normal nucleus (p. 81). Most of the data in the present chapter is taken from experiments in which colchicine was applied to the gametes at fertilisation, or to the egg at the first cleavage division. After these treatments many polyploid embryos were found (p. 21-80). Sub-nuclei were not seen in any of the embryos from the colchicine experiments; the rule that one nucleus equals one cell can therefore be regarded as true for these embryos.

Other polyploid embryos included in the data were found after normal mating or artificial insemination of untreated sperm (p. 2). One triploid embryo was obtained from a female homozygous for the factor 'silver'; this factor causes an incidence of spontaneous heteroploidy in 3½ day old embryos (Beatty and Fischberg, 1951a). All these probably arose spontaneously. Two haploid and two triploid embryos were found after trypaflavine or toluidine blue treatment of the sperm *in vitro* before fertilisation (pp. 151 & 174). These embryos were

probably cleaving normally. Many haploid and heteroploid embryos were found after the <u>in vitro</u> treatment of sperm with ultra-violet or X-rays before fertilisation. All of them have been excluded from the data because of their abnormal cleavage and sub-nuclei. The intention of the analysis is to compare the nuclear and cleavage numbers of haploid, diploid, and heteroploid embryos which are cleaving normally; in this lies the justification for the exclusion of the abnormal embryos.

Possible sources of error are due to bad squashing or damage during preparation of the embryos. These embryos and others in which the number of nuclei could not be counted with accuracy have not been included in the data. Beatty and Fischberg (1951b) have analysed the cell number of 3½ day old haploid, diploid and polyploid mouse embryos occurring spontaneously in their 'silver' strain or induced experimentally by the heat treatment of eggs in the fallopian tubes.

RESULTS.

The Euploid Embryos.

The cell numbers of all the haploid and polyploid embryos (i.e. those embryos with Xn chromosome sets where X is an integer) which were countable with accuracy are given in Table 21, together with their origin. For statistical treatment, these embryos have been grouped into four classes. One class includes embryos taken from females injected with colchicine before mating whether a single or repeated injections were given (p. 21 & 65). The second class comprises the 'residual' embryos (p. 62). The third class includes all mice which received colchicine

injections after mating (p. 72). The second and third classes are combined for the analysis of variance of the cell numbers of triploid embryos, the triploids in these two classes probably arising through a similar mechanism. These classes are not combined in the analysis of variance of the cell numbers of the diploid embryos.

The fourth class is made up of various non-colchicine treatments which produced polyploids; and where the effect of the treatment was such that, apart from the altered chromosome number, no other restrictions on embryonic development were obvious. Data from one female included in this class has not been given elsewhere. She came from a strain of mice in which the gene 'silver' was segregating, and gave three diploid and one triploid embryo following natural mating. The other embryos included in this class of the analysis have been given on a previous page.

The first purpose of the analysis was to trace any difference within the diploids, haploids, or each polyploid class resulting from the various treatments. An analysis of variance, using a one-way classification, was made on the nuclear counts of each of the above chromosomal types. The within treatment sum of squares was used as error term for the between treatment sum of squares. Analysis of the polyploids was made by two methods: first by the use of cell numbers of all the embryos; second, where a female had two or more of one polyploid type, the mean cell number of the polyploid type was taken with each female, the data not being weighted. In the analysis of the diploids, the mean cell number within females was the only method used because two or more

diploids per female were usually obtained (Table 21). The analysis of variance of the cell numbers of the diploids was calculated on both unweighted and weighted data.

Two of the classes used (the injection of colchicine before and after mating) contained a continuous variate (i.e. the concentration of colchicine injected). In neither case has a regression of the cell number of the various chromosomal types on concentration been calculated; by inspection, no repression line would be significant.

Analysis of variance of cell numbers of haploid, triploid, and tetraploid embryos.

Results for the three types were:

1. Analysis on all embryos

	Haploids			Triploids			Tetraploids		
	Sum of squares	DF	Mean square	Sum of squares	DF	Mean square	Sum of squares	DF	Mean squares
Within treatments	518.0	4	129.5	4260.9	24	177.5	693	10	69.3
Between treatments	321.4	2	160.7	641.2	2	320.6	192.7	2	96.4
Total	839.4	6		4902.1	26		885.7	12	

2. Analysis on means within females

	Haploids			Triploids			Tetraploids		
Within treatments				2015.3	19	106.1	616.0	8	77.0
Between treatments	As above			710	2	355	166.7	2	83.4
Total				2725.3	21		782.7	10	

In all cases, F was not significant. The test indicated that within each polyploid type no differences existed between

the cell numbers of the embryos resulting from the various treatments.

Analysis of variance of the diploid cell numbers.

The test for differences in the cell numbers of the diploid resulting after various treatments was made on only those diploids which were taken from mice known to have been affected by the treatment. Knowledge of the effect of colchicine comes primarily from the observation of heteroploid embryos. For this analysis, the cell numbers of diploid embryos were therefore taken only from females which had produced one or more polyploid embryos. The embryos included were therefore a selected group which should show the maximum effect of the treatment. One further limitation was necessary. Any type of heteroploid embryo indicated an effect of the treatment, but, as the results of this analysis were to be used later, only mice producing one or more haploid or polyploid embryos were included, and females with non-euploid heteroploids were ignored.

Analysis was made between the four classes previously described. Means within females were tested, first without weighting, and second by weighting the mean by the number of diploids per mouse. The figures used in this analysis are given in detail in Table 21. Results were:

	Not weighted			Weighted		
	Sum of squares	DF	Mean square	Sum of squares	DF	Mean square
Within treatments	4214.5	30	140.5	9669.2	30	322.3
Between treatments	644	3	214.7	1643.2	3	547.7
Total	4858.5	33		11312.4	33	

Neither F was significant. It was concluded that no difference had been shown to exist between the mean cell numbers of the diploid embryos following various treatments.

As no difference was traced between the means within females of the diploid embryos, the diploids were considered as a homogeneous sample. Similarly, as no difference within the haploids, triploids, or tetraploids were found, they were also considered as homogeneous groups. The entire data was therefore used to calculate the ratios obtained from the division of the mean number of cells in haploid, triploid, or tetraploid embryos by the mean number of the diploids. Thus, a 1n/2n ratio for haploids, 3n/2n for triploids, and 4n/2n for tetraploids was obtained.

Calculation of the haploid/diploid and polyploid/diploid ratios.

In the following calculations, data was taken only from females which produced at least one diploid and one haploid or polyploid embryo. The mean cell numbers of the diploids and of the haploids, triploids, or tetraploids were calculated within each female, and the analysis was made on the means. Details of the females and of the cell numbers of the embryos used in the calculations are given in Table 21.

For the calculation of each ratio, a positive correlation between the mean cell number within females of the diploid and the mean cell number of the particular polyploid type was necessary. This was to ensure that both chromosome types in each ratio were similarly affected by the treatment given. The calculation of this correlation, and of the polyploid/diploid

ratios were made as follows, using the triploid/diploid correlation as the example. Only the embryo cell counts from females which produced at least one triploid and one diploid embryo were taken, and the mean cell number of each of these two types for each female was calculated. The correlation and the ratio were calculated on these unweighted means. Similar claculations were made on the haploid and tetraploid cell numbers.

