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Sperm-egg interactions in the pig: monospermy, extensive polyspermy, and the formation of chromatin aggregates

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INTRODUCTION

Monospermic fertilization is an essential feature of the reproductive process in mammals, since penetration of the vitellus by more than one spermatozoon is invariably pathological (Austin & Bishop, 1957; Beatty, 1961; Piko, 1961; Austin, 1963, 1965), leading to failure or abnormal development of the early embryo. Although the rôle of the female reproductive tract in reducing the chances of polyspermic fertilization has been emphasized for many years (Chang & Pincus, 1951; Austin & Braden, 1952; Braden, 1953; Braden & Austin, 1954; Austin, 1964), and attention has been drawn to a putative function of the cumulus oophorus in regulating the number of spermatozoa reaching the surface of the unfertilized egg (Chang & Pincus, 1951; Austin, 1961), the nature of the defence mechanism instigated by the egg membranes against polyspermic fertilization is not fully understood. The block to polyspermy has been shown to be vested in the zona pellucida of most species of mammal so far examined (Braden, Austin & David, 1954), including the pig (Thibault, 1959; Pitkjanen, 1961), but a physiological block operating at the level of the vitelline membrane is also known to exist in several species (Austin & Braden, 1956), most notably the rabbit. In both forms of the block to polyspermy, the contents of the cortical granules (Austin, 1956; Yanagimachi & Chang, 1961; Szollosi, 1962, 1967) that are released into the perivitelline space at the time of sperm penetration are considered to be instrumental in the modifications to the zona pellucida and/or the vitelline surface, and more recent experiments have demonstrated a direct effect of isolated cortical granule material on the substance of the zona (Barros & Yanagimachi, 1971; Gwatkin, Williams, Hartmann & Kniazuk, 1973).

In oocytes of the domestic pig, polyspermy has been reported under a variety of experimental conditions, including post-ovulatory ageing of the eggs (Hancock, 1959; Thibault, 1959; Hunter, 1967*a*), and those found after systemic injections of progesterone (Day & Polge, 1968) or micro-injections of this hormone locally beneath the serosal layer of the Fallopian tube (Hunter, 1972*a*). On the basis of the latter study, the suggestion was advanced that one of the major factors influencing the incidence of polyspermy, at least so far as the pig is concerned, is the number of capacitated spermatozoa at the site of fertilization. In support of this contention, a remarkably high proportion of polyspermic eggs (33·8 %) was found when excessive numbers of spermatozoa were instilled directly into the Fallopian tubes some 12 hours before

ovulation (Hunter, 1973). The more extensive form of polyspermy described in the present communication has been obtained by following the same principle of increasing the population of capacitated spermatozoa available at the site of fertilization. The highly polyspermic oocytes resulting from this treatment have been used as a model for examining specific aspects of sperm–egg interactions, particularly the degree of metamorphosis of the sperm head within the vitellus. This experimental situation has been compared and contrasted with that found in normal monospermic fertilization. Ultrastructural features of the polyspermic condition will be the subject of a separate publication (Szollosi & Hunter, in preparation).

MATERIALS AND METHODS

The animals used in this study were either pure bred Large White gilts or cross bred Large White × Landrace, aged 6–9 months and weighing 100–140 kg. Full details of the experimental management of such animals have been presented in previous publications (Hunter, 1974; Hunter & Hall, 1974*a*, *b*). Techniques essential to the present work were (*a*) control of the time of ovulation and (*b*) surgical insemination of a known number of spermatozoa directly into the Fallopian tubes or proximal end of the uterine horn.

Ovulation was induced by means of a single intramuscular injection of 500 i.u. HCG (Chorulon, Organon) given in 4 ml physiological saline during late pro-oestrus and was assumed to occur some 41–42 hours later (Hunter, 1967*b*). Semen was obtained from boars of proven fertility, the gelatinous material removed by filtering the ejaculate through cotton gauze, and known volumes of the filtered semen deposited in the female tract using a disposable hypodermic syringe attached to a blunted 20 gauge needle. Estimates of sperm density in the ejaculate were made with a haemocytometer slide (Neubauer ruling), these ranging from 1·01 to $3{\cdot}75 \times 10^8$ cells per ml. The volume of semen instilled into the lower 1·0 cm of the tubal isthmus varied from 0·02 to 0·1 ml, whereas approximately 5 ml was deposited into the uterine lumen close to the utero-tubal junction. Insemination was performed between 10 and 19 hours before ovulation.

These surgical procedures were accomplished under general anaesthesia induced by intravenous injection of pentobarbitone sodium, and maintained by closed-circuit administration of halothane and oxygen. Access to the reproductive tract was gained via mid-ventral laparotomy. Following insemination, the animals remained in the heated surgical building (18 °C), and were slaughtered some 2 hours 50 minutes to 23 hours after completion of ovulation. Eggs were recovered by flushing the Fallopian tubes with Tyrode's or Eagle's medium, prepared as whole mounts (Chang, 1952), and examined in the living condition to estimate the number of spermatozoa that had attached to or penetrated the zona pellucida. After fixation in 25 % acetic alcohol for at least 24 hours, followed by immersion in absolute ethanol for 15 minutes, the preparations were stained with 0·5 % orcein in 45 % acetic acid. Detailed examination took place under a Leitz phase-contrast microscope, and suitable preparations were photographed using a medium green filter.

RESULTS

The following observations are based on examination of 437 eggs recovered from the Fallopian tubes of 39 animals. The number of recently formed corpora lutea counted in individual animals ranged from 9 to 16, with a mean of 12·7.

