INDUCTION AND INHIBITION OF THE SECOND POLAR DIVISION IN THE RAT EGG AND SUBSEQUENT FERTILIZATION

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Summary

Among 227 eggs from 23 untreated oestrous rats, the first polar body had persisted in three eggs (1.3 per cent.) and the second polar division had occurred spontaneously in two eggs (0.9 per cent.).

Emission of the second polar body was induced, in about an hour, by subjecting the rat to anaesthesia with ether, chloroform, ethyl chloride, ethyl alcohol, paraldehyde, nitrous oxide, or intraperitoneal "Nembutal." Ether and nitrous oxide influenced the most eggs. "Nembutal," administered subcutaneously, caused no polar body formation. The effect of anaesthetics is probably related to the production of cellular anoxia, and not to the fall in body temperature, for both ether and subcutaneous "Nembutal" depressed the body temperature to the same extent.

Cold-shock treatment (ice) led to the formation of the second polar body in virtually all the eggs, and hot-shock treatment (45°C) in none of them.

In untreated rats, the changes undergone by the male and female elements were closely correlated during the early stages of fertilization. A coordinating influence was also evident in the restoration of the normal relationship after it had been disturbed by experimental treatment.

Shrinkage of the vitellus, involving a reduction of 13 per cent. in volume, was found after sperm penetration, and sometimes after cold-shock treatment.

Sperms can readily penetrate into eggs that have emitted the second polar body under artificial stimulation; the block to polyspermy is unaffected and seemingly normal fertilization and cleavage may follow.

Resumption of the second polar meiosis was inhibited by treatment with hot shock or with colchicine. The influence of hot shock was transient whereas that of colchicine was more lasting. A high incidence of polyspermy (16 per cent.) was observed after hot-shock treatment, due evidently to interference with the block to polyspermy.

The ways in which the experimental treatments could induce heteroploidy in rat embryos are discussed.

The development of the block to polyspermy, the shrinkage of the vitellus, and the emission of the second polar body are seen as independent processes, capable of being evoked separately. Only the block to polyspermy appears to be a specific response to sperm penetration.

I. INTRODUCTION

In the eggs of mammals, as in those of many invertebrates and lower vertebrates, the immediate consequences of sperm penetration include the development of a block to polyspermy, the shrinkage of the vitellus, and the resumption

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of the second maturation division leading to the abstriction of the second polar body. These reactions are held to indicate the occurrence of "activation," or the arousal of the egg from a dormant state. Normally, these changes also mean that the entry of further sperms is precluded and that the chromosome number is reduced to the haploid state, preparatory to syngamy. In the absence of sperm penetration, the animal egg may yet be induced to undergo activation by artificial means, when the same changes may also occur and may even lead to parthenogenetic development, although variations occur between species and according to the treatment used.

Among mammalian eggs those of the rabbit have been chiefly used for studies on artificial activation and it has been shown that several of the agents known to be effective on the eggs of lower animals also induce activation in the rabbit egg when applied *in vitro* (Pincus and Enzmann 1935, 1936; Pincus 1936, 1939; Pincus and Shapiro 1940; Thibault 1947*a*, 1948, 1949). The agents include hyper- and hypotonic solutions, butyric acid, heat, and cold. Cold treatment was found to be effective *in vivo*, when ice was applied to the fallopian tubes shortly after ovulation. The activated rabbit egg generally showed a shrinkage of the vitellus, but the abstriction of the polar body was not common, the spindle giving place instead to a single (diploid) or two (presumably haploid) nuclei. Regular cleavage often followed activation, but Thibault (1949) considered that only eggs which form diploid nuclei could undergo such parthenogenetic development. Thibault (1947*b*, 1949) and Thibault and Ortavant (1949) also studied the reactions of sheep and rat eggs to cold shock. They found that the sheep egg behaved similarly to the rabbit egg, but that the rat egg differed, for it regularly extruded the second polar body and was never seen with reformed nuclei or undergoing cleavage. Thibault (1949) observed that, in the rat, ether anaesthesia or barbiturate narcosis, without other treatment, would induce the activation of a proportion of the eggs in the intact animal. He ascribed this effect partly to a direct action of ether on the eggs and partly to the drop in body temperature that occurred during anaesthesia or narcosis.

In the eggs of many of the lower animals, concomitantly with the shrinkage of the vitellus, a fertilization membrane is elevated after sperm penetration and this is not penetrable to sperms. The elevation of the membrane also constitutes one of the earliest and surest criteria of activation when this is provoked by artificial means. Consequently, eggs that are artificially activated are rendered incapable of normal fertilization. A fertilization membrane has not been described in the mammalian egg, except for one possible instance in *Cricetus* (Venable 1946), but shrinkage of the vitellus commonly occurs, and it may be argued with Thibault (1949) that artificially activated mammalian eggs are unfertilizable. This effect, however, does not appear to have been demonstrated experimentally.

The investigations to be described in the present paper were made in order to obtain further information on the activation of rat eggs by sperms, anaesthetics, and other agents, to determine whether eggs could be penetrated by sperms after artificial activation, and, should this be possible, to study the cytology of fertilization in these eggs.

II. Methods

Oestrous rats were selected between 8.00 and 9.00 a.m. by inspection of the vaginal smear. If required for studies on fertilization, the rats were placed with males for ½ hr between 9.00 and 10.00 a.m., and those that mated were detected by the presence of copulation plugs. It has previously been shown that oestrous rats in this colony have generally completed ovulation by 7.00 a.m. and that, if they then copulate, their eggs will be fertilized after an interval of at least 2 hr (Austin 1952). Under these circumstances, it is possible to subject the eggs to experimental treatment after mating and ovulation but before the time when fertilization would begin. Treatments were therefore applied between 10.00 and 11.00 a.m. The rats were killed the same afternoon, or the following morning if the later stages of fertilization were required.

