OBSERVATIONS ON THE PENETRATION OF THE SPERM INTO THE MAMMALIAN EGG

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Summary

A brief review is given of the literature, particularly that relating to the attempts made to effect the fertilization of the mammalian egg *in vitro*. It is considered that the evidence so far put forward for the fertilization *in vitro* of mammalian eggs is inconclusive.

Observations on eggs recovered at intervals after induced ovulation in mated rats indicate that sperm penetration of the zona pellucida occurs very rapidly and, generally, very soon after ovulation. As a rule, the sperm enters the vitellus immediately after passing through the zona, but quite often it remains for a period in the perivitelline space before entering the vitellus. The slit or potential hole the sperm makes in penetrating the zona persists and may be demonstrated at later stages.

Sperm entry into the vitellus has been observed *in vitro*; the process appears to be largely a function of the vitellus as the sperm is often motionless at the time.

When sperms were introduced into the fallopian tube of the rabbit *before* ovulation, most of the eggs subsequently recovered were fertilized. However, if the sperms were introduced shortly *after* ovulation the eggs rarely showed signs of penetration.

When sperms were introduced into the peri-ovarian sac of the rat shortly after ovulation, sperm penetration did not occur until four or more hours later, although sperms were regularly found about the eggs at two hours and later.

It appears therefore that the sperm must spend some time in the female tract before it is capable of penetrating the zona. These results and observations are discussed with the object of deriving a working hypothesis on the mechanism of sperm penetration through the zona pellucida.

I. Introduction

When it has reached the site of fertilization in the fallopian tube, the sperm of most species of mammals must yet pass three distinct barriers before it enters the egg to play its part in fertilization.

Much attention has been given to the nature of the first barrier, the cumulus oophorus, and the means whereby the sperm traverses it. Schenk (1878) noticed that the rabbit cumulus and that of the guinea pig were broken up when a suspension of sperms was added to it *in vitro*. It was not, however, until much later that Yamane (1930, 1935), Pincus (1930), and Pincus and Enzmann (1932, 1935) studied the reaction more closely and concluded that an agent resembling a proteolytic enzyme was involved. McClean and Rowlands (1942), Fekete

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and Duran-Reynals (1943), and Leonard and Kurzrok (1945) identified the enzyme as hyaluronidase, which has the effect of depolymerizing hyaluronic acid, the cement substance of the cumulus. The enzyme was shown to be carried by the sperm (Swyer 1946, 1947) and it was thought to function by denuding the egg as a preliminary to sperm entry. Leonard, Perlman, and Kurzrok (1947) and Austin (1948*a*, 1948*b*) pointed out that the action of the enzyme was probably limited to enabling the individual sperm to penetrate the intact cumulus.

There is little useful information available on the nature of the next two barriers, the zona pellucida and the surface of the vitellus, or on the manner in which the sperm traverses them.

The composition of the zona has been examined by several authors with varying results. Wallraff and Beckert (1939) detected polysaccharide in it, by the Bauer reaction. Leach (1947) considered the zona to be a mucoprotein, while Wislocki, Bunting, and Dempsey (1947) and Leblond (1950) described it as a mucopolysaccharide. Harter (1948), on the other hand, stated that glycoprotein could be demonstrated in the zona.

Some of the physical properties of the zona have also been investigated. Mayer (1842) observed that the zona was dissolved in a solution of potassium hydroxide of unstated concentration, and concluded that it was a single solid membrane, and not two thin membranes separated by a layer of protein. The removal of the zona from rabbit eggs after treatment with osmic acid and Muller's fluid (which contains potassium dichromate) was noted by Van Beneden (1875). Lams (1913) remarked that the guinea pig zona was occasionally dissolved by Zenker's and Hermann's fluids, both of which contain acetic acid. Huber (1915) observed the absence of the zona in many of the rat eggs fixed in Carnoy's fluid, which also contains acetic acid. More recently, Hall (1935), following this lead, studied the effect of pH on the mouse zona and found that it was dissolved readily at pH 3.7, more slowly at less acid reactions up to pH 5.4. Harter (1948) suggested that the lowered pH produced by the metabolism of the sperm in the immediate vicinity of the head might assist penetration of the egg by its solvent action on the zona.