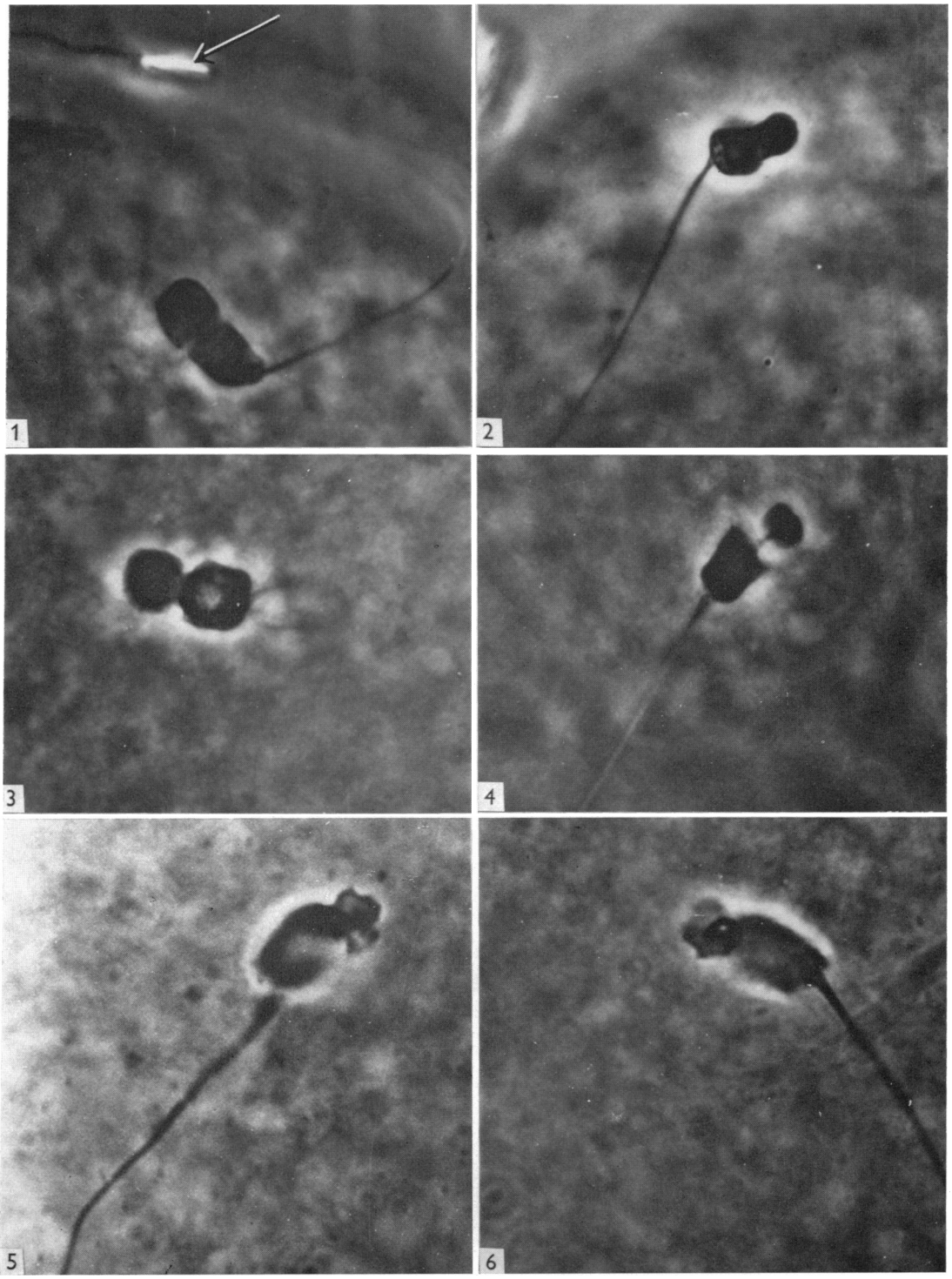
No evidence of sperm attachment or penetration could be detected in 13 eggs, and the chromosomes in each of these secondary oocytes were arranged at metaphase on the second meiotic spindle, or showed a configuration arising from degeneration of the metaphase plate. A further three eggs were primary oocytes, in each of which the peripherally located dictyate nucleus displayed the chromatin arranged around the nucleolus in the form of an uneven ring, horseshoe or crescent (Hunter & Polge, 1966). Two of these primary oocytes were highly polyspermic containing more than 60 unswollen sperm heads in the vitellus.

Among the remaining 421 eggs with spermatozoa attached to or within the substance of the zona pellucida, the number of spermatozoa per egg varied from 6 to more than 200. Resumption and/or completion of the second meiotic division arising from monospermic penetration of the vitellus had occurred in 78 (19%) of these eggs, but activation in the great majority (81%) was associated with polyspermy.

Monospermic fertilization

A number of eggs revealed the sperm head during critical stages of incorporation at the vitelline surface (Figs. 1–6), or during the early stages of transformation within the cytoplasm (Figs. 7–10). The most noticeable feature during incorporation was the alteration in morphology of the sperm head, the equatorial region of the head developing a modified and 'twisted' or 'waisted' appearance (see Hunter & Dziuk, 1968) during fusion with the vitelline membrane, although the relative proportions of the anterior and posterior regions of the head were, as yet, little changed. Coincident with these alterations in shape, the dimensions of the sperm head undergoing this first phase of incorporation appeared to have doubled when compared with those of spermatozoa on the surface, or in the substance, of the zona pellucida (see Fig. 1). It would seem that as soon as cytoplasmic contact is established with the sperm nucleus, but before the nucleus is surrounded by egg cytoplasm, vitelline factors are able to promote a preliminary expansion of the nuclear material. During the initial stages of this transformation, in which the sperm head frequently assumed the appearance of a dumb-bell, the mid-piece of the flagellum was apparently still attached to the sperm head (Figs. 1–4); whereas by the time the head was judged to be fully within the cytoplasm, a separation of the structural components in this region was usually distinct (Figs. 7, 8). Despite this separation, a substantial portion of the flagellum entered, and could be detected, in the vitellus of nearly all penetrated eggs (Figs. 7–10).

In a small number of preparations examined at this latter stage of sperm incorporation, membranous material appeared to have been displaced directly from the anterior region of the sperm head during entry into the cytoplasm (Figs. 5, 6). Likewise, in a few instances, there was evidence suggesting a slight remnant of membranous material associated with, or just in the surface of, the egg at the site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of sperm entry (Fig. 11). Such material probably represents vestiges of the inner acrosomal membrane and associated components, which apparently do not enter the vitellus. In comparison with spermatozoa on the surface of the egg, an overall enlargement of the sperm head could be detected during and immediately upon entry into the cytoplasm. However, as judged by a reduction in the dimensions of the head in stained preparations, this initial enlargement may be followed by a brief period of contraction in the sperm nucleus (Figs. 8, 11, 12), possibly during a re-orientation of the chromatin filaments, before formation of a typical pronucleus (Figs. 9, 10).

Changes also became conspicuous in the mid-piece at this stage. Characteristically, there was a bifurcation arising in the anterior portion of the mid-piece, with the open arms of the split projecting from the rostral end (Figs. 11, 12), this condition of the flagellum frequently being visible until the time of synkaryon formation.

In the early stages of decondensation and expansion of the sperm head chromatin, the nuclear material appeared bounded by edges of some contrast, indicating the formation of a limiting membrane (Figs. 8, 9). The development of small nucleoli was also noted shortly after this stage; these arose near the inner surface of the nuclear membrane, and coalesced during enlargement and migration of the male pronucleus, now surrounded by a distinct membrane (Fig. 10). A relatively close association continued to exist between the pronucleus and the flagellum during the period of migration; the possibility of an aster-like organization in pig eggs during the pronuclear stage has been considered elsewhere (Thibault, 1959; Szollosi & Hunter, 1973).

Following completion of the second meiotic division, and as observed under the light microscope, the female pronucleus developed in a manner essentially similar to that of the male. Two significant differences, though, were that there was slight

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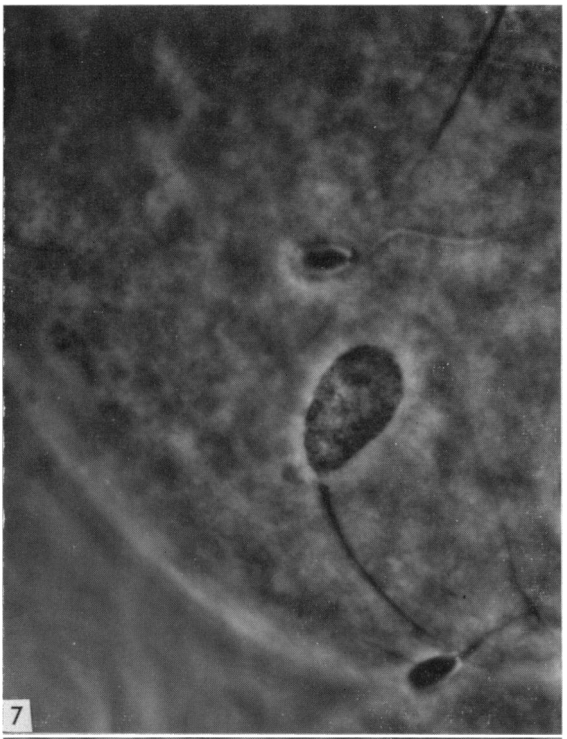
Figures 1–28 are phase-contrast photomicrographs at a magnification of ×c. 1500 of whole-mount preparations of pig eggs recovered from the Fallopian tubes of inseminated animals at various stages of the process of fertilization. The preparations were fixed for 24 hours in 25 % acetic alcohol, and stained with 0·5 % orcein in 45 % acetic acid. The zona pellucida was removed by this staining procedure, although some spermatozoa liberated from the zona remained in contact with the egg.