Results of the correlations were:

2n/3n:	$r = 0.28$	2n/4n:	$r = -0.55$	1n/2n:	$r = 0.81$
	$n = 19$		$n = 8$		$n = 7$

The 1n/2n correlation is significant at 1% level, neither of the others are significantly different from zero. The calculations of the ratios which follow will therefore vary in accuracy, the 4n/2n ratio being the least reliable.

If, within the same females used for the correlation, the mean cell number of the diploid and of each polyploid type are summed, the division of each polyploid sum by each respective diploid sum will give the arithmetic ratios of polyploid/diploid cell numbers. The means and ratios obtained from this calculation were:

$$1n/2n: \frac{25.71}{45.99} = 0.56 \quad 3n/2n: \frac{28.30}{44.18} = 0.64$$

$$4n/2n: \frac{18.13}{52.69} = 0.34$$

But this method fails to take differences between females into acount, and gives excessive prominence to the higher figures. It was only an approximate ratio.

A better ratio was obtained by the following method. No

differences between the diploids resulting from the various treatments had been traced by the analysis of variance of their cell numbers. The mean cell number of the diploid embryos within each female was therefore used as a control, and for each mouse this figure was divided by the mean cell number of its various polyploid embryos. For each mouse a ratio 2n/3n, or 2n/4n, or 2n/1n, was obtained, the ratios all being greater than unity because the diploid mean was the highest in all cases. The ratio was then transformed to logarithms to the base 10 to minimise the differences due to different development stages of the embryos (the development stages tending to geometric progression 2, 4, 8, 16, etc. in cell number). The mean log ratio and its standard error were calculated.

The standard error was multiplied by the value of t corresponding to the number of mice contributing to the mean log ratio. The fiducial limits for the value of the log mean were then obtained by the addition to and the subtraction from the log mean of the calculated t value. This gave the mean log ratio 2n/Xn for the three polyploid types, with an upper and lower fiducial limit. Bytaking reciprocals, each ratio and its limits were converted to Xn/2n, and these were transformed by antilogarithms.

The mean ratios, and the upper and lower fiducial limits calculated at the 5% and 1% levels of significance were:

	1n/2n	3n/2n	4n/2n
Mean ratio:	0.51	0.56	0.31
5% limits:	0.38 - 0.69	0.45 - 0.70	0.16 - 0.58
1% limits:	0.32 - 0.79	0.42 - 0.76	0.12 - 0.78
	n = 7	n = 19	n = 8

The number of cleavages of diploid and polyploid embryos.

The cell number of the $3\frac{1}{2}$ day old embryos is the result of a series of cleavages, each of which doubles the number of cells in the embryo. The time between cleavages diminishes slightly with successive cleavages (see p. 9). Conversion of the cell numbers of the $3\frac{1}{2}$ day old embryos to cleavage numbers will therefore convert the data from a geometric scale to an approximate arithmetic scale. Transformation of the cell number to cleavage number is obtained by the following equation (see p. 10):

$$x = \frac{\log N}{\log 2}$$ where x is the number of cleavages, and N is the number of nuclei in the embryo.

All the data on cell numbers of the diploid and polyploid embryos which has previously been analysed has been re-analysed on the basis of numbers of cleavages. The same analyses of variance, correlations and polyploid/diploid ratios have been calculated, using the same classes and data as before. The results obtained were:

Analysis of variance of cleavage number of each polyploid type:

1. Analysis on all embryos.

	Haploids			Triploids			Tetraploids		
	Sum of squares	DF	Mean square	Sum of squares	DF	Mean square	Sum of squares	DF	Mean square
Between treatments	2.32	2	1.16	1.69	2	0.85	0.98	2	0.49
Within treatments	2.17	4	0.54	13.58	24	0.57	5.93	10	0.59
Total	4.49	6		15.27	26		6.91	12	

2. Analysis on means within females.

	Haploids			Triploids			Tetraploids		
Between treatments				1.78	2	0.89	0.88	2	0.44
Within treatments	As above			8.27	19	0.43	5.19	8	0.65
Total				10.05	21		6.07	10	

Analysis of variance of the diploids:

	Not weighted			Weighted		
	Sum of squares	DF	Mean square	Sum of squares	DF	Mean square
Between treatments	0.37	3	0.12	1.65	3	0.55
Within treatments	5.04	30	0.17	9.52	30	0.32
Total	5.41	33		11.17	33	

None of the F values were significant.

Calculation of the polyploid/diploid ratios.

The correlations of the diploid and polyploid means within females for each polyploid type were:

1n/2n: r = 0.92 3n/2n: r = 0.39 4n/2n: r = -0.54

n = 7 n = 19 n = 8

The 1n/2n correlation is significant at the 1% level; neither of the others are significantly different from zero.

The calculations of the polyploid/diploid ratios and their fiducial limits made on the ratios within each female gave the following results:

	1n/2n	3n/2n	4n/2n
Mean ratio:	0.82	0.87	0.71
5% limits:	0.74 - 0.91	0.81 - 0.92	0.56 - 0.86
1% limits:	0.68 - 0.96	0.79 - 0.94	0.49 - 0.93
	n = 7	n = 19	n = 8

One further calculation was made on the cleavage numbers. Within the same females as above (i.e. those producing at least one diploid and one polyploid embryo) the overall mean of the means within females for each polyploid type and for their

Cleavage Numbers: Mean diffs + SE of same.

D – Tr

		Weight $\frac{n_0 n_T}{n_0 + n_T}$		
5.5 – 4.2	1.3	5/6	.83	1.08
5.6 – 4.8	0.8	2/3	.67	.53
5.9 – 5.7	0.2	2/3	.67	.13
5.7 – 2.8	2.9	1/2	.5	1.45
6.0 – 5.1	0.9	2/3	.67	.60
5.9 – 4.9	1.0	2/3	.67	.67
5.2 – 4.7	0.5	4/5	.8	.40
4.5 – 3.6	0.9	1/2	.5	.45
5.0 – 4.4	0.6	5/6	.83	.50
5.5 – 4.8	0.7	3/4	.75	.53
5.5 – 5.2	0.3	12/7	1.71	.51
5.8 – 5.5	0.3	2/3	.67	.20
5.9 – 5.7	0.2	4/5	.8	.16
4.9 – 4.9	0	2/3	.67	0
5.4 – 4.9	0.5	3/4	.75	.38
5.5 – 3.8	1.7	6/7	.86	1.46
5.6 – 4.8	0.8	2/3	.67	.53
5.0 – 4.9	0.1	5/6	.83	.08
4.7 – 4.4	0.3	3/4	.75	.23
			14.60	9.89

Σx^2 8.2109

$\Sigma x^2 = 3.063$

Best diff: $\frac{9.89}{14.60} = .677$

$V = .1702$

$\sigma = .413$

$S.E. = \frac{.413}{\sqrt{14.60}} \quad \frac{}{3.821} = 0.108$

[crossed out]

Best diff $= \frac{\Sigma WD}{\Sigma W}$

$SE = \frac{\sigma}{\sqrt{\Sigma W}}$

D – Tel.