The claims of some early workers, notably Barry (1840), that the continuity of the zona was broken by an orifice or "cleft," by means of which it was supposed the sperm might enter, have not been supported. In the mature ovarian egg, the zona appears as a thick, refractile membrane and, in fixed material, fine radial canals containing processes from the follicle cells may be seen crossing it, as has been described by Heape (1886), Nagel (1888), Sobotta (1895), Fischer (1905), and many other workers subsequently. The processes from the follicle cells may also be seen in living ovarian eggs (Austin and Smiles 1948). The zona of the tubal egg, however, appears to be homogeneous (Lams 1913) or may show fine radial striations (Heape 1886) or a faint, concentric layering (Corner 1928). There is, therefore, no sound evidence of any form of selected path or easy way for the passage of the sperm through the zona.

Concerning the nature of the third barrier, the surface of the vitellus, there has also been some difference of opinion. The earlier workers, from Barry (1839) to Lams (1913), were almost unanimous in referring to a vitelline membrane as a structure which surrounded the vitellus in both fertilized and unfertilized mammalian eggs. There is, however, no good reason for supposing the existence of an actual membrane around the vitellus, analogous to that in a bird's egg (Corner 1928). Nevertheless, it has long been recognized that the vitelline surface may present a distinct obstacle to sperm penetration in that it becomes modified in some way after the penetration of the first sperm so as normally to exclude the entrance of later sperms. The nature of this change is still a matter for speculation. Pincus and Enzmann (1932) and Gilchrist and Pincus (1932) showed that the vitellus in the rabbit and rat eggs suffers a shrinkage after sperm penetration, but, although the appearance of this change may suggest it, there is no evidence for the formation of a fertilization membrane as described in certain invertebrate eggs.

Undoubtedly the study of the mechanisms involved in the penetration of the zona and vitelline surface would be greatly facilitated if the fertilization of mammalian eggs could be achieved *in vitro*. During the past century, several workers have claimed success in this procedure, but it is difficult to decide whether these claims were properly founded. The main difficulty lies in distinguishing between effects resulting from sperm penetration and those due to parthenogenetic activation. There is also a danger that sperms introduced accidentally during the sectioning of the eggs may be mistaken for sperms that have entered the eggs in the normal way.

Schenk (1878) treated the ovarian eggs of rabbits and guinea pigs with sperms *in vitro* and noted the formation of a polar body and, following culture, the division of the egg.

Onanoff (1893), in a posthumous communication in which only conclusions were published, made a remarkable series of claims. He stated that rabbit and guinea pig eggs, taken from the uterus, could be fertilized *in vitro*, and that their development would proceed to the 8-cell stage. Eggs fertilized *in vitro* and transferred to the peritoneal cavity of males or females of either species would develop into embryos of the primitive streak stage.

Long (1912) described the break-up of the cumulus and the formation of the second polar body in rat eggs treated with sperms *in vitro*.

Frommolt (1934) mentioned the shrinkage of the vitellus as the sole criterion of fertilization in the rabbit eggs to which he had added sperms *in vitro*.

Krasovskaja (1934, 1935*a*, 1935*b*) claimed the fertilization *in vitro* of rabbit eggs with the sperms not only of the rabbit, but also the rat. The evidence included the abstriction of the second polar body, the formation of pronuclei and the division of the egg when cultured. She did not apparently observe the presence of any sperms within the eggs. Pincus (1936) remarks that the nuclear configurations shown by Krasovskaja can occur when eggs are cultured *in vitro* without the addition of sperms.

Yamane (1935), who studied the dispersion of the follicle cell mass by a suspension of sperms *in vitro,* considered that several of the eggs so treated had sperms within the vitellus. Two of the eggs are illustrated in Yamane's paper and as judged from these the evidence is not convincing. There appear to be several sperm heads in, or partly in, the vitellus, and these show no change towards pronucleus formation. The whole appearance strongly suggests that the heads in reality overlie the section of the vitellus and that they were carried there in the cutting of the section.

The most extensive experiments on the problem of fertilization *in vitro* have been carried out by Pincus (1930, 1939) and Pincus and Enzmann (1934, 1935). These authors added ejaculated and epididymal sperms to rabbit eggs *in vitro.* Both ovarian and tubal eggs were used and the fertilization of many of these was claimed. The evidence submitted included the shrinkage of the vitellus, the extrusion of the second polar body, the presence of sperms in the perivitelline space and vitellus as seen in histological sections, the formation of two pronuclei, the segmentation of the eggs, and the birth of young. These authors also made the point that, if sperm penetration is to occur, there must be a relatively high concentration of sperms (25,000 per cu. mm. or more) about the eggs. At lower concentrations the follicle cell mass surrounding the eggs was not completely removed and fertilization did not take place.

