THE REACTION OF THE ZONA PELLUCIDA TO SPERM PENETRATION

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Summary

The observations described concern the number of sperms seen within the eggs of the rat, mouse, rabbit, sheep, and dog.

A frequency distribution is presented for the eggs of 251 rats and 20 mice, according to the number of sperms that they were found to contain.

Considering the high proportion of penetrated eggs in both the rats and the mice, the number of eggs that contained more than one sperm (about 20 per cent.) was much smaller than would be expected if the distribution were controlled by chance alone. This is interpreted to mean that the penetrability of the zona pellucida to sperms decreases after the entry of the first sperm. Such a reaction has not previously been demonstrated in the zona pellucida. In eggs that contained two sperms, the points of entry through the zona were seen much more frequently in opposite hemispheres than in the same hemisphere, an observation that suggests the zona reaction is a propagated change. It is estimated that the mean time the reaction takes to reach completion is not less than 10 min or more than 1½-2 hr.

In the rabbit the penetrability of the zona pellucida is not influenced by sperm entry. The mean number of sperms in the perivitelline space of the rabbit egg increased from 0·2 10 hr after coitus to 61·8 16 hr after coitus; thereafter there was no significant change.

No sperms were detected in the perivitelline space of 12 fertilized sheep eggs or in 12 fertilized dog eggs, although numerous sperms adhered to the surface of the zona pellucida. The zona pellucida of both sheep and dog eggs apparently reacts to the entry of the fertilizing sperm in such a way as to preclude further penetration.

Published reports on the number of sperms within the eggs of various mammals are reviewed. It is concluded that, except in the eggs of the rabbit and perhaps the mole, the zona pellucida of mammals reacts to sperm penetration in such a way that few, if any, extra sperms can enter the eggs. The importance of this reaction in relation to fertility is discussed.

I. INTRODUCTION

The zona pellucida of the mammalian egg is an apparently structureless membrane that surrounds the vitellus but is separated from it by the perivitelline space. It is mucoprotein in nature (Braden 1952). The zona persists during the cleavage of the egg in the fallopian tube but disappears shortly after the egg enters the uterus. In spite of its temporary and apparently passive function as a protective membrane, the zona pellucida is nevertheless capable of exhibit-

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ing changes in state which are reflected in variations in its penetrability to sperms. It has been found recently that, in the rat, sperms do not enter the eggs immediately after ovulation. Evidently the egg membranes must first undergo some change, which represents perhaps a terminal phase in maturation (Austin and Braden 1954*b*). When this change has been effected, a sperm may readily traverse the zona at any time during the subsequent 12 hr. However, data have now been obtained indicating that, in many mammals, the entry of the first sperm into the egg initiates a reaction in the zona the effect of which is to preclude the entry of further sperms. This reaction resembles, in its consequence, the change that occurs in the vitellus, the so-called "block to polyspermy." Evidence for the occurrence of such a reaction in the zona, and an assessment of its speed, are the subjects of the present communication.

II. Methods

The experimental animals included rats of albino and hooded strains, albino mice, cross-bred rabbits, Merino ewes, and mongrel bitches.

The rats were mated before, during, or after ovulation, which has been shown to occur between midnight and 4 a.m. (Austin and Braden 1954*b*). "Normally" mated rats were those that were left with males overnight from 4 p.m. Coitus generally occurred in the early evening. Rats that mated were recognized by the presence of copulation plugs when examined the next morning. Other groups of rats were placed with males at 10 p.m., 3 a.m., or 8 a.m. and were permitted 1 hr for copulation. Normally mated rats and those mated at 10 p.m. and 3 a.m. were killed between 11 a.m. and 4 p.m. on the day of ovulation. Rats mated at 8 a.m. were killed at 10 a.m. on the following day. The eggs were recovered from the fallopian tubes by dissection under normal saline solution and were examined with the phase-contrast microscope.

Mice were left with males overnight and those with copulation plugs next morning were killed during the course of the day.

Rabbits were permitted to copulate, each female with two males, and killed 10, 13, 16, and 20 hr later. The eggs were recovered by flushing the tubes with normal saline solution and were fixed for several hours in 10 per cent. acetic-alcohol. They were embedded by Minchin's albumen method (Gatenby and Beams 1950), cut in 7-μ sections, and stained by the Feulgen technique. The sperm heads in the eggs could thus be readily recognized and counted.

The ewes were run with a raddled ram and were inspected each night and morning for signs of mating. Those that mated were killed 18-36 hr later. The eggs were recovered by the same method as was used for the rabbit and were examined in the fresh state.

The genital tracts of two bitches were removed by laparotomy 3-4 days after coitus. The eggs were recovered in the same way as for the rabbit. Eight eggs were examined in the fresh state; the remainder were fixed in 10 per cent. formol-saline and then embedded, sectioned, and stained in the same way as were the rabbit eggs.

III. Observations

(*a*) *Frequency Distribution of Rat and Mouse Eggs According to the Number of Penetrating Sperms*

From 87 normally mated rats, 826 eggs were obtained; 810 (98·1 per cent.) had been penetrated by sperms, most of them (76·1 per cent.) by one sperm only. The frequency with which eggs were seen to contain more than one sperm diminished progressively with larger numbers of penetrating sperms. Thus 135 eggs (16·7 per cent.) had two sperms, 25 eggs (3·1 per cent.) had three sperms, and 12 eggs (1·5 per cent.) had four sperms. Only 10 eggs were found to contain five or more sperms; two eggs were exceptional for they had about 25 and 50-60 sperms respectively, and were degenerate in appearance (Plate 1, Fig. 6). Ten of the eggs with two or more sperms were polyspermic, i.e. two sperms had entered the vitellus; all the other sperms that were present in excess of one for each egg were in the perivitelline space and had not penetrated the vitellus (Table 1).

Similar observations were made on rats mated at 10-11 p.m., 3-4 a.m., and 8-10 a.m., and similar frequency distributions were apparent among the eggs recovered. The only notable differences were that both the number of eggs containing two or more sperms, and the number of polyspermic eggs, increased with the later times of mating (Table 1). From these rats, four exceptional eggs were seen that contained 10, 12, 16, and 18 sperms respectively; the eggs with 16 and 18 sperms were clearly degenerate.

