Polyspermy-preventing Mechanisms in the Golden Hamster Egg

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ABSTRACT The zona pellucida of a fertilized egg recovered from an artificially inseminated female reacts to capacitated spermatozoa differently from the zona pellucida of an unfertilized egg. The zona of a fertilized egg shows a sperm-refractory response, "zona reaction," so that capacitated spermatozoa neither firmly attach to nor penetrates it. On the other hand, the zona of an unfertilized egg shows no such sperm-refractory response and many spermatozoa attach to and penetrate it. The zona reaction was observed not only in recently penetrated eggs but also throughout cleavage.

Judging from the earliest fertilization stage at which we observed a complete zona reaction, it is estimated that this reaction is completed in less than 15 minutes after sperm attachment to the vitellus.

One hundred per cent of the fertilized eggs recovered from females five hours after artificial insemination were refertilized *in vitro* after removal of the zona pellucida by trypsin-treatment. The incidence of refertilization decreased when the eggs were recovered at later times. Only 3% of the eggs recovered at nine hours after artificial insemination were refertilized. Judging from the fertilization stage at which eggs were no longer refertilizable, it has been estimated that the completion of the vitelline surface block to polyspermy is established in not less than two nor more than three to three and one-half hours.

Possible causes of polyspermy encountered with *in vitro* fertilized eggs are discussed.

Fertilization of mammalian eggs is normally monospermic; that is only one spermatozoon enter the egg cytoplasm. The head of this spermatozoon develops later into a male pronucleus. The entry of more than one spermatozoon into the egg cytoplasm (polyspermy) results in death of embryo during development (Bomsel-Helmreich, '65; Piko, '61a, '69).

The low incidence of polyspermy in mammals (for reviews, see Piko, '61a and Austin, '69) has been attributed to the low number of spermatozoa reaching the site of fertilization (Austin and Braden, '52; Braden and Austin, '54a) and to two other independent mechanisms that keep excess spermatozoa outside the egg. The first mechanism is the so-called "zona reaction" (Braden et al., '54). Austin and Braden ('56) postulated that the contents of the cortical granules, which are released from the egg into the perivitelline space soon after entry of the fertilizing spermatozoon into the egg cytoplasm, modify the substance of the zona pellucida rendering it less penetrable to excess spermatozoa. Experimental evidence for this idea has been presented by Barros and Yanagimachi ('71). The second mechanism is the vitelline surface block to polyspermy which is set up after sperm attachment to the vitelline surface (Austin and Braden, '56).

Austin ('56c) reported that among 519 fertilized hamster eggs recovered from mated females, only five contained supplementary spermatozoa (spermatozoa that crossed the zona pellucida but failed to enter the vitellus; Austin and Walton, '60). In another study, however, Austin and Bishop ('58) found one egg containing a supplementary spermatozoon among 500 fertilized eggs. These facts would indicate that the hamster egg exhibits a strong zona reaction as well as a vitelline

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surface block to polyspermy, but the latter would appear to be redundant (Austin and Walton, '60). In the rabbit, the situation seems to be somewhat different. One may find as many as 50 or more spermatozoa in the perivitelline space of an egg, but only one spermatozoon is able to enter the egg cytoplasm (Braden et al., '54). The apparent lack of a zona reaction in the rabbit would indicate that the block to polyspermy resides on the vitelline surface.

The incidence of polyspermy can be increased under various experimental conditions such as delayed mating in the rat (Austin and Braden, '53; Odor and Blandau, '56; Braden, '58; Piko, '58) and hyperthermia or local application of heat in the rat and mouse (Austin and Braden, '54; Braden and Austin, '54b; Austin, '56b; Braden, '58; Piko and Bomsel-Helmreich, '60). In the hamster, neither delayed mating nor local application of heat seems to increase the incidence of polyspermy (Austin and Braden, '56; Chang and Fernandez-Cano, '58). However, *in vitro* studies of fertilization of hamster eggs show a high incidence of polyspermy not only with regard to the number of polyspermic eggs but also, with regard to the number of spermatozoa *per* egg (Yanagimachi and Chang, '64; Barros and Austin, '67; Barros, '68; Yanagimachi, '69). Barros and Austin ('67) thought this high incidence of polyspermy observed under *in vitro* conditions was due to some deleterious effect of the incubation medium upon the egg mechanisms responsible for excluding extra spermatozoa and also to the use of immature or somewhat aged eggs. While we cannot exclude these factors as causes of polyspermy, another possibility is that under *in vitro* conditions the completion of the zona reaction and the vitelline surface block to polyspermy is not fast enough to prevent the entry of excess spermatozoa.

To the best of our knowledge, the only report available regarding the time required for the development of the zona reaction is that of Braden et al. ('54), who estimated that the zona reaction in rat and mouse eggs would take not less than ten minutes nor more than one-and-a-half to two hours to fully develop. No data is available in the literature regarding the time necessary for full development of the vitelline surface block to polyspermy.

The purpose of this work was to determine the time required for the development of the zona reaction and the vitelline surface block to polyspermy in hamster eggs. Evidence will be presented showing that in the hamster, the zona reaction is fully developed in less than 15 minutes after sperm attachment to the vitellus and that the vitelline surface block to polyspermy is developed in not less than two nor more than three to three-and-a-half hours.

MATERIALS AND METHODS

Adult female golden hamsters were kept in an air-conditioned room with 12 hours light and 12 hours dark (light from 6 AM until 6 PM) and their estrous cycles were determined according to Orsini's ('61) method, except that we considered the day of the post-estrous discharge as day 1.

