AN INVESTIGATION OF POLYSPERMY IN THE RAT AND RABBIT

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Summary

A review is presented of published reports on polyspermy in mammals.

The cytology of living polyspermic rat and rabbit eggs, as seen with the phase-contrast microscope, is described. Polyspermic rat eggs have been seen in various stages of fertilization and early cleavage; observations on rabbit eggs are restricted to the later phases of pronuclear growth.

In the polyspermic rat egg during fertilization there is generally a close similarity between the male pronuclei; in the rabbit egg variations in pronuclear size are common.

In the polyspermic rat egg, the chromosome complements from the female pronucleus and the male pronuclei all take part in the formation of the first cleavage spindle. Polyspermic rat eggs at the 2-, 4-, and 8-cell stages are quite normal in appearance, except for the presence of the extra sperm mid-pieces.

The incidence of polyspermy under normal mating conditions as observed in 826 penetrated eggs from 87 rats was about 1.2 per cent. Among 69 pronucleate eggs from 12 rabbits the incidence was about 1.4 per cent.

When rats were mated at 10.0 p.m. (ovulation 12.0 midnight to 4.0 a.m.) the incidence in 353 penetrated eggs was about 3.7 per cent.; when mated at 3.0 a.m., the incidence in 315 penetrated eggs was about 7.9 per cent.; and when mated at 8.0 a.m., the incidence in 411 eggs was about 8.8 per cent. In 955 penetrated eggs from 75 immature rats, in which ovulation was artificially induced, the incidence of polyspermy was about 2.1 per cent. Altogether 112 polyspermic rat eggs were seen, including 109 dispermic and three trispermic.

When rabbits were mated at about the time of induced ovulation, 11 presumed polyspermic eggs (about 16.4 per cent.) were observed among 67 pronucleate eggs from 17 rabbits. The 12 polyspermic eggs seen in normal and delay-mated rabbits included nine probably dispermic and three doubtful eggs.

The results obtained from the rats are interpreted as showing that the susceptibility to polyspermy increases rapidly after ovulation, but approaches a limit within 2-3 hr. At this point, the number of eggs that become polyspermic is between one-quarter and one-third of all eggs having a chance of becoming polyspermic. Beyond 3 hr there is little further increase in susceptibility.

The probability that polyspermy gives rise to triploidy in rats, and the fate of the polyspermic rat egg, are discussed.

I. Introduction

From the intensive studies on fertilization which were made in the last quarter of the nineteenth century, it became apparent that, although only one

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sperm nucleus normally conjugates with the egg nucleus, more than one sperm may enter the egg and form a pronucleus. In some animals, notably among the insects, elasmobranchs, amphibians, reptiles, and birds, the entry of additional sperms, "polyspermy," was found to be a regular occurrence. The extra pronuclei in no way interfere with the subsequent development of the egg, unless a very large number of sperms is involved.

In other species, however, the penetration of the first sperm evokes the elevation of a membrane, or an alteration of the egg surface, which excludes other sperms. This block to polyspermy is not, however, fully efficient so that polyspermy does sometimes occur, and is then known as "pathological polyspermy." Its consequences are discussed by Wilson (1925); they vary according to the behaviour of the extra pronuclei. In *Ascidia* and the frog, only one of the male pronuclei conjugates with the female pronucleus, but whereas the polyspermic ascidian egg soon degenerates the frog egg may continue development and sometimes even reach the tadpole stage. The difference seems to depend on the fact that, in the frog, the mitotic figures from the extra pronuclei remain separate in the cytoplasm and more or less regular cleavages can take place. However, three or more blastomeres may result from the first cleavage and many of the blastomeres are binucleate, one nucleus being haploid and the other diploid. In the polyspermic sea-urchin egg, two or more male pronuclei may conjugate with the female pronucleus, giving rise to multipolar spindles and thus to multiple cleavage. Occasionally a dispermic egg will develop as far as a free-swimming larva, but most often there is early degeneration.

Information on polyspermy in mammals is scarce and confused, but several authors have declared it to be pathological in this class of animal (Lams 1913; Wilson 1925; Hartman 1934).

Rein (1883) stated that, in his attempts to obtain the fertilization *in vitro* of rabbit and guinea pig eggs, he had several times seen a number of sperms penetrate into ovarian eggs and there execute vigorous movements. He was not sure, however, if the eggs were still alive at the time. Rather similar are the observations of Lams (1913) and Blandau and Odor (1952). Lams described a guinea pig egg, the only "polyspermic" one among 108 eggs that he examined, saying that it was degenerate in appearance, with the chromatic material scattered throughout the vitellus. It contained several entire sperms, unchanged in form, in addition to the fertilizing sperm, the head of which was partially transformed into a pronucleus. Blandau and Odor report that they have seen, in freshly recovered rat eggs, accessory sperms penetrating the vitellus although it already contained a fertilizing sperm. According to these authors, the accessory sperms may enter and leave the vitellus a number of times, though none were seen to be retained within the vitellus. In another communication, Odor and Blandau (1949) found no polyspermic eggs in a total of 423 rat eggs which they had examined.

To a different category belong the four mouse eggs described by Sobotta (1895) among the 1140 fertilized eggs that he studied. Mice were found to mate a second time, 24 hr after the first mating, and in some of the eggs from these animals Sobotta saw objects resembling early pronuclei or sperm heads

undergoing change in the egg cytoplasm. He considered that a second and later penetration of the eggs had occurred. Rubaschkin (1905) made rather similar observations on guinea pig eggs, in which he occasionally saw, besides the two normal pronuclei, a chromatic body staining strongly with haematoxylin or haemalum. These bodies closely resembled early male pronuclei. They were found also at later stages, such as the diaster stage of the first cleavage division. Rubaschkin thought they might indeed be accessory sperm nuclei, from a second and later penetration, but added that there was no certain evidence for this view. Kremer (1924) too recorded instances of a second and later sperm penetration in rat eggs, wherein he said it was more common than in mouse eggs. One of his figures shows a rat egg which has two well-formed pronuclei and, in addition, an apparently unchanged sperm head in the peripheral cytoplasm. One of the polyspermic eggs illustrated by Amoroso and Parkes (1947), obtained after inseminating rabbits with X-irradiated sperms, should be noted here for it also contained several unchanged sperm heads in the vitellus.

The observations of Gatenby and Hill (1924) suggest that polyspermy may be regarded as a normal process in *Ornithorhynchus*. This fact they consider to be consistent with the occurrence of physiological polyspermy in other megalecithal eggs, such as those of reptiles and birds.

Several authors have reported mammalian eggs with three pronuclei: two rat eggs, by Tafani (1889); one out of 36 pronucleated eggs in the cat, by R. van der Stricht (1911); two out of 11 pronucleated eggs in the cat, by Hill and Tribe (1924); one mouse egg, by Kremer (1924); one out of 71 pronucleated eggs in the ferret, by Mainland (1930); one rabbit egg, by Amoroso and Parkes (1947); one mouse egg, by Pesonen (1949); and three rat eggs, as well as one with four pronuclei, by Austin (1951). These authors considered the eggs to be polyspermic, except for Kremer, who thought that the phenomenon was due to the fertilization by one sperm of a binucleate egg, and Pesonen, who believed that the second maturation spindle, by completing its meiosis away from the surface of the egg, had given rise to two female pronuclei.