Figures 1–6 illustrate changes in the morphology of boar spermatozoa after passage through the zona pellucida and during fusion with and incorporation at the vitelline surface.

Fig. 1. Note that incorporation of the sperm head by the egg cytoplasm has commenced in the equatorial region of the sperm head and that the flagellum is still attached to this structure. Although the relative proportions of the anterior and posterior parts of the sperm head appear little changed, the overall dimensions of the head are seen to have increased when compared with those of the spermatozoon (arrowed) that was in the zona pellucida.

Figs. 2, 3 and 4. Preparations of different eggs showing spermatozoa at progressively more advanced stages of incorporation into the cytoplasm. Development of the 'waisted' condition of the sperm head is particularly noticeable in Fig. 3. The flagellum still appeared to be attached to the sperm head in these preparations, in each of which the chromosomes were arranged at anaphase or telophase on the second meiotic spindle.

Figs. 5 and 6. Spermatozoa at critical stages of the incorporation process, showing the membranous material that is displaced from the anterior region of the head. This phase is rarely seen clearly, but is thought to represent displacement of vestiges of the inner acrosomal membrane at the vitelline surface.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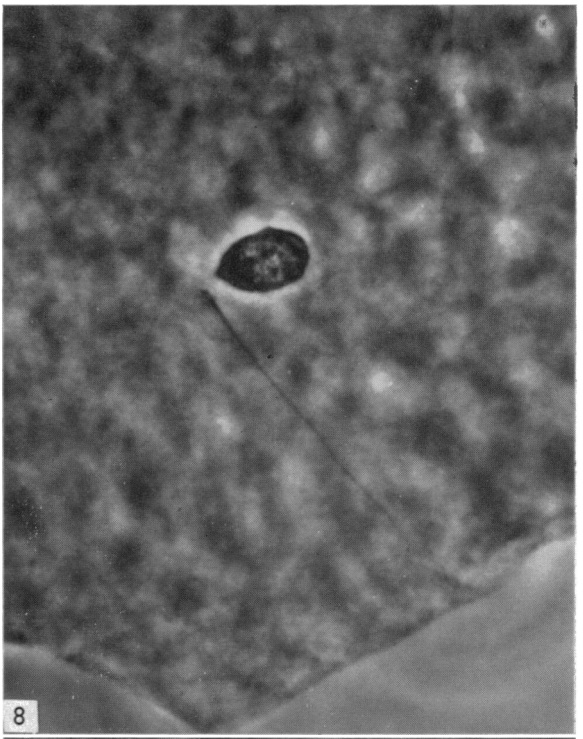
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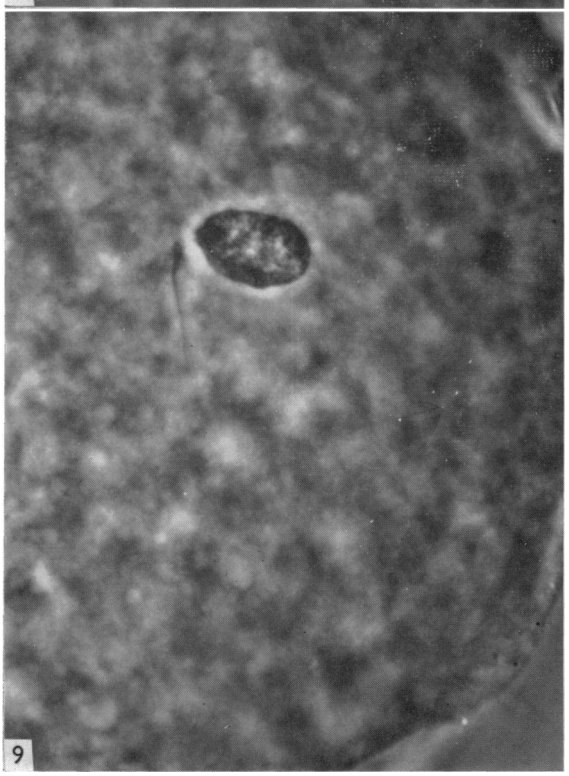
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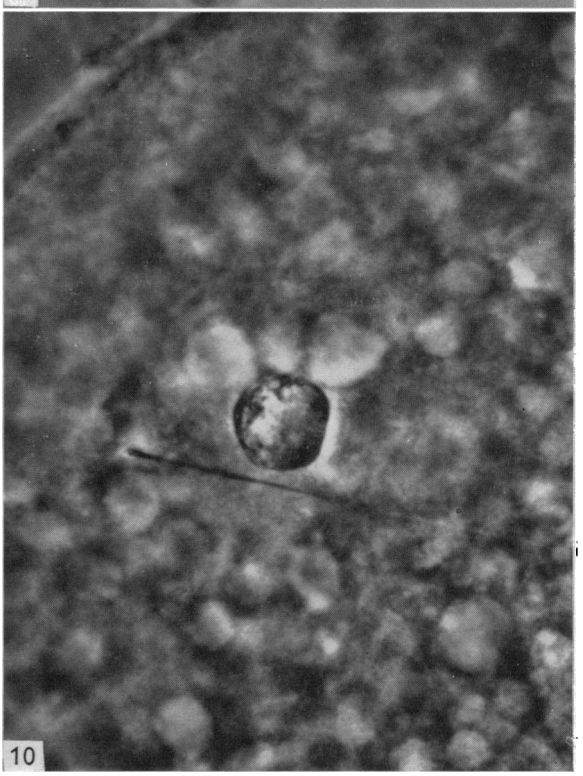
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asynchrony during expansion of the nuclear elements, the female structure appearing somewhat retarded and smaller; and the distribution of the chromatin became noticeably asymmetric in the female pronucleus, the rearrangement being pronounced at the commencement of pronuclear apposition (Hunter, 1972*b*, 1974).

Polyspermic fertilization

The number of male elements within the vitellus of the 343 polyspermic eggs ranged from 2 to > 80, with a mean of approximately 28.

The extent of morphological transformation of the sperm head into a male pronucleus during the first few hours after penetration seemed to be associated with the degree of polyspermy. Thus, in eggs containing less than 11–15 vitelline spermatozoa, some evolution of the sperm head towards a membrane-bounded pronuclear structure was invariably observed; in these instances, most sperm heads were at a closely comparable stage of transformation (Fig. 17), suggesting either an almost synchronous penetration of the oocyte, or else an arrested development of the sperm nuclei. Substantial portions of the flagellum could be detected in the vicinity of the sperm heads, with bifurcation of the anterior extremity of the mid-piece usually apparent, but other forms of structural alteration in the mid-piece were noted. These ranged from a parallel disposition of portions of the flagellum, to separation of one or more coarse fibres, but with the split orientated in the reverse manner towards the posterior region of the mid-piece (Figs. 13–16). All these configurations were presumed to represent different degrees of the same basic phenomenon of an initial decomposition and then degeneration of the mid-piece within the vitellus. Leaving this interpretation aside, ultrastructural evidence indicates that various organelles of the boar sperm mid-piece, including the mitochondria, do degenerate during the process of fertilization (Szollosi & Hunter, 1973; and unpublished), such degeneration frequently being quite advanced by the time of synkaryon formation.