Artificial insemination of females and recovery of fertilized eggs

Females were injected with 30 i.u. of HCG in the evening (7 to 9 PM) of day 3. In these animals, ovulation occurred consistently between 11 and 14 hours after the HCG injection (Yanagimachi, '69). At 14 hours after injection, females were anesthetized with ether then their uteri were exposed by mid-ventral incision. Two-tenths milliliter of a dense suspension of cauda epididymal spermatozoa in Tyrode's solution were injected into each uterine horn through a number 25 gauge needle attached to a tuberculine syringe. At various times between 5 and 72 hours after artificial insemination, animals were killed and the oviducts were flushed with Tyrode's solution. Whenever the recovered eggs were still in the cumulus, they were treated with 0.1% hyaluronidase (Bovine testicular hyaluronidase, NBC Lab.) in Tyrode's solution for about five minutes. The cumulus-free eggs were thoroughly rinsed in fresh Tyrode's solution.

Recovery of spontaneously activated eggs

A group of females were not treated with hormones but rather allowed to ovu-

late spontaneously. These animals were killed at 8 PM on the day of the post-estrous discharge (day 1) and the eggs (approximately 17–21 hours old) were flushed out of the oviducts with Tyrode's solution. Eggs recovered were always free of cumulus cells and about 80% of them were found to be spontaneously activated as reported by Austin ('56a) and Yanagimachi and Chang ('61).

Preparation of in vitro *capacitated spermatozoa*

A cauda epididymis of a fertile male was dissected out, incised in several places and placed under mineral oil in a sterile plastic petri-dish. When 1.6 ml of Tyrode's solution were put around the epididymis, spermatozoa dispersed into the solution to make a dense, homogeneous suspension. Ten minutes later, the epididymis was removed from the dish. The density of spermatozoa in the suspension was determined by means of a hemocytometer and 1×10^6 spermatozoa were added to 100 μl of a human blood serum fraction which was prepared as follows.

Blood drawn without heparinization from the cubital vein of human males was placed in pre-chilled siliconized centrifuge tubes, allowed to clot at room temperature for one to two hours, then centrifuged for ten minutes at 400 g. The serum thus obtained was heated at 70° C for one hour. When the coagulated serum was homogenized and centrifuged again for 30 minutes at 37,000 g, a clear supernatant was obtained, which has been found to be free of gamma globulins. When a small drop of a dense sperm suspension in Tyrode's solution is added to this serum fraction and incubated under mineral oil at 37° C, the spermatozoa maintain good progressive motility for several hours and become fully capacitated in about three hours (Barros and Garavagno, unpublished experiments).

Evaluation of the zona reaction

The eggs flushed out of the oviducts at various hours after artificial insemination were added to 100 μl of the human serum fraction containing fully capacitated spermatozoa. Within five minutes after insemination, the behavior of spermatozoa toward the zona pellucida was carefully observed under both a high power dissecting microscope and a phase-contrast microscope. After further incubation at 37° C for one to two hours, the eggs were re-examined with a phase-contrast microscope for evidence of sperm penetration through the zona pellucida. An egg was considered as having completed the zona reaction by the time of recovery if the zona pellucida of the already fertilized egg was not penetrated by spermatozoa *in vitro*.

Evaluation of the vitelline surface block to polyspermy

Fertilized eggs recovered from the oviducts at various hours after artificial insemination were freed from the zona pellucida by treatment for 30 to 60 seconds with 0.01% trypsin (hog pancreatic trypsin, 1–300, NBC Lab.) in Tyrode's solution containing 0.1% bovine serum albumin (Fraction V, Armour Pharmaceutical Co.). The zona-free eggs thus obtained were thoroughly rinsed with Tyrode-albumin solution then placed in a drop (50 μl) of incubation medium (Biggers et al., '71). To this drop was added 1 μl of the human serum fraction containing approximately 200 to 400 fully capacitated spermatozoa. After one hour of incubation under mineral oil at 37° C, the eggs were examined with a phase-contrast microscope to determine whether these eggs, which had already been penetrated by a fertilizing spermatozoon by the time of recovery were further penetrated by excess spermatozoa (refertilization). The presence of more than one sperm head showing signs of chromatin dispersion was used as a criterion for refertilization. When an egg showed any signs of refertilization, we considered that this egg had not completed its vitelline surface block to polyspermy by the time of recovery from the oviduct.

RESULTS

Timetable of early events of fertilization

It seems expedient at this point to describe the time sequence of the early

events of fertilization, which have been used in this work as landmarks to determine the time required for full development of the zona reaction and the vitelline surface block to polyspermy. Since *in vivo* fertilization is not synchronous, eggs recovered from mated females cannot be used to time the different stages of fertilization. This problem can be overcome by using the *in vitro* fertilization technique. Therefore in the present work we studied the time required for the sperm to cross the zona pellucida by inseminating cumulus-free eggs *in vitro* with capacitated spermatozoa. Zona-free eggs were used to time the remaining stages of fertilization.

In the fresh unfertilized egg, the second meiotic division is arrested at metaphase (fig. 1) and the cortex of the egg is rich in cortical granules (fig. 2). When cumulus-free eggs are mixed with capacitated spermatozoa, sperm attach to the zona immediately; however, the spermatozoa remain at the zona surface for at least five minutes before they begin to cross it (fig. 9). About 15 minutes after attachment, we were able to see some spermatozoa in the perivitelline space.