In a single instance, a cleaved polyspermic egg has been identified (Austin and Smiles 1948). This had two sperm mid-pieces in the cytoplasm and a small subnucleus in one of the blastomeres, but was otherwise normal in appearance.

From this brief review it can be seen that several different types of "polyspermy" have been described in mammalian eggs. Physiological polyspermy apparently occurs in *Ornithorhynchus*. Among the Eutheria, the phenomena described include eggs containing several unchanged sperms, others with three or more pronuclei at approximately equivalent stages of development, and others again in which early pronuclei were said to exist in the later stages of fertilization or in the blastomeres of 2-cell eggs. Little is known concerning the incidence of polyspermy and information on the fate of polyspermic eggs is lacking.

The investigations that are described in this paper were made with the purpose of obtaining a better understanding of the polyspermic mammalian egg,

through studies on rats and rabbits. A preliminary account of the results has already been submitted (Austin and Braden 1953*a*): the present communication represents a full report on the work done.

II. Methods

Randomly mated albino rats, and albino and cross-bred rabbits, were used in these studies.

In the adult rats in this colony, ovulation was found to occur principally between 12.0 midnight and 4.0 a.m., during the period of these investigations (Braden, unpublished data). For convenience, the day before ovulation will be referred to in the text as day 0, and the day on which ovulation occurs as day 1. The time of normal mating has been found to be during the late afternoon of day 0, but if oestrous female rats are placed with the males later, even as late as 9.0 a.m. on day 1, mating generally occurs.

Ovulation was induced in immature rats (35-50 days old) by the method of Rowlands (1944), except that B.D.H. "Serogan" and "Gonan" were used as sources of the gonadotrophin of pregnant mare's serum and chorionic gonadotrophin, respectively. When this procedure is employed, ovulation occurs mainly between 11 and 14 hr after the second injection (Austin 1951). The day of ovulation in the immature rat will be referred to in the text as day 1.

In rabbits, ovulation was induced by the intravenous injection of 50 I.U. of "Gonan"; it occurs about 10 hr after the injection.

Eggs were recovered from the rat fallopian tube by dissection under normal saline solution, and from the rabbit fallopian tube by flushing with saline. The eggs were examined with a phase-contrast microscope by the method previously described (Austin and Smiles 1948). Sometimes rat eggs under observation were fixed with aceto-carmine or 10 per cent. acetic alcohol and stained with methylene blue while compressed between coverslip and slide. Passage of the reagent to the egg is easily effected by applying drops of the solution to one side of the coverslip and a piece of filter paper to the opposite edge. The fluid is thus drawn under the coverslip.

III. Observations

(*a*) *Cytology of Polyspermic Rat Eggs*

In the present series, 109 dispermic and three trispermic rat eggs have been recovered and in these were represented most of the stages of fertilization and early cleavage. The earliest recorded is that shown in Plate 1, Figure 1; only two polyspermic eggs in this stage have been observed. Two sperm mid-pieces can be seen as they lie side by side in the superficial cytoplasm of the egg, and nearby are the two acrosomes. The nuclear parts of the sperm heads have undergone change and are at the stage when the nuclear material cannot be distinguished from the egg cytoplasm with the phase-contrast microscope.

Figures 2-5 show the pronuclei at various stages of development. In each of these eggs, two sperm mid-pieces are visible, and it is noticeable that the

two male pronuclei are almost identical in size and structure. Rarely, polyspermic eggs are seen in which the correlation between the male pronuclei is not quite as close as this. In Plate 1, Figures 4 and 5, the later stages of pronuclear growth are represented; the latter shows the two male pronuclei in apposition.

Plate 1, Figure 6, illustrates a polyspermic egg which has been penetrated by two extra sperms and is therefore described as trispermic. All three male pronuclei can readily be discerned as well as some evidence of the three sperm mid-pieces. Unlike the great majority of polyspermic eggs, the male pronuclei in this trispermic egg are not identical in size and form, but the differences are not great.

Polyspermic rat eggs, recovered at later stages, have shown some of the phases involved in the formation of the first segmentation spindle. Plate 2, Figure 7, shows an egg which, when first seen, had three pronuclei in the terminal stages of their development. The egg was kept under observation and, within half an hour, three groups of chromosomes could be discerned in the cytoplasm; these are indicated in the figure. The egg was left for another hour in the hope that spindle formation would occur, but as little further change could be detected it was fixed and stained. The three groups of chromosomes could then be seen very clearly (Plate 2, Fig. 8). There is a suggestion of chromosome scatter which may have been due to an interference to spindle formation provided by the *in vitro* conditions. Another dispermic egg, which was observed to have three groups of chromosomes, was immediately fixed and stained, and then compressed very firmly beneath the coverslip. By this procedure a better view was obtained of the chromosome groups (Plate 2, Fig. 9).

Several polyspermic eggs have been seen in which only a single group of chromosomes could be discerned. The egg shown in Plate 2, Figure 10, is one of these, after it had been compressed by the method just described. Apart from this prophase chromosome group, no other basophilic material could be observed in the egg. Plate 2, Figure 11, shows a prophase in another polyspermic egg treated in the same way. No polyspermic rat eggs have been seen in which there was any indication that the chromosome groups from all pronuclei did not together take part in the formation of the first cleavage spindle. Polyspermic eggs with the anaphase or telophase stages of the first cleavage spindle have rarely been found, but one showing an early telophase is illustrated in Plate 2, Figure 12. This egg was fixed and stained but not squashed; the spindle fibres are perceptible in this preparation and the spindle as a whole appears normal.

A number of polyspermic eggs have been seen in the 2-, 4-, and 8-cell stages of cleavage. At these stages the diagnosis of polyspermy is more difficult than during pronuclear development for it depends entirely upon the recognition that two or more sperm mid-pieces are present within the cytoplasm of the blastomeres. Ofter "supplementary" sperms, that have penetrated the zona pellucida but have not entered the vitellus, can be seen in the perivitelline space during fertilization and segmentation. Quite frequently too, the heads become detached from these supplementary sperms and the headless mid-pieces then superficially resemble the mid-pieces of fertilizing sperms. The distinction can,

however, be readily made by careful examination under the high power of the microscope. In the 2-cell egg, the mid-piece of a fertilizing sperm shows distinct signs of disintegration; much of its original opacity is lost, it may offer a granular appearance, and it is usually split for a variable proportion of its length. The disintegration becomes progressively greater in 4-cell and later stages of cleavage. By contrast, the mid-piece of a supplementary sperm remains uniformly smooth, opaque, and intact. Even if these structural differences are ignored, however, it is nearly always possible to decide definitely, after careful focusing, whether the mid-piece is in the cytoplasm or in the perivitelline space.