The pattern of the frequency distributions seemed to show that the proportion of eggs with only one sperm was higher than would be expected if the number of sperms that entered an egg was controlled by chance alone. This possibility was investigated by selecting from the normally mated rats a group that had been killed in the course of a single experiment, and arranging the numbers of eggs from these animals in the form of a frequency distribution based upon the number of penetrating sperms. Thus from 10 rats, 91 eggs were recovered, of which five were unpenetrated, 68 contained one sperm, 15 contained two sperms, one contained three sperms, and two contained four sperms. There were no eggs with five or more sperms. A theoretical or expected distribution was then calculated for the same total number of eggs on the assumption that chance alone controlled the number of sperms that entered the eggs (Table 2). The two distributions, the observed and the expected, differed widely from each other. In both, the largest group was made up of the eggs that contained only one sperm, but the number of eggs in this group was much lower in the expected series (33·1) than in the observed (68). On the other hand, the number of unpenetrated eggs, and the numbers of eggs containing two or more sperms, were much higher in the theoretical distribution. Clearly, a much greater proportion of eggs was observed with only one sperm than would be expected if chance alone controlled the number of sperms which penetrated the zona. This is interpreted to mean that, as a result of the penetration of the first sperm, some change occurs in the zona pellucida that tends to prevent the penetration of later sperms. From

TABLE 1

FREQUENCY DISTRIBUTION OF RAT AND MOUSE EGGS THAT CONTAINED VARIOUS NUMBERS OF SPERMS WITHIN THE ZONA PELLUCIDA

Species	Time of Mating	Number of Animals	Total Eggs	Unpenetrated Eggs	Eggs Penetrated by i Sperms									Polyspermic Eggs*	Total Penetrated Eggs
					$i=$ 1	2	3	4	5	6	7	8	>8		
Rats	Normal	87	826	16	628	135	25	12	3	0	4	1	2 (25, 50-60)	10	810
Rats	10-11 p.m.	69	656	9	482	126	21	13	2	1	1	0	1 (10)	18	647
Rats	3-4 a.m.	22	235	8	158	45	13	5	3	2	1	0	0	19	227
Rats	8-10 a.m.	73	649	55	396	128	42	15	6	2	2	0	3 (12, 16, 18)	51	594
Mice	Normal	20	178	9	138	27	2	1	0	1	0	0	0	2	169

* The polyspermic eggs are distributed through the other columns according to the total number of sperms within the zona pellucida.

the data in Table 1, it will be seen that similar conclusions may be drawn, not only for the complete results from rats mated at the normal time, but also from those mated at later periods.

The expected distribution was determined in the following way. Consider any one tube. If the assumption is made that the chances of sperm penetration into an egg are unaffected by the entry of the first sperm, then the penetrating sperms may be supposed to be randomly distributed amongst the eggs. Let there be s penetrating sperms and n eggs. If each egg has the same chance of being penetrated, this chance will be $1/n$, and the probability of any one egg having i sperms in it will be given by the binomial term

$$\binom{s}{i}\left(1-\frac{1}{n}\right)^{s-i}\left(\frac{1}{n}\right)^{i} \qquad (0\leqslant i\leqslant s).$$

Hence the expected number of eggs with i sperms will be

$$\binom{s}{i}\left(1-\frac{1}{n}\right)^{s-i}\left(\frac{1}{n}\right)^{i-1}.$$

From this expression it is possible to obtain expected frequencies corresponding to the observed frequencies. Since, however, both s and n are small for any one fallopian tube, it is necessary to pool the results from a number of tubes, by adding the expected numbers of eggs with i sperms, and in a similar manner the corresponding observed numbers. The results are set out in Table 2.

TABLE 2

OBSERVED AND EXPECTED FREQUENCIES OF RAT AND MOUSE EGGS CONTAINING VARIOUS NUMBERS OF SPERMS

Species	Frequency	Number of Eggs Containing i Sperms						Total Eggs
		$i=$ 0	1	2	3	4	$\geqslant 5$	
Rats	Observed	5	68	15	1	2	0	91
	Expected	26·8	33·1	20·7	7·8	2·1	0·6	91·1
Mice	Observed	3	42	10	0	1	0	56
	Expected	15·3	22·4	12·8	4·4	1·0	0·2	56·1

So far, the assumption has been made that eggs do not differ in their penetrability. It is reasonable to suppose that this is not so, as a few eggs, for example, were found with very large numbers of sperms. However, the effect of allowing for differences in penetrability will be simply to increase both ends of the theoretical distribution, i.e. it would increase the numbers of eggs expected to have either no sperms or more than four sperms. This further enlarges the discrepancy between observed and expected values. The effect as regards the zero class ($i=0$) is discussed in some detail by Stevens (1937).

From similar considerations as those just described for the rats, it can be shown that in mice also the observed frequency distribution of eggs according to the number of sperms they contain differs significantly from the expected distribution. Twenty mated female mice yielded 178 eggs, of which 94·9 per cent. were penetrated, 82·6 per cent. by only one sperm (Table 1). Two of the eggs (1·2 per cent.) were polyspermic. The observed and expected distributions of mouse eggs are set forth in Table 2. The results are interpreted to mean that the zona pellucida of the mouse egg, like that of the rat egg, becomes less penetrable shortly after the entry of the first sperm.

(*b*) *Disposition of Points of Entry of Sperms that have Passed through the Zona Pellucida in the Rat*

Confirmatory evidence for the occurrence of a zona reaction was sought by noting the disposition of the small slits or potential holes made by sperms passing through the zona. The appearance of these slits has been described by Austin (1951), who found that they persisted at least until the cleavage of the egg. Slits were very often difficult to discern, but could usually be clearly shown by rolling the egg about so that some of the vitelline material exuded through them. Observations were confined to eggs that contained two sperms and subjective assessments by two independent observers were made of the angle subtended at the centre of the egg by the two slits. Sometimes one or both slits could not be demonstrated, and these eggs were discarded. Eggs in which only one slit could be found were not recorded as representing zero angle, for although two sperms may conceivably enter by one slit the slit itself cannot be regarded as a potentially reactive zone. Assessments of angles to the nearest 10° were made on a total of 29 eggs and the results were: 20-30°, two eggs; 40-50°, two eggs; 80-90°, four eggs; 100-110°, 10 eggs; 120-130°, seven eggs; 140-150°, three eggs; 160-170°, one egg.

On the assumption that the zona has a spherical surface, that both sperms have a chance of entering by any given area on the surface, and that this chance is proportional only to the area, the probability of both sperms penetrating the same hemisphere is 1/2. This probability will clearly be reduced if the passage of the first sperm induces a reduction in the penetrability of the zona that spreads from the point of entry. Out of 29 eggs, only eight were found with both sperms in the same hemisphere. This is significantly different from equipartition ($\chi^2 = 5{\cdot}83$, $P < 0{\cdot}05$). However, in the fallopian tube the eggs are generally clumped together and sperms would tend first to penetrate the hemisphere of the egg that faces outwards. Therefore the probability of both sperms entering the same hemisphere would, in fact, be greater than 1/2, and the significance of finding only eight eggs penetrated in the same hemisphere would be higher than that just obtained. Thus it can be concluded that the change resulting from sperm penetration is propagated through the zona.

