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Sperm Capacitation and Fertilization in Mammals

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There are many weaknesses in our present understanding of the morphology and physiology of mammalian fertilization. This situation is, in part, a reflection of the difficulties engendered by the relatively small numbers of eggs available for study, by the internal site of fertilization, and by the failure for many years to recognize the existence of a special functional dependence of sperm upon the female reproductive tract, prior to fertilization. This latter relationship was first recognized independently by Chang (1951), and by Austin (1951), who reported that the sperm of fertile rats and rabbits must first reside in the female tract for some hours before they may penetrate the zona pellucida. Cognizance of the fact that a subtle functional change, now termed "capacitation" (Austin, 1952), occurs in sperm as a result of exposure to secretions of the female tract, has allowed more fruitful studies of fertilization in mammals by experimental manipulation *in vivo*, as well as the development in species such as the rabbit, hamster and mouse, of repeatable techniques for the fertilization of ova *in vitro*. The basis of the development of this dependent relationship between sperm and the female tract is not entirely clear. The spermatozoon is a highly differentiated cell which has little cytoplasmic reserve or capacity for *de novo* synthesis of its cellular components. Since mammalian spermatozoa are often subjected to a period of storage in the cauda epididymidis after completion of their maturation in the male, this might require the development of a particularly stable cell surface. In this context it would be interesting to examine the capacitation requirement in the mammalian testiconda which do not appear to have developed any extensive capacity for sperm storage. Alternatively, the noncapacitated state may reflect a stable condition which has a protective value, particularly for the acrosomal apparatus, needed to withstand exposure to the biochemically complex female tract for some hours before arrival of the egg.

In the last decade, there have been several comprehensive reviews of fertilization in mammals (see for example: Austin and Walton, 1960; Austin, 1965, 1968; Blandau, 1961; Hancock, 1962, domestic animals; Piko, 1969). Current ideas relating to the preliminaries to fertilization, including capacitation, have also been discussed in recent years by several investigators (Chang, 1955a, 1955b, 1959; Austin and Bishop, 1957; Austin, 1964, 1967; Bishop, 1961; Parkes, 1960; Restall, 1967; Bedford, 1967a) and in the *Journal of Reproduction and Fertility*, Suppl. 2 (1967). The ready availability of the texts cited above makes it unnecessary here to cover all of the wider aspects of fertilization. The present treatment attempts, therefore, to bring together and to assess critically current evidence, ideas, and speculation relating to the nature, the biological significance of, and the environment required for capacitation. In the second part of this discussion the probable place of capacitation in the process of fertilization is considered in conjunction with the most recent evidence of the mode of sperm passage through the zona pellucida and its entry into the vitellus.

THE NEED FOR CAPACITATION IN DIFFERENT MAMMALS

Since the original observation in the rat and rabbit of the need for capacitation, a feature

which apparently does not occur in the invertebrates used so commonly in studies of fertilization, there has been a question as to the ubiquity of this phenomenon in mammals.

So far, it has been possible to determine the need for capacitation by critical experiment in only a few species. In the rabbit, sperm which pass from the uterus to the fallopian tube can become fully capacitated in about 5–6 hr (Chang, 1955; Adams and Chang, 1962b). In the fallopian tube alone, a period of 10–11 hr is necessary for functional capacitation (Adams and Chang, 1962b). Experiments to determine the time after insemination at which rabbit uterine sperm first acquire the ability to penetrate eggs placed in the uterine cavity (Bedford, 1968b, 1969a), or *in vitro* (Seitz *et al.*, 1970), indicate, however, that capacitation in the rabbit uterus alone is not completed before 10–11 hr rather than 6 hr, as previously had been thought.

In the rat, the observations of Austin (1951), Austin and Braden (1954), and Noyes (1953), based on studies of the time relations of fertilization *in vivo*, provide good evidence of the need for capacitation in this species and that it is achieved in 2–3 hr.