In evaluating this evidence the following points should be noted. Gilchrist and Pincus (1932) showed that, in rat eggs, shrinkage of the vitellus could be induced by incubating the eggs with a suspension of dead sperms. Pincus and Enzmann(1936), Pincus (1939), and Pincus and Shapiro (1940) reported that, by subjecting rabbit eggs *in vitro* to supra- or subnormal temperatures, hyper- or hypotonic solutions, butyric acid solution, or culturing in a moist chamber, varying degrees of activation were induced. They have recorded that the extrusion of the second polar body, the formation of two pronuclei, the segmentation of the egg, and even the birth of young could all be induced in the absence of sperms. More recently Thibault (1947*a,* 1947*b,* 1948) has studied the artificial activation of the rabbit egg by cold and noted that the egg may subsequently show the formation of a single, diploid nucleus or two nuclei closely resembling normal pronuclei. He also stated that the first polar body, following cold treatment of the egg, may divide so that the egg appears to have two normal polar bodies. Furthermore, it is known that even under normal condition *in vivo* the unfertilized eggs of the rat, mouse, and ferret may fragment in such a manner as closely to resemble segmented fertilized eggs (Austin 1949; Chang 1950*a*).

With regard to the high concentrations of sperms required and the removal of the follicle cell mass before fertilization, it has been observed by other authors that these conditions do not apply to normal fertilization *in vivo.* Thus, remarkably few sperms are to be found *in vivo* in the vicinity of the recently fertilized egg (Tafani 1889; Sobotta 1895; Sobotta and Burckhard 1910; Hammond 1925; Hammond and Walton 1934; Austin 1948*b*; Blandau and Odor 1949). More-

over, removal of the follicle cell mass is not necessary for fertilization (Lewis and Wright 1935; Leonard, Perlman, and Kurzrok 1947; Austin 1948*a*, 1948*b*; Blandau and Odor 1949).

The fertilization *in vitro* of human ovarian eggs, which were also matured *in vitro*, is claimed by Rock and Menkin (1944) and Menkin and Rock (1948). Their evidence consists of an identification of sperm heads within the bounds of the zona in histological sections, and the segmentation of some of the eggs during subsequent culture.

It is clear that there is only one unequivocal sign that permits a distinction to be drawn between non-specific activation and fertilization, and that is the presence of the sperm within the egg. This evidence, however, is difficult to establish positively in histological material where any small basophilic object may be interpreted as a sperm head, and where true sperm heads may have been introduced into the egg section accidentally.

It cannot be said that any useful information on the mechanism of sperm penetration of the zona and vitellus, or on the conditions influencing this process, has come from the reports on *in vitro* fertilization reviewed above. Recently, however, attempts have been made to define certain of the conditions required for penetration (Moricard and Bossu 1949*a*, 1949*b*; Moricard 1949, 1950). These authors stated that if eggs, still enclosed in pieces of fallopian tube, were treated with sperms under a layer of "vaseline oil," and incubated for periods up to seven hours, sperm heads could be identified in the zona, perivitelline space, and vitellus. No penetration was observed when the experiment was carried out under aerobic conditions and in the absence of fallopian tube. The authors had observed that a reducing potential existed within the normal fallopian tube and conclude that this is an important requirement for sperm penetration.

The observations now to be described are recorded in the hope that they may throw some light on the mechanisms involved in the passage of the sperm through the zona and vitelline surface.

II. Methods

Observations were made with adult and 40-55-day old immature rats, and with adult rabbits.

Ovulation was induced in the immature rats by injection of 20 I.U. of pregnant mare's serum (B.D.H. "Serogan") followed 48-56 hours later by 20 I.U. of chorionic gonadotrophin (B.D.H. "Gonan"), according to the procedure described by Rowlands (1944). In the rabbits, ovulation was induced by injecting 50 I.U. chorionic gonadotrophin intravenously.

For the surgical work on both the rats and rabbits ether anaesthesia was found most satisfactory, accompanied in the rabbits by atropine premedication.

The eggs were examined with a phase-contrast microscope, by the method described by Austin and Smiles (1948).

III. Observations

(a) *Time Relations of Sperm Penetration after Mating in Immature Rats*

For the assessment of time relations, the immature rat was selected because of the reliability with which ovulation may be induced in this animal, and the high suitability of the eggs for phase-contrast microscopy.

In the initial experiments, which were designed to show the time of ovulation, rats were killed at hourly intervals from 11 to 14 hours after the injection of chorionic gonadotrophin. The fallopian tubes were removed and examined by dissection under normal saline. The presence of eggs in the tubes was taken as the criterion of the occurrence of ovulation. The results (Table 1) show that ovulation occurs mostly 12-13 hours after the injection of chorionic gonadotrophin.