To determine the effect of anaesthetics, the rats were anaesthetized with ether, chloroform, ethyl chloride, ethyl alcohol, paraldehyde, "Nembutal," or nitrous oxide. The anaesthesia was controlled at sufficient depth to permit of operative procedures as judged by the disappearance of cutaneous and corneal reflexes. The volatile anaesthetics and nitrous oxide were administered by inhalation, paraldehyde was given orally, and "Nembutal" was injected subcutaneously or intraperitoneally. Nitrous oxide was administered with a carbon dioxide-oxygen mixture, and the "secondary saturation" technique (Hewer 1948) was employed. Adequate doses of "Nembutal" were found to be 3.5-4.0 mg/100 g body weight (subcutaneously) or 3.0 mg/100 g (intraperitoneally) and of paraldehyde to be 0.3 ml/100 g.

Cold-shock treatment was effected by holding a piece of ice against the fallopian tube for about 1 min, with the rat under subcutaneous "Nembutal" anaesthesia. For the application of hot shock, the ovaries and fallopian tubes were brought to the exterior through dorsolateral incisions, and the rat was suspended with its back on the surface of the water in a water-bath. Small clips were attached to the juxta-ovarian fat and these, by their weight, ensured that the fallopian tubes were totally immersed in the water. The temperature of the water was maintained at 44.5-45.5°C and treatment was applied for 8-12 min.

The colchicine solution was freshly prepared each morning and was injected intraperitoneally at the rate of 0.05-0.1 mg/100 g body weight.

Sperm extracts were prepared by suspending rat epididymal sperms in normal saline solution and passing the mixture through a glass bacterial mill.

All eggs were examined in the fresh state; they were dissected from the fallopian tubes under saline, transferred to a slide, slightly compressed under a coverslip with greased edges, and studied with a phase-contrast microscope.

III. Observations

(*a*) *General Effects of the Selected Anaesthetics*

Among the anaesthetics tried, ether, "Nembutal," and paraldehyde were found to be the most satisfactory for rats, in that surgical anaesthesia of at least 15-30 min duration could be maintained without much risk. On the other hand,

chloroform, ethyl chloride, and nitrous oxide were more difficult to use and the resuscitative measures which were frequently needed would have interfered seriously with operative procedures.

TABLE 1

MEAN RECTAL TEMPERATURE OF FIVE ADULT FEMALE RATS BEFORE, DURING, AND AFTER 15 MIN ETHER ANAESTHESIA

	Rectal Temperature (°C)								
Before Anaesthesia	During Anaesthesia			After Anaesthesia					
	5 Min	10 Min	15 Min	5 Min	10 Min	15 Min	30 Min	1 Hr	2 Hr
38·0	34·9	33·0	32·1	31·2	31·5	32·5	35·7	36·9	37·3

Body temperature seemed to fall with all the forms of anaesthesia, but actual measurements were made only when ether or subcutaneous "Nembutal" were used. With ether anaesthesia, body temperature fell progressively and reached a minimum shortly after the end of administration. The temperature then rose gradually until it was approximately normal about 2 hr later. Mean figures for the rectal temperature of five female rats subjected to anaesthesia for 15 min are shown in Table 1. The maximum depression of temperature, observed 5 min after anaesthesia, was about 7°C. With "Nembutal" anaesthesia, the maximum depression of body temperature observed in five

TABLE 2

MEAN RECTAL TEMPERATURE OF FIVE ADULT FEMALE RATS BEFORE AND AFTER SUBCUTANEOUS INJECTION OF "NEMBUTAL"

	Rectal Temperature (°C)					
Before Injection	After Injection					
	½ Hr	1 Hr	2 Hr	3 Hr	4 Hr	5 Hr
37·9	34·7	32·0	31·0	33·8	35·3	36·4

female rats was only reached about 2 hr after injection, but was of the same magnitude, nearly 7°C (Table 2). Recovery of body temperature was also slower, and even 5 hr after injection the mean temperature was still 1.5°C below that recorded prior to the injection of the "Nembutal."

Ethyl alcohol was administered in the manner of an anaesthetic, not with this purpose in mind but to achieve a lasting concentration in the body fluids without the risk of lethal intoxication. Rats subjected to alcohol vapour did reach a degree of unconsciousness sufficient for surgical work but only after 1 hr of treatment. The animals were maintained in this state for 2 hr without showing evidence of impending respiratory failure.

(*b*) *The Contraction of the Vitellus*

The volume of the vitellus was determined from measurement of the diameter of uncompressed eggs with an eyepiece micrometer. The mean diameter of 46 eggs from five unmated oestrous rats was 75.5 μ (range 72.8-81.3), equivalent to a volume of 225,000 cu. μ. The effect of sperm penetration upon the volume of the vitellus was observed in five mated rats. The 43 eggs obtained all had sperms within the vitellus. Their mean diameter was 72.1 μ (range 68.5-74.9), equivalent to a volume of 195,000 cu. μ. The difference between the means for diameters is 3.4 μ or about 5 per cent. ($P < 0.01$) and between the volumes 30,000 cu. μ or 13 per cent. Differences of this order were observed also after cold-shock treatment in the eggs from some unmated oestrous rats; the eggs in one fallopian tube were treated while those in the other tube served as controls. Results, however, were not consistent, for, in other rats, both treated and untreated eggs were found to have similar diameters, although second polar body abstriction had occurred in the treated eggs and not in the untreated.