When actively moving capacitated spermatozoa were mixed with zona-free eggs, many collided with and attached to the eggs, soon becoming motionless. Within two and one-half to five minutes after sperm attachment, the cortical granule breakdown had already taken place (fig. 3) indicating that egg and sperm membranes had already fused. Ten minutes after sperm attachment, the egg chromosomes begin their anaphasic movements (fig. 4). At this time the sperm head seems to be resting in a depression of the egg surface; however, the sperm chromatin does not show any signs of dispersion (fig. 10). Twenty minutes after sperm attachment, the spindle of the second meiotic division has already rotated so that the female chromosomes are now parallel to the egg surface. The beginning of the abstriction of the second polar body is indicated by a cleft originating at the egg surface (fig. 5). At 20 minutes after sperm attachment, sperm chromatin dispersion is becoming evident, and by 30 minutes is very distinct in the middle region of the sperm head (fig. 11). Most of the chromatin is dispersed by 40 minutes and appears as a less granular area in the egg. However, the most apical end of the sperm head (the perforatorium, Franklin et al., '70; Yanagimachi and Noda, '70) remains dense (fig. 12). Meanwhile the formation of the second polar body has advanced. The second meiotic division is completed and the female chromosomes are in their respective locations, *i.e.*, the future polar body and the egg cortex. Sixty minutes after sperm attachment, the whole sperm head has dispersed and the developing male pronucleus is acquiring a spherical shape (fig. 13). The female pronucleus is already distinct and shows a few small nucleoli (fig. 6). Nucleoli begin to appear in the male pronucleus about 90 minutes after sperm attachment (fig. 14), while the female pronucleus now shows a few large nucleoli. By two hours after sperm attachment, the male and female pronuclei are similar in appearance (figs. 7, 15) and the entire middle piece of the sperm tail is within the egg cytoplasm. Within the next hour, the nucleoli decrease in number and increase in size so that by three hours after sperm attachment there is usually one to three large nucleoli within each pronucleus (figs. 8, 16). At this time the sperm tail is almost completely inside the egg cytoplasm.

Zona reaction

When the eggs recovered from the oviducts at different times after insemination were mixed *in vitro* with fully capacitated spermatozoa, two types of eggs were clearly distinguished within five minutes (fig. 17). One type had a cloud of actively moving spermatozoa strongly attached to the zona pellucida (egg a). After these eggs were cleaned by strongly pipetting them through a small bore pipette, examination with a phase-contrast microscope revealed that none of them contained spermatozoa either in the perivitelline space or in the vitellus. It was evident that this type of egg had not been penetrated prior to the time of recovery from the oviducts. In the other type of egg, no spermatozoa were found firmly attached to the zona pellucida (egg b), although many actively moving spermatozoa were continuously col-

liding with it. Examination of these eggs with a phase-contrast microscope revealed that each had already been penetrated by one spermatozoon (fig. 18) by the time of recovery from the oviducts and that the cortical granules had completely broken down. Such a sperm-refractory response of the zona pellucida was observed in all the fertilized eggs except three, two of which were recovered at five hours and the other at six hours after artificial insemination (see table 1). Careful examination of these three eggs revealed that each had one spermatozoon attached to the vitelline surface. Judging from the condition of the spermatozoon within each egg and the complete breakdown of the cortical granules, it was believed that fusion of the egg and spermatozoon had taken place shortly before the egg was recovered from the oviduct.

These observations suggest that the sperm-refractory response of the zona pellucida is a fairly reliable criterion for distinguishing fertilized from unfertilized eggs. When eggs were separated using this criterion, and allowed to incubate for another one to two hours with capacitated spermatozoa, the zonae pellucidae of the already fertilized eggs were never penetrated by excess spermatozoa; while all the unfertilized eggs became heavily polyspermic (see figs. 19, 20). The zonae pellucidae of these latter eggs showed so many slits left by penetrating spermatozoa that they had a saw-like appearance.

Eighty-one per cent of the 100 eggs recovered from unmated females approximately 17 to 21 hours after the estimated time of ovulation, showed spontaneous activation. Fifty-nine per cent of the activated eggs contained one pronucleus, 40% contained two pronuclei and 1% was at the two-celled stage. Cortical granules were always present in such eggs regardless of the condition of activation, although the density of granules seemed to be somewhat reduced. After 120 such spontaneously activated eggs were inseminated with capacitated spermatozoa and incubated for two hours, 92% of them contained spermatozoa in the perivitelline space only and 2% contained spermatozoa both in the perivitelline space and in the vitellus.

TABLE 1

In vivo *fertilized eggs showing positive and negative zona reaction after incubation* in vitro *with capacitated spermatozoa*

Eggs recovered		No. of fertilized eggs showing	
Hours after insemination	No.	Negative zona reaction	Positive zona reaction
5	121	2	21
6	111	1	60
7	93	0	74
9	21	0	21
24	31	0	31
48	21	0	21
72	9	0	9

Vitelline surface block to polyspermy

As soon as actively moving capacitated spermatozoa were mixed with *in vivo* fertilized zona-free eggs, many of them collided with and attached to the vitelline surface. Many of these spermatozoa became motionless and could not be removed by mild pipetting.

After one hour of incubation, the eggs contained many spermatozoa in the vitellus with different degrees of chromatin dispersion (see figs. 21, 22). By changing the plane of focus, it was possible to identify the pronucleus and tail of the spermatozoon that fertilized the egg *in vivo*. It was usually at a more advanced stage than the refertilizing spermatozoa. In all cases there were many spermatozoa attached to the egg surface but only in some of the eggs did the spermatozoa show signs of chromatin dispersion.