Table 1

TIME OF INDUCED OVULATION IN IMMATURE RATS

Time Killed After Injection of Chorionic Gonadotrophin (hr.)	Total Numbers of Rats Used	Rats in which Ovulation had Occurred	
		No.	Percentage
11	25	3	12
12	29	18	62
13	23	19	83
14	21	20	95

In the next series of tests, the immature female rats were placed with adult males immediately after the injection of chorionic gonadotrophin and examined for the presence of the copulation plug 10-16 hours later. Mated rats were killed at intervals ranging from 11 to 32 hours after the injection. The eggs were examined for evidence of sperm penetration and the results are shown in Table 2.

All the rats that mated and provided eggs had sperms in at least a proportion of the eggs. Data were obtained from 10 rats at each of the times selected, except at 11 hours after chorionic gonadotrophin when the frequency of ovulation is very low. At 11 hours a little over one-third of the eggs contained sperms in the perivitelline space and in the vitellus whereas at later times from 61 to 87 per cent. of eggs showed sperms in these locations. At 12 hours most of the eggs recovered had sperms within the vitellus.

In a total of 54 eggs there were sperms in the perivitelline space but not in the vitellus; 42 of these eggs came from rats killed 12-18 hours after chorionic gonadotrophin and only seven from the rats killed at 20-32 hours. Pronuclei were seen in the eggs from seven of the 10 rats killed as early as 12 hours after chorionic gonadotrophin.

Supernumerary sperms (i.e. sperms in the perivitelline space of eggs that have also a sperm in the vitellus) were frequently seen. The largest number of supernumerary sperms observed was 23. Plate 1, Figure 4, shows this egg, but only about 20 sperms can be identified at the focal plane selected. These sperms are all in the perivitelline space although in the photograph they appear to be within the vitellus.

(*b*) *Sperm Penetration after Injection of Sperms into the Peri-ovarian Sac of Immature Rats*

Ovulation was induced in groups of immature rats by the method described. At 16-17 hours after the injection of chorionic gonadotrophin (i.e. about 3-5 hours after ovulation) a small volume (0.001-0.01 ml.) of a suspension of epididymal sperms was injected with a fine needle into the peri-ovarian sac on each side. Runner (1947) obtained normal pregnancy in the mouse by introducing sperms in this manner. In the present investigation the sperm suspension was made with an isotonic phosphate buffer solution at pH 7.2 and kept under paraffin. The rats were killed at intervals from 2 to 8 hours and at 24 hours after the injection of sperms, and the eggs were examined for evidence of penetration.

TABLE 2

DISTRIBUTION OF SPERMS IN THE EGGS FROM MATED RATS KILLED AT INTERVALS AFTER THE INJECTION OF CHORIONIC GONADOTROPHIN

	Hours After Injection when Killed						
	11	12	14	16–18	20	24	28–32
Number of eggs having sperms in perivitelline space but not in vitellus	5	10	16	16	5	1	1
Sperms in vitellus	8	115	92	140	168	185	167
Total number of eggs containing sperms	13	125	108	156	173	186	168
Total number of eggs examined	35	157	175	254	201	243	194
Number of rats providing eggs	2	10	10	10	10	10	10

The results (Table 3) show that none of the eggs obtained before 4 hours after the injection of sperms showed any penetration. At 4 hours only one egg in a total of 301 had a sperm within. After 4 hours eggs quite frequently contained sperms, the highest proportion being observed in the rats killed at 24 hours. These provided 203 eggs, of which 34 contained sperms. In all rats, including those killed at 2, 3, and 4 hours after the injection of sperms, numerous sperms were observed in the tubes and surrounding the eggs. When the larger volumes of sperm suspension were used, as in groups 1, 2, 8, and 11 (Table 3) all eggs were free of follicle cells when recovered.

(*c*) *Sperm Penetration after Introduction of Sperm Suspensions into the Fallopian Tubes of Rabbits*

Sperm suspensions were introduced into the fallopian tubes of rabbits under ether anaesthesia. The sperms were obtained from the epididymis of adult male rabbits and used either undiluted or diluted with a buffered saline.