5.9 – 3.3	2.6	3/4	.75	1.95
5.9 – 2.6	3.3	2/3	.67	2.20
5.5 – 4.0	1.5	3/4	.75	1.13
5.3 – 5.1	0.2	6/7	.86	0.17
5.2 – 4.8	0.4	6/7	.86	0.34
5.6 – 3.3	2.3	1/2	.5	1.15
6.5 – 3.9	2.6	2/3	.67	1.73
5.6 – 4.8	0.8	7/8	.89	0.7
			5.95	9.37

[crossed out] Σx^2 14.8693

Σx^2

Best diff $= \frac{9.37}{5.95}$

$= 1.574$

V 0.5563

σ .746

$SE = \frac{.746}{\sqrt{5.95}} \quad \frac{}{2.439}$

$= .306$

D – H

5.7 – 4.8	0.9	2/3	.67	0.6
5.7 – 4.7	1.0	9/10	.9	0.9
5.8 – 5.1	0.7	6/7	.86	0.6
5.9 – 5.2	0.7	2/3	.67	0.47
4.8 – 3.3	1.5	1/2	.5	0.75
5.5 – 5.3	0.2	1/2	.5	0.1
5.0 – 3.3	1.7	5/6	.83	1.42
			4.93	4.84

Σx^2 4.3398

Σx^2

Best diff $= \frac{4.84}{4.93}$

$= .982$

V .166

σ .407

$SE = \frac{.407}{\sqrt{4.93}} \quad \frac{}{2.220}$

$= .183$

FORAB D – Tel.

5.6 – 4.8	0.8	5/6	1.2	0.9[cut off]
6.3 – 5.5	0.8	8/6	1.33	1.06
4.8 – 3.9	0.9	1/2	.5	0.45
5.7 – 5.6	0.1	3/4	.75	0.08
5.2 – 3.5	1.7	8/9	.89	1.51
5.3 – 3.6	1.7	3/4	.75	1.2[cut off]
			5.42	5.31

Σx^2 6.096

Σx^2

V .279

Best diff $= \frac{5.31}{5.42}$

$= .980$

σ .528

$SE = \frac{.528}{\sqrt{5.42}} \quad \frac{}{2.328}$

.227

Comb. Ave.

Σ Weight $= 11.37$ $\quad \Sigma$ Diff × W $= 14.68$ $\quad n = 14$

Best diff: $\frac{14.68}{11.37}$

$= 1.291$

[crossed out]

$\Sigma x^2 = 20.9660$

Σx^2

$V = .429$

$\sigma = .655$

$SE = \frac{.655}{\sqrt{11.37}} = \frac{}{3.3719}$

$= .194$

diploid sibs was:

1n:	4.53	2n:	5.49	Arithmetic 1n/2n ratio:	0.83
3n:	4.69	2n:	5.43	Arithmetic 3n/2n ratio:	0.86
4n:	3.98	2n:	5.69	Arithmetic 4n/2n ratio:	0.70

To test the accuracy of the difference between the diploids and the polyploids in cleavage number, the difference between the mean number of cleavages of the diploid and polyploid embryos within each mouse was calculated. For each mouse, the difference was weighted by the formula $\frac{n_1 n_2}{n_1 + n_2}$ where n_1 is the number of diploid, and n_2 the number of polyploid embryos contributing to the means. The best estimate of the mean difference is given by:

$$\text{Best difference} = \frac{\text{S(Weighted difference within mice)}}{\text{Sum of the weights}}$$

$$\text{with a standard error: } \frac{\text{standard deviation of differences between mice}}{\text{Sum of the weights.}}$$

The values obtained were:

Difference in number of cleavages between:-

Diploids and haploids	0.98 ± 0.18
Diploids and triploids	0.68 ± 0.11
Diploids and tetraploids	1.57 ± 0.31

Expressed in this way the data permits direct comparison between diploid and non-diploid development, diploid being superior to haploid by one cleavage, to triploid by 0.7 cleavages and to tetraploid by 1.6 cleavages, at 3½ days development.

The Aneuploid and Mosaic embryos.

For simplicity, these embryos have been grouped into hypo-diploids, hyper-diploids, mosaics, and others. The source,

chromosome complement, cell number, and the mean of diploid embryos from the same mouse, are given in Table 22. Apart from the mean diploid cell number within each female, no calculations have been made on the figures.

The hypo-diploids were always behind the diploids, but despite this three embryos reached cell counts of 36, 34 and 25 respectively. These embryos were classified on a single mitosis except for the embryo with 34 chromosomes which had two clear mitoses. The hyper-diploids appeared to be slightly more advanced in cell numbers, two of them having more cells than the diploid mean. Five of these embryos were classified on two mitoses, the other three on a single one. Three of the six hypo-diploids, and all the hyper-diploids were blastocysts when judged by cell number; the chromosome complement of these embryos apparently had little effect on development to the blastocyst stage.

More than one mitosis per embryo was necessary to recognise the mosaics. Three haploid/diploid mosaics were observed, two coming from the same female which had been injected before mating with 1/30,000 colchicine, the other arising from a 'residual' female which was injected after mating. The latter had two haploid and one diploid mitosis, the two former had one haploid mitosis with one and three diploid mitoses respectively. One of the mosaics had a higher cell count than the diploid mean, the others were behind the diploids. All the diploid/tetraploid mosaics had one tetraploid and one or more diploid mitoses; the number of cells in these embryos was consistently below the diploid mean. The number of cells in mosaics may have been

influenced by the time of doubling of the haploid or diploid blastomeres to diploid or tetraploid respectively.

Five other mosaics, among which three had three different chromosome counts, are listed in Table 22. In addition four embryos which belonged to none of the other classes are given. Two of the latter were found with a group of below-haploid chromosomes, the other two had chromosome counts between triploid and tetraploid. Each of these nine embryos had fairly high nuclear numbers, some of them possessing more than their diploid sibs. Once again, it appeared that the chromosome complement of these embryos had not severely affected their development to the blastocysts.

DISCUSSION.

In terms of number of nuclei, the tetraploid embryos possessed one-third, and the haploids and triploids just over one-half, of those of the diploid embryos. Conversion of the data to the cleavage number increases these ratios to 0.71, 0.82 and 0.87 respectively. Of the two methods of comparison, the cleavage number is more convenient as it approximates to an arithmetical progression; in the following discussion all references will be to the cleavage number unless otherwise stated.

The haploid-diploid and the triploid-diploid means within females were positively correlated, the tetraploid-diploid mean was negative. This implied that the tetraploid embryos were delaying further behind their diploid sibs as development proceeded; however some of them were only slightly behind the diploids. The slowly-developing tetraploids had probably

suffered from an excess of colchicine as perusal of the actual cell numbers indicates (Table 21); these embryos may therefore be expected to accentuate the difference in number of cleavages between the tetraploids and their diploid sibs. Despite this, the difference between the two was only 1.57 ± 0.31 cleavages. But in the tetraploids, the first cleavage of the embryo was suppressed by the colchicine. One cleavage division should therefore be subtracted from the above difference to indicate the actual difference in number of cleavages of the two types; the difference becomes only 0.5 approximately.