The incidence of refertilization of the eggs recovered at various hours after artificial insemination is shown in table 2. The highest incidence (100%) was obtained in eggs recovered from the oviduct at five hours after artificial insemination. At that time the eggs were at the stages of fertilization depicted in figures 3 through 6 and 10 through 14. Eggs recovered at six hours after artificial insemination were also at different stages of fertilization, including eggs with pronuclei corresponding to a developmental age of two hours or more. When eggs recovered at this time were inseminated *in vitro* some of them already showed completion of the vitelline surface block

TABLE 2

Number of in vivo *fertilized eggs refertilized* in vitro *after removal of the zona pellucida*

Hours after insemination	Fertilized eggs recovered	Refertilized		Non-refertilized	
	No.	No.	%	No.	%
5	56	56	100	0	0
6	110	42	38	68	62
7	34	20	58	14	42
8	70	5	7	65	93
9	32	1	3	31	97

to polyspermy, because only 38% of the eggs were refertilized. Some eggs of a developmental age of at least two hours were refertilized, while others of an apparently similar age were not. This was also true for eggs recovered at later times. Eggs recovered at nine hours after artificial insemination contained well formed pronuclei (see figs. 8, 16) and the sperm tail was wholly inside the egg cytoplasm. These eggs were at a developmental age of three to three and one-half hours. Following incubation with capacitated spermatozoa only 3% of the eggs were refertilized.

DISCUSSION

Zona reaction

Braden et al. ('54) reported for the first time the occurrence of the zona reaction in mammalian eggs. They arrived at this conclusion after a statistical analysis of the frequency distribution of normally fertilized rat and mouse eggs containing more than one spermatozoon. They found that this distribution was smaller than that expected if chance alone had controlled the number of spermatozoa entering the eggs. Recently Barros and Yanagimachi ('71) presented direct experimental evidence for the occurrence of this reaction.

Possible involvement of cortical granule material in the induction of the zona reaction was first suggested by Austin and Braden ('56) and experimentally endorsed by Barros and Yanagimachi ('71) who induced apparently normal zona reaction by exposing the zonae pellucidae of unfertilized eggs to the cortical granule material released from fertilized eggs. This also seems to be supported, though indirectly, by the apparent lack of zona reaction in aged, spontaneously activated hamster eggs, in which the majority of the cortical granules are present. Yanagimachi and Chang ('61) earlier reported that unfertilized, spontaneously activated hamster eggs recovered 18 hours after ovulation did not have PAS-positive cortical granules, while the perivitelline space was rich in PAS-positive material. They inferred that the loss of fertilizability of aged hamster eggs was due to spontaneous breakdown of cortical granules which could have induced the zona reaction. The apparent disagreement between the present study and Yanagimachi and Chang's study may be explained in terms of the different techniques used in both works to evaluate the fertilizability and the presence of cortical granules in aged eggs (*in vivo* insemination and cytochemical examination of cortical granules, Yanagimachi and Chang vs. *in vitro* insemination and phase-contrast microscope examination of cortical granules, the present study).

To estimate the lower and upper limits of the time required for full development of the zona reaction, we have to use certain landmarks in the early events of fertilization. For the lower limit we have used the time at which cortical granules have already broken down. We saw that zona-free eggs could exhibit cortical granule breakdown as early as 2.5 minutes after insemination. This time might not represent the actual time required *in vivo*, since in our system many spermatozoa could have attached and initiated the cortical response at the same time in several places, thus speeding it up. When we used a very diluted sperm suspension, the cortical granules were absent from the egg cortex at five minutes but not at 2.5 minutes after insemination. Therefore we can place the lower limit for the time required for the development of the zona reaction between 2.5 and five minutes after sperm attachment to the vitellus or 17 and 20 minutes after effective contact with the outer edge of the zona pellucida.

For the upper limit, we have used as a criterion, the stage of fertilization at

which we were able to observe a positive zona reaction. This was a stage at which the cortical granules had already broken down, but the spermatozoon attached to the vitelline surface showed no signs of chromatin dispersion, *i.e.*, about 20 minutes after sperm attachment to the vitellus or about 35 minutes after effective contact with the outer edge of the zona pellucida. However, it is very likely that eggs at this stage had completed their zona reaction at an earlier time since they were incubated for five minutes before they were examined with the phase-contrast microscope. We believe that the zona reaction is fully developed in less than 15 minutes after sperm attachment to the vitellus. Even though somewhat wider limits were estimated by Braden et al. ('54) for the development of the zona reaction in the rat (not less than 10 minutes nor more than 1.5 to 2 hours), it is possible that the actual time in the rat is similar to that demonstrated for hamster eggs.

Vitelline surface block to polyspermy

Barros ('68) found that *in vitro* fertilized hamster eggs, although highly polyspermic, contained very few supplementary spermatozoa. He concluded that the block to polyspermy operating at the vitelline surface was either very slow or that it could be completely absent. The results herein reported would support the idea of very slow development of the vitelline surface block to polyspermy. Based on the stage of fertilization at which eggs were no longer refertilizable, we have judged that in hamster eggs, the vitelline surface block to polyspermy takes not less than two nor more than 3 to 3.5 hours after sperm entry into the vitellus to develop.