Early attempts to demonstrate capacitation time in the mouse were fraught with difficulty, as analysis of the time relations of fertilization in this species seemed either to indicate no need for capacitation or that a very short time is involved (Austin and Braden, 1954). As an additional complication, there is a suggestion that the cumulus and also the egg matures for a period after ovulation in the mouse, (Braden, 1959; Zamboni, 1970). Recently, Whitingham (1968) achieved fertilization *in vitro*, with sperm recovered from the mouse uterus 1–2 hr after mating, and Iwamatsu and Chang (1969) were successful likewise using mouse sperm capacitated in bovine follicular fluid. In this medium, capacitation of mouse sperm requires about 2 hr.

In turning to the hamster, observations on fertilization *in vivo* (Strauss, 1956; Chang and Sheaffer, 1957;), and data on *in vitro* fertilization (Yanagimachi and Chang, 1954; Yanagimachi, 1966; 1969a, b; Barros and Austin, 1967; Barros, 1968 a,b) show that golden hamster sperm require a minimal capacitation period of 2½–3 hr before sperm penetration first becomes possible. Although, in a single study, Chinese hamster sperm were capacitated in golden hamster fluids, (Pickworth and Chang, 1969), the time relations of fertilization were similar to those in the golden hamster. This probably indicates that a like period of about 3 hr is required for capacitation in this less commonly used species.

After natural mating in the ferret, ovulation does not normally occur until about 30 hr later (Hammond and Walton, 1934), yet epididymal sperm can first begin to penetrate a few tubal eggs only 3½ hr after their insemination via the ostium directly into the tubal ampulla (Chang and Yanagimachi, 1963). Since sperm were found earlier on the zonae of unfertilized eggs, this time of 3½ hr may be taken as the minimal period for capacitation in the ferret oviduct alone.

In the pig, penetrated tubal ova have been found only 2 hr after vaginal insemination of ejaculated sperm (Hunter and Dziuk, 1968). If capacitation occurs in this species, which seems probable, it must be a rapid process after natural mating when sperm pass from the uterine environment to that of the tube.

The only definite data available for the sheep appear to be those of Mattner (1963), who found that sperm develop the capacity to penetrate tubal ova at about 1½ hr after their tubal insemination, but not before this time. In the cow, Edwards (quoted by Austin, 1969) has observed that ejaculated sperm would not fertilize cow eggs until the sperm had been incubated with follicular fluid for 6–7 hr; this approach, an extrapolation from techniques developed in studies with the hamster, suggests that capacitation is necessary in this species.

The question of the need for capacitation in primates, including man, has yet to be

resolved. The different types of data obtained by indirect methods, which have been advanced by Dukelow and Chernoff (1969) as quasi–evidence for the need for capacitation in monkeys, are in no way conclusive. Since a pronucleate egg was present among a total of three penetrated eggs collected from rhesus monkeys only 6 hr after insemination (Marston and Kelly, 1968), capacitation in this species, if it occurs, must occupy not more than about 3 hr. The situation in man has remained somewhat cloudy, particularly so in view of several rather dubious claims for successful *in vitro* fertilization with ejaculated sperm, in which little attention had been given to the possibility of artificial activation. Most convincing is the recent report by Edwards *et al.* (1969), who seminated oocytes matured *in vitro* with washed ejaculated spermatozoa for periods ranging from 6–31 hr. Later examination suggested that a proportion of these oocytes had been penetrated, and that this was favored by preincubation of sperm with follicular fluids. This claim seems well founded in light of the further demonstration in some cases of the presence of the fertilizing sperm tail in the ooplasm (Bavister *et al.*, 1969).

In conclusion, we now have definite or suggestive evidence in several mammals of the need for a period of capacitation before sperm penetration can occur. Species differences exist in the time needed to reach a state of functional capacitation, and perhaps in the environmental factors required for its completion (see below).