In view of the very slight difference in cleavages between the diploids and the colchicine-produced tetraploids, analysis of the tetraploid data resulting after hot-shock treatment of first-cleavage eggs by Beatty and Fischberg (1952) was carried out using cleavage numbers. Six tetraploid-diploid comparisons were given by these authors; these were first analysed alone, then in combination with the colchicine tetraploid-diploid comparisons. The results were:

	Hot-shock	Hot-shock & colchicine
Number of comparisons	6	14
Correlation	0.81	0.05
4n/2n mean of means within females	0.82	0.76
5% fiducial limits	0.69 - 0.94	0.66 - 0.85
1% fiducial limits	0.63 - 1.00	0.62 - 0.89
Overall mean of means within females	2n: 5.48	2n: 5.60
Weighted difference between 2n and 4n	0.98 ± 0.23	1.29 ± 0.19

The positive correlation between the tetraploid and diploid means following the hot-shock is significant at the 5% level. These embryos are therefore more comparable than those resulting from the colchicine treatment. The mean of means within females for the diploids and tetraploids resulting from the hot-shock have a difference of exactly one cleavage; the weighted difference is 0.98 cleavages. When one cleavage is allowed to the tetraploids for the first cleavage which was suppressed, there is therefore no difference between the two. But two assumptions have to be made for this conclusion: first, that the cleavage after the suppressed first cleavage in the tetraploids occurs at the same time as the second cleavage in the diploids; second, that the diploids are not retarded due to the hot-shock or to the colchicine. The latter is probably true for the mean cleavage number in diploid embryos taken from females which were naturally mated without any experimental treatment was almost identical at 5.6. No evidence can be given on the first assumption, but as the chromosomes must duplicate themselves before the first division of the tetraploid, the time interval is probably identical. If these assumptions are valid, the mathematical evidence indicates that the rate of cleavage in tetraploid embryos is the same as the rate in diploid embryos.

The results of similar calculations made on the haploid and triploid embryos resulting from colchicine treatment before fertilisation have been given; the haploids were 0.98 ± 0.18 cleavages behind the diploids, and the triploids 0.68 ± 0.11 cleavages behind the diploids, both being weighted differences. In the case of the tetraploids, the missing cleavage could be

attributed to the treatment;in the haploids and triploids there must be a different reason. If tetraploid cleavages occur at the same rate as diploids, then triploid mitoses probably also occur at this rate. A possible objection here is that the number of centrioles in the triploids is unbalanced, whereas this is not so in tetraploids. But, as triploids can develop to 9½ days gestation (Fischberg and Beatty 1951) without any abnormality in their mitoses, the effect of the centriole unbalance is not noticeable. If the rate of triploid cleavage is identical to diploids, then the cause of the delay must be before these mitoses begin, i.e. in the pro-nuclear stages. Some experimental evidence in support of this suggestion has been given, both haploids and triploids probably spending longer periods in the pronuclear stages than their diploid sibs (p. 43).

Other data, comparable to the data analysed above, is again available from the heteroploids resulting from the hot-shock treatment of the fallopian tubes, and from mice giving a high incidence of spontaneous embryos, both reported by Beatty and Fischberg (1951b). The data concerning diploid-triploid comparisons has been re-analysed as the number of cleavages which had occurred in these embryos. For reasons given later, the 3½, 4½, and 5½ days development results have been analysed separately. The results were:

	3½ days	4½ days	5½ days
Number of comparisons	42	4	1
Correlation	0.30	0.93	-
3n/2n mean of means within females	0.94	0.91	-
5% fiducial limits	0.90-0.97	0.85-0.98	-
1% fiducial limits	0.89-0.98	0.80-1.02	-
Overall mean of means within females	2n: 5.37 3n: 5.00	2n: 6.90 3n: 6.38	2n: 8.1 3n: 8.3
Weighted difference between 2n and 3n	0.38±0.07	0.64±0.16	-

It is again evident that the number of cleavages which have occurred in the triploids is only slightly behind that of the diploids; the difference in these embryos being less than in those produced by the colchicine treatment. If the delay in cleavage is attributable to the pronuclear stages, it follows that, with increasing numbers of cleavages, the ratio of the number of cleavages in triploids to that in diploids should approach a value of one. This is because the triploids will always be a fixed amount behind the diploids, and this fixed amount will have less and less effect as the numbers of cleavages increase. In the data of Beatty and Fischberg just analysed, the ratio at the 4½ day stage should be nearer to value one than the 3½ day stage. In fact, it is not so, but the fiducial limits more than encompass the difference. Only one comparison was available for the 5½ day embryos, the triploid mean being higher than the diploid mean. But any result from these comparisons may be invalidated because, at the later stages of development, the cleavage number is probably meaningless due to asynchronous

divisions and differentiation within the embryos. Attempts to plot the ratio against the stage of development for all three types of polyploid yielded no definite result; the differences in time of development between different mice were not sufficiently great to indicate if the curve was approaching unity.

Once the numbers of cleavages for each polyploid type are known, it is possible, by converting the cleavage number to a time scale, to assess their 'developmental age'. The difference between diploid and the various polyploids in cleavage number will, when converted to time, indicate the extra time required by the polyploids to catch up to normal development (assuming that the diploids represent normal development). The 'developmental age' and time behind normal development for the three polyploids resulting from the treatments, assuming the sixth cleavage occurs 72 hours after sperm entry, are as follows:-

	Colchicine	Hot-shock
Haploids:		
Developmental age	56.4 hrs.(2n 66.0 hrs.)	
Behind normal	9.6 hrs.	
Triploids:		
Developmental age	54.0 hrs. (2n 64.8 hrs.)	60 hrs.(2n 64.8 hrs.)
Behind normal	10.8 hrs.	4.8 hrs.
Tetraploids:		
Developmental age	48 hrs.(2n 68.4 hrs.)	54.0 hrs.(2n 66.0 hrs.)
Behind normal	20.4 hrs.	12 hrs.

In experiments designed to follow the further development of polyploid embryos it may be necessary to allow for the times shown above so that the polyploids can make up for their delayed development. The two most obvious methods for achieving this seem to be: first, by transplanting treated embryos to females in an earlier stage of their pregnancy cycle; and second, by the use of

lactating females as the experimental animals, to utilise the delayed implantation which occurs in them (Ensmann, Saphir, and Pincus, 1932).

If further work confirms that polyploid, especially tetraploid, mitoses occur at the same rate as diploid mitoses, then the mouse embryo behaves similarly to the frog _Rana pipiens_ and where the first three cleavages occur at the same rate in diploid and triploid embryos (Briggs, 1947). But the mouse egg may differ from _Rana pipiens_ and _Triturus viridescens_ (Fankhauser, 1945) in that triploid eggs spend a longer period of time in the pronuclear stages than diploids. In later stages of Amphibian development, the number of cells present is inversely proportional to the number of chromosome sets present (Fankhauser, 1945; Fischberg, 1944, 1948; Briggs, 1947). In these larvae, growth and differentiation is very advanced, and is in contrast to the position in the early embryonic divisions of the egg. In the mouse egg, very little increase in size if any has occurred up to $3\frac{1}{2}$ days development, the process thus far having been largely, if not wholly, a division of a pre-existing mass of material. Few effects of growth and differentiation will have therefore affected the picture of cleavage described; the cleavage rate being solely due to factors within the cell. Under these conditions, tetraploid, triploid, and haploid mitoses probably occur at a similar rate. It is interesting to note that the nuclear-cytoplasmic ratio of the whole embryo is the same for tetraploids as it is for diploids; only the distribution of the parts is altered by tetraploidy. This ratio will be different in triploids, where the second polar body chromosomes are included

within the egg, and in haploids where only one gamete is represented.

