SPERM PENETRATION OF THE ZONA PELLUCIDA
OF THE PIG EGG

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INTRODUCTION

In most mammals that have been studied, the first spermatozoon which penetrates an egg stimulates the egg to undergo a reaction—the 'zona reaction'—which bars further spermatozoa from penetrating through the zona pellucida (Braden, Austin & David, 1954). The sequel to the zona reaction may be classified into two categories, according to species: species in which spermatozoa are entirely prevented from entering the zona, e.g. the hamster, and species in which spermatozoa can penetrate into the zona but cannot traverse it, e.g. the pig. In the present study advantage has been taken of the occurrence of this phenomenon in the pig; spermatozoa in the thickness of the zona were studied, presuming that such observations may furnish clues about the mechanism of sperm penetration of the zona. The structure of the zona was also studied.

MATERIALS AND METHODS

Twenty-six Yorkshire or cross-bred Yorkshire-Duroc gilts were injected intramuscularly with 500 i.u. human chorionic gonadotrophin (HCG) on the day preceding oestrus. Twenty-four hours later (i.e. on the first day of oestrus), the gilts were artificially inseminated with 90 ml. of fresh undiluted semen. Forty-eight hours after the HCG injection, the gilts were slaughtered. Previous experience (Dziuk & Baker, 1962) has shown that gilts ovulate approximately 40 hr. after an HCG injection given during pro-oestrus. Ovulation was therefore estimated to occur about 8 hr. before slaughter. That ovulation did occur as estimated was verified in most cases by examination of the ovaries, by the finding of eggs in the oviducts and by observing the stages of development of the recovered eggs. In some gilts the ovulation time was not accurately estimated because of an error made in predicting the day of oestrus. In such cases the HCG was apparently injected after the gilt had already released her own gonadotrophin, so that ovulation resulted from the endogenous rather than the exogenous gonadotrophin, and ovulation occurred at an earlier time than expected.

The eggs were recovered by flushing the oviducts with physiological saline (0.9% NaCl) and were first examined under the dissecting microscope. The eggs were then mounted between a glass slide and a coverslip supported by a vaseline-paraffin mixture, and were examined with the ordinary and the phase-contrast microscope.

Of the 26 gilts treated, 24 ovulated. A total of 271 eggs were recovered: 36 were in
cumulus; 198 were 1-cell and denuded; 34 were 2-cell; and 3 were 4-cell. One or more spermatozoa were embedded in the zonae of 92 denuded eggs. Such eggs were assumed to be fertilized or about to be fertilized.

OBSERVATIONS AND DISCUSSION

Structure of the zona pellucida

The zona cannot be seen well in eggs still surrounded by cumulus cells and in eggs whose zonae contain a large number of spermatozoa. Denuded eggs whose zonae contained few or no spermatozoa were therefore used.

The zona was observed to consist of three concentric layers (Pl. 1, fig. 1), here referred to as the inner-zona, the middle-zona, and the outer-zona. The three layers cannot always be distinguished as clearly as in the egg shown in Pl. 1, fig. 1; the zona may appear to consist of two layers, or even of only one layer. Pl. 1, fig. 2, and Pl. 2, fig. 6, depict eggs in which the zona seems to consist of only two layers, the inner-zona and the middle-zona appearing as one layer. However, careful focusing on such specimens usually revealed the zona to consist of three layers.

In addition to the three layers of the zona proper the outer-zona is sometimes surrounded by a thin remnant of the cumulus-matrix. Occasionally this remnant of cumulus-matrix is seen as a chunk in a particular area outside the zona (Pl. 1, fig. 3).

A multi-layered zona is not a unique feature of the pig egg; in other species, too, more than one layer have been described (see Dickmann, 1963). We do not know whether or not the course of the spermatozoon through the zona is influenced by the particular structure of the zona.

The slit in the zona made by the fertilizing spermatozoon

The fertilizing spermatozoon makes a narrow slit in the zona by penetrating it (Pl. 2, figs. 4 and 5). Such a slit is difficult to detect for the following reasons. First, in a zona there is only one slit, the location of which is random, since the fertilizing spermatozoon can penetrate the zona at any point. Secondly, a slit can only be seen if it is orientated in a particular way to the light passing through the microscope. For this reason careful rolling of an egg under the coverslip is required in order to bring a slit into full view. Such full views were obtained in eight eggs; in additional eggs part of the slits were seen, but no further attempts were made to obtain a full view of them.

The shape of all the slits observed was very similar; we refer to it as 'the penetration curve'. In the pig, this curve closely resembles that described in the jird and guinea-pig (Austin & Bishop, 1958), and in the rabbit (Dickmann, 1964).