Introduction was effected by means of a fine glass tube attached to a micrometer syringe. Two groups of rabbits were used: in the first group (13 rabbits), sperms were introduced 12-14 hours after the injection of chorionic

TABLE 3

NUMBER OF EGGS SHOWING SPERM PENETRATION EXPRESSED AS FRACTIONS OF TOTAL EGGS RECOVERED FROM RATS KILLED AT INTERVALS AFTER INJECTION OF SPERM SUSPENSION INTO PERI-OVARIAN SAC

Expt. No.	Time After Injection of Sperms (hr.)							
	2	3	4	5	6	7	8	24
1	0/15		0/10					
2	0/15		0/14		0/3			
					5/13			
3								7/12
								0/18
								4/10
								0/1
								4/7
4			0/31	0/25	0/8	2/16		
					0/19	10/25		
5								7/23
								0/33
								3/15
								6/28
6		0/38	0/11	0/17	5/21	1/11		
7				0/47	0/21	0/28	1/26	
							3/36	
							4/35	
8		0/16	0/61	0/9	1/13	4/13		
						0/21		
9	0/14	0/7	0/35	1/47	0/7			
10	0/20	0/16	0/9	2/15	0/7			
11	0/65	0/13	0/29	0/23	0/5	1/43	2/10	
12	0/6	0/41	1/11	0/14		3/38	1/9	
13	0/40	0/18	0/61	0/10	0/17			2/21
	0/8							1/15
								0/3
								4/17
14	0/35	0/9	0/29	0/52	2/25			
		0/20		1/18				
Total	0/218	0/178	1/301	4/277	13/159	21/195	11/116	34/203
Percentage	0	0	0.3	1.4	8.1	10.8	9.5	16.7

gonadotrophin (about 2-4 hours *after* ovulation); in the second group (6 rabbits), sperms were introduced 3-5 hours after the injection of chorionic gonadotrophin (about 5-7 hours *before* ovulation). The rabbits were killed, mostly more than 30 hours after ovulation, and the eggs examined for evidence of penetration and fertilization. The results (Table 4) show that where the

sperms were introduced after ovulation only two eggs in a total of 63 contained sperms. On the other hand (Table 5), when sperms were introduced before ovulation, sperm penetration occurred in 19 of the 25 eggs recovered.

TABLE 4

RESULTS OBTAINED FROM INTRODUCING SPERM SUSPENSION INTO THE FALLOPIAN TUBES OF RABBITS SHORTLY AFTER OVULATION

Rabbit No.	Sperm Suspension Introduced		Hours After Ovulation when Killed	Results
	Dilution	Volume		
1	Undiluted	0.03 ml.	7½	7 × 1-cell eggs. No sperms within
2	Undiluted	0.03 ml.	6½	3 × 1-cell eggs. No sperms within
3	Undiluted	0.03 ml.	32	7 × 1-cell eggs. No sperms within. Sperms numerous in "albumen"
4	Undiluted	0.03 ml.	51	5 × 1-cell eggs. No sperms within. Sperms numerous in "albumen"
5	1 : 10	0.03 ml.	32	4 × 1-cell eggs. No sperms within. Sperms numerous in "albumen"
6	1 : 10	0.03 ml.	33	5 × 1-cell cells. No sperms within. Sperms numerous in "albumen"
7	Undiluted	0.005 ml.	32	3 × 1-cell eggs. No sperms within
8	Undiluted	0.0025 ml.	33	4 × 1-cell eggs. No sperms within. 1 × 16 cell egg, with 1 sperm trasersing the zona
9	1 : 8	0.002 ml.	32	3 × 1-cell eggs. No sperms within. 1 × 2 cell egg, with 1 sperm in zona and 4 in perivitelline space
10	1 : 8	0.001 ml.	33	6 × 1-cell eggs, 1 egg fragmented. No sperms within
11	Undiluted	0.001 ml.	31	4 × 1-cell eggs. No sperms within. Some sperms in "albumen"
12	1 : 10	0.003 ml.	31	4 × 1-cell eggs. No sperms within. Some sperms in "albumen"
13	1 : 10	0.003 ml.	32	5 × 1-cell eggs. No sperms within. Some sperms in "albumen"

(*d*) *General Observations on Sperm Penetration* in vivo

Examination of eggs recovered from mated rats soon after ovulation has shown that a sperm may occupy any one of five positions in the egg. It may lie (*a*) totally within the vitellus, (*b*) with a variable part of the mid-piece or tail still in the perivitelline space, or (*c*) with the tail still projecting through the zona into the surrounding medium. It may also lie (*d*) totally within the perivitelline space or (*e*) with its tail still projecting through the zona. By far the commonest form observed is (*a*), but (*d*) is quite frequently seen. The other forms are somewhat rare. An egg showing form (*c*) is illustrated in Plate 1, Figure 1.

Eggs examined later on, however, towards the time of the first segmentation, very rarely have sperms in any position except totally within the vitellus,

for the fertilizing sperm, or totally within the perivitelline space, for any supernumerary sperms.