TABLE 1

TOTAL NUCLEAR AND NUCLEOLAR VOLUMES AND NUMBER OF NUCLEOLI IN NORMAL AND POLYSPERMIC 2-CELL RAT EGGS

	Egg Number	Total Nuclear Volumes (cu. μ)	Total Nucleolar Volumes (cu. μ)	Total Number of Nucleoli
Normal	1*a*	4990	744	8
	2*a*	5064	589	15
	3*a*	5952	584	7
	4*a*	7702	677	9
	5*a*	7484	819	5
	6*a*	4834	562	13
	7*a*	4582	702	10
	Total	40608	4677	67
	Mean	5801	668	9·6
Polyspermic	1*b*	5846	704	8
	2*b*	4796	607	11
	3*b*	6709	619	9
	4*b*	5106	632	15
	5*b*	7891	793	18
	6*b*	4752	572	14
	7*b*	5932	666	7
	Total	41032	4593	82
	Mean	5862	656	11·7

Aside from the presence of two or more mid-pieces within the cytoplasm, cleaved polyspermic eggs were found to be remarkably free from any evidence of abnormality. Polyspermic 2-cell rat eggs are shown in Plate 3, Figures 13 and 14. Parts of the two sperm mid-pieces can be seen in each egg, and the cytoplasm and nuclei are plainly very similar to those of monospermic 2-cell eggs. Plate 3, Figures 15 and 16, shows more clearly the two sperm mid-pieces as found in polyspermic 2-cell eggs. As is often the case, the two mid-pieces are apparently held together by a ring-shaped structure which is illustrated more clearly in Plate 3, Figure 16. This structure is commonly seen about the mid-piece of the sperm in normal 2-cell eggs, but is not invariably present.

With successive cleavage stages it becomes more difficult to discern the two sperm mid-pieces upon the presence of which the diagnosis of polyspermy depends. The danger is that an extra sperm mid-piece may be missed and not that a supplementary sperm in the perivitelline space may be taken for an extra penetrating sperm, for the difference between these two is very distinct in the later cleavage stages. Plate 3, Figure 17, shows the two sperm mid-pieces which were found in an otherwise normal 8-cell egg. They were much thinner than the sperms seen in the 2-cell egg (Plate 3, Fig. 16), apparently through loss of an outer granular layer; they were also much reduced in length.

In Plate 3, Figures 18, 19, and 20, are shown eggs which exhibit features that are unusual and may have a bearing upon some of the cases of "polyspermy" reported in the literature. Plate 3, Figure 18, shows a monospermic egg containing two female pronuclei which may have originated through the failure to extrude the second polar body. Quite often, segmented eggs are seen that have a small subnucleus in one blastomere in addition to the normal nucleus (Plate 3, Fig. 19). Sometimes the subsidiary nucleus is somewhat larger than this, but only once has an egg been seen which had two nuclei of about the same size and structure in one blastomere and a normal nucleus in the other (Plate 3, Fig. 20). Neither of these eggs was polyspermic.

Observations were also made on the total volumes of nuclei and the number and total volumes of nucleoli, in seven normal and seven polyspermic 2-cell eggs. The normal 2-cell eggs came from the same fallopian tubes as the polyspermic eggs and were measured so as to serve as controls. The method of measurement, together with data on normal values for segmented eggs, are described by Austin and Braden (1953*b*). The results are shown in Table 1: mean total nuclear volumes were 5801 cu. μ for the normal, and 5862 cu. μ for the polyspermic eggs, and mean total nucleolar volumes were 668 and 656 cu. μ respectively. The normal eggs had a mean of 9.6 nucleoli per egg, with a range from five to 15, and the polyspermic eggs a mean of 11.7, with a range from seven to 18. There was no significant difference in any of these values between normal and polyspermic eggs.

(*b*) *Cytology of Polyspermic Rabbit Eggs*

Although it is easy to see sperms in the perivitelline space of the living rabbit eggs, they can seldom, in the authors' experience, be detected in the cytoplasm. Consequently the diagnosis of polyspermy in the rabbit has had to rest solely upon the presence of more than two pronuclei. Normal eggs in the course of fertilization are shown in Plate 4, Figures 21 and 22; in each egg two well-formed pronuclei can be seen. In one egg the pronuclei are about the same size, whereas in the other they are distinctly dissimilar in size. Each pronucleus contained a few small nucleoli. Two presumptive polyspermic eggs are depicted in Plate 4, Figures 23 and 24. Each of these has three objects which are considered to be pronuclei, for they also contained a few small dark bodies like nucleoli and otherwise closely resemble the pronuclei in Plate 4, Figures 21 and 22. In one egg, however, two pronuclei are smaller than the third, whereas in the other egg two are larger than the third. The majority

of the polyspermic rabbit eggs referred to later in this paper resembled one or other of these two types of eggs in approximately equal numbers. However, two eggs were found with six objects closely resembling pronuclei of various sizes. One of these eggs is shown in Plate 4, Figure 25; only four of the "pronuclei" can be seen, the other two being at a lower focal plane. So far as could be determined by careful focusing, there was no continuity between any of these "pronuclei" and thus no suggestion that the smaller ones were only lobules projecting from other "pronuclei."

(*c*) *Incidence of Polyspermy in Rats*

To determine the incidence of polyspermy under varying conditions, adult rats were mated and killed according to five different schedules:

(*A*) Female rats were mated under normal conditions, i.e. they were left with males overnight from 4.0 p.m., and those with copulation plugs the next morning were killed during the course of the day (day 1).

(*B*) Female rats were left with males for 1 hr only, between 10.0 and 11.0 p.m.; those with copulation plugs were killed during the next day (day 1).

(*C*) Oestrous female rats were selected about midnight by means of the vaginal smear and left with males for 1 hr between 3.0 and 4.0 a.m. of day 1; those with copulation plugs were killed during the course of the same day.

(*D*) Oestrous female rats, selected by vaginal smear, were left with males for 1-2 hr between 8.0 and 10.0 a.m. on day 1; those with copulation plugs were killed on the morning of day 2.

(*E*) Oestrous female rats, selected by vaginal smear, were left with males for 1 hr between 9.0 and 10.0 a.m. on day 1; those with copulation plugs were killed during the afternoon of day 4.

Observations were also made on the incidence of polyspermy in immature rats, and these were treated according to schedule *F*: Immature female rats received injections for the induction of ovulation and were left with adult males overnight from the time of the second injection (3.0-4.0 p.m., day 0); those with copulation plugs the next morning were killed at various times on days 1 and 2.

The numbers of rats used in each group, together with the principal observations made in these experiments, are set forth in Table 2.

The group of rats which were allowed to mate under normal conditions (schedule *A*) was made up of 87 animals, and these yielded a total of 826 eggs, of which 810 (98.1 per cent.) had been penetrated by sperms. Ten eggs, or 1.2 per cent. of the penetrated eggs, were polyspermic; in each of these one extra sperm had entered the vitellus. It was notable that in all the polyspermic eggs the two male pronuclei were closely similar in size and structure.