In eggs showing very considerable polyspermy (approximately 30–50 vitelline spermatozoa), no such discrete evolution of the male nuclei was seen. The excessive penetration had produced a situation within the vitellus in which neighbouring sperm heads were frequently in contact (Figs. 21–26). Even in limited instances of

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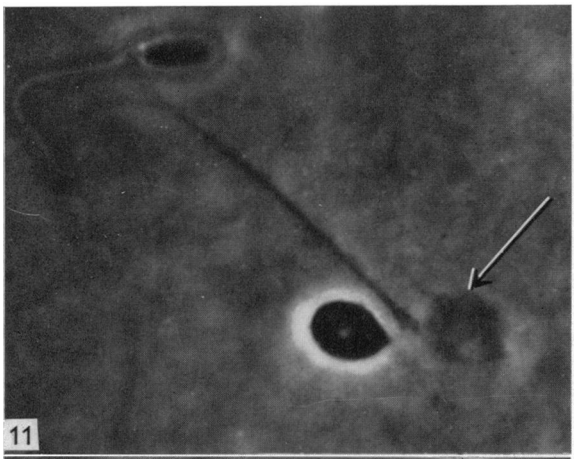
Figures 7–10 illustrate changes in spermatozoa soon after penetration into the egg cytoplasm.

Fig. 7. A recently penetrated egg showing expansion of the sperm head (cf. with spermatozoa in the zona pellucida). The mid-piece of the flagellum does not appear to have separated completely from the head.

Fig. 8. A slightly later stage in the fertilization process. The sperm head is located deeper in the cytoplasm and has assumed a more spherical shape. The flagellum is now completely separated from the head structures. The dimensions of the head at this stage suggest that the sperm nuclear chromatin has undergone a brief period of contraction leading to some head shrinkage.

Fig. 9. Following the temporary phase of shrinkage, the sperm nucleus has now embarked on a second phase of swelling during formation of a pronucleus. A nuclear membrane appears to have evolved, and small nucleoli can be discerned arising near its periphery.

Fig. 10. A later stage in formation of the male pronucleus. Note that dispersion and rearrangement of much of the sperm head chromatin has occurred, and that the nucleoli seem to arise close to the inner surface of the nuclear membrane. The flagellum remains in close proximity to the male element.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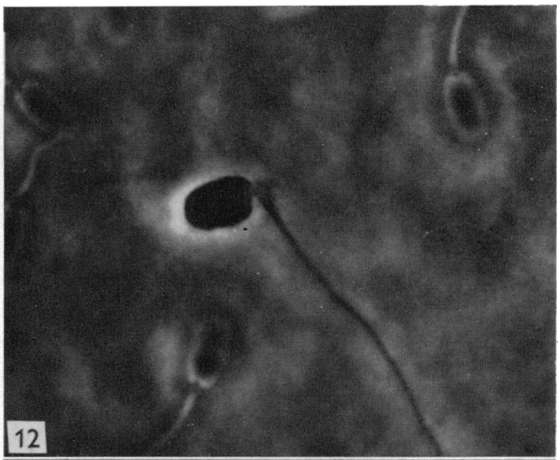
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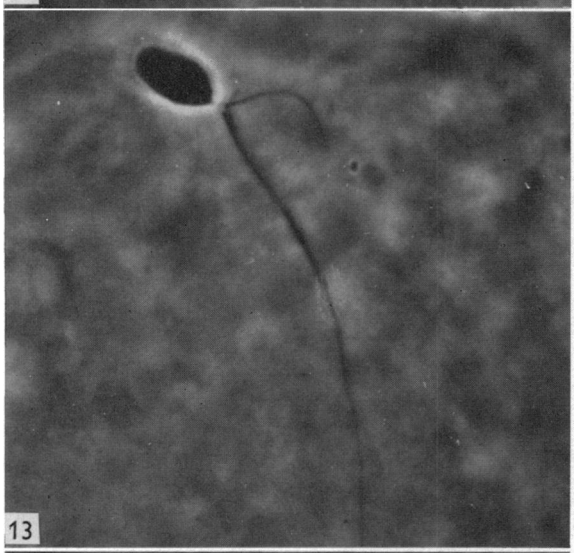
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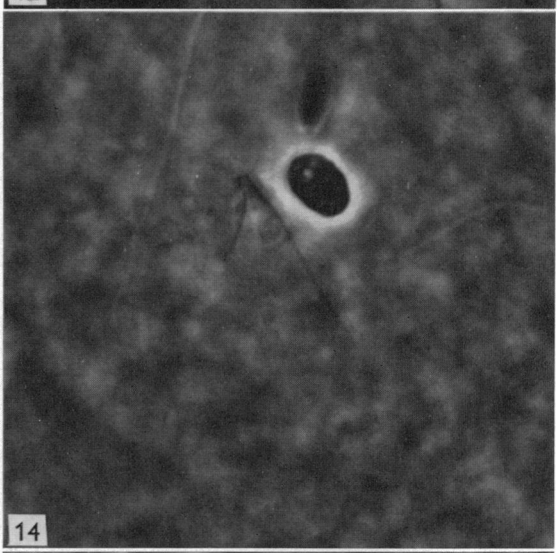
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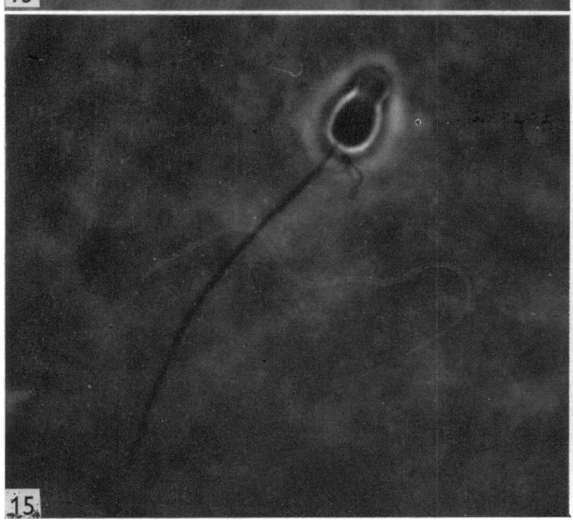
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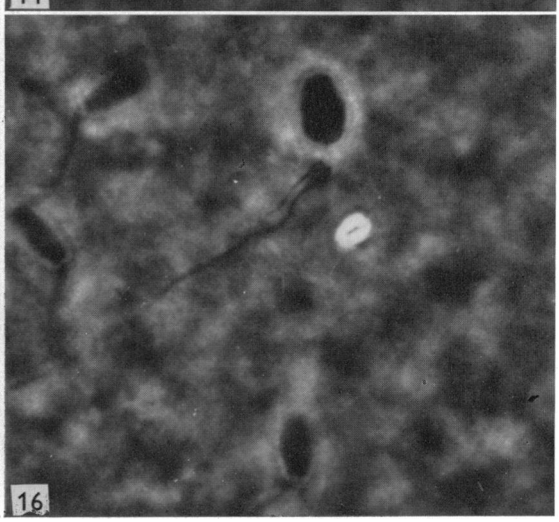
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such contact (Figs. 21, 22), the developing association between haploid groups of chromatin suggested the formation of a complex of sperm head material. The number of associated flagella was usually the most accurate indication of the number of male elements involved, particularly when some differential swelling of the chromatin had obscured the contour of individual sperm heads.