(*c*) *The Induction of Second Polar Body Abstriction*

The artificial induction of abstriction of the second polar body was studied in a total of 120 rats that were treated with anaesthetics and other agents (Table 3). Eggs from 23 untreated rats were also examined, the animals being killed at about the same time after ovulation as those in the test groups; among the 227 eggs that could be analysed, the second maturation division in metaphase was seen in all except two eggs which exhibited polar bodies instead. Three other eggs had polar bodies in addition to the metaphase chromosome group (Plate 1, Fig. 1). There was thus a total of five eggs (2.2 per cent.) with polar bodies; in three eggs (1.3 per cent.) the first polar body had persisted and in two eggs (0.9 per cent.) the second polar body had been formed spontaneously.

A group of 20 rats was treated with ether anaesthesia for 15 min, and provided a total of 186 eggs, of which 183 were analysable (Table 3). Sixty-four per cent. of these eggs had polar bodies; they were clearly second polar bodies, for metaphase chromosomes could not be seen and in most of the eggs the remains of the spindle could still be identified (Plate 1, Fig. 2). The second maturation spindle, in metaphase, was present in the remaining eggs except for one in which the meiosis had not been resumed but the chromosomes had become scattered through part of the cytoplasm. It is possible that chromosome scatter had occurred also in the three eggs that could not be analysed, but it is a difficult process to recognize with certainty.

Some evidence on the rate at which the second polar division occurs was obtained with this group by killing the animals at different times after anaesthesia. Two of the rats were killed 1 hr after the end of anaesthesia. They yielded 17 eggs of which eight exhibited stages in the resumed meiotic division; anaphase was evident in one egg, early telophase in six eggs, and the polar body in one egg. Five other rats were killed at 1½ hr after the end of anaesthesia. These provided 47 eggs, in 32 of which there was a freshly formed polar body; in the remaining 15 the second maturation spindle was still in metaphase. In eggs with recent polar bodies, the group of chromosomes remaining within the vitellus was in the form of a dense rounded mass (Plate 1, Fig. 2). The other 13 rats that received ether anaesthesia were killed 2-3 hr after the end of anaesthesia, and from them were obtained 186 eggs, of which 77 had polar bodies. In most of the eggs with polar bodies, the chromosomes remaining within the eggs were still arranged in a dense mass, but in some a certain amount of scatter had occurred (Plate 1, Fig. 3).

TABLE 3

PROPORTION OF EGGS WITH POLAR BODIES AFTER APPLICATION OF VARIOUS AGENTS *IN VIVO*

Agent	Number of Rats	Total Eggs	Analysable Eggs	Eggs with Chromosome Scatter	Eggs with Polar Bodies	
					Number	Fraction (%)
None	23	230	227	0	5	2·2
Ether: 15 min	20	186	183	1	117	64
"Nembutal" (subcutaneous)	10	89	86	0	1	1·2
"Nembutal" (intraperitoneal)	10	96	89	2	23	26
Chloroform: 15 min	10	95	91	3	40	44
Ethyl chloride: 15 min	10	89	82	1	8	9·8
Ethyl alcohol: 2 hr	10	97	80	15	6	7·5
Paraldehyde (oral)	10	93	83	4	33	40
Nitrous oxide: 15 min	10	93	82	3	45	55
Nitrous oxide: 30 min	10	95	80	0	59	74
Cold shock (ice)	10	91	83	0	82	99
Hot shock (44·5-45·5°C)	10	86	64	10	0	0
Sperm extract	10	77	68	1	7	10

In a second experimental group, rats were given "Nembutal" subcutaneously. Only one of the 86 analysable eggs had a polar body (Table 3). No chromosome scatter was recorded, but it may have occurred in the three eggs that could not be analysed. On the other hand, when "Nembutal" was administered intraperitoneally, 26 per cent. of the analysable eggs had polar bodies and in two eggs with polar bodies chromosome scatter was observed (Table 3).

Similar observations were made on the effects of anaesthesia with chloroform (15 min), ethyl chloride (15 min), ethyl alcohol (2 hr), paraldehyde, and nitrous oxide (15 and 30 min); 10 rats were used with each agent (Table 3). The proportion of analysable eggs that contained polar bodies varied greatly, from 7.5 per cent. with ethyl alcohol to 74 per cent. with 30-min nitrous oxide. With each of these anaesthetics, there were some eggs that lacked polar bodies but in which chromosome scatter could be distinguished. Particularly was this so with alcohol; rats treated with this agent gave 15 eggs that showed chromosome scatter but no spindle or polar body.

The most effective method for inducing the emission of the second polar body was found to be cold-shock treatment. Ten rats so treated gave 83 analysable eggs, of which all but one had polar bodies (Table 3); the single exception was a degenerate egg.

TABLE 4

CORRELATION BETWEEN MALE AND FEMALE ELEMENTS DURING THE EARLY STAGES OF FERTILIZATION IN 387 EGGS FROM ADULT RATS

Female / Male	Chromosomes in Metaphase	Chromosomes in Anaphase	Chromosomes in Telophase	Polar Body Formed	Early Female Pronucleus Formed	Total
Sperm in perivitelline space	93					93
Sperm in vitellus. Head unchanged	24	13	4			41
Sperm in vitellus. Head changing	5	14	51	14		84
Sperm in vitellus. Head invisible			8	70	1	79
Early male pronucleus formed				9	81	90
Total	122	27	63	93	82	387

Rats treated with anaesthetics and with cold shock were killed at intervals between 1 and 4 hr thereafter, and in this way it has been possible to examine eggs at various times after the induction of polar body abstriction. Observations at 1 and 1½ hr were those made after ether anaesthesia and have already been described. With the other agents also, polar body formation was complete in less than 2 hr and the chromosomes remaining in the vitellus were generally compacted into a dense mass (Plate 1, Fig. 2). In some eggs, chromosome scatter, following polar body abstriction and resembling that shown in Plate 1, Figure 3, was observed. When eggs were examined later, 3-4 hr after treatment, increasingly more of them showed chromosome scatter, and in progressively more eggs with polar bodies no chromosomes could be distinguished, probably because they were scattered through portion of the vitellus.