Refertilization has also been reported in the rat after treating fertilized eggs with EDTA (Piko, '61b, '69) or injecting EDTA or other substances into the oviduct (von der Borch, '67). Piko ('69) found that when fertilized eggs were treated with EDTA, supplementary spermatozoa were incorporated into the egg cytoplasm as late as the time close to the first cleavage. He has suggested that the contents of the cortical granules may coat the egg surface preventing extra spermatozoa from binding with the egg membrane. The treatment with EDTA perhaps alters or changes the coating substance, probably by removing Ca ions, allowing spermatozoa to establish an effective binding with the egg membrane. Also, Sugiyama ('51) previously found with sea urchin eggs that treatment with Ca and Mg-free sea water immediately after fertilization allowed the eggs to become refertilized. Moreover he also found that following removal of the fertilization membrane, treatment with Ca and Mg-free sea water rendered the eggs refertilizable even at the two-cell stage. It seems then, that removal of Ca and Mg ions reverses the polyspermy-preventing mechanism operating at the vitelline surface of sea urchin and very likely of rat eggs.

The results reported here, however, seem to indicate that we are not dealing with a reversion of the vitelline surface block to polyspermy but rather with its gradual establishment. This is supported by the fact that higher incidences of refertilization were obtained at earlier times after entry of the first spermatozoon, then as time passed those incidences gradually decreased. Tyler et al. ('56) were able to refertilize eggs of sea urchins (*Lytechinus pictus* and *L. variegatus*) up to 40 minutes after the original fertilization by simple mechanical removal of the fertilization membranes. A similar situation is encountered with fish eggs. There, monospermy is assured by a thick and tough chorion with a micropyle, which allows entry to the egg cytoplasm of only one spermatozoon at a time. After the fertilizing spermatozoon has entered the egg, any extra spermatozoa within the micropyle are pushed out by a colloidal material possibly derived from the cortical alveoli (Sakai, '61). Mechanical removal of the chorion within 20 minutes after fertilization renders the egg refertilizable (Yanagimachi, '57).

The cause of polyspermic fertilization of hamster eggs under in vitro *conditions*

The high incidence of polyspermy observed in studies of *in vitro* fertilization of hamster eggs has been attributed to some adverse effect of the culture medium upon

the polyspermy-preventing mechanisms; and also to the use of immature or somewhat aged eggs (Barros and Austin, '67). On the other hand, it has been suggested that polyspermy may result simply because of too many capacitated spermatozoa around the eggs (Yanagimachi and Chang, '64; Barros, '68; Yanagimachi, '69). This latter idea would be supported by the following facts: (1) hamster eggs depend mainly upon the zona reaction (as an intrinsic mechanism) to avoid polyspermy; (2) the zona reaction takes 17 to 30 minutes to be fully developed after the spermatozoon makes effective contact with the outer edge of the zona pellucida; (3) the number of effective collisions between sperm and egg during *in vitro* fertilization is extremely high as compared with the number found *in vivo;* (4) *in vitro* fertilized hamster eggs lose their cumulus and corona cells in less than one hour (sometimes as early as 10 minutes) after insemination, while *in vivo* fertilized eggs lose these cells several hours after sperm penetration; so that *in vivo* fertilized eggs are more protected against effective collisions with spermatozoa than *in vitro* fertilized eggs.

It has also been found that sperm penetration into hamster eggs *in vitro* is continuous for a period of at least four hours after insemination with fully capacitated spermatozoa (Barros et al., '72). This apparent lack of a zona reaction for *in vitro* fertilized eggs may be explained by assuming that the substance of the cortical granules can escape through the many slits left by the penetrating spermatozoa and thus not reach the threshold concentration required to induce the reaction.

The female genital tract reduces the number of spermatozoa reaching the site of fertilization at any one time so that the chances for polyspermy are reduced without reducing the chances for fertilization (Chang, '51; Austin and Braden, '52; Braden, '53; Braden and Austin, '53; Braden and Austin, '54a). We believe that the intrinsic polyspermy-preventing mechanisms of mammalian eggs are adequate only if the premise stated above is compiled with, otherwise both the zona reaction and the vitelline surface block to polyspermy are inadequate mechanisms.

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PLATE 1

EXPLANATION OF FIGURES

Development of the female pronucleus.

1 Unfertilized zona-free egg showing the second meiotic metaphase. × 3,500.

2 Surface of unfertilized zona-free egg showing the cortical granules. × 3,500.

3 Zona-free egg 2.5 minutes after insemination. Note the disappearance of the cortical granules. × 3,500.

4 Zona-free egg ten minutes after insemination. The female chromosomes have started their anaphasic movements. × 3,500.

5 Zona-free egg 20 minutes after insemination. The abstriction of the second polar body is beginning and it is evidenced by a cleft originating at the surface of the egg. × 3,500.

6 Zona-free egg 60 minutes after insemination. The female pronucleus is already formed and contains a few small nucleoli. × 3,500.

7 Zona-free egg two hours after insemination. The female pronucleus with numerous medium sized nucleoli. × 3,500.

8 Zona-free egg three hours after insemination. The number of nucleoli has decreased, while the size has increased. × 3,500.