(c) *Assessment of the Time Taken for Completion of the Change in the Zona Pellucida of the Rat Egg Consequent upon Sperm Penetration*

To obtain a measure of the rate at which the zona pellucida undergoes change after the entry of the first sperm, mated rats were killed during the period when sperm penetration was taking place and also 24 hr later, when all penetration would have ceased. In each fallopian tube the number of sperms that were present in eggs in excess of one per egg, and the proportion of penetrated eggs, were determined. The zona reaction time was assessed from the mean time between the entry of the first and subsequent sperms into an egg.

Results were obtained from three groups of rats. In the first group, oestrous rats were mated after the time of ovulation; they were placed with males for 30 min between 8.30 a.m. and 9.30 a.m., and those that had copulation plugs were killed 2½, 3, 3½, or 4 hr later. Of the 128 rats killed, 72 yielded penetrated eggs. The data are recorded for the fallopian tubes according to the number of eggs and the number of penetrating sperms in excess of one per egg (Table 3). The mean number of extra penetrating sperms was 0·04 per egg in tubes in which up to 40 per cent. of the eggs were penetrated.

TABLE 3

NUMBER OF EGGS CONTAINING EXTRA SPERMS, AND TOTAL NUMBER OF EXTRA SPERMS IN THE EGGS, FROM FALLOPIAN TUBES OF RATS KILLED WHILE PENETRATION WAS IN PROGRESS, OR 24 HR LATER

Type of Mating	Time of Killing	Percentage Penetration per Tube	Number of Tubes	Number of Eggs		Total Number of Extra Sperms	Ratio of Extra Sperms to Penetrated Eggs
				Penetrated	With Extra Sperms		
Delayed	While penetration proceeding	0+-40 40+-100	41 74	53 313	2 113	2 167	0·04 0·53
	24 Hr after mating	93·1	124	511	159	236	0·46
Normal	While penetration proceeding	0+-40 40+-100	24 29	33 108	2 19	2 25	0·06 0·23
	24 Hr after mating	98·1	174	808	180	264	0·33

A second group of rats was mated under the same conditions as the first. There were 62 that had copulation plugs and these were killed 24 hr after mating when all sperm penetration would have ceased. The mean number of extra sperms per egg was 0·46 (Table 3).

The rats that made up the third group had been kept under conditions of controlled illumination (Austin and Braden 1954*b*) which was arranged so

that ovulation occurred shortly after midday. They were caged with males and mating took place at a normal time in relation to ovulation. Fifty animals were found with copulation plugs and they were killed at 2, 3, 4, or 5 p.m. In the fallopian tubes in which up to 40 per cent. of the eggs were penetrated, the mean number of extra sperms per egg was 0·06 (Table 3). An estimate of the mean number of extra penetrating sperms per egg (0·33) in normally mated rats killed 24 hr after mating is also included in Table 3. This figure is derived from the rats mentioned in Section (*a*) and Table 1.

Upper and lower limits for the rate of change in the zona pellucida were calculated in the following way:

(i) *Lower Limit of Mean Zona Reaction Time.*—A lower limit of T—the time required for the completion of the zona reaction—was derived from the consideration that in fallopian tubes with a low percentage of penetrated eggs at the time of killing (0+-40 per cent.), a smaller proportion of eggs with extra sperms would be expected than in tubes in which penetration was more advanced, for in the latter the eggs had, in general, been penetrated longer. It was assumed that this difference was due only to the stage at which the animals were killed so that the proportion p' of penetrated eggs that would have had extra sperms could be estimated from data on the rats killed after all penetration had ceased.

In any egg, owing to the change in the zona caused by the first penetration, the probability $p(t)\delta t$ of an extra sperm entering during the time t to $t+\delta t$ from penetration will tend to decrease, reaching virtually zero after time T. Clearly, for a given value of p', a lower limit of T is obtained by taking $p(t)$ constant ($=p$) for time T and zero thereafter. Then the probability $G(t)$ for an egg penetrated by a single sperm to acquire extra sperms within time t can be shown to be given by

$$G(t) = 1-e^{-pt}, \qquad (0\leqslant t\leqslant T)$$
$$=1-e^{-pT} \qquad (t>T)$$

so that p can be estimated from the relation

$$p' = 1-e^{-pT}.*$$

Next, an expression was found for the corresponding probability *at the time of killing*. In any one tube there would generally be one, sometimes two, penetrated eggs, when 0+-40 per cent. of the eggs are penetrated. These would have been liable to penetration by extra sperms for different unknown lengths of time, t. The distribution $f(t)$ of this 'waiting time' may be taken as approximately exponential, viz.

$$f(t) = ae^{-at} \qquad (0\leqslant t\leqslant\infty),$$

where $1/a$ is the mean waiting time. The value of $1/a$ can be estimated in the following way. It has been shown that in rats mated after ovulation the mean interval between the penetration of the first and last egg in any one fallo-

* This expression for $G(t)$ depends on the assumption, made throughout this section, that sperms penetrate the egg independently.

pian tube is about 1 hr (Austin and Braden 1954*b*). Since the mean number of eggs per tube was found to be five, the average interval between the penetration of any two eggs would have been about 15 min. A conservative estimate, therefore, of the mean waiting time, $1/a$, would be 5 min. Then the required probability P is

$$\begin{aligned} P &= \int_0^\infty G(t)f(t)\mathrm{d}t \\ &= \int_0^T (1-\mathrm{e}^{-pt})f(t)\mathrm{d}t+(1-\mathrm{e}^{-pT})\int_T^\infty f(t)\mathrm{d}t \\ &= \frac{p}{p+a}(1-\mathrm{e}^{-(p+a)T}). \end{aligned}$$

From the data on the rats mated after ovulation (Table 3) it is seen that in the 0+-40 per cent. class two eggs out of 53 have extra sperms. With $p' = 159/511$ and $1/a = 5$ min, it is easy to calculate for specified values of T the probability P' for the occurrence of extra sperms in two or fewer eggs out of 53. P' is found to be less than 0·05 for $T = 10$ min, which may thus be taken as a lower limit of the mean zona reaction time. Similar calculations for the rats that were kept under conditions of controlled illumination and mated before ovulation show that for $T = 10$ min, P' is less than 0·15. In these rats the mean interval between the penetration of the first and last egg in any one fallopian tube was taken to be 2 hr (Austin and Braden 1954*b*).