Rats that received the hot-shock treatment provided 64 eggs that could be analysed and none of these showed a polar body. In 10 eggs neither spindle nor polar body could be seen but scattered chromosomes were discernible in the cytoplasm.

Sperm extracts were injected into the peri-ovarian sac of 10 rats. Among the 68 analysable eggs recovered, seven had polar bodies and one egg showed chromosome scatter.

(*d*) *Correlation of Male and Female Elements in the Early Stages of Fertilization*

Information on the correlation between the changes undergone by the sperm head and its derivatives on the one hand and the egg chromosomes and their derivatives on the other during fertilization has already been reported (Austin 1951). The observations were made on eggs recovered from immature rats after induced ovulation. Data have now been obtained on the eggs of adult rats. One hundred and forty-four adult female rats were mated between 9.00 and 9.30 a.m. and killed 2½-4 hr and 7 hr after mating. The successive changes shown by the male and female elements were classified in a similar manner to that used by Austin (1951), except that an extra stage was introduced: that in which the sperm head, having undergone some degree of metamorphosis, is temporarily indistinguishable by phase-contrast microscopy as it lies in the cytoplasm.

The data are set forth in Tables 4, 6, and 7. Sixty-seven rats provided a total of 387 eggs in the early stages of fertilization. There were 93 eggs in which sperms were present only in the perivitelline space, and in all these eggs the second maturation spindle was still in metaphase. In 41 eggs, sperms had entered the vitellus but had not undergone any detectable change; many of these eggs (24) displayed metaphase chromosomes but in others the meiosis had advanced to anaphase (13 eggs) or to telophase (four eggs). The early changes that the sperm head undergoes in the vitellus were seen in 84 eggs, the majority (51) of which had the second maturation spindle in telophase. In some (14) of the eggs, however, polar body abstriction had been completed, whereas in others there was evidence of delay in the response of the egg to sperm penetration, for the meiosis was still in anaphase (14 eggs) or even in metaphase (five eggs). The stage in which the sperm head is indistinguishable was noted in 79 eggs and in most (70) of these the polar body had been formed but the group of chromosomes remaining within the egg had not yet given place to pronuclei. The female pronucleus was evident in only one egg in this stage, while in eight others the chromosomes were still arranged in telophase. Early male pronuclei were recorded in 90 eggs altogether and in 81 of these the female pronucleus was also discernible. The remaining nine eggs had a polar body but no female pronucleus.

(*e*) *Induction of Polar Body Abstriction before Fertilization*

The capability for fertilization of eggs in which polar body formation had

been artificially induced was tested by the use of ether, cold shock, and nitrous oxide. The results are shown in Table 5.

A control group of 36 rats, mated under the same conditions as the animals in the test groups, yielded a total of 327 eggs, of which 294 had been penetrated by sperms, 115 of them by more than one sperm. All the penetrated eggs were undergoing apparently normal fertilization or early cleavage, except for 27 eggs (9.2 per cent.) that were polyspermic but otherwise normal (25 dispermic, two trispermic). There were 88 eggs in the pronuclear stages of fertilization.

TABLE 5

EFFECT ON FERTILIZATION OF ANAESTHETICS AND OF COLD-SHOCK TREATMENT WHEN APPLIED TO MATED RATS BEFORE SPERM PENETRATION

Agent	Number of Rats	Total Eggs	Penetrated Eggs		Eggs with Pronuclei			Eggs in Other Stages of Fertilization or in Cleavage
			Number	Fraction (%)	Total	Normal ♂ Pronuclei	Normal ♀ Pronuclei	
None	36	327	294	90	88	88	88	206
Ether: 15 min	21	196	91	46	30	29	24	61
"Nembutal" (subcutaneous)	10	89	71	80	17	16	16	54
Cold shock	15	130	52	40	25	23	17	27
Nitrous oxide: 30 min	6	58	47	81	23	23	17	24
Colchicine	16	129	97	75	43	40	32	54
Hot shock	17	168	137	82	55	52	41	82

Ether anaesthesia of 15 min duration was administered to rats after mating and these provided 91 penetrated eggs. In most of the eggs in the pronuclear stages of fertilization both the male and female pronuclei were adjudged to be normal. Some, however, showed pronuclear abnormalities; in one egg both pronuclei were degenerate and in another five, one pronucleus, considered to be the male pronucleus, was normal whereas the female pronucleus was either absent altogether or replaced by one to three subnuclei. The appearance of subnuclei is shown in Plate 1, Figure 5, and Plate 2, Figures 8 and 9; they are smaller than pronuclei and may contain only one nucleolus. The rats in this group also provided eggs that either showed stages in the formation of the first cleavage spindle or else had undergone cleavage and were in the 2-cell stage; all appeared quite normal.

A third group of rats was treated similarly to the second group, except that they received a subcutaneous injection of "Nembutal" in place of ether anaesthesia. This group provided 71 penetrated eggs that were in various stages of fertilization or early cleavage, and only one egg showed any abnormality. It was polyspermic, for there were two sperm mid-pieces in the vitellus, but it

had only a single large oval nucleus and five subnuclei (Plate 1, Fig. 4). The oval nucleus may possibly have been formed by the fusion of two male pronuclei.