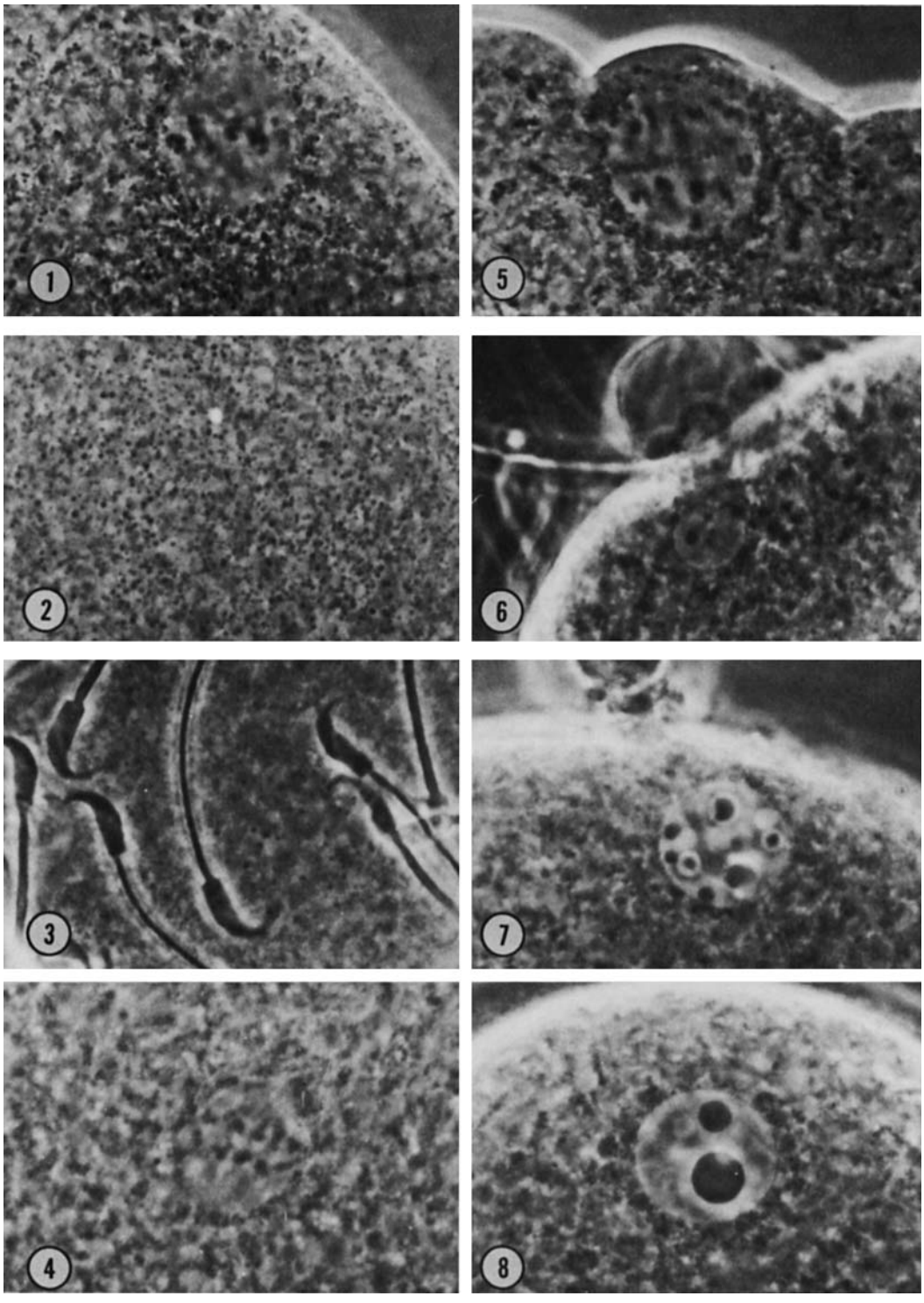
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PLATE 2

EXPLANATION OF FIGURES

Development of the male pronucleus.

9 Spermatozoon in the thickness of the zona pellucida (zp) of a cumulus-free egg. Ten minutes after insemination. × 3,750.

10 Zona-free egg showing a spermatozoon attached to the vitelline surface and probably fused with the egg. Ten minutes after insemination. × 3,500.

11 Zona-free egg with a spermatozoon showing chromatin dispersion in the middle region of the head (arrows). Thirty minutes after insemination. × 3,500.

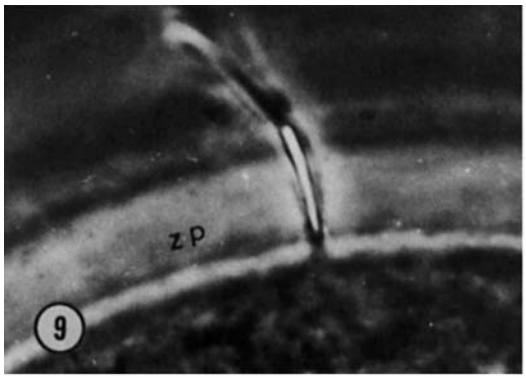
12 Zona-free egg showing a spermatozoon with almost completely dispersed chromatin. Part of the subacrosomal material still remains undispersed (arrows). × 3,500.

13 Zona-free egg showing the developing male pronucleus 60 minutes after insemination. × 3,500.

14 Zona-free egg 90 minutes after insemination. Small nucleoli have appeared within the male pronucleus. × 3,500.

15 Zona-free egg two hours after insemination. The number and size of nucleoli have increased within the male pronucleus. × 3,500.