(ii) *Upper Limit of Mean Zona Reaction Time.*—A somewhat different approach was used to obtain an upper limit of T. Let the rate of penetration of extra sperms be $r(t)$ per minute per egg at time t from the first penetration. Suppose that $r(t)$ is given by the exponential decay curve

$$r(t) = r_0\mathrm{e}^{-ct} \qquad (0 \leqslant t \leqslant \infty).$$

This allows penetration over an infinite period. T is defined such that all but 5 per cent. of extra sperms will have entered the eggs within time T, so that

$$\int_T^\infty r(t)\mathrm{d}t = 0{\cdot}05\int_0^\infty r(t)\mathrm{d}t,$$

that is

$$\mathrm{e}^{-cT} = 0{\cdot}05,$$

or

$$cT = 3{\cdot}00.$$

c is given by the relation

$$\int_0^\infty r(t)\mathrm{d}t = f,$$

where f is the ratio of extra sperms to penetrated eggs for killings after the cessation of all penetration, an estimate of which is available from Table 3.

Thus

$$r_0/c = f,$$

so that

$$T = 3{\cdot}00\, f/r_0.$$

Now it seems reasonable to suppose that the actual decay curve does not decrease more rapidly than the above exponential. An upper limit of T may thus be found from a lower limit of r_0.

As already noted, the mean interval between the penetration of the first and last egg in any one fallopian tube, in rats mated after ovulation, is about 1 hr. During this time the average penetration rate may therefore be taken as at least one sperm per hour per egg. Assuming no sudden change in the zona of an egg at the moment of the penetration of the first sperm, this will give also a lower limit of the initial rate r_0, viz. $r_0 = 1/60$. With $f = 0{\cdot}46$ (Table 3) the upper limit of T is found to be about 1½ hr. The result obtained from the rats kept under controlled illumination was similar, namely about 2 hr.

Finally, it may be pointed out that in both groups of rats a lower limit for T of about 10 min is obtained by the present method if $r(t)$ is defined by

$$\begin{aligned} r(t) &= r_0 \qquad (0 \leqslant t \leqslant T) \\ &= 0 \qquad (t > T), \end{aligned}$$

with $r_0 = 1/20$, $1/40$ respectively. As these values correspond to reasonable upper limits of r_0, this result provides a confirmation of the estimate already given.

TABLE 4

NUMBERS OF SPERMS IN THE EGGS OF RABBITS KILLED 10-20 HR AFTER COITUS

Interval Between Mating and Killing (hr)	Number of Animals	Number of Eggs			Number of Sperms in Perivitelline Space		Number of Sperms in Zona	
		Recovered	Examined	Penetrated	Mean	Range	Mean	Range
10	4	19	9	5	0·2	0-2	0·6	0-2
13	4	30	19	19	12·6	0-29	4·3	0-9
16	6	48	28	28	61·8	10-169	9·3	2-29
20	5	29	18	18	82·5	18-240	9·7	1-29

(d) Number of Penetrating Sperms in the Eggs of the Rabbit, Sheep, and Dog

Nine eggs were examined from rabbits killed 10 hr after coitus, five were penetrated. The mean number of sperms in the perivitelline space was 0·2 and, in the thickness of the zona, it was 0·6 (Table 4). The mean number of sperms at both sites was much higher in eggs from rabbits killed at 13 hr: 12·6 and 4·3 sperms respectively. The values increased still further in the next 3

hr: 28 eggs examined 16 hr after mating had a mean number of 61·8 sperms in the perivitelline space and 9·3 sperms in the thickness of the zona. The differences between the mean numbers in the perivitelline space at 10 and 13 hr, and also at 13 and 16 hr were significant. There was, however, no significant increase in the mean number in the perivitelline space between 16 and 20 hr.

Twelve eggs were recovered from 16 ewes killed 24-48 hr after service. Of these, six eggs were in various stages of fertilization, three eggs were in the 2-cell stage, and three were in the 4-cell stage. They were observed in the fresh state by microscopy after slight compression under the coverslip. Subsequently they were fixed and stained while still under the coverslip. Several hundred sperms were regularly observed attached to and, in some instances, partially embedded in the zona pellucida, but none was seen in the perivitelline space (Plate 1, Fig. 5).

Twelve eggs in the 6- to 8-cell stage were recovered from the tubes of two bitches 3-4 days after coitus. Eight eggs were examined in the fresh state and four after fixing, sectioning, and staining. As was noted with the sheep eggs, numerous sperms were seen adhering to the external surface of the zona pellucida but none could be detected in the perivitelline space (Plate 1, Figs. 3 and 4).

IV. Discussion

It is rare for more than one sperm to enter the vitellus in the eggs of the marsupial and placental mammals, though under certain experimental conditions the incidence of this phenomenon, polyspermy, may be greatly increased (Austin and Braden 1953*a*, 1953*b*, 1954*a*). The low incidence of polyspermy in normal circumstances has generally been attributed to a propagated change, 'the block to polyspermy,' in the cortex of the egg, which is initiated by the entry of the first sperm and which precludes the entry of subsequent sperms. A similar reaction to sperm penetration is well known in the eggs of invertebrates, where it has been studied in some detail (Rothschild and Swan 1949, 1951, 1952; Runnström and Kriszat 1952). By contrast, the occurrence of a change in the zona pellucida consequent upon sperm penetration does not seem to have been suspected.

In rats and mice, penetration of the zona pellucida by a second sperm occurs much more frequently than penetration of the vitellus by two sperms; in normally mated animals in the present experiments the incidence of polyspermy for both species was 1·2 per cent., whereas in 22·0 per cent. of rat eggs and in 17·4 per cent. of mouse eggs two or more sperms had passed through the zona pellucida. Examination of the distribution of the extra sperms that penetrated the zona showed a departure from what would be expected if chance alone operated; considering the low proportion of eggs with no sperms, the number that had more than one sperm was much smaller than expected. This departure from the theoretical distribution is highly significant and leads to the conclusion that, in rats and mice, the passage of the first sperm through the zona pellucida evokes a reaction which greatly reduces the chances of a sub-

sequent penetration. The reaction could either be a change in the nature of the zona that renders it less penetrable or it could involve a loss of attractiveness of the egg for the sperm. The first alternative seems the more likely because the second implies the existence of chemotaxis between egg and sperm, and there is apparently no sound evidence for this phenomenon in any species of animal (Buller 1903; Tyler 1948; Rothschild 1951). Moreover it is difficult to conceive how a loss of attractiveness could spread in a relatively short time through the large mass of hyaluronic acid in which the eggs are embedded. It is, therefore, highly probable that the zona pellucida of rat and mouse eggs reacts to sperm penetration by becoming less penetrable to further sperms. The evidence obtained also suggests that the reaction is propagated through the zona from the point of entry of the sperm.