In a fourth experimental group the eggs were subjected to cold-shock treatment *in vivo*, between the times of mating and anticipated sperm penetration. Here again, distinct abnormality could only be seen in some of those in the pronuclear stages of fertilization. There were 17 eggs in which both male and female pronuclei were considered to be normal, and six in which only the male pronucleus was normal, the female pronucleus being replaced by one to three subnuclei. One exceptional egg had 13 subnuclei of various sizes and another had no distinct nuclei but simply a number of nucleoli scattered through the cytoplasm. The rats in this group also provided eggs that showed the first cleavage spindle or had entered the 2-cell stage; all were quite normal in appearance.

TABLE 6

PROPORTION OF EGGS WITH POLAR BODIES AT TWO DIFFERENT STAGES OF FERTILIZATION IN UNTREATED RATS AND AFTER TREATMENT WITH COLCHICINE OR HOT SHOCK

Treatment	Eggs with Sperm Head Invisible			Eggs in Pronuclear Stage			
	Total	With Polar Body		Total	Pronuclei Normal		
		Number	Fraction (%)		Total	With Polar Body	
						Number	Fraction (%)
None	79	71	90	88	88	88	100
Colchicine	34	4	12	43	32	3	9
Hot shock	28	8	29	55	41	37	90

In the fifth group, rats anaesthetized for 30 min with nitrous oxide yielded 40 penetrated eggs in which fertilization appeared to be proceeding normally. Two other penetrated eggs, however, contained only one pronucleus (considered to be the male), and four had an apparently normal male pronucleus but one or more subnuclei in place of the female pronucleus. An egg with three visible subnuclei is illustrated in Plate 1, Figure 5.

Among the 190 penetrated eggs from the rats that received ether or nitrous oxide anaesthesia or cold-shock treatment there were 19 polyspermic eggs, of which 15 were dispermic and four trispermic. This incidence does not differ significantly from the 9.2 per cent. observed in the control group of rats.

(*f*) *Inhibition of the Second Maturation Division*

An attempt was made to prevent the occurrence of the second maturation division, which leads to the abstriction of the second polar body and which normally follows sperm penetration. This was done by giving the rats an intraperitoneal injection of colchicine shortly after mating, or by subjecting the eggs to hot-shock treatment *in vivo,* between the times of mating and fertilization. The rats were killed in the late afternoon or on the following morning. The results are set forth in Tables 5, 6, and 7.

TABLE 7

NUMBERS OF EGGS PENETRATED BY MORE THAN ONE SPERM IN UNTREATED RATS AND AFTER COLCHICINE AND HOT-SHOCK TREATMENTS AND THE PROPORTIONS OF THOSE EGGS THAT WERE POLYSPERMIC

Treatment	Total Penetrated Eggs	Eggs Penetrated by >1 Sperm		Polyspermic Eggs		*B/A* (%)
		Number, *A*	Fraction (%)	Number, *B*	Fraction (%)	
None	294	115	39	27	9·2	23
Colchicine	97	28	29	9	9	32
Hot shock	137	34	25	22	16	65

The rats that received colchicine yielded 97 penetrated eggs, of which nine were polyspermic. The polyspermic eggs represented 32 per cent. of eggs that contained more than one sperm. Among the eggs undergoing fertilization, there were 34 eggs wherein the head of the sperm had reached the stage at which it is indistinguishable in the cytoplasm, but in the majority of these no polar body had been formed. Clearly, polar-body abstriction had been inhibited by the colchicine, and this effect was evident also in 32 apparently normal pronucleate eggs, none of which had polar bodies. Abnormal eggs included three in which only subnuclei or groups of nucleoli were present, five that showed a single large, presumably male, pronucleus (Plate 2, Fig. 7), and three eggs that had one or two subnuclei in place of the female pronucleus (Plate 2, Fig. 9).

From the rats treated by the hot-shock method, 137 penetrated eggs were recovered. Thirty-four of the penetrated eggs had more than one sperm within the bounds of the zona pellucida, and 22 (65 per cent.) of these had more than one sperm within the vitellus, that is, they were polyspermic (19 eggs were dispermic and three trispermic). Of the eggs that showed the stage of fertilization at which the sperm head is invisible, the majority (71 per cent.) had no polar bodies, but of the eggs that were recovered later, in normal pronuclear stages, nearly all (90 per cent.) possessed polar bodies. The inhibitory effect of hot shock, unlike that of colchicine, appears to pass off in a few hours. Fourteen normally cleft 2-cell eggs were obtained from rats in this group; 10 of the eggs had polar bodies. The abnormal eggs included seven with only one

large nucleus (like that in Plate 2, Fig. 7), presumed to be the male pronucleus, and seven with apparently normal male pronuclei with one or more subnuclei in place of the female pronucleus.

IV. Discussion

In confirmation of the findings of Sobotta and Burckhard (1910), Kirkham and Burr (1913), and Huber (1915), the first polar body was rarely seen in the recently ovulated eggs of untreated rats. The incidence observed in 227 eggs was 1.3 per cent. Sometimes the second maturation meiosis is resumed spontaneously, for in 0.9 per cent. of the eggs the second polar body was evident. The two types of polar body have the same significance for the interpretation of many of the observations described in this paper, since it was not possible to distinguish between them in eggs that showed chromosome scatter or were in the later stages of fertilization. Moreover, the incidence of polar bodies in the eggs from the untreated rats was low, and any significant increase in the incidence could therefore be attributed to the emission of the second polar body resulting from the treatment applied.