When eggs having sperms traversing the zona are examined from above the point of penetration, the sperm tail is seen to project through what appears to be an elliptical hole in the zona. Often, as a result of the pressure exerted by the overlying cover-slip, some of the contents of the egg exude through the hole past the sperm tail. A similar slit or potential hole can usually be recognized in eggs that contain sperms even though the tails do not project through. It has never been seen, however, in eggs that do not contain sperms. If more than one sperm lies within an egg, it is usually possible to discern more than one slit in the zona, and often as many as there are sperms within. The number of slits has never exceeded the number of sperms. The appearance of the slit is shown in Plate 1, Figure 2. When this egg was rolled under the cover-slip so that the slit came to the free surface at the side of the egg, the cytoplasm of the vitellus immediately exuded, as may be seen in Plate 1, Figure 3. These signs of sperm entry have been seen in eggs recovered just before segmentation and may well persist for much longer.

TABLE 5
RESULTS OBTAINED FROM INTRODUCING SPERM SUSPENSION INTO THE FALLOPIAN TUBES OF RABBITS ABOUT SIX HOURS BEFORE OVULATION

Rabbit No.	Sperm Suspension Introduced		Hours After Ovulation when Killed	Results
	Dilution	Volume		
14	Undiluted	0.001 ml.	42	1 × 1-cell egg. No sperms within
15	Undiluted	0.001 ml.	42	3 × 1-cell eggs. No sperms within. 1 × 16-cell egg, sperms seen within
16	1 : 10	0.001 ml.	42	2 × 16-cell eggs. Sperms seen within
17	1 : 10	0.001 ml.	42	5 × 16-cell eggs. Sperms seen in all eggs
18	1 : 10	0.001 ml.	42	1 × 1-cell egg. 6 × 16-cell eggs, sperms seen within
19	1 : 10	0.001 ml.	42	1 × 1-cell egg. 5 × 16-cell eggs, sperms seen within

(*e*) *Observations made on Eggs* in vitro

Eggs have been obtained within three or four hours of ovulation from both rats and rabbits, and have then been subjected to certain tests. In the main these have involved suspending the eggs in a variety of media and adding the appropriate sperms. With adequate sperm concentrations the follicle cell mass about both species of eggs is rapidly broken up. The rat egg is easily denuded but the rabbit egg retains a layer of follicle cells (the "corona"), which is only removed by very high concentrations of sperms. When rabbit eggs are treated with sperm supensions *in vitro* the sperms adhere to the zona by the anterior part of the head, so that, with high concentrations, the entire

surface of the egg becomes covered with firmly adherent sperms. This does not happen to the rat egg; only occasionally are sperms found attached to the zona and even then the attachment is very weak and the sperms are readily brushed off. No concentration of sperms has been found to have any observable effect upon the zona in either species.

Penetration of the sperm through the zona was never observed. Unsuccessful tests included the removal of rat eggs from the fallopian tube under paraffin so that the eggs were suspended only in tubal fluid. Epididymal sperms and sperms obtained from the uteri of mated rats were added to these eggs, and the preparations were incubated at 37°C. for up to 7 hours. No penetration occurred.

In confirmation of Hall's (1935) statement, the rat zona has been found to pass into solution in weakly acid media; readily at a pH less than 5, more slowly and uncertainly at a pH between 5.5 and 6.5.

With both rat and rabbit eggs, the zona is dissolved in solutions of some reducing agents at pH 7-8, as well as at acid reactions. Thus 0.01M glutathione and 0.01M cysteine in Tyrode solution caused rapid dissolution of the zona whereas 0.01M ascorbic acid was almost without effect.

Penetration by sperms through the surface of the vitellus has now been observed *in vitro* on several occasions. Eggs obtained from mated, immature rats within a few hours after ovulation frequently have sperms in the perivitelline space but no sperm, as yet, in the vitellus (as indicated above in (*a*) and Table 2). A number of these eggs have been kept under observation at 30-37°C. for several hours, and in most of them the penetration of the head into the vitellus was seen. Usually this occurred with the head lying flat upon the surface of the vitellus but sometimes the pointed extremity of the hook-shaped head preceded the rest. Penetration generally happened within an hour from the commencement of observations, but in one case it took place near the end of the third hour. The process of penetration involved simply the gradual sinking of the sperm head into the substance of the vitellus. Sometimes the sperm continued to show some motility during its entry into the vitellus, but more often it was quite motionless. First the mid-piece and then the tail slowly followed the head into the vitellus.