Data secured from rats after both normal mating and delayed mating have been used to estimate the time required by the zona reaction to reach completion. From both sets of results the conclusion drawn is that the period is between 10 min and 1½-2 hr. It is considered that the assumptions made in arriving at these figures were particularly conservative and that the mean zona reaction time is very unlikely to lie outside the stipulated limits. It would be of interest to have some idea of the relation of this period to the time required for the change in the vitellus known as 'the block to polyspermy.' Direct evidence is virtually impossible to obtain at present, but indirect evidence suggests that, under normal conditions, the reaction time of the vitellus is shorter than that of the zona. The fact that the incidence of polyspermy in rats and mice is much lower than the incidence of eggs containing more than one sperm is consistent with this suggestion. In the sea-urchin the block to polyspermy is completed in about 1 min (Rothschild and Swan 1952) and it is reasonable to suppose that a similar time may pertain in mammalian eggs. In the rabbit the zona apparently does not react to sperm penetration, yet the incidence of polyspermy after normal mating is no higher than in rats (Austin and Braden 1953*a*, 1953*b*).

As just noted, the zona pellucida of the rabbit egg, in contrast to that of rat and mouse eggs, showed no evidence of a decrease in penetrability for the first 6 hr after ovulation. Indeed the rate of sperm penetration of the rabbit egg increased from six sperms per egg per hour over the first 3 hr to 18 per egg per hour over the second 3 hr. By 6 hr after ovulation a thin layer of mucoprotein ("albumen") begins to accumulate on the eggs (Braden 1952, 1953) (see Plate 1, Fig. 1). This layer presumably prevents the entry of further sperms, as postulated by Hammond (1934), for the mean number of sperms in the eggs at 20 hr was not significantly higher than at 16 hr.

The rabbit appears to be practically unique among mammals in that its eggs commonly have more than 50 sperms in the perivitelline space. The appearance of the rabbit egg is shown in Plate 1, Figures 1 and 2. Information is available for the eggs of 32 species of mammal from nine different orders (Appendix I), and it appears that the eggs of mammals generally display few or no sperms in the perivitelline space, although about 20 sperms have been

reported in the mole (Heape 1886) and "several" to "numerous" sperms were noted in eggs of the pocket gopher (Mossman and Hisaw 1940). As was noted in the present investigation, a small proportion of rat and mouse eggs contained up to 10 sperms, and similar observations have been made for the guinea pig (Hensen 1876; Lams 1913), the cat (Hill and Tribe 1924), the ferret (Hamilton 1934), and several species of bats (Van Benedin 1867, 1911; Van Benedin and Julin 1880; Van der Stricht 1910). In all the remaining species, although they were commonly seen in large numbers on the surface of the zona or within its substance, no sperms were found in the perivitelline space. The 12 sheep eggs and the 12 dog eggs examined by the present authors were like this. It appears highly probable therefore that, in most species of mammal, the zona pellucida changes after the entry of the first sperm in such a way as to reduce greatly the chances of penetration by further sperms. However, the rate at which this change occurs in different species evidently varies. Rat eggs, for instance, commonly had sperms in the perivitelline space, whereas sheep eggs had none, and this was so in spite of the fact that the frequency of sperm-egg collisions is of approximately the same order in both the species (Braden and Austin 1954).

As the reaction of the zona to sperm penetration is so widespread among mammals, it is presumably of some significance for the normal development of the fertilized egg. Just as the reaction of the vitelline surface to sperm penetration is important in protecting the egg against polyspermy, so the existence of a similar reaction in the zona pellucida would protect the egg from the entry of excessive numbers of sperms into the perivitelline space. The zona reaction may possibly also provide an additional bar to polyspermy, but only in species in which the reaction occurs most rapidly, for example the dog and the sheep. The effect that large numbers of sperms in the perivitelline space would have upon the egg is not clear, however. Rat eggs with more than 15 sperms were all degenerate in appearance, and Mossman and Hisaw (1940) reported in the pocket gopher an egg containing about 65 sperms that was plainly degenerating. On the other hand, the rabbit egg, and to a less extent, the mole egg, can evidently tolerate the presence of numerous sperms.

In conclusion, it may be pointed out that three mechanisms have now been demonstrated which together ensure that, in mammals, with the exception of the monotremes, the eggs will be fertilized without there being too great a danger of penetration by an excessive number of sperms. Firstly, the chances of meeting of egg and sperm are controlled by the restrictive action of the female tract on sperm passage, so that relatively few sperms are present at the site of fertilization (Austin and Braden 1952; Braden 1953; Braden and Austin 1954). Secondly, the zona pellucida has now been shown to react to the entry of the first sperm so as greatly to reduce the chances of a subsequent penetration. Finally, the entry of the fertilizing sperm into the vitellus evokes a reaction in the cortex that tends to exclude further sperms; evidence for the operation of this 'block to polyspermy' in mammalian eggs has been discussed by Austin and Braden (1953*b*).

V. References

Amoroso, E. C., Griffiths, W. F. B., and Hamilton, W. J. (1939).—*Vet. Rec.* **51**: 1009.

Amoroso, E. C., Griffiths, W. F. B., and Hamilton, W. J. (1942).—*J. Anat.* **76**: 377.

Assheton, R. (1899).—*Quart. J. Micr. Sci.* **41**: 329.

Austin, C. R. (1951).—*Aust. J. Sci. Res.* B **4**: 581.

Austin, C. R., and Braden, A. W. H. (1953*a*).—*Nature* **172**: 82.

Austin, C. R., and Braden, A. W. H. (1953*b*).—*Aust. J. Biol. Sci.* **6**: 674.

Austin, C. R., and Braden, A. W. H. (1954*a*).—*Aust. J. Biol. Sci.* **7**: 195.

Austin, C. R., and Braden, A. W. H. (1954*b*).—*Aust. J. Biol. Sci.* **7**: 179.

Barry, M. (1843).—*Phil. Trans.* **133**: 33.

Bischoff, T. L. W. (1845).—"Entwicklungsgeschichte des Hundeeies." (F. Vieweg u. Sohn: Braunschweig.)

Black, W. G., Ulberg, L. C., Christian, R. E., and Casida, L. E. (1953).—*J. Dairy Sci.* **36**: 274.