Since the slit is uniform in width and since it is considerably narrower than the width of the sperm head, it may be inferred that the spermatozoon does not rotate about its longitudinal axis while passing through the zona. Rikmenspoel (1962) observed that, as regards motility, there are two main types of bull spermatozoa: those that swim in a fairly straight direction and rotate about their longitudinal axes, and those that swim in small circular paths and do not rotate. It is of interest to note that in both Rikmenspoel's and our own observations the two features of sperm motility—curved paths and non-rotation—are concomitant.
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In this study only one spermatozoon was seen in the inner-zona (Pl. 2, fig. 6). That this was the fertilizing spermatozoon was deduced from the observation that the apex of the sperm head was deep inside the inner-zona, a region reached only by the fertilizing spermatozoon (see below), and by the fact that no other spermatozoa were present in this egg. By comparing Pl. 2, fig. 6, with Pl. 2, figs. 4 and 5, it will be noted that the shape of the penetration curve of the fertilizing spermatozoon is very similar to that of the slit.

The position of spermatozoa embedded in the zona

Spermatozoa other than the fertilizing spermatozoon, here referred to as ‘extra spermatozoa’, do not normally traverse the zona, but they can penetrate it. Sperm penetration of the zona follows a particular pattern, which is illustrated in Pl. 2, figs. 7, 8 and 9. Pl. 2, fig. 7, shows a spermatozoon whose head had just entered the outer-zona. Attention is drawn to the fact that the longitudinal axis of the sperm head is almost parallel to the surface of the zona. Pl. 2, fig. 8, depicts a spermatozoon whose head is just inside the middle-zona; the sperm head forms a small, but noticeable angle with the surface of the zona. The sperm head shown in Pl. 2, fig. 9, (indicated by arrow) is just outside the outer boundary of the inner-zona. This sperm head forms a still larger angle with the surface of the zona than that formed by the sperm head depicted in Pl. 2, fig. 8. Thus the extra spermatozoa shown in progressively deeper positions within the zona (Pl. 2, figs. 7, 8 and 9) illustrate that they follow a curved path very similar to that of the fertilizing spermatozoon. Extra spermatozoa have never been observed in the inner-zona (see Pl. 3, fig. 10), indicating that in this layer the zona reaction is normally absolute. We do not know why spermatozoa are impeded at various depths in the middle-zona and the outer-zona. It is unlikely that a weak zona reaction takes place in these two layers, because many zonae were observed containing numerous spermatozoa in which it seemed that, except for spatial limitation in the two outer layers (Pl. 3, fig. 10), no block had occurred.

The sperm-penetration filament (SPF)

If a spermatozoon, embedded in the zona, is properly orientated towards the light passing through the microscope, a fine filament can be seen extending from the tip of its head (Pl. 3, figs. 11, 12 and 13). The length of filaments as measured on photographs (including photographs not shown in this paper) ranged between 6 and 14 μ. However, the filament could be longer than indicated if (1) the anterior section was not seen due to its curved course, or (2) the anterior section was so attenuated that it could not be resolved with the light microscope.

Extra spermatozoa are normally barred from entering the inner-zona. The filament, however, has been seen to extend into the inner-zona, and in a number of cases it was seen extending all the way to the inner boundary of the inner-zona. It is evident from the photographs (Pl. 3, figs. 11, 12 and 13) that the filament leads the spermatozoon on its curved course. We suggest, therefore, that the sperm path through the zona is determined by the filament, and thus propose to call this structure sperm-penetration filament (SPF).

The only other mammal in which a SPF has been observed is the rabbit (Dickmann, 1964). On the other hand, a structure analogous to the SPF—the acrosomal filament
—has been described in several marine invertebrates (Dan, 1952, 1956; Colwin & Colwin, 1955, 1957; Rothschild & Tyler, 1955; Afzelius & Murray, 1957).

Critical microscopic examination revealed that the sperm head, viewed on edge, tapers in a non-symmetrical fashion, and that the SPF extends from the tapered tip; furthermore, that the flat side of the sperm head always faces the vitellus as the spermatozoon enters the zona. These features are illustrated diagrammatically in Text-fig. 1.

Austin & Bishop (1958) observed that in several rodents the acrosome is lost from the sperm head prior to its entry into the zona. On the basis of their observations they suggested that removal of the acrosome prior to penetration of the zona may be a phenomenon common to all mammals. In our study we have neither confirmed nor refuted Austin & Bishop’s hypothesis. We therefore have no information as regards the organelle in the sperm head in which the SPF originates. In the lower marine forms, in which acrosomal filaments have been described, apparently the acrosome plays a major role in sperm penetration of the outer membrane of the egg. The acrosomal filament originates in the acrosome, as its name indicates.