Results also confirm Thibault's (1949) observation that ether anaesthesia causes the emission of the second polar body. He found polar bodies in 76 per cent. of the eggs after 15-20 min anaesthesia; in the present investigation 64 per cent. of the eggs had polar bodies after 15 min anaesthesia. Thibault ascribed the effect partly to a direct action of ether upon the eggs and partly to the fall in body temperature that occurs during the anaesthesia. The authors' observations favour a direct action upon the eggs, or upon the immediate environment of the eggs, for the fall in body temperature was equally profound and even more protracted when narcosis was induced with subcutaneously injected "Nembutal" and yet the proportion of eggs with polar bodies was not increased above that seen in untreated rats. When the "Nembutal" was given by the intraperitoneal route, however, a moderately high proportion (26 per cent.) of the eggs was found to have polar bodies. By this method of administration, the "Nembutal" may well achieve a higher concentration in the immediate environment of the eggs and a direct action upon this environment or even upon the eggs themselves becomes more likely. The precise nature of the action is uncertain but may involve local anoxia. A high proportion of eggs with polar bodies was observed after nitrous oxide anaesthesia, and with this agent the predominating, perhaps exclusive, effect at the cellular level is the production of anoxia, particularly when the secondary saturation technique is employed. Various proportions of eggs were found with polar bodies after treatment with other anaesthetics, corresponding perhaps to differing degrees of cellular anoxia produced.

Both hot and cold stimuli have been found to induce activation, including the emission of the second polar body, in the eggs of some invertebrates and lower vertebrates (Tyler 1941; Peacock 1943), and in the rabbit (Pincus and Enzmann 1936; Pincus 1939). The two forms of temperature shock had very different effects on rat eggs, for whereas cold shock induced polar body forma-

tion in virtually all the eggs, as Thibault (1949) also reported, no eggs were found with polar bodies after the hot-shock treatment. Indeed, it was shown that hot shock will inhibit, for a while, the second polar meiosis that normally follows sperm penetration.

In some of the eggs from rats treated with anaesthetics and other agents, there was no sign of a resumption of the second maturation meiosis, but instead the chromosomes were found to have become scattered through the neighbouring cytoplasm. This was seen particularly after treatment with ethyl alcohol and hot shock. Chromosome scatter is not a normal consequence of sperm penetration and has not been observed to occur spontaneously during the 24 hr after ovulation. It is evidently an aberrant reaction on the part of the egg to the stimulus applied.

A close correlation between the changes undergone by the male and female elements during the early stages of fertilization was found in untreated adult rats, and in general the results were similar to those previously reported for immature rats (Austin 1951). The chief difference was that the eggs of adult rats appear less sensitive to the stimulus of sperm entry than do those of immature rats, for in no instance did the resumption of meiosis occur before the sperm had penetrated the vitellus. Indeed in 29 eggs the second maturation spindle was still in metaphase although the sperm head lay in the vitelline cytoplasm and in five of these eggs the sperm head had even begun its early metamorphosis.

Confirming earlier observations (Austin 1952), sperm penetration was noted in a large proportion (90 per cent.) of the eggs from rats that were not permitted to mate until several (5-9) hours after ovulation. The incidence of polyspermy in these eggs was 9.2 per cent., a figure identical with that recently reported for another group of rats mated after ovulation (Austin and Braden 1953*a*). This high incidence of polyspermy is associated with delayed mating; after normal mating the figure was only about 1.5 per cent. (loc. cit.).

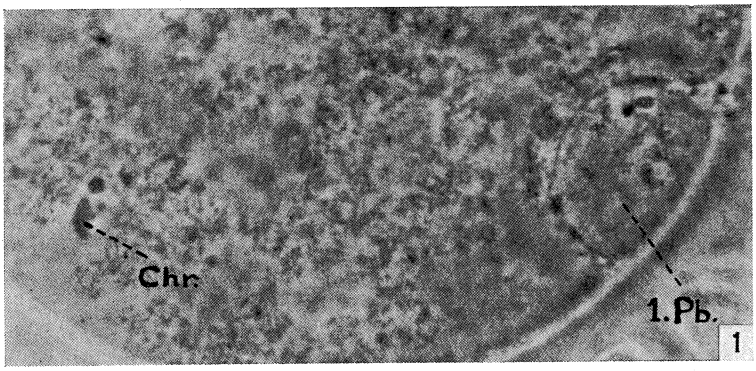
Procedures designed to induce second polar body abstriction (ether or nitrous oxide anaesthesia, or cold shock) were applied to mated rats before the time of sperm penetration and resulted in a reduction in the number of eggs penetrated. Nevertheless, it was quite clear from the results that sperms could penetrate into eggs that had already formed the second polar body. Moreover, the mechanism underlying the block to polyspermy was evidently little affected, for the incidence of polyspermy in these rats (10 per cent.) differed insignificantly from that observed in the control group. Apparently normal fertilization may also occur, in spite of the initial disturbance in the correlation between the male and female elements. It would seem that this disturbance was corrected before the formation of the female pronucleus, but only if chromosome scatter had not taken place. The development of subnuclei in place of normal female pronuclei was probably the result of chromosome scatter. Of interest is the fact that the rabbit egg also, according to Chang (1952), may be fertilized and show early cleavage after chilling *in vitro*, but Chang could not be certain that the emission of the second polar body had preceded sperm penetration.