When rats were mated at 10.0-11.0 p.m. on day 0 (schedule *B*) a sudden increase in the proportion of polyspermic eggs was obtained: 3.7 per cent. of

TABLE 2

INCIDENCE OF POLYSPERMIC EGGS IN ADULT RATS MATED AT VARIOUS TIMES AND IN IMMATURE RATS AFTER INDUCED OVULATION

Schedule	Time of Mating	Time of Killing	Number of Rats	State of Eggs	Total Eggs	Total Penetrated Eggs	Polyspermic Eggs	% Polyspermic of Penetrated Eggs	Remarks
A	Day 0* 4 p.m.	Day 1* 11 a.m.-4 p.m.	87	Fertilization stages	826	810	10	1·2	All polyspermic eggs were dispermic
B	Day 0 10-11 p.m.	Day 1 11 a.m.-3 p.m.	39	Fertilization stages	366	353	13	3·7	All polyspermic eggs were dispermic
C	Day 1 3-4 a.m.	Day 1 10 a.m.-3 p.m.	32	Fertilization stages	323	315	25	7·9	Two trispermic eggs included
D	Day 1 8-10 a.m.	Day 2 9.30-10 a.m.	52	Fertilization and first cleavage stages	456	411	36	8·8	All polyspermic eggs were dispermic; one was degenerating
E	Day 1 9-10 a.m.	Day 4 2-5 p.m.	21	4-cell to 8-cell stages of cleavage	166	143	8	5·8	Polyspermic eggs were four 4-cell and four 8-cell; all dispermic
F	Day 0 4 p.m.	Day 1 and Day 2	75	Fertilization and first cleavage stages	1130	955	20	2·1	*Immature Rats.* One polyspermic egg was trispermic and one was degenerating

* Ovulation occurs between midnight on day 0 and 4 a.m. on day 1 in adult rats, and was induced between 2 a.m. and 6 a.m. on day 1 in the immature rats.

penetrated eggs. This figure is significantly different from the value obtained after normal mating ($P < 0.02$). There were 39 rats in this group and from them a total of 366 eggs was obtained, of which 353 (96.4 per cent.) were penetrated. As with the first group, all polyspermic eggs seen were dispermic.

In schedule *C*, 32 rats were mated between 3.0 and 4.0 a.m. on day 1, and these provided 323 eggs, of which 315 (97.5 per cent.) were penetrated. Twenty-five eggs, 7.9 per cent. of the penetrated eggs, were polyspermic, there being 23 dispermic eggs and two trispermic. This proportion of polyspermic eggs represents a significant increase above that observed after the 10.0 p.m. mating ($P < 0.05$).

All the penetrated eggs seen in schedules *A*, *B*, and *C* were undergoing fertilization; most of them were in the pronuclear stages.

The 52 rats mated, according to schedule *D*, at 8.0 to 10.0 a.m. on day 1, and killed 24 hr later, gave 411 penetrated eggs in a total of 456 (90.1 per cent.), including 36 polyspermic eggs (8.8 per cent. of penetrated eggs). This proportion of polyspermic eggs is not significantly different from the 7.9 per cent. noted in the rats mated at 3.0 to 4.0 a.m. (schedule *C*). No trispermic eggs were seen, all the polyspermic eggs having only one additional sperm within them. When recovered, the polyspermic eggs were in the terminal stages of pronuclear development (four eggs), in various phases of the first cleavage mitosis (13 eggs), or had undergone cleavage and were in the 2-cell stage (18 eggs). One undivided polyspermic egg showed nuclear fragmentation and was probably degenerating.

The polyspermic eggs seen in these four groups (schedules *A-D*) were well distributed among the rats. In general, only one polyspermic egg was found amongst the eggs from any one rat. However, one rat in schedule *A*, three in schedule *B*, three in schedule *C*, and six in schedule *D* provided two polyspermic eggs each. In addition, one rat in schedule *D* yielded three polyspermic eggs and another gave a total of four.

Observations were also made in these four groups of rats on the number of eggs which had sperms in the perivitelline space ("supplementary" sperms) in addition to one or more sperms in the vitellus. This material will form part of another communication but may be considered briefly here, since the number of eggs containing more than one sperm represents the total number of eggs in which polyspermy could have occurred under the conditions prevailing. It was found that, in the rats in schedule *A*, 22.5 per cent. of the penetrated eggs contained more than one sperm, while the figures for the other schedules were 27.8 per cent. for *B*, 30.4 per cent. for *C*, and 32.4 per cent. for *D*. The figures for the delay-mated groups do not differ significantly among themselves, but together represent a level which is very significantly different ($P < 0.001$) from the figure for the normally mated group in schedule *A*. The incidence of polyspermy in these groups was 1.23, 3.68, 8.37, and 8.76 per cent. respectively. The figure for group *C* was obtained by omitting the results from 10 rats which are included in the results shown in Table 2; these were omitted because supplementary sperms had not been counted in these animals. The incidence of

actual in relation to possible polyspermy was calculated from the data just given and found to be 5.5, 13.3, 27.5, and 27.1 per cent. for the four groups *A-D* respectively (Table 3). A significant difference ($P < 0.05$) exists between 5.5 and 13.3 per cent., and between 13.3 and 27.5 per cent., but not between 27.5 and 27.1 per cent.

TABLE 3

NUMBER OF POLYSPERMIC EGGS, EXPRESSED AS A PROPORTION OF THE PERCENTAGE OF EGGS PENETRATED BY MORE THAN ONE SPERM, IN NORMAL AND DELAY-MATED RATS

Time of Mating	(1) Total Penetrated Eggs	(2) No. Eggs Penetrated by >1 Sperm	(3) Percentage of Eggs Penetrated by >1 Sperm	(4) No. Polyspermic Eggs	(5) Percentage Polyspermic Eggs	(6) (5) Expressed as Percentage of (3)
A, normal	810	182	22·5	10	1·23	5·5
B, 10.0-11.0 p.m.	353	98	27·8	13	3·68	13·3
C, 3.0-4.0 a.m.	227*	69	30·4	19*	8·37*	27·5
D, 8.0-10.0 a.m.	411	133	32·4	36	8·76	27·1

* The data for 3.0-4.0 a.m. mating in this table exclude results from 10 rats, included in Table 2, because supplementary sperms were not counted in the eggs from these animals.

In schedule *E*, 21 rats that had mated between 9.0 and 10.0 a.m. on day 1 were killed 3 days later, during the afternoon of day 4. From these animals 166 eggs were obtained, of which 143 (86.1 per cent.) had been penetrated. Eight eggs (5.8 per cent. of the penetrated eggs) were judged to be polyspermic; there were four 4-cell and four 8-cell eggs. Each had one extra sperm mid-piece in the cytoplasm of the blastomeres, but were otherwise quite normal in appearance. The sperm mid-pieces were often difficult to discern but once seen were easily distinguished from supplementary sperms lying in the perivitelline space, for they showed the advanced disintegration normally seen in the mid-pieces of fertilizing sperms, particularly in the later cleavage stages. The lower incidence of polyspermy found in this series may be misleading because some polyspermic 6-8-cell eggs may have been missed.

The 75 immature rats, treated according to schedule *F*, gave a total of 1130 eggs, of which 955 eggs (84.5 per cent.) were penetrated. There were 20 polyspermic eggs (2.1 per cent. of the penetrated eggs). This is not a significantly higher frequency than the 1.2 per cent. noted for the adult rats under normal mating conditions (schedule *A*). Nine of the polyspermic eggs were undergoing fertilization and 11 were in the 2-cell stage, including one egg which was trispermic. One of the polyspermic 2-cell eggs did not appear normal and was classed as degenerating.

(*d*) *Incidence of Polyspermy in Rabbits*

To obtain an indication of the frequency of polyspermy in rabbits under normal circumstances, 12 rabbits were mated and then killed 16-20 hr later. From these animals a total of 72 eggs was obtained; all of these eggs showed evidence of sperm penetration, and 69 (96 per cent.) had pronuclei. Only one egg (1.4 per cent. of the eggs with pronuclei) could be regarded as polyspermic, and this was a doubtful case since the "pronuclei" were ill-defined and may in reality have been pronuclear fragments.