The mechanism of the passage of a spermatozoon through the zona

Based in part on our observations and in part on speculation, we propose that the sequence of events in the passage of a spermatozoon through the zona is as follows. First, the spermatozoon attaches itself to the zona by the flat surface of its head; it then ejects the SPF into the zona. The SPF, in turn, guides the spermatozoon on its curved course through the zona. While passing through the zona, the spermatozoon does not rotate about its longitudinal axis.

The present study has elicited several points as regards the process of sperm passage through the zona. However, to obtain a more fundamental understanding of this process, in the pig and in mammals in general, several pertinent questions will have to be answered. (1) Is removal of the acrosome a prerequisite for sperm entry into the zona (as was shown to be the case in several rodents, by Austin & Bishop, 1958)? (2) When attached to the zona does the spermatozoon secrete a substance which conditions the zona to allow the sperm to enter it (as was suggested by Dickmann,
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1964)? (3) Is there an enzyme attached to the leading surface of the sperm head which makes it possible for the spermatozoon to penetrate the zona (as was suggested by Austin & Bishop, 1958)? (4) Does ejection of a filament occur in mammals other than the pig and the rabbit (Dickmann, 1964)? (5) While passing through the zona, does the spermatozoon depend on its tail for forward propulsion, or is there some mechanism by which the spermatozoon is drawn into the egg? Dan (1954) suggested that the acrosomal filament of certain starfishes penetrates the jelly coat (the outer coating of the egg) and the vitelline membrane, and stimulates the egg cortex, which then draws into the egg the filament with the attached spermatozoon. (6) Can 'capacitation' (for review of the subject 'capacitation of spermatozoa' see Chang, 1959; Noyes, 1959; Austin & Walton, 1960) be equated with the ability of the spermatozoon to eject the SPF?

SUMMARY

1. Pig eggs were examined with the ordinary and phase-contrast microscope in search for clues about the process of sperm penetration of the zona pellucida.

2. The following observations were made. (i) The zona pellucida consists of three concentric layers—the inner-zona, the middle-zona, and the outer-zona. (ii) The fertilizing spermatozoon makes a narrow slit in the zona by penetrating through it. The slit has a particular shape referred to as 'the penetration curve'. (iii) Extra spermatozoa (i.e. spermatozoa other than the fertilizing spermatozoon) enter the zona and follow a course very similar to that of the fertilizing spermatozoon, but they can penetrate it only as far as the middle-zona. Extra spermatozoa have not been observed in the inner-zona, indicating that in this layer the 'zona reaction' is normally absolute. (iv) A filament extending from the apex of the sperm head has been observed in spermatozoa embedded in the zona. It is suggested that the sperm path through the zona is determined by this filament, which is therefore termed the sperm-penetration filament (SPF).

3. On the basis of these observations the following sequence of events in the passage of a spermatozoon through the zona is suggested. First, the spermatozoon attaches to the zona; it then ejects into the zona the SPF. The SPF, in turn, guides the spermatozoon on its curved course through the zona.

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REFERENCES


EXPLANATION OF PLATES

PLATE 1

Fig. 1. An egg showing that the zona pellucida consists of three concentric layers (the inner-zona, the middle-zona, and the outer-zona). The outer boundary of each of the layers is indicated by an arrow. × 560.

Fig. 2. An egg in which the inner-zona and the middle-zona appear as one layer; the outer-zona is clearly differentiated. × 560.

Fig. 3. An egg with a remnant piece of cumulus-matrix (arrow). × 290.

PLATE 2

Fig. 4. A profile view of a slit in the zona made by the fertilizing spermatozoon. × 560.

Fig. 5. The same specimen as in Fig. 4 after the egg had been somewhat compressed between coverslip and slide. Phase contrast. × 560.

Fig. 6. The fertilizing spermatozoon in the thickness of the zona. × 560.

Figs. 7-9. Spermatozoa in progressively deeper positions in the zona. Fig. 7. Sperm head in the outer-zona (part of the tail, outside the zona, is seen). × 820. Fig. 8. Sperm head in the middle-zona (part of the tail is inside and part outside the zona). × 560. Fig. 9. The sperm head (arrow) is in a somewhat deeper position in the zona than the sperm head depicted in Fig. 8. × 560.

PLATE 3

Fig. 10. An egg whose zona had been penetrated by a large number of spermatozoa (estimated at several hundreds). Note that the inner-zona is sperm-free. × 560.

Fig. 11. Three spermatozoa within the zona. In the one on the right the sperm-penetration filament (SPF) can be seen extending from the apex of the sperm head. In the spermatozoon on the left the SPF is slightly out of focus. The spermatozoon in the middle is not in focus. × 560.

Fig. 12. Three spermatozoa within the zona. Reference is made to the middle one in which the SPF can be seen. × 560.

Fig. 13. A spermatozoon within the zona showing its SPF. Phase contrast. × 560.