In more extreme cases of polyspermy, formation of aggregates of sperm head material had occurred (Figs. 27, 28), and in these situations the chromatin seemed to have established continuity between adjoining sperm heads and to have gone on to further coalescence. As judged from the number of flagella in the vicinity of the chromatin, at least as many as eight or nine sperm heads could participate in the formation of such aggregates. The subsequent evolution of these structures was difficult to determine accurately, since the condition of the cytoplasm in oocytes showing excessive polyspermy suggested that its degeneration had already commenced. However, the aggregates did not show features characteristic of pronucleus formation, such as swelling and reorganization as a spherical structure, the appearance of a distinct nuclear membrane and nucleoli, or migration through the substance of the vitellus; nor in eggs containing chromatin aggregates were pronuclear structures observed that might have formed from discrete male elements. Similarly, a characteristic female pronucleus could not be distinguished in these eggs, although the extruded second polar body was visible in stained preparations.

Several separate aggregates of sperm head chromatin were frequently present in eggs containing these structures, but male elements that had not become fused in this manner could also be detected elsewhere in the cytoplasm, usually in the peripheral region. In a number of instances, a form of pycnosis was visible in individual sperm heads in such eggs.

DISCUSSION

The above study has revealed several novel features. Although it is known that spermatozoa penetrating the zona pellucida undergo a conventional acrosome reaction with vesiculation developing between the plasma and outer acrosomal membranes (Barros, Bedford, Franklin & Austin, 1967; Bedford, 1968; Yanagimachi & Noda, 1970; Szollosi & Hunter, 1973), the fate of the inner acrosomal membrane and of any residual complement of acrosomal enzymes has remained uncertain, at least for spermatozoa of the domestic species. However, the finding in a number of eggs of a conspicuous displacement of membranous material from the sperm head

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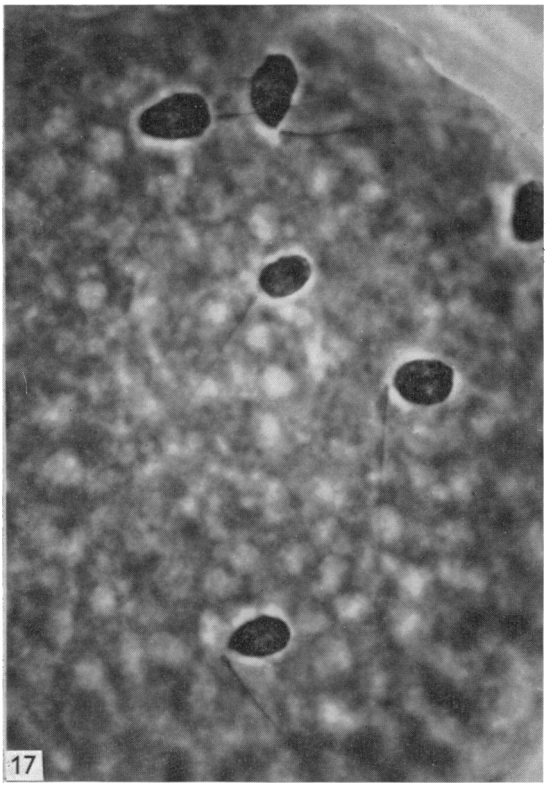
Figures 11–16 show preparations of activated eggs, illustrating some of the configurations assumed by the mid-piece in monospermic and polyspermic pig eggs.

Figs. 11 and 12. As judged by their dimensions relative to spermatozoa on the surface of the egg, the sperm head in each of these preparations is undergoing the transitional phase of shrinkage (contraction) that is thought to follow full incorporation into the vitellus. The mid-piece in both preparations shows commencement of the split condition, i.e. bifurcation at its rostral end. Fig. 11 also suggests a slight remnant of membranous material (arrowed, but out of focus) in the surface of the egg at the site of sperm entry.

Figs. 13, 14, 15 and 16. Each of these spermatozoa has been photographed within a polyspermic egg. Note the various configurations of the mid-piece associated with early stages in the disintegration of this portion of the flagellum, and the breaking away of one or more coarse fibres.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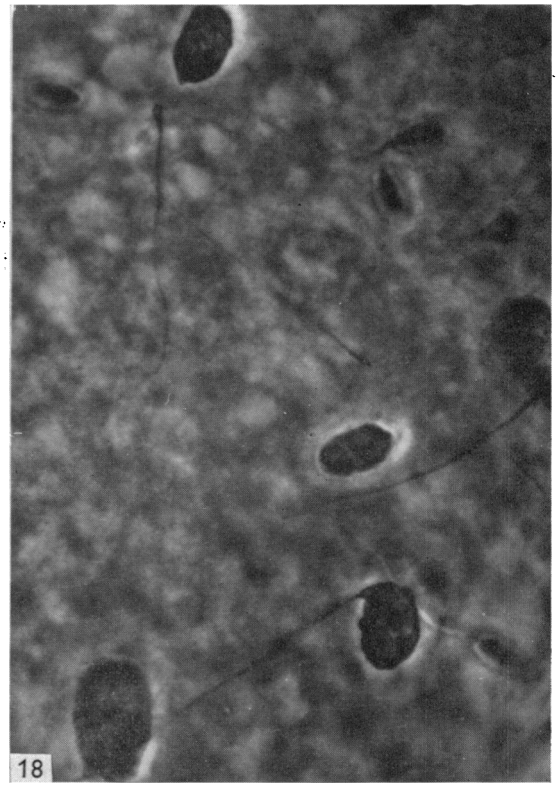
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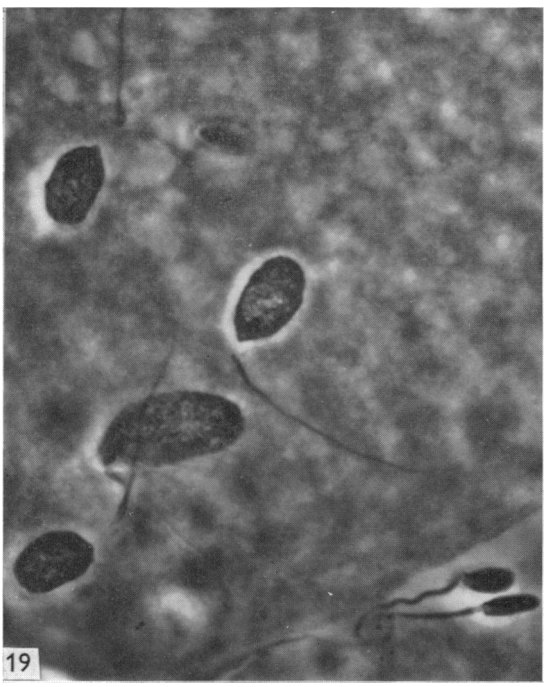
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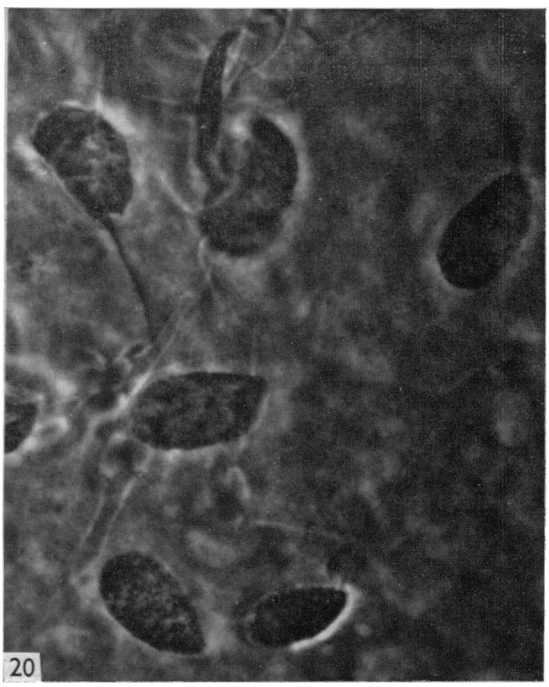
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during incorporation from the perivitelline space into the egg cytoplasm strongly suggests that this further loss of head membranes is a standard feature accompanying incorporation of boar spermatozoa. The implications are twofold: first, that bound acrosomal enzymes are unlikely to enter the vitellus, and secondly that divesting the sperm of this inner membranous component may facilitate a rapid reaction between sperm nuclear material and the egg cytoplasm.