16 Zona-free egg three hours after insemination. The number of nucleoli have decreased, while the size has increased. × 3,500.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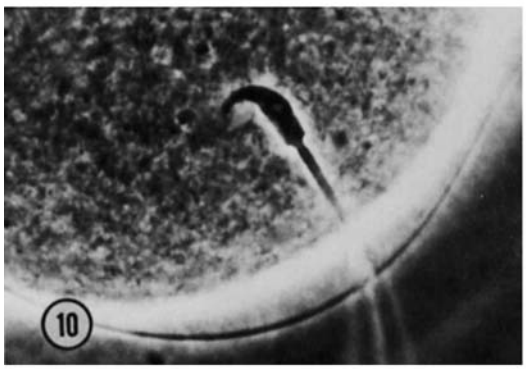
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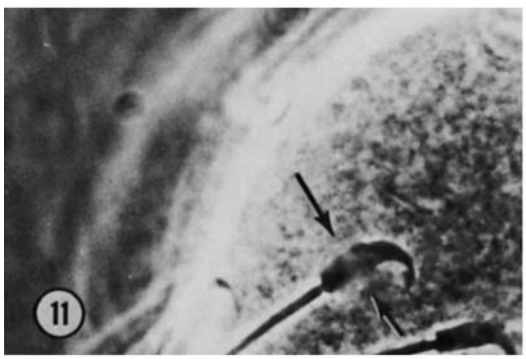
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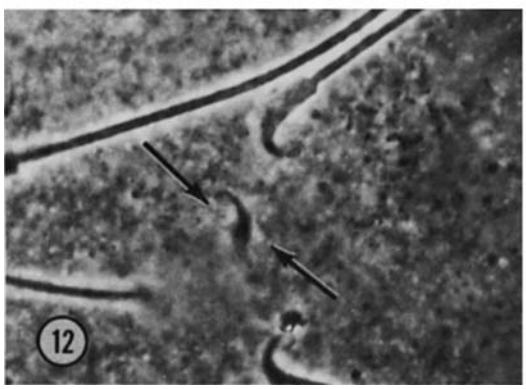
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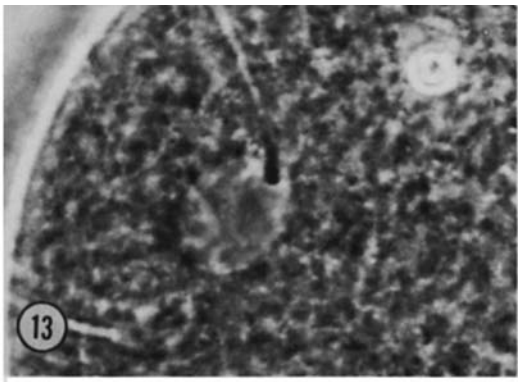
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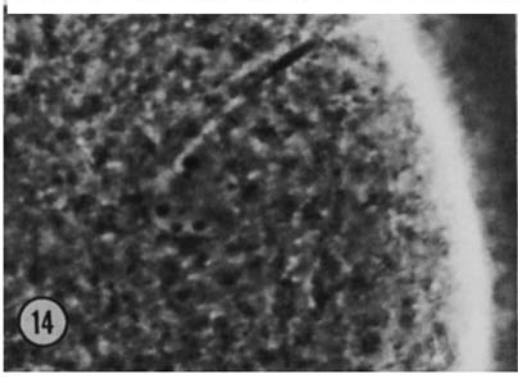
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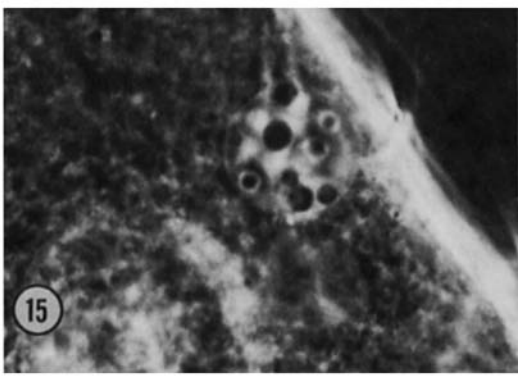
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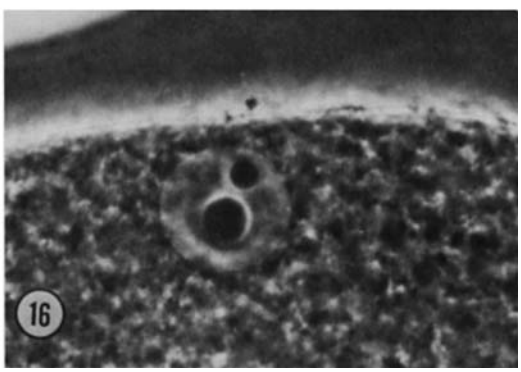
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PLATE 3

EXPLANATION OF FIGURES

17 Two eggs recovered from a female hamster five hours after artificial insemination and mixed *in vitro* with capacitated spermatozoa. One egg (a) has many spermatozoa attached to the zona pellucida (negative zona reaction), while the other (b) does not have spermatozoa firmly attached to the zona surface (positive zona reaction). In this latter egg it is possible to see the tail of the fertilizing spermatozoon (arrow). × 400.

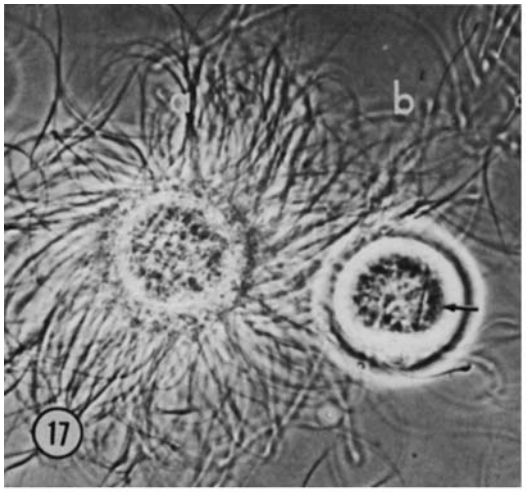
18 High magnification of a fertilized egg with positive zona reaction. × 1,120.