ANALYSIS OF THE FACTORS IMPORTANT FOR CAPACITATION

The data relating to the environmental aspects of capacitation are still fragmentary and have been obtained for only a few mammals. It appears that the requirement for capacitation differs in various species, both as regards time and also possibly in the quality of the environment. This is not surprising, since it is true that spermatozoa are deposited at different sites (vagina or uterus) and that there are species variations in the time of their transport and in the time relationship between insemination and ovulation. Furthermore, in rodents especially, the configuration of the ovarian bursa and fimbria may ensure that much of the follicular fluid released at ovulation mixes with and contributes to the activity of the tubal fluids in the post ovulatory period.

There are several problems which have made it difficult to assay accurately the capacitation potential of different sites for rabbit spermatozoa. Not more than about 1–2 million sperm are normally found in the rabbit uterus after natural mating and it is doubtful if any significant volume of seminal plasma passes into the uterus at this time. Yet, experimentally, large numbers of sperm have been placed in the uterus, with or without seminal plasma, by injection into the cervix or directly through the uterine wall. Rarely has it been taken into account, however, that these manipulations might disturb the capacitation potential of the environment, though Soupart and Orgebin-Crist (1966) have pointed out that double ligation of the uterus with accumulation of uterine fluid did depress the rate at which sperm were capacitated in this site.

The same criticism applies to experiments aimed at defining the capacitating activity of the fallopian tube; for it has not been possible to avoid disturbance of the tubal environment involved in placement of relatively large numbers of sperm in a fluid medium into the tubal lumen.

Only very recently have the techniques for *in vitro* fertilization in the rabbit been mastered to the extent that these can now be used for the assay of capacitation systems. In the rabbit, assessment of capacitation has mostly been carried out *in vivo*, by finally transferring the experimental sample to the fallopian tube and allowing the sperm to fertilize tubal eggs. In several instances, in-

vestigators have failed to take into account the fact that the timing of these tubal inseminations allowed sperm to experience the tubal environment for several hours before termination of the fertile life of the egg. Hence, there is little doubt that in several claims for capacitation in particular experimental situations, *in vivo* and *in vitro,* all or part of the capacitation process was achieved in the oviduct of the "assay" female; this has led to contradictions and inconsistencies in the literature. Bearing these points in mind, the present treatment of this topic, which is necessarily centered around the rabbit and hamster, attempts to outline what is known of the environment needed for capacitation and the factors which modify its activities.

Role of the Female Tract and Follicular Secretions

Initial experiments (Chang, 1951, 1955; Austin, 1951) showed that capacitation can be achieved in the uterus or fallopian tube of the rabbit. Later it became apparent that about 11 hr are required for capacitation in the fallopian tube (Adams and Chang, 1962b). Since sperm taken from the uterus 6 hr after insemination fertilized tubal eggs (Chang, 1955) and sperm inseminated into the uterus were able to pass into the oviduct and there penetrate ova about 6 hr later (Adams and Chang, 1962b), the idea has grown that capacitation is achieved in the rabbit uterus in about 6 hr, and thus more quickly than in the oviduct. Contrary to this concept, recent experiments in which sperm were exposed to the uterus alone showed that much more time is required for completion of capacitation in the rabbit uterus since eggs placed in the uterus at various times after vaginal insemination were not penetrated by sperm until about 10–11 hr after insemination; after this time, penetration and pronuclei formation occurred normally and promptly (Bedford, 1969a). Using another approach, Seitz *et al.* (1970) studied the fertilization of ova *in vitro* with sperm recovered from the uterus at different times after mating. No fertilization was obtained with samples collected from the uterus earlier than 8 hr after mating. Those sperm collected at 8 hr fertilized only a small proportion of eggs, but no estimate was made of the time of penetration *in vitro;* this would give an accurate idea of whether capacitation was completed *in vitro* during the 3–4 hr for which the egg remained fertilizable under these conditions. This possibility is suggested by the fact that much higher fertilization rates were obtained with uterine sperm samples recovered 12 hr and, optimally, 16 hr after mating. There is a real likelihood that individual spermatozoa are capacitated at different rates (Dziuk, 1965); these last results fit well with the prediction that rabbit uterine samples for *in vitro* fertilization might with advantage be collected as late as 15-16 hr, assuming that the first sperm to become functionally capacitated appear in the uterus only 10–11 hr after mating (Bedford, 1969a).