During the first phase of incorporation of the sperm head, and prior to separation from the mid-piece, the dimensions of the sperm nucleus are seen to have increased when compared with those of spermatozoa on or in the zona pellucida. The nucleo-cytoplasmic contact that promotes this change also results in a morphological transformation of the sperm head from a dorso-ventrally flattened structure into a more spherical or cylindrical body. Our phase-contrast observations suggest the occurrence then of a transitory period during which nuclear shrinkage is found before the formation of a pronucleus, this shrinkage possibly being associated with a re-arrangement of chromatin fibres.

Whereas, under conditions of monospermic fertilization, the evolution from a recognizable male gamete into a pronuclear structure encompassed by a distinct nuclear membrane proceeds along a course and at a rate that can be characterized (Hunter, 1972*b*, 1974), a series of anomalies is observed in the pattern of development following polyspermic penetration. The situation in pig eggs under conditions of mild polyspermy (e.g. dispermy and trispermy) has been documented elsewhere (Hunter, 1972*a*, 1973); accessory male pronuclei are formed, but syngamy between the female and more than one male pronucleus rarely ensues. Fragmentation appears to be the fate of most of these eggs. By contrast, in situations of multispermic penetration of pig eggs (> 20 spermatozoa), only a limited degree of swelling of the sperm heads is found within the vitellus. Moreover, although the head separates from the mid-piece very soon after penetration, the nuclear structures in such highly polyspermic eggs invariably remain in the cortical region of the cytoplasm. Whether this failure of central migration is principally due to an adverse effect of polyspermy on the organelles that regulate such movement, or whether the formation of a pronuclear structure is essential for migration, has yet to be resolved. Failure of the sperm heads to undergo normal transition in highly polyspermic eggs also remains perplexing. On the basis of Austin's (1961) suggestion, it was reasoned that failure of sperm head swelling might be associated with the utilization of a cytoplasmic component present in only limited amounts (Hunter, 1967*c*). Whilst this interpretation is still considered to be reasonable, the disorganized condition

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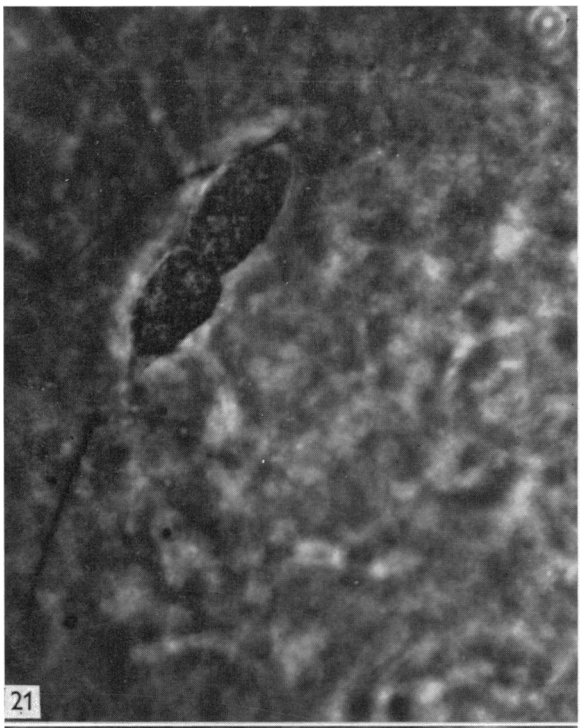
Figures 17–20 show preparations of polyspermic pig eggs illustrating the variable extent of the polyspermic condition and also the state of the male elements in such eggs.

Fig. 17. An egg that has been penetrated by nine spermatozoa. Note that the sperm heads have separated from their respective mid-pieces and that some morphological transformation of the sperm nuclei has occurred. Judged from the degree of swelling of the sperm heads, penetration of the egg membranes must have been approximately synchronous.

Figs. 18, 19 and 20. A range of polyspermic conditions in eggs penetrated by large numbers of spermatozoa. Note that some swelling of the sperm head has occurred, that separation of the mid-piece has not been completed in a number of instances, and that individual sperm heads are in close proximity in the eggs illustrated in Figures 19 and 20.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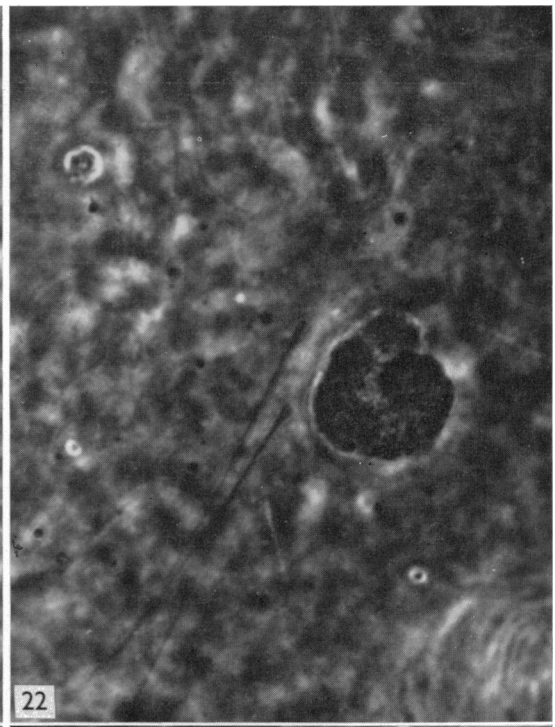
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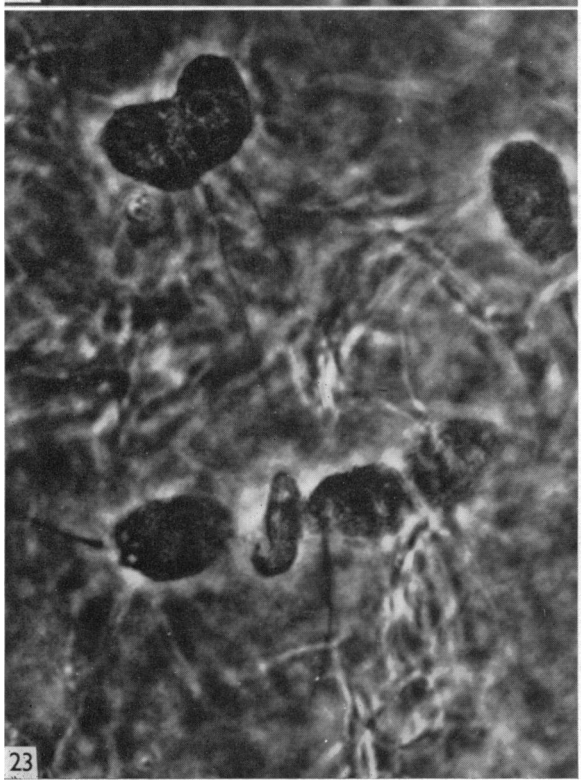
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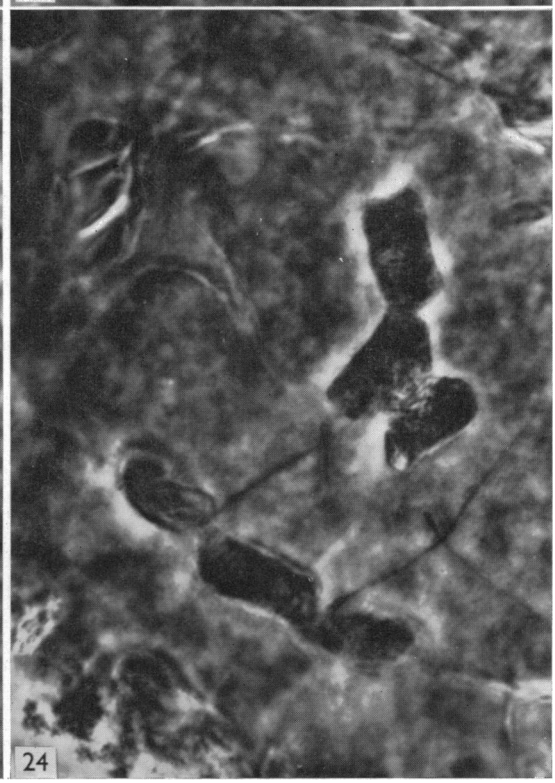
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of the cytoplasm observed in many of these polyspermic eggs needs also to be invoked to explain the restricted interaction between the vitellus and male elements.