Another group, consisting of 17 rabbits, received an intravenous injection of 50 I.U. of chorionic gonadotrophin and were mated 10 hr later, i.e. at about the time of the induced ovulation. These rabbits were killed 8-12 hr after mating and from them a total of 112 eggs was recovered. Eighty-two eggs (73 per cent.) showed evidence of sperm penetration, and 67 eggs (60 per cent.) were found to have pronuclei. There were 11 possibly polyspermic eggs (16.4 per cent. of the eggs with pronuclei), most of them (nine eggs) having three well-formed pronuclei. In each of the remaining two eggs, six closely grouped objects were found which resembled pronuclei of different stages. Among the eggs which had three pronuclei, the pronuclear size varied: in four eggs, one pronucleus was larger than the other two, in four other eggs, one pronucleus was smaller than the other two, and in one egg the pronuclei were graded in size. Observations on the incidence of polyspermy in rabbit eggs are summarized in Table 4.

TABLE 4

INCIDENCE OF POLYSPERMY IN EGGS FROM RABBITS AFTER NORMAL MATING AND AFTER MATING AT THE TIME OF OVULATION

	No. of Rabbits	Total No. of Eggs	No. of Penetrated Eggs	No. of Eggs with Pronuclei	No. of Polyspermic Eggs	Per-centage*	Remarks
Normal mating	12	72	72	69	1	1·4	Polyspermic egg doubtful; possibly nuclear fragmentation
Late mating	17	112	82	67	11	16·4	Two polyspermic eggs doubtful; possibly nuclear fragmentation

* Polyspermic eggs as percentage of eggs with pronuclei.

IV. DISCUSSION

(*a*) *Cytological Features*

The observations described in this paper provide information on a number of features that characterize the polyspermic eggs of rat and rabbit, more especially the former. Polyspermic rat eggs have been seen in most phases of the formation and development of the pronuclei, in the formation and divi-

sion of the first cleavage spindle, and in the 2-, 4-, and 8-cell stages of embryonic development. Observations on rabbit eggs have been limited to the later phases of pronuclear growth.

In the rat egg, diagnosis of the polyspermic state could be made with reasonable certainty because the two, or rarely three, sperm mid-pieces were regularly seen and often the extra acrosomes could also be discerned. A close correlation existed in the degree of development of the two male pronuclei and they were nearly always strikingly similar to one another in appearance.

Supposedly polyspermic rabbit eggs, on the other hand, may show either one small and two large pronuclei, or one large and two small pronuclei. Both these forms are referred to here as polyspermic although, without the evidence which the sperm mid-pieces would provide if they were clearly discernible, the diagnosis of polyspermy in rabbit eggs is uncertain. If the larger pronucleus is the male, as Amoroso and Parkes (1947) indicate, then eggs with one large and two small pronuclei may not be polyspermic. It is conceivable that, in such eggs, penetration by a single sperm has occurred after the second maturation spindle has moved inwards from the surface, because of delay in penetration. Completion of the mitosis would then give rise to two female pronuclei. This mechanism was considered by Pesonen (1949) to have been the mode of origin of the three pronuclei he had observed in a mouse egg, and a probable instance of the same phenomenon has been noted in the present series (Plate 3, Fig. 18). Actually the inward migration of the spindle is more likely to occur in the rabbit egg (Thibault 1951). On the other hand, the male pronuclei in polyspermic rabbit eggs may not always be similar in size or always larger than the female pronucleus as they apparently are in rat eggs. Some variation in the relative sizes of male and female pronuclei in normal rabbit eggs was in fact noted in the present series, and one trinucleate egg was seen in which the pronuclei were graded in size. Moreover, Amoroso and Parkes (1947) picture a rabbit egg with one large and two small pronuclei, indicating that they considered the large pronucleus and one of the smaller pronuclei to be male. Provisionally, it seems expedient to regard all the trinucleate rabbit eggs observed in this investigation as polyspermic.

Rather more problematic is the nature of the two rabbit eggs each of which was found to contain six apparent pronuclei of various sizes. This state may have arisen through the male pronuclei differing in their rates of growth, but it is also possible that the smaller "pronuclei" were produced by a process of lobulation and separation from a large pronucleus.

The evidence obtained indicates that, in polyspermic rat eggs, the female and both male pronuclei eventually give place in a normal way to groups of chromosomes which can be identified in the living eggs. The chromosomes then gather into a single prophase group from which the metaphase of the first cleavage spindle is formed. The steps involved in these processes, as seen in living monospermic rat eggs, have previously been described (Austin 1951). By fixing and staining polyspermic eggs in the appropriate stage, it has now also been shown that no chromatic material can be discerned in the vitellus, except that which composes the prophase or metaphase group of chromosomes. There seems to be no doubt, therefore, that all three pronuclei contribute their

chromosome complements to the first segmentation spindle. Only one telophase stage has so far been seen, but to judge from this the mitosis of the first cleavage spindle may well proceed in a normal manner.

As with the pronuclear stages, the polyspermic 2-cell rat egg is quite normal in appearance, except that two sperm mid-pieces are present in the cytoplasm. Total nuclear volumes and total nucleolar volumes, as well as the total number of nucleoli, were closely similar in monospermic and polyspermic eggs. Quite often, the sperm mid-pieces in the polyspermic 2-cell egg both pass through a curious ring-shaped structure which is also commonly seen about the sperm in monospermic 2-cell eggs. It is possible that this ring arises during the process of cytoplasmic cleavage. When the constriction of the cytoplasm occurs at cell division, the sperm mid-pieces lying in the cytoplasm would tend to be brought together and then left with the ring-like relic still about them.

(*b*) *Previous Reports on Polyspermy*

Previous observations on polyspermic eutherian eggs are of a varied nature. The trinucleate eggs of Tafani (1889), van der Stricht (1911), Hill and Tribe (1924), and Mainland (1930) were almost certainly polyspermic, although the substantiating evidence of extra sperm mid-pieces in the vitellus was lacking. The trinucleate eggs reported by Kremer (1924) and Pesonen (1949) may also have been polyspermic, in spite of these authors' alternative suggestions. On the other hand, several of the instances recorded in the literature cannot properly be termed polyspermy, notably the grossly degenerate egg mentioned by Lams (1913). The eggs described by Sobotta (1895) and Rubaschkin (1905) may have suffered a second and delayed penetration as they suggest, but this seems unlikely. These authors may well have mistaken for early pronuclei the small basophilic objects of unknown nature which have been referred to in mammalian eggs, under such terms as "pseudochromosomes" or "corps enigmatiques," by Lams and Doorme (1908), van der Stricht (1910), van der Stricht (1911), Lams (1913), and Hill and Tribe (1924).

Alternatively, Sobotta may have mistaken for early pronuclei the small subnuclei often seen in apparently normal monospermic 2-cell eggs; an example in a rat egg is shown in Plate 3, Figure 19, and Blandau (1952) describes similar subnuclei in eggs from rats inseminated after ovulation. The subnucleus noted in a polyspermic 2-cell rat egg by Austin and Smiles (1948) was almost certainly another example of the same thing, and not the residue of a male pronucleus as they suggest. The apparent presence of unchanged sperm heads in the vitellus of pronucleate or 2-cell eggs, as reported by Kremer (1924), may have been due to artefacts arising during fixation or the cutting of the sections. Certainly, the evidence offered seems inadequate to support the idea that a second sperm could penetrate an egg so long after the first.