Since rabbit sperm exposed to both uterus and oviduct can be functionally capacitated in 5–6 hr, and thus more quickly than in the uterus or oviduct alone, this implies the involvement of at least two factors, one contributed to a greater degree by the uterus, and the other by the fallopian tube. During the residence of rabbit sperm in the female after mating, these activities must synergize to produce the most efficient rate of capacitation as sperm pass from the uterus to the oviduct. Separation of the uterotubal junction seemed to delay the appearance of functionally capacitated uterine sperm until about 15 hr after mating, as judged by the time of penetration of eggs in the isolated uterus (Bedford, 1969a). Thus, elements from the oviduct may exert an influence on capacitation of sperm in the uterus. The evidence that sperm are apparently not capacitated for about 15 hr in the isolated uterus also implies that the rabbit uterus alone is not wholly adequate for capacitation in a biological sense, for rabbit ova begin to lose their fertilizability

at about 16 hr after mating (Chang, 1952; Adams and Chang, 1962a). These facts, together with recent evidence of factors involved in *in vivo* capacitation of rodent sperm (Hunter, 1968) and the knowledge that the initial phases of the process in the rabbit can be achieved outside the uterus (see below), led the author to conclude that "the primary factors provided by the fallopian tube have a greater specific importance for capacitation than those arising in the uterus" (Bedford, 1969a).

On the other hand, the idea that the physical environment alone may create optimal conditions for accomplishment of capacitation has arisen also during experiments aimed at defining the conditions required for *in vitro* capacitation of hamster sperm (Bavister, 1969). In fact, relatively little is known about the contribution of the various regions of the hamster female tract to capacitation *in vivo*, since major attention has been devoted so far to *in vitro* capacitation. However, studies of the time relations of sperm penetration *in vivo* indicate that capacitation is not completed in the hamster uterus (Hunter, 1969). This is suggested also by the failure to fertilize eggs in the uterus of the hamster (Hunter, 1968) or rat (Bedford, unpublished) and may hold for other species in which the uterus is filled with whole semen for several hours after mating. It is interesting that in the hamster, mouse, and rat, the largest numbers of sperm appear to pass into the ampulla of the oviduct at the time of and after ovulation (Braden and Austin, 1954; Yanagimachi and Chang, 1963), during which time the tube would be filled with a follicular/tubal fluid mixture. This fluid is competent to capacitate hamster epididymal sperm *in vitro* in 2–3 hr (Barros and Austin, 1967) and Yanagimachi (1969a) has suggested that the follicular and tubal fluids may act synergistically in this respect. There is still a question, however, as to the specific value of these fluid components. For, while Yanagimachi (1969a) has concluded that "an efficient capacitation of the (hamster) spermatozoa seems to occur only when a high concentration of follicular fluid is present in the incubation medium," a consistently high fertilization rate has nevertheless been obtained in other experiments in which the ratio of the defined physiological medium to cumulus mass was as high as 50:1 (Bavister, 1969). In these latter experiments, in which pH appeared as a critical factor, Bavister has inferred that "tubal or follicular fluids may not capacitate spermatozoa directly, but simply create suitable environmental conditions for spontaneous occurrence of this process." It is, perhaps, relevant to draw attention here to recent experiments which show that under defined conditions of sperm numbers, calcium concentration, and "fertilisin" stimulation, there is a clearly defined pH optimum (pH 8.0) for a maximal acrosome reaction in *Arbacia punctulata* spermatozoa (Gregg and Metz, 1969). It has been suggested that capacitation might possibly require cell-to-cell contact between the sperm and endometrium (Soupart and Orgebin-Crist, 1966). This view has been endorsed by Hamner and Sojka (1968), who failed to capacitate sperm within a Millipore chamber in the rabbit uterus; they have concluded that rabbit uterine and oviduct secretions do not contain all the factors necessary for capacitation and that contact with an epithelium is necessary. In contradistinction to these ideas, sperm have now been capacitated successfully within a Millipore chamber in the rabbit uterus (Abney and Skipper, 1969), and a significant degree of capacitation has been accomplished in rabbit uterine fluid alone (Kirton and Hafs, 1965; Bedford, unpublished). Furthermore, *in vitro* capacitation of mouse and hamster spermatozoa has been obtained consistently with follicular or tubal fluids (Barros and Austin, 1967; Yanagimachi, 1969a, b; Iwamatsu and Chang, 1969); and apparently even in a relatively simple physiological medium (Bavister, 1969). In the author's opinion, these positive results militate strongly against the notion that a sperm–