Aggregates of sperm nuclear chromatin do not appear to have been reported previously for mammalian oocytes. The basic reason underlying their formation was judged to be the very close proximity of sperm head material in the vitellus of highly polyspermic eggs, rather than any form of specific attraction between the nucleo-proteins of spermatozoan origin. A similar conclusion was reached upon surveying the distribution of sperm tails in the egg cytoplasm in relation to the male nuclear elements. Nevertheless, once contact was established, an affinity between adjacent sperm heads became clear because they subsequently coalesced. The degree of morphological transformation that can be achieved by these multinucleate structures remains uncertain, as does their ability to attract the formation of a nuclear membrane and associated annulate lamellae (see Szollosi & Hunter, 1973). These fundamental limitations apart, the potential for further development of these aggregates as polyploid nuclei would also be severely compromised by the degenerate condition of the cytoplasm and the disorganized state of many of the cell organelles.

As stated in the introduction to this paper, there is now good evidence to indicate that the incidence of polyspermy in mature pig eggs can be elevated very significantly by increasing the population of capacitated spermatozoa present at the site of fertilization (Hunter, 1973). The most satisfactory means of achieving this situation is by surgical insemination directly into the Fallopian tubes at an appropriate interval before ovulation. Although capacitation can be accomplished within three hours of artificial insemination (Hunter & Dziuk, 1968) or natural mating of pigs (Hunter, 1972*b*), situations in which the spermatozoa are exposed sequentially to the uterine horns and Fallopian tubes, expression of the capacitated state is significantly delayed after deposition of aliquots of boar semen directly into the Fallopian tubes, and in this situation takes some 5–6 hours (Hunter & Hall, 1974*a*). However, in order to ensure that freshly ovulated eggs are confronted by a population of capacitated spermatozoa sufficiently large to promote extensive polyspermy, tubal insemination is best performed some twelve hours (Hunter, 1973) or more (present study) before ovulation. This finding therefore suggests some limit on the capacitation potential of the Fallopian tube in oestrous pigs, a conclusion which would be in line with the observations of Bedford (1970) in the rabbit.

An important point in support of the contention that the polyspermic condition arises from closely synchronous penetration of the zona pellucida was the finding

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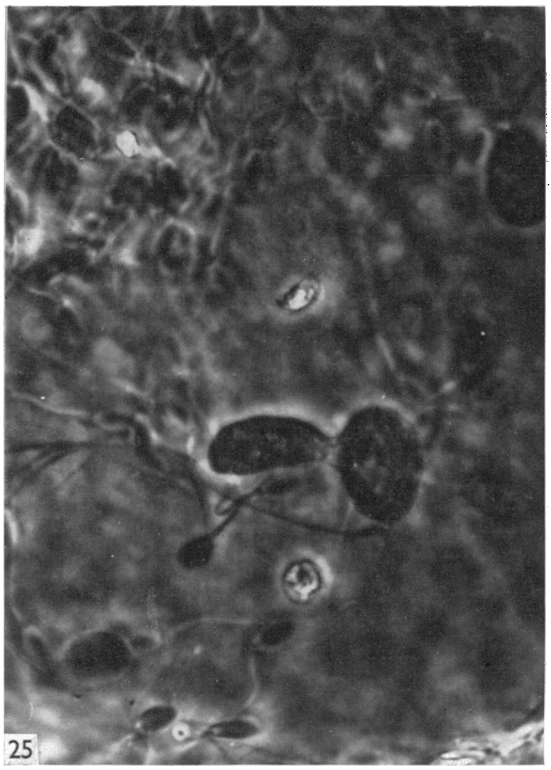
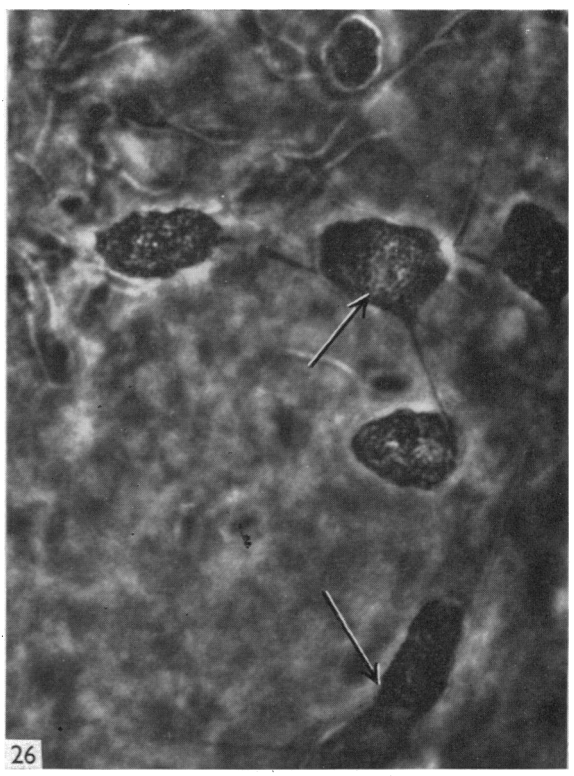
Figures 21–24 illustrate the relationship between pairs or groups of spermatozoa in the cytoplasm of pig eggs showing excessive polyspermy.