When the features of polyspermic rat eggs are compared with those of certain invertebrates and lower vertebrates, some major differences are seen to exist. The rat egg is unlike the ascidian or frog eggs in that the extra male pronuclei do not remain separate in the cytoplasm and give rise to separate spindles, but instead come into contact with the female pronucleus and all then

contribute to the first cleavage spindle. In this particular, the rat egg is like that of the sea-urchin in which the chromosomes from all pronuclei may become involved in the first cleavage spindle. Under these circumstances a multipolar spindle is formed in the sea-urchin egg, the number of poles being related to the number of extra centrosomes introduced by the sperms. No suggestion of multipolar spindles was seen in polyspermic rat eggs—an observation which raises some doubt about the function of centrosomes in the cleavage of fertilized rat eggs.

In polyspermic eggs of both frogs and sea-urchins, the first cleavage may result in the formation of three or more blastomeres; there is no indication of such a process with rat eggs. Binucleate blastomeres are commonly found in the cleavage stages of polyspermic frog eggs but have not been seen in polyspermic rat eggs. One rat egg with a binucleate blastomere was indeed seen in the present series (Plate 3, Fig. 20), resembling in this way a bat egg described by van der Stricht (1910), but it contained only one sperm mid-piece and so could not have been polyspermic.

(*c*) *Increased Incidence of Polyspermy after Delayed Mating*

The incidence of polyspermy in rat and rabbit eggs under normal mating conditions was low, about 1.2 and 1.4 per cent. respectively, which is consistent with the small number of previously reported cases in these and other species. In immature rats after normal mating the incidence of polyspermy (2.1 per cent.) was not significantly higher than in adult rats, which seems to indicate that the artificial induction of ovulation does not appreciably influence the resistance of the eggs to the entry of more than one sperm into the vitellus. The increase in the incidence of polyspermy that occurred as a result of delayed mating in adult rats and rabbits is striking, both for the rapidity of increase and for the high incidence finally reached. An increase in incidence of about three times (to 3.7 per cent.) occurred in rats mated at 10.0 p.m., which is about 2-6 hr before the expected time of ovulation. This is remarkable because the time of penetration of eggs in these animals could hardly differ appreciably from that obtaining after a normal mating, and it does not seem possible for the eggs to have undergone any change. However, the sperms at the site of fertilization would presumably be fresher and more vigorous at the time of ovulation, in rats mated at 10.0 p.m., than in rats after normal mating. This is likely because with normal mating the sperms would have to spend several more hours in the female tract before the eggs reach the tube. The greater vigour of the sperms may, therefore, be the main reason for the sharply increased incidence of polyspermy observed with 10.0 p.m. mating. In support of this contention is the fact that the proportion of rat eggs containing more than one sperm is about the same after the 10.0 p.m., 3.0 a.m., and 8.0 a.m. matings (27.8, 30.4, and 32.4 per cent.) and that these three values are on a level which is significantly greater than that after normal mating (22.5 per cent.).

When rats were mated at 3.0-4.0 a.m. the incidence of polyspermic eggs was found to be 7.9 per cent., and when mated at 8.0-9.0 a.m., the incidence was 8.8 per cent.; the figures do not differ significantly from one another.

These values are a little more than twice that obtained for the 10.0 p.m. mating, and between six and seven times that for normal mating. As with the 10.0 p.m. mating, the high incidence of polyspermy is to some extent referable to the greater vigour of the sperms, but this explanation does not seem adequate to account for the increase in incidence of polyspermy beyond that noted for the 10.0 p.m. mating. Moreover, after the 3.0 a.m. and 8.0 a.m. matings, the number of eggs that became polyspermic represented a larger proportion (27.5 and 27.1 per cent., respectively) of the total number of eggs that theoretically could have become polyspermic under the circumstances prevailing. These considerations point to the occurrence of some change in the egg itself that renders it more susceptible to polyspermy. The susceptibility increases rapidly after ovulation but apparently reaches a limit early, at a point where between one-quarter and one-third of the eggs that have a chance of becoming polyspermic, actually do so. It is shown elsewhere that when rats were mated at 3.0 a.m. the penetration of most of the eggs occurred 2-3 hr later than in rats normally mated (Braden and Austin, unpublished data). The change responsible for the increased susceptibility to polyspermy must therefore take place principally in this short period, because no further increase in the incidence of polyspermy was found with the 8.0 a.m. mating. A rapid rise in susceptibility of a similar nature is known in some sub-mammalian forms: the change in *Asterias* eggs, for example, was found to be such that over 80 per cent. became polyspermic when inseminated after standing in sea-water for less than 2½ hr (Clark 1936).

A striking contrast with the findings presented in this paper is provided by the recently published observations of Blandau (1952) who artificially inseminated 78 rats in four groups: before ovulation and at 3-5, 6-8, and 9-12 hr after ovulation. Blandau describes a number of interesting abnormalities in eggs undergoing fertilization and in the 2-cell stage, which could be imputed to the entry of sperms into aging eggs. However, in not one of the 777 penetrated eggs that he examined had more than one sperm entered the vitellus. The absence of polyspermy from Blandau's material seems remarkable and an adequate explanation for it is difficult to find; it may perhaps be referable to the use of artificial insemination in place of natural mating.

If the diagnosis of polyspermy in the rabbit is correct, and this question has already been discussed, the incidence after mating performed at the time of ovulation (16.4 per cent.) is much higher than with normal mating and higher even than that seen after delayed mating in rats. This is consistent with the fact that, compared with rat eggs, a much larger proportion of rabbit eggs contains supplementary sperms in the perivitelline space and the numbers of supplementary sperms are also larger (Braden and Austin, unpublished data). There would therefore be much greater chances of polyspermy in rabbit than in rat eggs. The increase in polyspermy after delayed mating in rabbits may provide an explanation for the occurrence of polyspermy when rabbits were inseminated with X-irradiated sperms (Amoroso and Parkes 1947), because irradiation of the sperms was observed to cause a delay of 3-10 hr in the penetration of the eggs.

(*d*) *Fate of the Polyspermic Embryo*

The increased incidence of polyspermy resulting from delayed mating may have important practical and theoretical implications. So far, however, there appears to be no information on the fate of these embryos. In the present study, polyspermic eggs have been found to develop at least to the 8-cell stage without showing any noticeable abnormality. The lower incidence of polyspermy observed in 4- and 8-cell eggs may not have any significance because diagnosis becomes progressively more difficult as cleavage proceeds, and it is possible that some polyspermic 8-cell eggs were missed.

The evidence obtained indicates that polyspermy may be an important source of triploidy in rats, for both male pronuclei apparently contribute to the formation of the first cleavage spindle. On the other hand, nuclear size, nucleolar number, and nucleolar volume in polyspermic 2-cell eggs do not differ significantly from normal 2-cell eggs, and it is commonly known that triploid cells often have larger nuclei and more numerous nucleoli than diploid cells. Decisive evidence depends on the determination of the actual number of chromosomes on the first, or on any subsequent, cleavage spindle, and attempts are at present being made here to obtain this information. If, as seems likely, polyspermy (dispermy) does in fact give rise to triploidy, then the problem of the fate of the polyspermic embryo becomes the problem of the fate of the triploid embryo. A triploid mouse embryo at 9½ days of development has been recorded by Fischberg and Beatty (1951), but there is as yet no certainty that triploid embryos can survive to birth (Beatty 1951).