Braden, A. W. H. (1952).—*Aust. J. Sci. Res.* B **5**: 460.

Braden, A. W. H. (1953).—*Aust. J. Biol. Sci.* **6**: 693.

Braden, A. W. H., and Austin, C. R. (1954).—*Aust. J. Biol. Sci.* **7**: (in press).

Buller, A. H. R. (1903).—*Quart. J. Micr. Sci.* **46**: 145.

Chang, M. C. (1951).—*Fert. Steril.* **2**: 203.

Clark, R. T. (1934).—*Anat. Rec.* **60**: 135.

Enders, R. K. (1952).—*Proc. Amer. Phil. Soc.* **96**: 691.

Gatenby, J. B., and Beams, H. W. (1950).—"The Microtomists Vade Mecum." (J. & A. Churchill: London.)

Gilchrist, F., and Pincus, G. (1932).—*Anat. Rec.* **54**: 275.

Graves, A. P. (1945).—*Amer. J. Anat.* **77**: 219.

Green, N. W., and Winters, L. M. (1946).—*J. Morph.* **78**: 305.

Hamilton, W. J. (1934).—*Trans. Roy. Soc. Edinb.* **58**: 251.

Hamilton, W. J. (1944).—*J. Anat., Lond.* **78**: 1.

Hamilton, W. J., Barnes, J., and Dodds, G. H. (1943).—*J. Obstet. Gynaec., Brit. Emp.* **50**: 241.

Hamilton, W. J., and Day, F. T. (1945).—*J. Anat., Lond.* **79**: 127.

Hamilton, W. J., and Laing, J. A. (1946).—*J. Anat., Lond.* **80**: 194.

Hammond, J. (1934).—*J. Exp. Biol.* **11**: 140.

Hartman, C. G. (1916).—*J. Morph.* **27**: 1.

Hartman, C. G. (1919).—*J. Morph.* **32**: 1.

Heape, W. (1886).—*Quart. J. Micr. Sci.* **26**: 157.

Hensen, V. (1876).—*Z. Anat. EntwGesch.* **1**: 213.

Heuser, C. H., and Streeter, G. L. (1929).—*Contr. Embryol. Carneg. Instn.* **20**: 1.

Hill, J. P. (1910).—*Quart. J. Micr. Sci.* **56**: 1.

Hill, J. P. (1918).—*Quart. J. Micr. Sci.* **63**: 91.

Hill, J. P., and Tribe, M. (1924).—*Quart. J. Micr. Sci.* **68**: 514.

Lams, H. (1913).—*Arch. Biol., Paris* **28**: 229.

Lams, H., and Doorme, J. (1908).—*Arch. Biol., Paris* **23**: 259.

Lewis, W. H., and Hartman, C. G. (1933).—*Contr. Embryol. Carneg. Instn.* **24**: 187.

Lewis, W. H., and Hartman, C. G. (1941).—*Contr. Embryol. Carneg. Instn.* **29**: 7.

Lewis, W. H., and Wright, E. S. (1935).—*Contr. Embryol. Carneg. Instn.* **25**: 113.

Longley, W. H. (1911).—*Amer. J. Anat.* **12**: 139.

Moricard, R., and Bossu, J. (1949).—*Bull. Soc. Gynéc. Paris* Seance **7** Feb. p. 30.

Mossman, H. W., and Hisaw, F. L. (1940).—*Amer. J. Anat.* **66**: 367.

Odor, D. L., and Blandau, R. J. (1949).—*Anat. Rec.* **104**: 1.

Pearson, O. P. (1944).—*Amer. J. Anat.* **75**: 39.

Rothschild, Lord (1951).—*Symp. Biochem. Soc.* No. 7: 40.

Rothschild, Lord, and Swan, M. M. (1949).—*J. Exp. Biol.* **26**: 164.

Rothschild, Lord, and Swan, M. M. (1951).—*J. Exp. Biol.* **27**: 400.

ROTHSCHILD, LORD, and SWAN, M. M. (1952).—*J. Exp. Biol.* **29**: 469.
RUNNSTRÖM, J., and KRISZAT, G. (1952).—*Exp. Cell Res.* **3**: 419.
SCHOOLEY, J. P. (1934).—*J. Morph.* **56**: 477.
SMITHBERG, M. (1953).—*Anat. Rec.* **117**: 554 (proc.).
SOBOTTA, J. (1895).—*Arch. mikr. Anat.* **45**: 15.
SOBOTTA, J., and BURCKHARD, G. (1910).—*Anat. Hefte* **42**: 433.
STEVENS, W. L. (1937).—*Ann. Eugen., Lond.* **8**: **57**.
TYLER, A. (1948).—*Physiol. Rev.* **28**: 180.
VAN BENEDIN, E. (1867).—*Mém. Acad. R. Belg.* **34**: 1.
VAN BENEDIN, E. (1875).—*Bull. Acad. Belg.* **40**: 686.
VAN BENEDIN, E. (1911).—*Arch. Biol.* **26**: 1.
VAN BENEDIN, E., and JULIN, C. (1880).—*Arch. Biol.* **1**: 551.
VAN DER STRICHT, O. (1910).—*Mém. Acad. R. Belg. Cl. Sci.* (2) **2** (3).
VAN DER STRICHT, O. (1923).—*Arch. Biol.* **33**: 229.
VAN DER STRICHT, R. (1911).—*Arch. Biol.* **26**: 365.
WRIGHT, P. L. (1948).—*Anat. Rec.* **100**: 593.

ADDENDUM

After this paper had been submitted for publication, confirmatory evidence of a change in the zona pellucida after sperm penetration was found in a note by Smithberg (1953). In his studies on the effects of proteolytic enzymes on the zona of mouse eggs, Smithberg noted that the zona was always removed more rapidly from unfertilized than from fertilized eggs, and concluded that the nature of the zona pellucida changes after fertilization.

EXPLANATION OF PLATE 1

Photographs of living eggs, except for that in Figure 4.

Fig. 1.—Rabbit egg examined without compression, showing numerous sperms in the perivitelline space, but none on the surface of the zona pellucida. This egg was recovered about 7 hr after ovulation and already has an appreciable layer of mucoprotein ("albumen") on the surface of the zona. × 400.

Fig. 2.—Rabbit egg compressed beneath coverslip showing four sperms between the vitellus and the zona, and one in the thickness of the zona. × 1000.

Fig. 3.—Dog egg in the 6-cell stage compressed beneath coverslip. Numerous sperms visible on the surface of the zona, but none seen between the vitellus and the zona. × 1000.

Fig. 4.—Section of 8-cell dog egg stained to show sperm heads; these are evident in large numbers on the surface of the zona, but are lacking between the vitellus and the zona. × 400.