Finally, one qualification must be made on the cell numbers and cleavage numbers of polyploid embryos. Though the means within females showed that diploid embryos were always more advanced than the polyploids, examination of the actual numbers themselves (Table 21) shows that four of the triploids and two of the tetraploids had more cells than one or more of their diploid sibs. The data therefore agree with the same observations of Beatty and Fischberg (1951b). Two of the three haploid-diploid mosaics and one diploid/tetraploid mosaic also possessed more cells than one or more of their diploid sibs. None of the haploids analysed in this work had greater cell counts than their diploid sibs, but Beatty and Fischberg give data showing that their only haploid had achieved this superiority.

SUMMARY.

1. The cell number and the number of cleavages of 3½ day old diploid and polyploid embryos of the mouse, *Mus musculus*, have been analysed.
2. No difference within the diploid embryos or within any of the polyploid types were detected between the various experiments contributing to the data.
3. Tetraploid embryos cleave at or nearly at the same rate as diploids, and haploids and triploids may also cleave at this rate.
4. Differences between diploid and polyploid embryos in cell and cleavage number may be due to delay in the pronuclear

stages in haploids and triploids, and solely to the suppressed first cleavage in the tetraploids.

5. Provided that the haploid complement is present, changes in the chromosome number alone do not appear to affect the development of the mouse egg to a blastocyst.

REFERENCES

BEATTY, R.A. & FISCHBERG, M. 1951a. Heteroploidy in mammals: I Spontaneous heteroploidy in pre-implantation mouse eggs. J. Genet. 50, 345-359.

__________ 1951b. Cell number in haploid, diploid, and polyploid mouse embryos. J. exp. Biol. 28, 541-552.

__________ 1952. Heteroploidy in mammals: III Induction of tetraploidy in pre-implantation mouse eggs. J. Genet. 50, 471-479.

BRIGGS, R. 1947. The experimental production and development of triploid frog embryos. J. exp. Zool. 106, 237-266.

ENZMANN, E.V., SAPHIR, N.R. & PINCUS, G. 1932. Delayed pregnancy in mice. Anat. Rec. 54, 325-341.

FANKHAUSER, G. 1945. The effect of changes in chromosome number on amphibian development. Quart. Rev. Biol. 20, 20-78.

FISCHBERG, M. 1944. Veränderungen der Chromosomenzahl bei *Triton alpestris* nach Kältebehandlung der Eier. Rev. suisse Zool. 51, 430-436.

__________ 1948. Experimentelle Auslösung von Heteroploidie durch Kältebehandlung der Eier von *Triton alpestris* aus verscheiden Populationen. Genetica 24, 213-329.

FISCHBERG, M. & BEATTY, R.A. 1951. Spontaneous heteroploidy in mouse embryos up to mid term. J. exp. Zool. 118, 321-336.

TABLE 21. DETAILS OF THE CELL NUMBERS OF DIPLOID, HAPLOID AND POLYPLOID 3½ DAY OLD MOUSE EMBRYOS TAKEN FROM VARIOUS FEMALES.

Data is taken only from females which yielded at least one diploid and one haploid or polyploid embryo.

Source of data		Diploids	Mean	Haploids	Mean	Triploids	Mean	Tetraploids	Mean
Colchicine injected before mating:- (Conc. x 1000)	1/100	67, 39	53	27					
	1/50	56, 53, 42, 38, 34	44.6			18			
	1/30	52, 49, 24	41.7						
	1/20	70, 59, 50	59.7					10	
		60, 37	48.5			28			
		60, 55	57.5			50		6	
		50	50			7			
		58, 47, 26	43.7					16	
	1/15	63, 57, 55, 53, 52, 49, 47, 43	52.4	25					
		62, 60, 54, 54, 46, 44	53.3	25					
		64, 60	62			35			
		60, 58	59	33		30			
		54, 41, 41, 36, 28, 27	37.8					33	
		47, 43, 33, 23	36.5			26			
	1/10	22	22			12			
		38, 34, 33, 32, 25	32.4			21			
		46	46			31, 30, 22	27.7		
		53, 50, 41, 34	44.5			70, 30, 8	36		
	1/15 repeated	61, 40, 37, 33, 33, 21	37.5					28	
Residual ♀♀		60, 50	55			45			
		67, 62, 62, 50	60.3			50			
		27	27	10					
Colchicine after mating	1/3.5	49	49					10	
		55, 38, 35	42.7						
		35, 26	30.5			30			
		67, 50, 47, 44	52						
	1/2	87	87					21, 9	15
Various origins	A	58, 53, 52, 52, 50, 43, 37	49.3					27	
	B	41, 41, 39	40.3			30			
		57, 56, 50, 41, 35, 30	44.8			14			
	C	46	46	40					
		56, 40	48			27			
	D	47, 39, 29, 24, 17	31.2	10		30			
	E	29, 25, 25	26.3			21			

Key to Source of Data:-

A Natural Mating
B Artificial insemination
C Trypaflavine on the sperm before fertilisation
D Toluidine blue on the sperm before fertilisation
E Natural mating of a 'silver' female.

TABLE 22. DETAILS OF THE CELL NUMBERS OF SEVERAL KINDS OF 3½ DAY OLD HETEROPLOID EMBRYOS FOUND IN VARIOUS EXPERIMENTS.

Experimental source	Chromosome complement of embryos (2n=40)	Number of nuclei in embryos	Mean number of nuclei in diploid sibs
A	Haploid/diploid mosaic	55	50.5
		31	50.5
B		28	42.7
A	Diploid/tetraploid mosaic	35	44.6
C		45	60.3
		46	60.0
B		32	42.7
		21	52.0
D		31	-
A	Between haploid & diploid:		
	34 chromosomes	34	-
	35 "	11	25.0
	38 "	25	30.4
	34 "	36	51.5
C	28 "	14	-
B	31 "	14	29
A	Between diploid & triploid:		
	46 "	31	-
	48 "	30	59.3
	42 "	65	42.0
	47 "	23	33.0
	50 "	28	-
	45 "	45	54.0
	43 "	42	37.5
C	50 "	35	55.8
A	Various mosaics: 46/40/30	49	-
	45/34	19	-
	40+/13	29	25.5
C	43/40/35	63	55.0
B	37/35/29	25	36.0
A	Various types: 72 chromosomes	31	37.8
	7 "	62	52.7
	10 "	43	51.7
	68 "	42	-

Key to Experimental Source

A. Colchicine injected into uterus before mating; B. Colchicine injected into uterus after mating; C. From 'residual' females; D. Trypaflavine injected into uterus before mating.

C H A P T E R V.

ASPECTS OF FERTILISATION AND OF THE CLEAVAGE OF DIPLOID AND HETEROPLOID EGGS IN THE MOUSE.

The results of various experiments on the mouse egg at fertilisation or the first cleavage have been given and discussed. So far, each of these experiments has been considered in isolation or in relation to similar experiments; the purpose of the present chapter is to combine and correlate certain aspects of all of them. For simplification, the discussion will be divided into six parts, namely: the sperm, the egg, sperm entry into the egg, pronuclear growth and syngamy, the mammalian centriole and development, and the chromosomes and cleavage.

The sperm.