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ADDENDUM

Since this paper was submitted for publication, the authors have been able to make analysable squash preparations of the prophase or early metaphase chromosome groups on the first cleavage spindle in four dispermic rat eggs. Estimates of the chromosome numbers were: 57 ± 5, 60 ± 5, 60 ± 5, and 60 ± 3 ($2n = 42$). These results clearly indicate the presence of a triploid number of chromosomes on the first cleavage spindle and further support the conclusion drawn in the paper, that polyspermy (dispermy) in rats probably gives rise to triploidy in the embryo.

EXPLANATION OF PLATES 1-4

A_1 and A_2: acrosomes detached from penetrating sperm heads. *Chr.*: chromosome groups. S_1, S_2, and S_3: sperm mid-pieces. ♂, ♀: male and female pronuclei.

PLATE 1

Polyspermic rat eggs at various stages of fertilization. Phase contrast. × 700.

Fig. 1.—An egg seen shortly after entry of the two sperms into the vitellus. The sperm heads have undergone some degree of change and are in the stage at which they are not discernible in the cytoplasm by phase-contrast microscopy. Both sperm mid-pieces and both acrosomes can be seen.

Figs. 2-5.—Eggs in stages of pronuclear development. Both sperm mid-pieces can be seen in three of these pictures. The close similarity in development of the two male pronuclei is striking.

Fig. 6.—A trispermic egg. The three male pronuclei and the three sperm mid-pieces can be discerned.

PLATE 2

Polyspermic rat eggs at stages in the formation and division of the first cleavage spindle. Figure 7 by phase-contrast microscopy; remainder by direct illumination of stained preparations.

Fig. 7.—This egg was first observed in the terminal stages of pronuclear development, and is shown here at a stage soon after the three pronuclei had given place to three groups of chromosomes. × 700.

Fig. 8.—The same egg after it had been kept *in vitro* for about 2 hr and had then been fixed and stained. There are three distinct groups of chromosomes and there was no other strongly basophilic material in this egg. × 700.

Fig. 9.—Squash preparation of a polyspermic egg showing three groups of chromosomes. × 1000.

Figs. 10 and 11.—Squash preparations of polyspermic eggs showing single groups of chromosomes, evidently in the prophase of the first cleavage spindle. Figure 10 × 1000, Figure 11 × 1500.

Fig. 12.—Early telophase spindle in a polyspermic rat egg. × 1100.

PLATE 3

Polyspermic eggs in early cleavage stages (Figs. 13-17) and some anomalies seen in monospermic eggs (Figs. 18-20). Phase contrast.

Figs. 13 and 14.—Polyspermic 2-cell eggs. Two sperm mid-pieces are easily seen in each egg. Both eggs appeared otherwise to be quite normal. × 700.

Fig. 15.—The two sperm mid-pieces in 2-cell eggs are often found arranged in the manner shown in this picture: they appear to be bound together by a ring-shaped structure. × 700.

Fig. 16.—The two sperm mid-pieces in another polyspermic 2-cell egg are seen more clearly to pass through the ring-shaped structure. × 3000.

Fig. 17.—The two sperm mid-pieces in a polyspermic 8-cell egg. They are reduced in diameter and lack the granular mitochondrial sheath. × 1500.

Fig. 18.—A monospermic egg showing one male and two female pronuclei. × 700.

Fig. 19.—A monospermic 2-cell egg with a small subnucleus in one blastomere in addition to the normal nucleus. × 700.

Fig. 20.—A monospermic 2-cell egg having two nuclei of similar size and appearance in one blastomere; the other blastomere had a single nucleus of the same form and dimensions. × 700.

PLATE 4

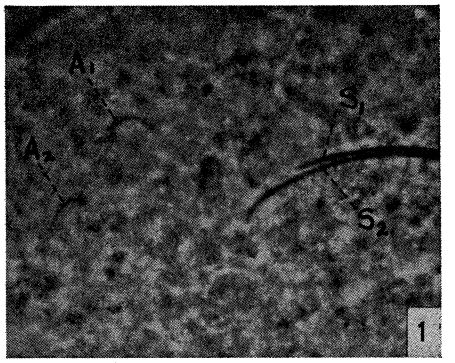
Pronuclei in normal and polyspermic rabbit eggs. Phase contrast. × 700.

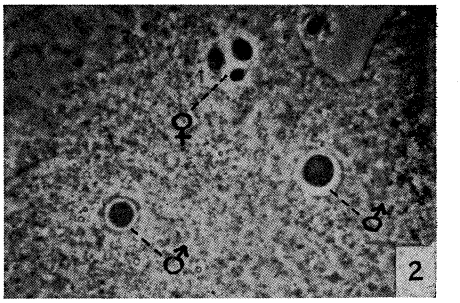
Figs. 21 and 22.—Rabbit eggs obtained from an animal after normal mating. In the first, the pronuclei are about the same size, but, in the second, one is nearly twice as big as the other.

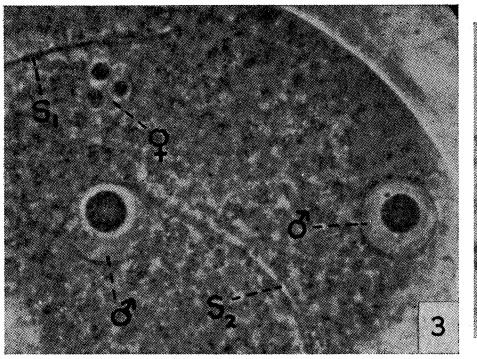
Figs. 23 and 24.—Eggs with three pronuclei. These were obtained after delayed mating. In one (Fig. 23), two pronuclei are smaller than the third, while in the other (Fig. 24), two are larger than the third.

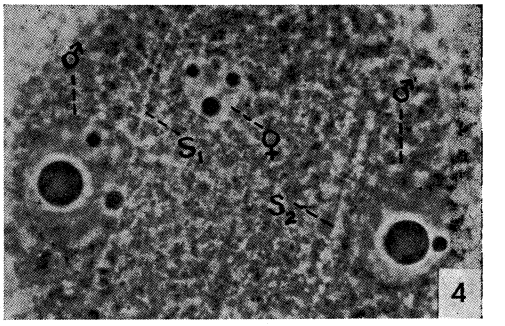
Fig. 25.—An egg with six possible pronuclei; two small ones are not visible at this focal plane.