Fig. 5.—Sheep egg in the 2-cell stage, slightly compressed. No sperms visible in the perivitelline space, but many are present on the zona. × 1000.

Fig. 6.—Degenerate rat egg containing 50-60 sperms within the bounds of the zona pellucida. Phase-contrast microscopy. × 400.

APPENDIX I

The following is a summary of information available on the occurrence of extra penetrating sperms in the eggs of mammals. Only reports on eggs that were uncleaved or were in the 2- to 16-cell stages are included.

MARSUPIALIA

Didelphis virginiana.—About 100 eggs examined. Sperms not reported in the perivitelline space (PVS) or the zona, although many were seen in the "albumen" layer (Hartman 1916, 1919). One egg "polyspermic" (Hill 1918).

Setonix brachyurus.—One egg, with no sperms in the PVS but several embedded in the zona; there were many sperms in the "albumen" layer (Sharman, personal communication 1953).

Dasyurus viverrinus.—About 20 eggs examined. No sperms reported in the PVS or the zona, although there were many in the "albumen" layer. One egg was "polyspermic" (Hill 1910).

INSECTIVORA

Talpa europea.—Three eggs illustrated, all shown with about 20 sperms in PVS (Heape 1886).

Blarina brevicauda.—About 40 eggs seen. Sperms often found in the corona but no mention of any in the PVS or zona (Pearson 1944).

CHIROPTERA

Vesperugo noctula.—About 100 eggs. One egg had one sperm in the PVS, the remainder had none (Van der Stricht 1910).

Vespertilio mystacinus.—Three eggs, one of which had 16 sperms in the PVS, the remainder had none (Van Benedin and Julin 1880).

Vespertilio murinus.—Twelve eggs examined. One egg had one sperm in PVS but none embedded in the zona, three eggs had "several" sperms in the PVS, two eggs had a sperm embedded in the zona, but none in the PVS, and the remaining six eggs had no sperms in the PVS or the zona (Van Benedin 1867, 1911; Van Benedin and Julin 1880).

Vespertilio dasycnemus.—Three eggs seen, one had several sperms embedded in the zona, but none in the PVS. The remaining two eggs contained no extra sperms (Van Benedin 1911; Van Benedin and Julin 1880).

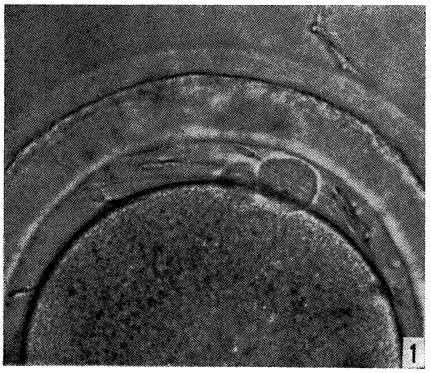
Rhinolophus ferrum-equinum.—Only one egg seen. It had several sperms embedded in the zona, but none in the PVS (Van Benedin and Julin 1880).

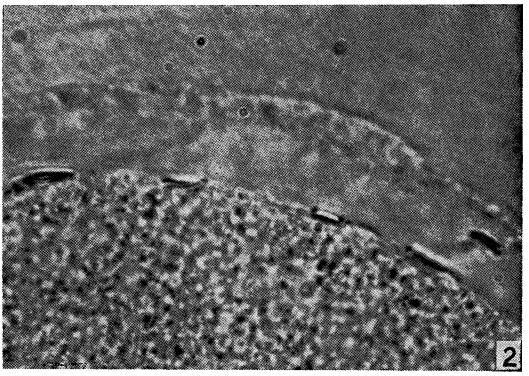
PRIMATES

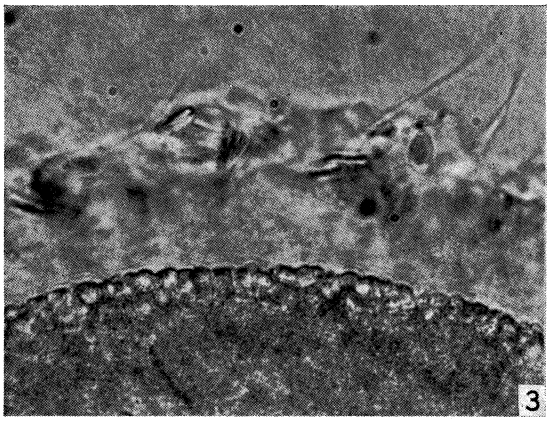
Macaca rhesus.—Four eggs seen. There were apparently no sperms in the PVS, but embedded in the zona were seven, five, and, in two eggs, "several" sperms (Lewis and Hartman 1933, 1941).

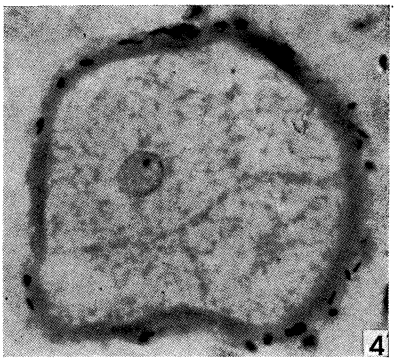
Homo sapiens.—Two eggs examined. No sperms reported in the PVS though there were many in and on the zona (Hamilton, Barnes, and Dodds, 1943; Hamilton 1944).

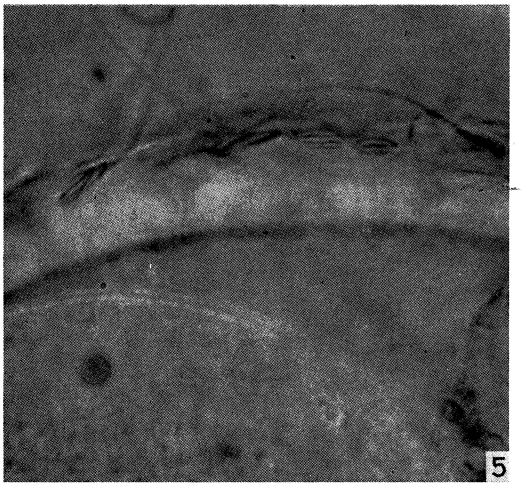
ZONA PELLUCIDA AND SPERM PENETRATION

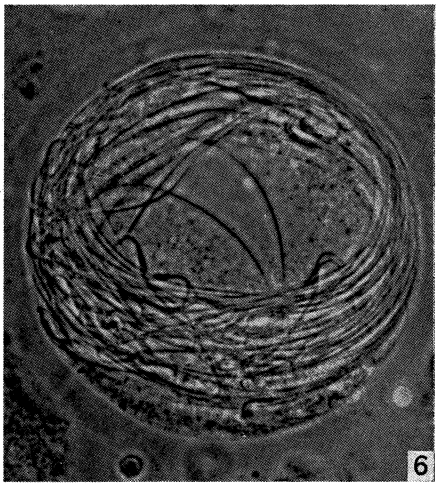












LAGOMORPHA

Oryctolagus cuniculus.—Eggs from two rabbits examined. In each egg "a number of sperms" were seen in the PVS (Barry 1843).