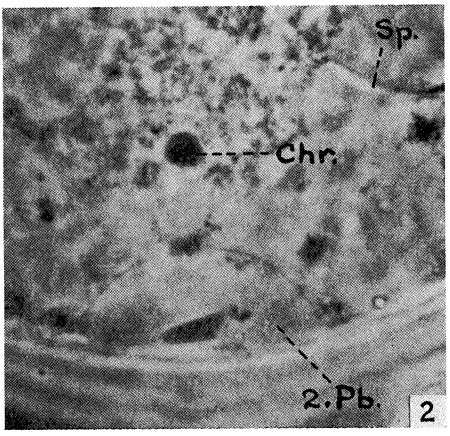
Emission of the second polar body normally follows sperm penetration but could be inhibited with either hot shock or colchicine. The results differed according to the method employed. The inhibitory influence of hot shock apparently passed off in most of the eggs, so that polar body abstriction, fertilization, and the first cleavage could then proceed in a normal manner. Once again the initial disturbance in the correlation between male and female elements seems to have been adjusted before pronuclei were formed. Sometimes subnuclei replaced the female pronucleus, presumably owing to chromosome scatter. The influence of colchicine, on the other hand, was more lasting, and, after treatment with this agent, the majority (90 per cent.) of eggs with seemingly normal pronuclei showed no sign of having emitted the second polar body. It may well be that such eggs contained a diploid female pronucleus and were thus capable of giving rise to a triploid embryo, but further work is required to explore these possibilities.

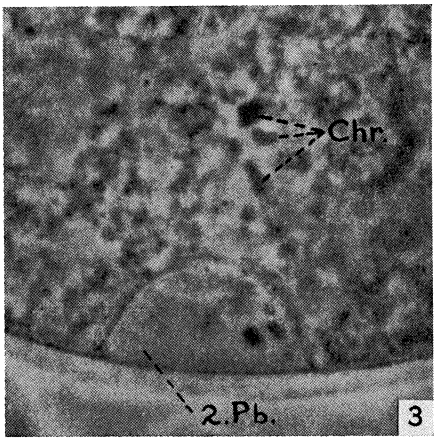
The effect of hot shock on rat eggs was unlike that observed by Fankhauser and Godwin (1948) in *Triturus*; in this species, the treatment caused submergence of the spindle and inhibited the formation of the second polar body but did not prevent the resumption of the meiosis following sperm penetration. Two female pronuclei were thus formed and both subsequently underwent syngamy with the male pronucleus. Fankhauser and Godwin showed that it was by this mechanism that hot shock led to the production of triploid embryos. Fischberg and Beatty (1952) found a large increase in the incidence of triploidy following hot-shock treatment in mice and thought the mechanism was probably the same as in *Triturus*. Results obtained in the present investigation do not support this hypothesis, at least for the rat egg. On the other hand, a striking effect of hot shock upon the rat egg was found to be an increase in the incidence of polyspermy; among the eggs so treated, 16 per cent. were polyspermic compared with 9.2 per cent. in the control group. Examination of the data shows that 65 per cent. of the eggs that contained more than one sperm were polyspermic, as compared with 23 per cent. in the control group. These facts are interpreted as showing that hot shock interferes with the operation of the block to polyspermy, and there is evidence that dispermy would lead to triploidy in the embryo (Austin and Braden 1953*b*). By contrast, colchicine had little if any influence on this response of the eggs to sperm penetration. It would seem that both colchicine and hot-shock treatment may give rise to polyploid rat embryos, the first by suppression of the second polar division and the second by rendering the egg more susceptible to polyspermy.

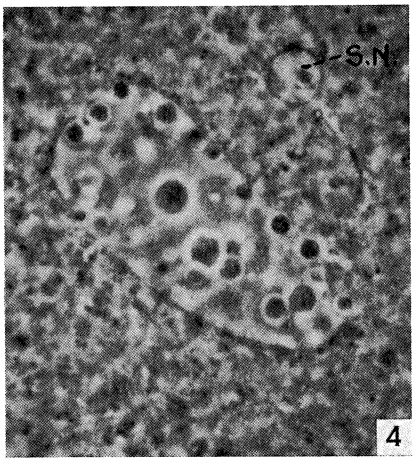
A small proportion of the eggs obtained from mated rats that had received treatment with anaesthetics, temperature shock, or colchicine were classed as abnormal. The fate of these eggs is worthy of speculation, for they were seldom actually degenerate in appearance and some may well have been capable of further development. Of particular interest were 15 eggs in which only a single large nucleus, considered to be the male pronucleus, was present (as in Plate 2, Fig. 7), and seven eggs that had a male pronucleus and two subnuclei in place of the female pronucleus (as in Plate 2, Fig. 8). Rat eggs having only the male pronucleus may be able to undergo subsequent cleavage, just as, apparently,