If spermatozoa from different inbred lines of mice are mixed in equal numbers, sperm from one inbred line fertilises many more eggs than sperm of the other. This difference may be due to pathological, physiological, or morphological characters of the sperm types, to an effect of one sperm type on the other, or to a favourable relationship between one sperm type and the eggs to be fertilised. The results (p. 18) indicate either of the first two of these three alternatives. If one sperm type is more active than another, the former may reach the site of fertilisation in larger numbers and fertilise more eggs. It is known that the number of sperms in the female tract is reduced from the considerable numbers at ejaculation to a mean of 16.9 sperms per ampulla (Braden and Austin, 1954b). If ascent up the fallopian tube, and penetration of the cumulus oophorus, are properties of the activity

of individual spermatozoa, most of the few sperms around the eggs could be of the more active type. This theory of selective fertilisation based on differential activity of spermatozoa ascending the female tract is analogous with the mechanism of selective fertilisation in plants caused by differential rates of growth of the pollen tube down the style.

The activity of spermatozoa is affected by experimental agents. Toluidine blue has a pronounced photochemical effect, reducing sperm activity to zero under certain conditions. Prolonged ultra-violet irradiation also inhibits sperm activity; high dosages of X-rays may do so.

The ovulated egg.

At fertilisation, the second maturation division of the egg is at metaphase. If fertilisation does not occur, the spindle and chromosomes remain identifiable for at least 42 hours. Degenerative processes are probably more rapid after this age.

Either prior to, or at fertilisation, the spindle of the egg can be destroyed by the action of colchicine or trypaflavine. The former has the more effective action. Destruction of the spindle sets free the dyads, and movements in the ooplasm may cause the chromosomes to become arranged round the periphery of the egg. The chromosomes may be completely expelled from the egg into small nucleated bodies which resemble undersized second polar bodies. Loss of the chromosomes may be accentuated at fertilisation due, for example, to shrinkage of the vitellus. The outwardly-directing movements of the egg cytoplasm may cease at sperm entry.

Sperm entry into the egg.

Fertilisation may be delayed after ultra-violet or X-irradiation of the sperm; delay may be caused by the reduced activity of the sperm after treatment by these agents. Normally, the sperm-head sinks sideways into the vitellus of the egg. After high irradiation dosages, the majority of sperm-heads enter the vitellus normally, but ultra-violet may cause some sperm-heads to assume a hooked shape in the vitellus. After high dosages of X-irradiation, the sperm-head may disintegrate in the ooplasm.

Sperm entry normally results in the development of a block to polyspermy by the zona reaction (Braden, Austin, and David, 1954), and in the completion of the second maturation division. X-irradiation or trypaflavine treatment of the sperm before fertilisation, and probably ultra-violet or toluidine blue treatment also, occasionally cause the failure of stimulation of the second maturation division at sperm entry. Polyspermy also occurs after these treatments. Similar phenomena have been reported in rabbit eggs after X-irradiation of the sperm (Amoroso and Parkes, 1947), and in sheep ova after trypaflavine treatment of the sperm (Thibault, 1949). The stimuli which cause the block to polyspermy and the completion of the second maturation division are probably related. Failure to cause stimulation may be due to damage to the cytoplasm of the sperm by these agents.

After natural mating, the second polar body of the egg may not form normally after sperm penetration. Instead of the normal division of the egg into a large ovum and a small second polar body, the egg may divide into two equal or nearly equal parts. One part contains one pronucleus, and is the analogue of

the second polar body; the other part contains two pronuclei, and is presumably the ovum with one male and one female pronucleus. Similar abnormalities have been reported by Braden and Austin (1954c) after heat-treatment of the mouse egg at fertilisation. These authors also reported a related phenomenon in the division into two halves of parthenogenetically stimulated mouse eggs; they termed this effect 'immediate cleavage' (Braden and Austin, 1954a).

Pronuclear growth and syngamy.

The male pronucleus may fail to be normally differentiated after heavy ultra-violet irradiation of the sperm before fertilisation. The normal swelling of the pronucleus is interrupted, and it remains as a greatly-swollen sperm-head. The stimulation of sperm-entry into these ova is sufficient to cause the normal growth of the female pronucleus. After high X-irradiation dosages, on the sperm, the male pronucleus may fail to differentiate in a normally stimulated ova, or it may grow alone in an ova which was not stimulated to complete its second maturation division at sperm entry. In the latter ova, the second maturation division of the ova remains at metaphase, and growth of the male pronucleus is very unusual and probably abortive. After trypaflavine treatment of the sperm, some sperm-heads which penetrate the vitellus fail to stimulate the ova and remain unchanged in the ooplasm. These effects may represent four increasingly abnormal aspects of sperm-egg relationships at fertilisation:- normal stimulation of the egg with normal growth of the male pronucleus; normal stimulation and growth of the

female pronucleus with abnormal formation or disintegration of the male pronucleus; failure to stimulate the egg with some differentiation of the male pronucleus; failure to stimulate the egg with no differentiation of the sperm-head into a male pronucleus. Blandau (1952) reported that, in rat ova fertilised between nine and twelve hours after ovulation, sperm entry and growth of the male pronucleus could occur without the normal growth of the female pronucleus; this phenomenon appears to be between the second and third aspects described above. Pincus and Enzmann (1936), and Amoroso and Parkes (1947), also reported degenerative changes in the male pronucleus after ultra-violet and X-irradiation respectively of rabbit sperm before fertilisation. Drebinger (1951) found that trypaflavine damaged the protoplasm of frog sperm, reducing its activity and fertilising ability.

After ultra-violet or X-irradiation of the sperm before fertilisation, many ova contained either one pronucleus which was presumably female, or a normal female pronucleus and an abnormal male pronucleus. After colchicine treatment at fertilisation, many ova contained a single pronucleus which was presumably female. Many of these gynogenetic and androgenetic ova failed to cleave; others probably achieved a retarded first cleavage. Eggs containing a single pronucleus are therefore usually highly abnormal in cleavage capacity compared with normal bi-pronucleate eggs, whether the single pronucleus is male or female.

After colchicine treatment at fertilisation, a large number of pronuclei can be seen in many eggs. One pronucleus is normal-sized, and is probably male. The others are small, and are probably female. If both sets of maternal chromosomes of the

second maturation division are retained in the egg at fertilisation, its complement will be triploid. The presence of up to five pronuclei in these eggs shows that pronucleus formation does not require a haploid chromosome set. The discovery of triploid blastocysts after colchicine treatment indicates that all these pronuclei may undergo syngamy. Statistical and cytological evidence suggests that multi-pronucleate ova may be delayed in syngamy and the first cleavage.

The mammalian centriole.

After colchicine treatment at fertilisation, many ova contain a single pronucleus which is probably male. Cleavage of these ova would produce haploid androgenetic embryos. After the same treatment, haploid blastocysts were identified at 3½ days gestation; some of these ova must therefore be capable of development. The haploid blastocysts were almost as advanced as their diploid sibs; the slight delay behind diploids may have been due to a longer development in the pronuclear stage. Cleavage in the haploid embryos was therefore probably the same as in diploids.