19 Egg with negative zona reaction after being incubated *in vitro* for two hours with capacitated spermatozoa. The egg was thoroughly cleaned by pipetting to eliminate spermatozoa from the zona pellucida. This egg contained many pronuclei (p) but only two are in focus. Note the many slits made by penetrating spermatozoa (arrows). × 1,120.

20 Egg with negative zona reaction which became highly polyspermic after one hour of incubation with capacitated spermatozoa. × 1,120.

21 Egg recovered from the oviduct of a female artificially inseminated six hours previously. The egg was treated with trypsin to digest the zona pellucida and incubated for one hour with capacitated spermatozoa. Note the male and female pronuclei. × 1,120.

22 Same egg as in figure 21, but at a different focal plane to show the refertilizing spermatozoa (arrows). × 1,120.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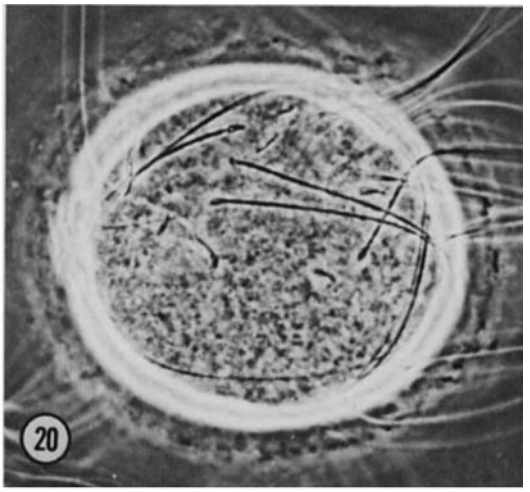
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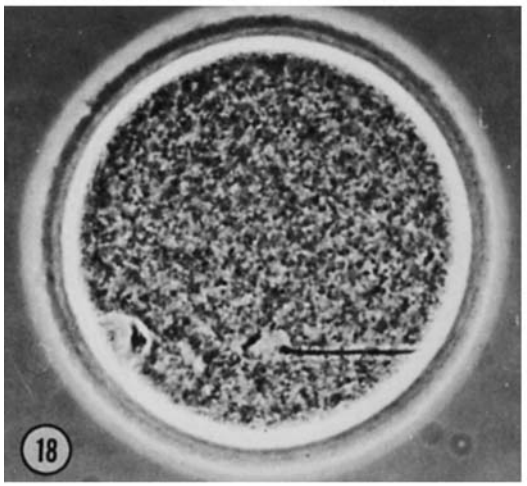
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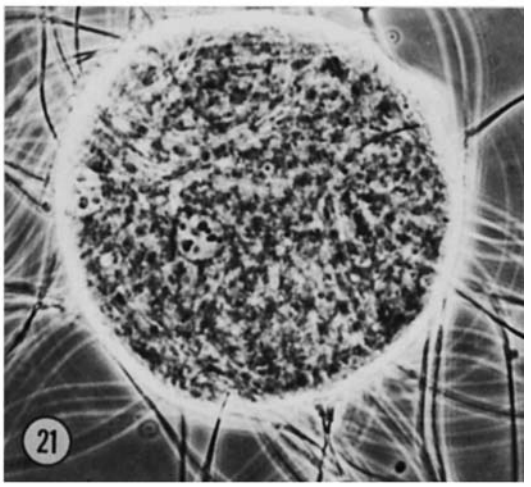
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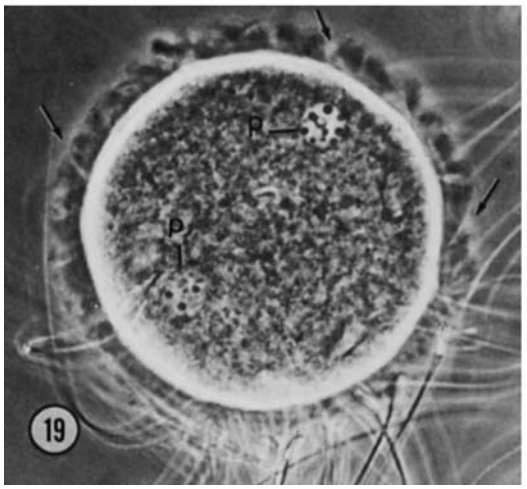
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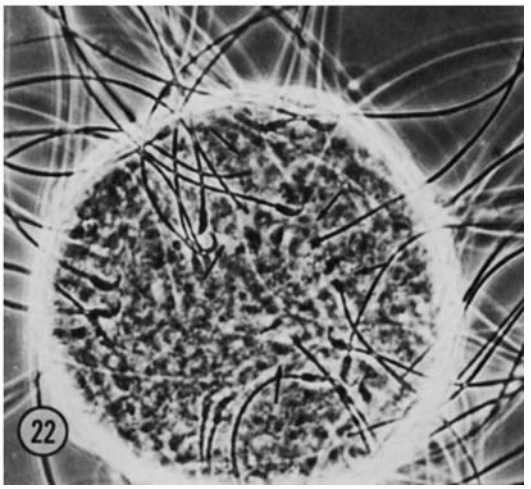
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