IV. Discussion

In this paper observations are described which relate to the penetration of the sperm into the egg of the rat and rabbit, attention being directed particularly to the passage of sperms through the zona pellucida and the surface of the vitellus.

Examination of eggs recovered at intervals after normal mating in rats provided data for the following conclusions:

(*a*) The penetration of the sperm into the egg is a very rapid process. It probably takes no more than a few minutes at most for the head of the sperm to pass through the zona, judging by the fact that among over 1,200 eggs

examined none showed a sperm head in the thickness of the zona. The frequency with which supernumerary sperms are seen and the number (as many as 23) in one egg show that the sperm has no difficulty in traversing the zona.

(*b*) Penetration into the egg generally occurs very shortly after the arrival of the eggs in the fallopian tube and in some instances may occur within a few minutes. This is shown by the frequent occurrence of well-formed pronuclei in eggs recovered at 12 hours after the ovulating injection, i.e. probably well within an hour of ovulation.

(*c*) Penetration into the egg can occur in either one or two stages: the sperm may pass straight through the zona and into the vitellus, or it may remain for a variable period in the perivitelline space before entering the vitellus. Penetration without pause appears to be the more general occurrence. The two-stage process, however, is not uncommon; between 10 and 20 per cent. of penetrated eggs, recovered within 5 or 6 hours of ovulation, had sperms in the perivitelline space but not in the vitellus. It seems clear that these sperms would later have entered the vitellus, as very few eggs recovered 8 or more hours after ovulation have sperms only in the perivitelline space.

(*d*) When the sperm passes through the zona of the rat egg, it leaves a slit or potential hole, which can readily be demonstrated. The slit has been seen as late as the stage of the first segmentation and may well persist for longer than this. It cannot constitute a serious point of weakness in the zona for the latter is a strong elastic membrane and the contents of the egg can only be made to flow through the slit when the egg is firmly compressed under a cover-slip. The sperm apparently penetrates the zona at any point on the surface; when several sperms have entered a single egg the slits are seen often at widely different parts of the zona.

The entry of the sperm head into the vitellus occurs readily *in vitro* and has been observed on several occasions. The head sinks into the vitellus and, within a few minutes, begins to undergo the series of changes leading to the formation of the male pronucleus (Austin 1951). The rest of the sperm is gradually taken into the vitellus in the same manner. During these events as observed *in vitro,* the sperm is often quite motionless. Penetration into the vitellus thus appears to be a function of the vitellus itself. However, nothing has been observed to suggest that the mid-piece or tail are ever taken into the vitellus before the head. There must therefore be some property of the head that results in its being absorbed into the vitellus first.

Penetration of the sperm through the zona pellucida is clearly a process of a different nature. Although the zona presents little or no obstacle to the sperm *in vivo* under normal circumstances, penetration *in vitro* has not been observed in any of the preparations, even when the use of artificial media was avoided. Furthermore, the evidence obtained from the animal experiments showed that when sperms were introduced into the female tract, in both rats and rabbits, they were not able immediately to penetrate the zona. In the rabbits, 19 out of 25 eggs were penetrated when sperms were introduced into the fallopian tubes before ovulation, but only two eggs out of 63 were

penetrated when the sperms were put in 2-4 hours after ovulation. Presumably the eggs become unfertilizable before the sperms acquired the capacity for penetration. Hammond (1934) considered that the eggs of the rabbit remained fertilizable for not more than 6 hours after ovulation.

The rats provided a slightly different picture, probably because rat eggs remain fertilizable for about 12 hours (Blandau and Jordan 1941; Soderwell and Blandau 1941). When sperms were introduced into the peri-ovarian sac 3-5 hours after ovulation no penetrated eggs were found until 4 hours after the operation. At 4 hours and later, 84 eggs out of a total of 1251 were found to contain sperms. This is not a high proportion, but it should be remembered that the actual times were close to the limit of the fertilizable life of the rat egg. In the rats killed 2 and 3 hours after operation, sperms were always seen among the eggs, often in quite large numbers, and yet, as just stated, none of these eggs was penetrated. Here again there seems to be a need for the sperms to spend some time, apparently a few hours, in the female tract before they can penetrate the zona.

The following further conclusions seem to be justified:

(*a*) Sperms freshly obtained from the epididymis are incapable of penetrating the zona immediately, even *in vivo* and under conditions that must closely resemble the normal.

(*b*) The sperms must remain within the female tract for a period before they are able to penetrate the eggs.

(*c*) The sperms need not pass through the uterus; it is sufficient that they reside for a period in the peri-ovarian sac or in the fallopian tube.