But the number of haploid embryos found at 3½ days gestation after colchicine treatment was considerably fewer than the number of unipronucleate ova induced by the treatment. Only 5% of them were found as haploid embryos. The remainder must have died very early in development. Recently, ova have been examined after the time of the first cleavage. Many unipronucleate ova could be seen to be dying without cleavage, their cytoplasm becoming greatly furrowed and fragmented. There was, therefore,

a very definite all-or-none response to colchicine treatment in the development of these ova. Cleavage was either normal or completely absent. Some knowledge of the factors causing the all-or-none effect can be inferred. The ova were subjected to a considerable amount of colchicine, and the death without cleavage could have been due to the toxic effect of the chemical. But only the unipronucleate eggs were missing at 3½ days gestation; almost all of the multi- and bi-pronucleate eggs also observed after this treatment could develop to blastocysts without difficulty. Also, the haploid embryos which did cleave were almost as successful as diploids, and showed not a trace of the toxic action of colchicine. It seems most unlikely that the toxicity of colchicine would affect only the haploids, and in such a manner that cleavage was either normal or completely suppressed.

The all-or-none response in development was probably due to other factors than toxicity of the chemical. By the nature of the action of colchicine on the spindle of the unfertilised egg, it is possible that other maternal constituents in addition to the chromosomes may be lost from the egg. If the loss of these constituents occurred independently to that of the chromosomes, then the maternal elements in the egg at fertilisation could include none, either, or both of the chromosomes and the other constituents. Presence of the maternal chromosomes is not necessary for cleavage, for the sperm chromosomes alone are sufficient. But absence of the other constituents prevents cleavage; they might therefore be the maternal centriole or centrioles. If the maternal centrioles are lost, the centriole obtained from the sperm-head is insufficient to organise cleavage

in the embryo. If one or both of the maternal centrioles remain in the fertilised ovum, cleavage can occur normally. Centrioles from both egg and sperm are necessary, therefore, in these ova.

The same rules must presumably govern gynogenetic development. All of the ova which were fertilised by irradiation sperm responded in the normal manner by extruding their second polar body. All of them possessed the normal maternal complement of chromosomes and centrioles. From the sperm, however, they received irradiated chromosomes and centrioles. After ultra-violet or X-irradiation, the sperm chromatin failed to condense into chromosomes, and the embryos commenced development with haploid maternal chromosomes only. Some of these gynogenones failed to cleave (see also Amoroso and Parkes, 1947, who found the same result after X-irradiation of rabbit sperm before fertilisation); the others had a very abortive development which failed after up to three cleavages. The chromosome complement of these embryos is unlikely to have prevented cleavage, for some of the haploids produced by colchicine, and others occurring spontaneously (Beatty and Fischberg, 1949 and 1951a; Beatty, 1954) developed without difficulty to blastocysts. Suppression of normal cleavages in the gynogenones may have been due to the presence of an irradiated male centriole which, depending on the amount of damage it had received, disabled the embryos at the first or an early cleavage.

So far, a single centriole has been postulated in the sperm-head, and one or more in the ovum. The work of Thibault (1949) clarifies the centriolar complement of the ovum. Thibault found

that if the rabbit egg was stimulated parthenogenetically, eggs which extruded their second polar body failed to cleave. Only some of those which retained their second polar body within the ovum succeeded in cleaving normally. The parthenogenones which cleaved to blastocysts were therefore diploid; this has also been found by Chang (1954). One of the conditions for parthenogenetic rabbit development is that the egg must retain all the contents of the second maturation division. The same general rule probably applies to other mammalian species. The usual response to parthenogenetic agents of the rat egg (Thibault, 1949) and the mouse egg (Braden and Austin, 1954a) is the extrusion of the second polar body. The haploid rat eggs usually fail to cleave (Thibault, 1949), though a few of them can divide to two-celled embryos (Austin, 1954). The arguments given previously concerning the normal cleavage of haploid mouse embryos probably applies to other mammalian species; the failure of the majority of these parthenogenones may therefore be due to their possession of only a single centriole. Only if both centrioles are retained can these eggs cleave; advanced parthenogenones in mammals are therefore diploid.

Three centrioles are therefore postulated in the gametes at fertilisation; the egg possesses two, one at each pole of the second meiotic spindle, the sperm possessing one. Any undamaged pair of these are necessary for normal cleavage. Under natural conditions, one egg centriole enters the second polar body; the other egg centriole and the sperm centriole remain in the ovum. The possession of all three, as presumably occurs in triploids, does not result in tripolar mitoses or in the uneven distribution

of chromosomes, and triploid embryos can be found up to implantation (Fischberg and Beatty, 1951). Thibault (1949) classifies mammal eggs into two types according to their response to agents which induce parthenogenesis: the depolarised type, e.g. rabbit and sheep, which do not necessarily extrude their second polar body, and the polarised type, e.g. rat, which do extrude it. In experiments attempting the production of viable parthenogenetic embryos, the depolarised type are normally used because of their superior development; parthenogenetic rabbits have been reported to full term by Pincus (1939) and Pincus and Shapiro (1940). The application of colchicine to the mouse egg, which is a polarised type, followed by the parthenogenetic stimulation of these eggs, may induce their development without the extrusion of the second polar body. Alternatively, the fertilisation of colchicine-treated mouse eggs by ultra-violet irradiated sperm may induce diploid gynogenesis.

<u>The chromosomes and cleavage.</u>

Haploid, triploid and tetraploid mouse embryos were first found at 3½ days gestation by Beatty and Fischberg (1949, 1951b) occurring either spontaneously or after heat-treatment of fertilised ova. Similar heteroploids were also found after colchicine treatment at fertilisation or at the first cleavage, and after various experimental agents were added to the spermatozoa before fertilisation (p. 81). Except for those of the latter group which were induced by ultra-violet or X-irradiation, the alteration in chromosome complement was probably the only alteration imposed on the development of these embryos. The irradiations probably induced additional damage to change in chromosome complement.

At $3\frac{1}{2}$ days gestation, untreated diploid embryos have undergone approximately 5.6 cleavages. Tetraploid embryos induced by experimental agents other than irradiations were retarded by approximately one cleavage behind their diploid sibs. But, to induce tetraploidy, one cleavage of a diploid embryo has presumably been suppressed. After the suppressed cleavage, therefore, further cleavages in tetraploids had probably occurred at the same rate as in diploids. Neither chromosome complement nor the possible doubling of the number of centrioles in each cell had retarded their rate of cleavage. The triploid and haploid embryos occurring spontaneously or found after the use of experimental agents other than irradiations were retarded by 0.7 and 1.0 cleavages respectively in comparison with their diploid sibs. Statistical and cytological evidence indicates that the lower cleavage number of haploids and triploids could have been due to delay in pronuclear growth. If so, their cleavage also occurred at the same rate as in diploids. If not, their cleavage was only slightly retarded behind diploids. Rate of cleavage up to $3\frac{1}{2}$ days gestation is therefore largely independent of the chromosome complement of the embryo.

After irradiation of the sperm before fertilisation, however, the resultant haploids were highly retarded in comparison with diploid sibs; some haploids had cleaved only once by $3\frac{1}{2}$ days gestation. These haploids were undoubtedly gynogenetic, whereas the other haploids described were probably androgenetic. It seems unlikely, however, that the difference between maternal and paternal chromosomes would cause this difference in behaviour. The few polyploids resulting from the irradiation experiments were

also retarded. If cleavage rate is normally independent of chromosome complement, the failure of cleavage in these embryos may have been due to their possession of an irradiated male centriole or cytoplasm. Cleavage appears to be largely dependent on the centriolar complement of the embryo. If the chromosomes of an embryo could be inactivated without damage to its centrioles, the achromosomal embryo may cleave. In Arbacia, this phenomenon has been observed (Harvey, 1940). Two systems may work in conjunction in early development: first, the duplication of nuclear and cytoplasmic materials in the cells leading to regular cleavage in the embryo; second, the genic control of development imposing an order and organisation on the cleaving cells.