One egg. In the PVS, 20 sperms were counted in an optical section (Van Benedin 1875).

About 30 eggs seen, and up to 50 sperms were counted in the PVS, and in addition many sperms were seen embedded in the zona (Hensen 1876).

Eight eggs examined. The mean numbers of sperms in the PVS and in the zona 4½-6 hr after ovulation were 37 and 34 respectively (Moricard and Bossu 1949).

About 20 eggs seen. The mean numbers of sperms in the PVS and in the zona 6-14 hr after ovulation were 17 and 10 respectively (Chang 1951).

Forty-six eggs examined. The mean numbers of sperms in the PVS and in the zona 6-10 hr after ovulation were 72 and 9 respectively (Present authors, Table 4).

RODENTIA

Cricetus auratus.—About 40 eggs were examined, but no extra penetrating sperms were seen (Graves 1945).

Geomys bursarius.—Eight eggs examined. There were "several" to "numerous" sperms in the zona and the PVS. One degenerated egg had more than 65 sperms in the PVS (Mossman and Hisaw 1940).

Citellus elegans.—Fourteen eggs seen. There were many sperms embedded in the zona, but none in the PVS (Schooley 1934).

Tamias striatus.—Six eggs seen. Many sperms in the zona, but none in the PVS (Schooley 1934).

Eutamias operarius.—Eight eggs seen. Many sperms in the zona, but none in the PVS (Schooley 1934).

Callospermophilus lateralis.—Eight eggs examined. Many sperms in the zona, but none in the PVS (Schooley 1934).

Mus musculus.—1350 eggs examined. Sperms were rarely seen in the PVS (Sobotta 1895).

Thirty-six eggs examined. Occasional eggs had one or two sperms in the PVS (Lams and Doorme 1908).

Fifteen eggs illustrated. Two eggs can be seen to have two sperms in the PVS, and another two eggs each have one sperm in the PVS (Lewis and Wright 1935).

Eggs examined, 169. Twenty-seven eggs contained one sperm in PVS, one egg had two sperms, one egg had three sperms, and another egg had five sperms in the PVS. Two eggs were dispermic (Present authors, Table 1).

Rattus rattus.—About 150 eggs examined. Sperms were "quite commonly" seen in the PVS (Sobotta and Burckhard 1910).

About 120 eggs seen. "More than one sperm may pass through the zona"; the extra sperms remained in the PVS (Gilchrist and Pincus 1932).

Eggs examined, 366. Fifty-seven eggs contained one sperm in the PVS, 20 eggs had two sperms, five eggs had three sperms, two eggs had four sperms, and one egg had five sperms (Odor and Blandau 1949).

Eggs examined, 810, of which 132 eggs had one sperm in the PVS, 24 eggs had two sperms, 11 eggs had three sperms, three eggs had four sperms, two eggs had five sperms, two eggs had six sperms, and one egg had seven sperms. Two abnormal eggs had 24 and 50-60 sperms respectively in the PVS. Ten eggs were dispermic (Present authors, Table 1).

Cavia porcellus.—About 20 eggs examined. A number of eggs had 1-10 sperms in the zona, but only four eggs had one sperm in the PVS (Hensen 1876).

Fifteen eggs seen. There were no sperms in the PVS (Lams and Doorme 1908).

Eggs seen, 108. Sperms occasionally found embedded in zona and, more rarely, in the PVS. One "polyspermic" egg (Lams 1913).

CARNIVORA

Mustela frenata.—Twenty-five eggs examined. No sperms were reported in the PVS or in the zona (Wright 1948).

Mustela furo.—Five eggs seen. One egg had one sperm in the PVS, another had five sperms, and the rest had none. Sperms were frequently seen in the zona (Hamilton 1934).

Mustela vison.—Four eggs examined. Many sperms were seen on and around the zona, but none reported in the PVS or the zona (Enders 1952).

Felis catus.—Twelve eggs illustrated. None of the eggs shown to have sperms in the PVS, but two had a number of sperms embedded in the zona. In addition, one dispermic egg was described (Van der Stricht 1911).

A small number of eggs was examined. Up to 400 sperms were seen on and around the zona but apparently no sperms were present in the PVS or zona (Longley 1911).

Eleven eggs described. One egg had "several" sperms in the PVS and six eggs had "numerous" sperms in the zona. Two eggs were dispermic (Hill and Tribe 1924).

Canis familiaris.—Seven eggs illustrated and described. Each had numerous sperms on the surface of the zona, but apparently none was seen in the PVS (Bischoff 1845).

Nineteen eggs illustrated. Sperms are shown on the surface of the zona, but not in the PVS (Van der Stricht 1923).

Twelve eggs examined. No sperms were seen in the PVS, although there were many on the surface of the zona (Present authors).

PERISSODACTYLA

Equus caballus.—Five eggs examined. There were a few sperms on the surface of the zona, but apparently none in the PVS (Hamilton and Day 1945).

ARTIODACTYLA

Sus scrofa.—Five eggs seen. Numerous sperms were found adhering to the zona, but none reported in the PVS (Assheton 1899).

Three eggs examined. "Numerous sperm heads in zona." Apparently no sperms in the PVS (Heuser and Streeter 1929).

Twenty-nine eggs examined. Sperms "associated with zona," but apparently no sperms seen in the PVS (Green and Winters 1946).

Bos taurus.—About 30 eggs examined. Many sperms were seen in the zona, but none reported in the PVS (Hamilton and Laing 1946).

Six eggs seen. A "large number" of sperms was reported in and on the zona but none mentioned as being in the PVS (Black *et al.* 1953).

Ovis aries.—Six eggs depicted. Numerous sperms shown on the zona, but none visible in the PVS (Clark 1934).

Twelve eggs examined. There were numerous sperms on the surface of the zona and a few embedded in the zona, but none in the PVS (Present authors).

Capra hircus.—Fourteen eggs described. Numerous sperms were seen on the surface of the zona, but apparently none in the PVS (Amoroso, Griffiths, and Hamilton 1939, 1942).