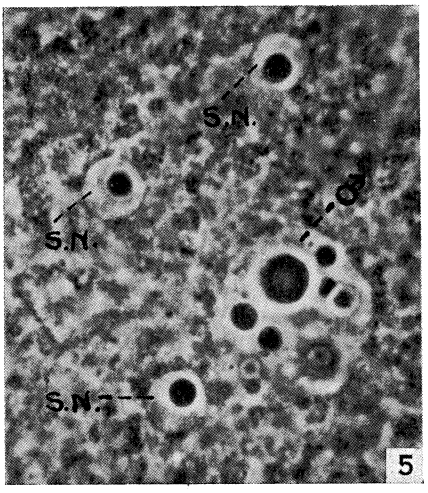
SECOND POLAR DIVISION IN THE RAT EGG



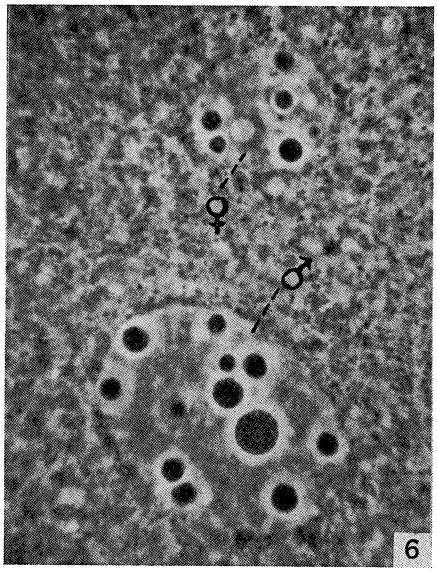


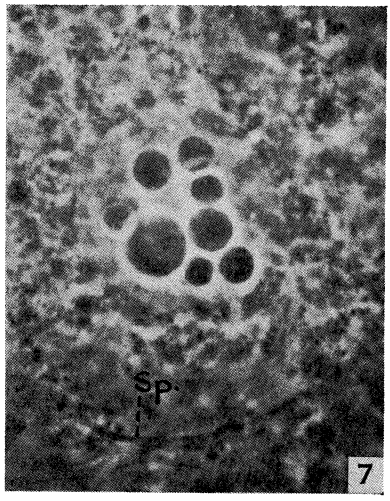


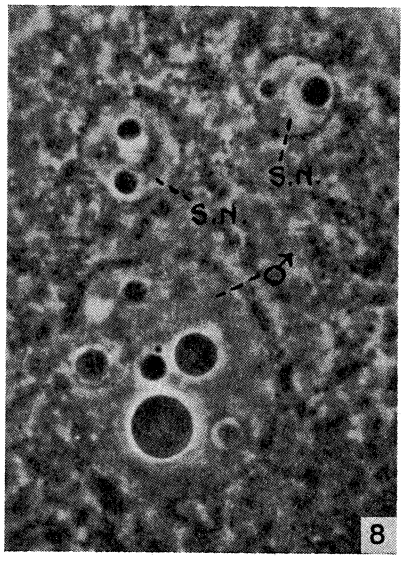


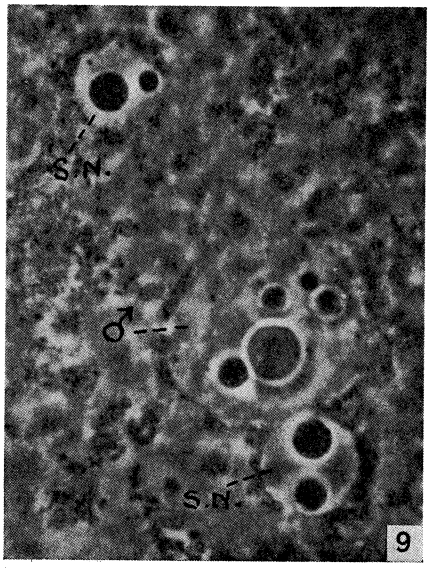


SECOND POLAR DIVISION IN THE RAT EGG









rabbit eggs can with only a female pronucleus (Thibault 1949), but whereas the rabbit eggs are diploid, the rat eggs are presumably haploid and could thus give rise to haploid embryos. Some of the eggs with two well-formed subnuclei in addition to the male pronucleus showed no evidence of having abstricted the second polar body. They could therefore have arisen in the manner described by Fankhauser and Godwin (1948) for *Triturus* and would theoretically be capable of giving rise to triploid embryos.

Observations show that in the rat the immediate consequences of sperm penetration, which may be referred to as the criteria of activation, are independent processes and are capable of separate evocation. Both the contraction of the vitellus and the emission of the second polar body can be induced by non-specific stimuli. Moreover, the resumption of the second maturation division may also be inhibited by artificial means. These things may be done, however, without seriously interfering with the penetrability of the vitellus to the sperm or with the operation of the block to polyspermy. As yet it has not been found possible to induce the block to polyspermy by artificial means. Only the entry of the sperm was observed to evoke this reaction in the rat egg, which thus differs from the eggs of certain lower animals.

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Explanation of Plates 1 and 2

All the photographs were obtained with the aid of a phase-contrast microscope. Magnification is × 1250 for Figures 1, 2, and 3, and × 1000 for Figures 4-9.

Plate 1

Fig. 1.—A persisting first polar body (1. *Pb.*) and the metaphase chromosome group (*Chr.*) of the second maturation division.

Fig. 2.—A newly formed second polar body (2. *Pb.*) in an egg undergoing fertilization. The dense rounded mass of chromosomes (*Chr.*) in the vitellus and the equatorial plate of the spindle are clearly visible. (*Sp.* = sperm mid-piece.)

Fig. 3.—A second polar body about 4 hr after its formation was artificially induced. The chromosomes (*Chr.*) in the vitellus are somewhat scattered and there is no trace of the spindle.

Fig. 4.—A polyspermic egg obtained from a rat subjected to narcosis by subcutaneous injection of "Nembutal." The single large oval nucleus may be the product of fusion of two male pronuclei. Apart from this, only subnuclei were present, one of which is visible in the figure.

Fig. 5.—Egg from a rat treated with nitrous oxide anaesthesia, showing the male pronucleus and three of the subnuclei (*S.N.*) that apparently replace the female pronucleus.

Plate 2

Fig. 6.—The male and female pronuclei in a normal egg undergoing fertilization; they are shown here for comparison with the pronuclei and subnuclei in Figures 7-9.

Fig. 7.—The sole nucleus to be seen in a penetrated egg from a rat treated with hot shock after mating. It is presumed to be the male pronucleus. (*Sp.* = sperm mid-piece.)

Figs. 8 and 9.—The apparently normal male pronucleus and the two large subnuclei (*S.N.*) that replaced the female pronucleus in eggs obtained from rats after treatment with cold shock (Fig. 8) and colchicine (Fig. 9).