REFERENCES

AMOROSO, E.C. & PARKES, A.S. 1947. Effects on embryonic development of X-irradiation of rabbit spermatozoa _in vitro_. Proc. Roy. Soc., B, 134, 57-78.

AUSTIN, C.R. & BRADEN, A.W.H. 1954. Nucleus formation and cleavage induced in unfertilised rat eggs. Nature 173, 999-1000.

BEATTY, R.A. 1954. Haploid rodent eggs. Proc. 9th Intern. Congr. Genetics, Bellagio.

BEATTY, R.A. & FISCHBERG, M. 1949. Spontaneous and induced triploidy in pre-implantation mouse eggs. Nature 163, 807.

__________ 1951a. Heteroploidy in mammals: I Spontaneous heteroploidy in pre-implantation mouse eggs. J. Genet. 50, 345-359.

__________ 1951b. Cell number in haploid, diploid and polyploid mouse embryos. J. exp. Biol. 28, 541-552.

BLANDAU, R.J. 1952. The female factor in fertility and infertility. I Effects of delayed fertilization on the development of the pronuclei of rat ova. Fertility and Sterility 3, 349-365.

BRADEN, A.W.H. & AUSTIN, C.R. 1954a. Reactions of unfertilised mouse eggs to some experimental stimuli. Exp. Cell Res. 7, 277-280.

________________ 1954b. The number of sperms about the eggs in mammals and its significance for normal fertilisation. Aust. J. Biol. Sci. 7, 543-551.

________________ 1954c. Fertilization of the mouse egg and the effect of delayed coitus and hot-shock treatment. Aust. J. Biol. Sci. 7, 552-565.

BRADEN, A.W.H., AUSTIN, C.R. & DAVID, H.A. 1954. The reaction of the zona pellucida to sperm penetration. Aust. J. Biol. Sci. 7, 391-409.

CHANG, M.C. 1954. Development of parthenogenetic rabbit blastocysts induced by low temperature storage of unfertilised ova. J. Exp. Zool. 125, 127-150.

DREBINGER, K. 1951. Kerngifte und Lichtstrahlung. Eine Studie an Froschspermien zur Wirkungsanalyse der Kerngifte. Arch. EntwickMech. 145, 174-204.

FISCHBERG, M. & BEATTY, R.A. 1951. Spontaneous heteroploidy in mouse embryos up to mid-term. J. exp. Zool. 118, 321-336.

HARVEY, E.B. 1940. A comparison of the development of nucleate and non-nucleate eggs of Arbacia punctulata. Biol. Bull. Wood's Hole, 79, 166.

PINCUS, G. 1939. Comparative behaviour of mammalian eggs in vivo and in vitro. IV Development of fertilised and artificially activated rabbit eggs. J. exp. Zool. 82, 85-129.

PINCUS, G. & ENZMANN, E.V. 1936. The comparative behaviour of mammalian eggs in vivo and in vitro: II The activation of tubal eggs of the rabbit. J. exp. Zool. 73, 195-208.

PINCUS, G. & SHAPIRO, H. 1940. The comparative behaviour of mammalian eggs in vitro and in vivo. VII Further studies on the activation of rabbit egg. Proc. Amer. Phil. Soc. 83, 631-647.

THIBAULT, C. 1949. L'oeuf des mammifères, son développement parthénogénétique. Ann. Sci. nat., Zool. 11, 136-219.

SUMMARY.

Heteroploidy in mouse embryos was induced by various treatments of the gametes at fertilisation or of the egg at the first cleavage.

If fertilisation occurred in the presence of colchicine, the second maturation spindle of some ova was destroyed by the chemical. In these ova, the dyads became scattered through the öoplasm. Three and a half day embryos developing from these ova had chromosome complements of between haploid and triploid; the haploids were probably androgenetic. Tetraploids, mosaics, and other heteroploids were also found in small numbers. After colchicine was applied to the egg at the time of the first cleavage, small numbers of tetraploids were found at 3½ days gestation.

Other treatments of sperm samples were intended to inactivate the male chromatin before fertilisation. Many resultant 3½ day old embryos were heteroploids; among them were some haploids which were presumably gynogenetic. Ultra-violet irradiation of the sperm was most successful in inducing haploidy; X-irradiation gave less success. After addition of trypaflavine or toluidine blue to the sperm before fertilisation, probably no gynogenetic development was induced. Insemination of rat or rabbit sperm into mouse females also failed to induce gynogenones to 3½ days gestation.

Histological events at fertilisation and the first cleavage were studied after ultra-violet, X-ray, or trypaflavine treatment of the sperm, and the observations were compared with those found

after normal mating. Ultra-violet irradiation suppressed the condensation into chromosomes of the male chromatin at syngamy; some of the eggs then developed as haploids, others degenerating without cleavage. X-irradiation did not usually prevent pronucleus formation, but the male chromosomes were lost into sub-nuclei during early cleavage. Trypaflavine reduced the penetrating power of the sperm, and most eggs were unfertilised. Abnormalities in sperm-egg relationships at fertilisation were seen after X-irradiation or trypaflavine treatment of the sperm.

The tetraploid embryos produced in the colchicine experiments were one cleavage behind diploids at $3\frac{1}{2}$ days gestation. When allowance is made for the suppressed cleavage which caused the tetraploidy, the mitotic rate of both types of embryo was probably identical. Triploid and haploid embryos found after colchicine treatment at fertilisation were less than one cleavage behind diploids. Syngamy and the first cleavage may have been delayed in triploid and haploid ova, but mitotic rate in them after the first cleavage was probably the same as in diploids. Many of the haploid, triploid, and tetraploid embryos were blastocysts. No heteroploids were found in the offspring of colchicine-treated mothers.

Irradiation of the sperm by ultra-violet or X-rays had severe effects on embryonic development. High dosages of either restricted development to the first cleavage. Lower dosages caused considerable mortality before and after implantation. None of the haploids found after these treatments had completed their fourth cleavage at $3\frac{1}{2}$ days gestation; many other heteroploids possessed sub-nuclei and/or cytological anomalies. Genetic

damage to the chromosomes by the irradiations was presumably responsible for embryonic death after the early cleavages; death in the early cleavage stages may have been due to cytoplasmic damage to the sperm.

Evidence from three sources: the failure of normal cleavage following irradiation of the sperm before fertilisation, the degeneration without cleavage of the majority of androgenetic haploid ova found after colchicine treatment at fertilisation, and the work of other authors, has been used to construct a theory concerning the numbers of centrioles in the gametes at fertilisation.

ACKNOWLEDGEMENTS

I am deeply indebted to Dr. R. A. Beatty, for his advice during the experimental work, and for his criticisms during preparation of the manuscript; and to Professor C. H. Waddington, F.R.S., for laboratory facilities.

I also wish to thank Mr. J. Isaacson and his staff for the liberal supplies of mice, and Mr. A. Graham and Mr. D. Pinkney for the preparation of the photographs.

This work was carried out during the tenure of post-graduate studentship from the University of Edinburgh.