Figs. 21 and 22. Preparations showing the manner in which the close proximity of sperm heads in the cortex of the vitellus leads to the formation of a complex of sperm head material. Two spermatozoa are involved in this condition in Fig. 21, whereas three have contributed to the structure shown in Fig. 22. In both preparations, the mid-pieces have separated from the sperm heads and the nuclear chromatin has undergone some swelling and re-arrangement.

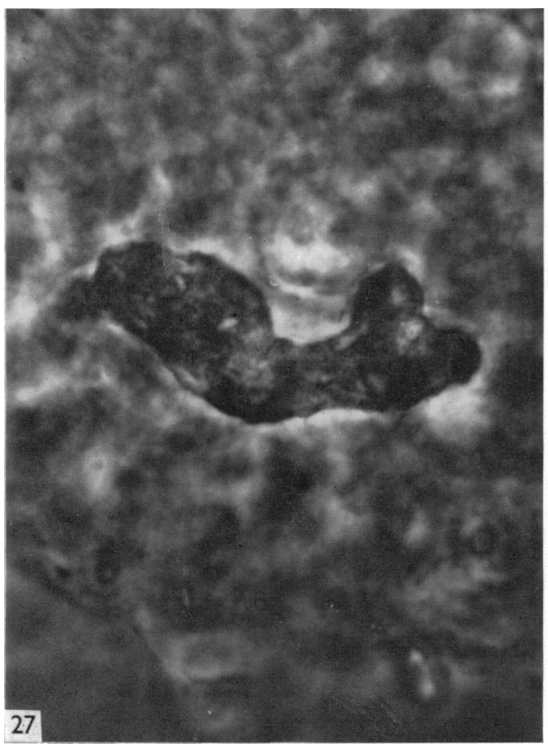
Figs. 23 and 24. Preparations illustrating other versions of sperm head transformation during formation of complexes of sperm nuclear material. Full separation of the mid-piece from the sperm head has failed to occur in several instances. The cytoplasm in these eggs appeared to be undergoing degeneration as a result of excessive penetration.



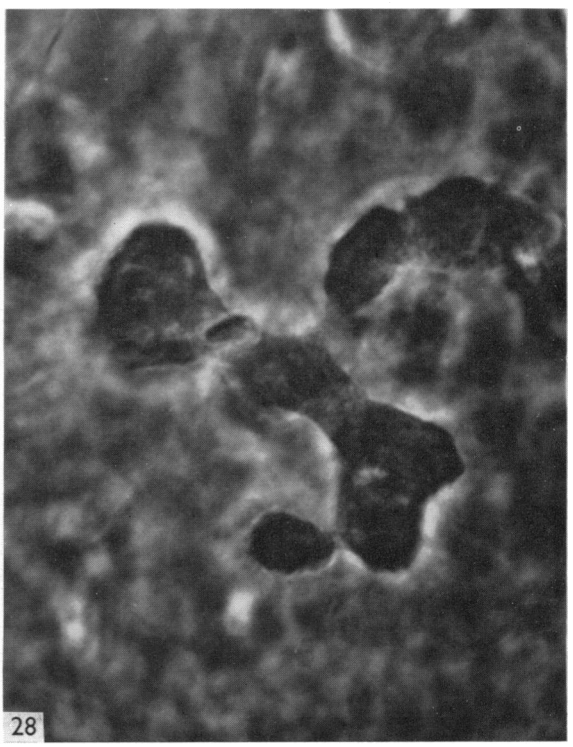
25



26



27



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of an apparently functional zona reaction in a high proportion of the polyspermic eggs observed in this study. As polyspermic eggs with large numbers of spermatozoa located in the substance of the zona, but none in the perivitelline space, were recovered several hours after the estimated time of sperm penetration and activation, it can be inferred that a block to polyspermy was established in these eggs – albeit too late to prevent the pathological condition from arising. An alternative interpretation of the data is that under the conditions prevailing in the Fallopian tube following instillation of very large numbers of spermatozoa, the block to polyspermy was retarded or rendered less efficient. But this explanation is not favoured since similar surgical inseminations performed *after* the time of ovulation do not lead to extensive polyspermy (unpublished observations). A recent estimate for the time required *in vivo* for establishment of the zona reaction in eggs of the golden hamster was less than 15 minutes after sperm attachment to the vitellus (Barros & Yanagimachi, 1972). If a similar time obtained in eggs of the domestic pig, this would certainly fit in with the extensive polyspermy and the subsequent establishment of a zona reaction in the present study.

As a concluding remark, attention was drawn in an earlier paper on the subject of polyspermy in pig eggs to the fact that the proportion of eggs exhibiting this abnormality in diverse experimental situations corresponds rather closely to the extent of embryonic failure in this species, and accordingly that a proportion of the eggs might be more susceptible to polyspermy. This led to the suggestion that some oocytes may have inherent defects rendering them prone to abnormal fertilization and/or to embryonic loss (Hunter, 1973). Although the present work does not lend support to this notion, since in a number of animals all the eggs recovered were polyspermic, this finding does not completely negate the earlier point of view. Subtle differences between oocytes, particularly those finding expression in membrane characteristics, most probably require a more sensitive indicator for their detection than is afforded by the appearance of extensive polyspermy.

SUMMARY

The process of incorporation and metamorphosis of the sperm head within the vitellus has been examined by phase-contrast microscopy in a large series of pig eggs exhibiting either normal monospermic fertilization or extensive polyspermy. This latter condition was induced *in vivo* after increasing the numbers of capacitated spermatozoa in the Fallopian tubes by pre-ovulatory surgical insemination.

Figures 25–28 illustrate stages in the formation of aggregates of sperm head chromatin in the vitellus of highly polyspermic pig eggs.

Fig. 25. Contact has been established, and coalescence is progressing, between two sperm heads from which the mid-pieces have detached and in which some swelling has occurred. The relative orientation of adjacent sperm heads does not appear to be critical for fusion to proceed.

Fig. 26. An egg containing several independent aggregates (arrowed) of sperm head chromatin, in addition to a large number of sperm heads whose condition suggested some form of pycnosis.

Figs. 27 and 28. Aggregates of sperm head chromatin that have formed as a result of the fusion of approximately eight or nine male elements in the cortical region of the vitellus. The potential for further transformation of these aggregates is unknown, but considering that the surrounding cytoplasm shows signs of degeneration, it is probably not great.

Attention was drawn in monospermic eggs to the initial fusion between the spermatozoon and vitelline surface which produced a characteristic constriction in the equatorial region of the head. Immediately following cytoplasmic contact with the sperm nucleus, an increase in size was detectable in this structure, remnants of the inner acrosomal membrane having apparently been displaced during incorporation. In fixed preparations of activated eggs, there was some evidence that the morphologically transformed sperm nucleus underwent a brief period of shrinkage before commencing pronuclear formation.

The most striking feature of the polyspermic condition was the number of spermatozoa that had entered the vitellus (2 to > 80), and the formation of aggregates of sperm head chromatin in eggs penetrated by more than 20–30 spermatozoa; the heads of at least 8 or 9 spermatozoa could participate in the formation of such an aggregate. Various unusual configurations were noted during breakdown of the midpiece in polyspermic eggs, and degeneration was also a general feature of the cytoplasm in situations of excessive polyspermy. Aspects of the block to polyspermy are discussed, and it is inferred that highly polyspermic pig eggs can still exhibit a zona reaction.

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