(*d*) Although eggs are normally penetrated whilst surrounded by an apparently intact cumulus, they may still be penetrated even though denuded some hours previously. This is indicated by the presence of sperms in eggs recovered 6, 7, and 8 hours after the injection of relatively large amounts of sperm suspension into the peri-ovarian sac (Expt. Nos. 1, 2, 8, and 11 in Table 3). All the eggs recovered at earlier hours in these groups were denuded. Evidently the physical presence of the cumulus is not required by the sperm when it begins to make its way through the zona, and the egg, in the rat at least, does not, as Chang and Pincus (1951) suggest, become unfertilizable when it is denuded.

It is of interest to note here that in the rat and rabbit, under normal circumstances, mating takes place much longer before ovulation than the time taken for the sperms to reach the site of fertilization. Thus in the rabbit, ovulation occurs 9-10 hours after mating (Barry 1839; Heape 1905) and the sperms reach the ovarian end of the fallopian tube in 4 hours (Heape 1905) or probably less. The corresponding times for the rat are 8-11 hours (Blandau, Boling, and Young 1939), and 15-30 minutes respectively (Blandau and Money 1944).

The principal conclusion arising from the work described in this paper is that, at least in the rat and rabbit, the sperm must undergo some form of preparation or capacitation before it can penetrate the zona, and that this process is normally effected in the fallopian tube.

Such a conclusion is plainly in conflict with the numerous claims on the attainment of *in vitro* fertilization reviewed in the introductory section of this paper. These claims, however, were based on evidence that must be considered inconclusive. It therefore seems highly probable that the fertilization of mammalian eggs *in vitro* has not yet been achieved. Consistent with this is the fact that some other workers have also reported negative results (Umbaugh 1949; Chang 1950*b*; Moricard 1950).

The work of Moricard and Bossu (1949*a*, 1949*b*) belongs to a different category, for, in spite of their use of the term *in vitro*, their experiments involved sperm penetration in the isolated tube and not strictly *in vitro*. These authors observed sperm penetration only after the sperms had been incubated in pieces of fallopian tube for several hours, and their findings are therefore also consistent with the observations recorded in the present paper.

The process whereby the sperm makes its way through the zona has yet to be determined. Harter's (1948) suggestion that an acid reaction about the sperm head is the active agent is unattractive for two reasons. Firstly, if it were true, one would expect the dissolution of the entire zona when the egg is incubated in the presence of a high concentration of sperms, and this has not been observed. Secondly, Harter's suggestion could not apply to the penetration of the rabbit zona, which is not removed by treatment with acid media down to pH 3 (Braden, personal communication 1950). In some respects there is a better case to be made out for a reducing potential as the active agent, because this, as noted in the text, is effective on both the rat and rabbit zona. However, this theory is also objectionable on the grounds that, if it were true, a high concentration of sperms should remove the entire zona.

In general, the data obtained seem to favour the possibility that a specific agent, in the nature of a mucolytic enzyme, is carried by the sperm and assists its penetration by digesting a path through the zona. It is further suggested that an inhibitor normally accompanies the sperm and must be removed before the agent can act upon the zona. The removal of the inhibitor by absorption through the mucosa of the tube may well be a slow process and this would account for the relatively long sojourn in the female tract required by the sperm after injection into the tube or peri-ovarian sac. It is quite possible that in the normal circumstances after mating the time required to be spent by the sperm in the female tract would be less, because the supposed inhibitor could be more effectively removed when the sperm must traverse the whole length of uterus and tube.

This hypothesis is, of course, highly speculative. More information is required, particularly on the nature of the processes involved in the capacitation of the sperm and on the composition and properties of the zona. These problems are now under investigation in this laboratory.

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EXPLANATION OF PLATE 1

Fig. 1.—Sperm mid-piece and tail projecting from the interior of the vitellus, through the zona, and out into the surrounding medium. In this egg the head of the sperm has already developed into a pronucleus. x1120.

Fig. 2.—The slit in the zona considered to have been made by a sperm in entering the egg. The surface of the vitellus, at a lower focal plane, is seen as a blurred background. x1120.

Fig. 3.—When the egg shown in Figure 2 was rolled beneath the cover-slip so that the slit came to the free surface of the egg, the vitelline material exuded as shown, thus demonstrating a break in the continuity of the zona. x1120.

Fig. 4.—An egg from an immature rat with 23 sperms in the perivitelline space: the largest number seen in a rat egg. x700.

PENETRATION OF SPERM INTO THE MAMMALIAN EGG