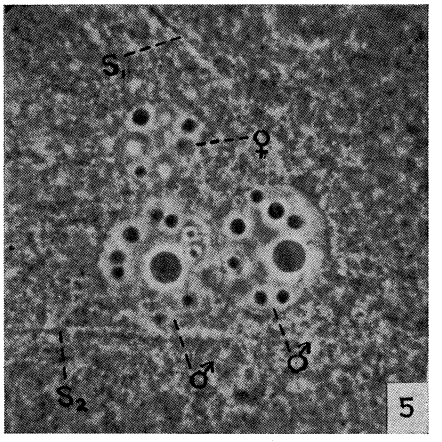
POLYSPERMY IN THE RAT AND RABBIT

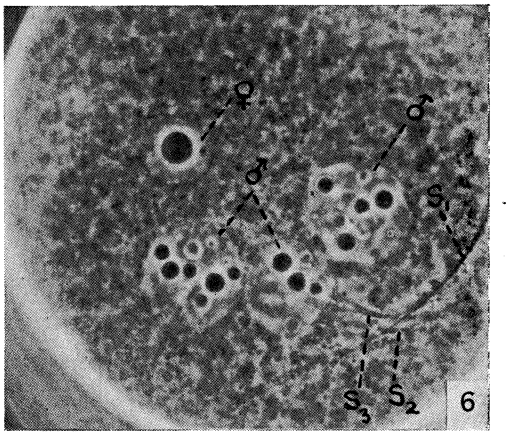












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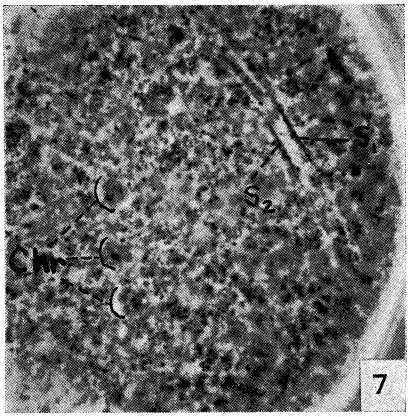

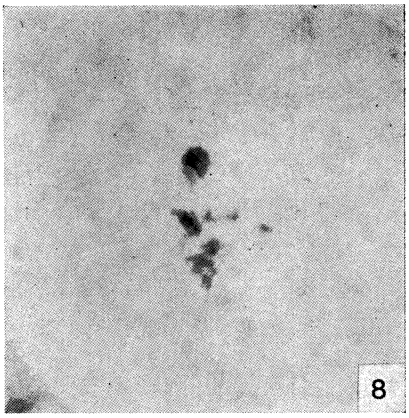

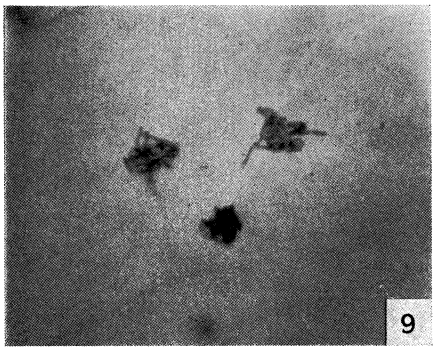

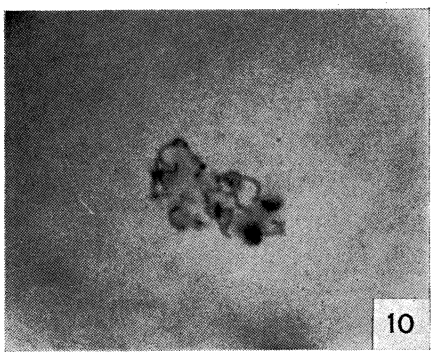

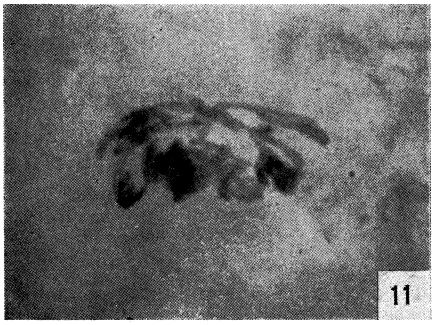

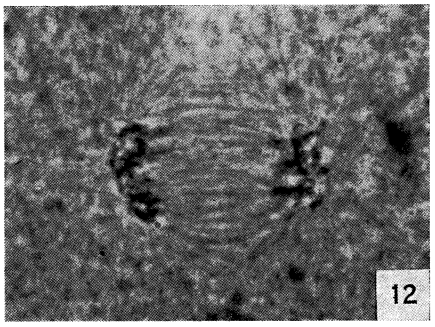

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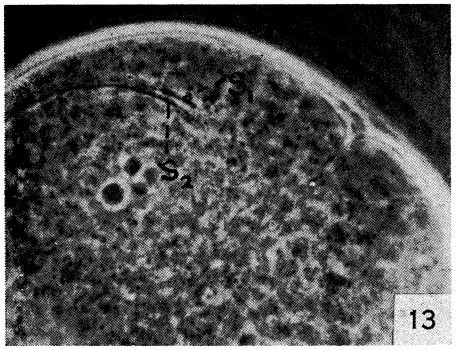

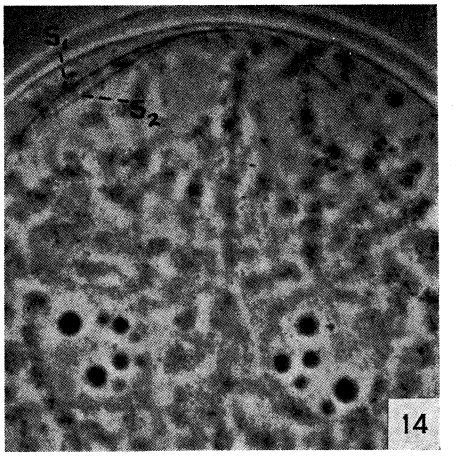

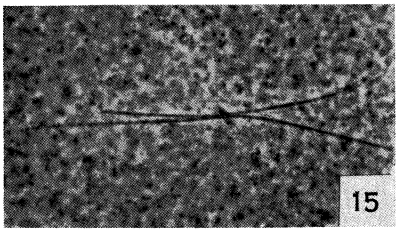

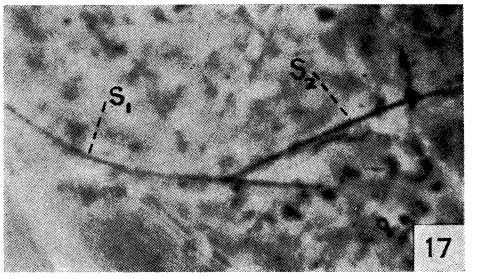

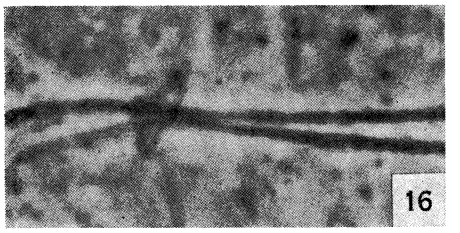

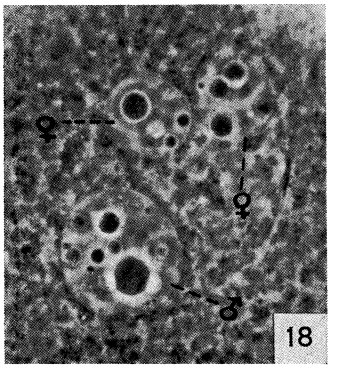

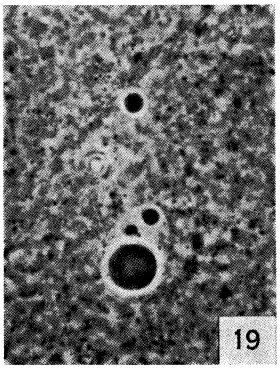

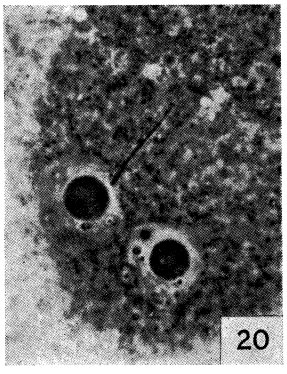

POLYSPERMY IN THE RAT AND RABBIT