epithelium contact is necessary for capacitation.

Specificity of Capacitation

The idea has been mooted that a species-specific requirement for capacitation may help to maintain the breeding purity of a species, but there does not seem to be any capacitation barrier between closely related species in which the physical and psychological aspects of mating might allow hybridization. Reciprocal capacitation is possible between sheep and goat, hare and domestic rabbit, cottontail rabbit and domestic rabbit, and mink and ferret (see Chang and Hancock, 1967). These and other recorded examples make it unlikely that the specificity of capacitation has been an important factor in speciation.

In the hamster, the capacitation factors are not species-specific, and fresh rat and mouse follicular fluid can bring about functional capacitation of hamster sperm, though not so efficiently as fluids from the hamster ovary (Barros and Austin, 1967; Barros, 1968a; Yanagimachi, 1969a). Untreated rabbit follicular fluid appears ineffective in this respect and cannot sustain the motility of hamster sperm (Yanagimachi, 1969a). However, when bovine follicular fluid was detoxified, this became competent to induce functional capacitation of hamster spermatozoa and to initiate the acrosome reaction Yanagimachi, 1969b).

Present evidence suggests that the capacitation requirement of rabbit sperm is rather more rigorous than that of hamster sperm. In the author's opinion, there is no good evidence for complete capacitation of rabbit sperm *in vitro*. The report of the efficacy of B-amylase treatment (Kirton and Hafs, 1965) has not yet been substantiated (Soupart, 1966; Chang, personal communication; Barros, 1968b). In agreement with another claim of Kirton and Hafs (1965), the present author has been able to achieve a significant degree of capacitation in rabbit uterine fluid *in vitro;* this was judged by the fertilization of ova after tubal insemination of recipient females at 12½ hr after administration of an ovulating injection of HCG. Nevertheless, in these experiments, as in those of Ericsson (1969) who incubated rabbit sperm with mule eosinophils, it is probable that capacitation was completed *in vivo* in the tube of the recipient. This whole question might now be approached using some of the techniques which have been developed successfully in the hamster.

The presently available data make it appear that capacitation of rabbit sperm depends to some degree on factors present in the female tract. An initial phase of capacitation can be set in train in ectopic *in vivo* sites such as the anterior chamber of the eye, the colon, and the bladder (Noyes *et al.*, 1958), and more efficiently in the isolated vagina (Bedford, 1967b) and in heterologous estrous uteri (Bedford and Shalkovsky, 1967; Hamner and Sojka, 1967). However, in none of these sites was capacitation completed. The finding that capacitation of rabbit sperm cannot be completed in the estrous uterus of the rat (Bedford and Shalkovsky, 1967) suggested that functional capacitation of rabbit sperm may require specific factors, confined at least to the Lagomorpha. However, as discussed above, the most recent ideas do not now necessarily accord the uterus a premier or total role in capacitation of rodent spermatozoa. Thus, the failure to completely capacitate rabbit sperm in the rat uterus may have been due to a general, and not to a species-specific inadequacy of the rat uterus. For this reason the specificity requirement of capacitation in the rabbit cannot be considered proved.

Influence of Hormones

Since the secretory activity of the female tract is governed to a considerable degree by the ovary, it is not surprising that the capacitation potential of the female can be modified by hormones. The capacitating activity of the uterus appears more sensitive

than that of the oviduct to the endocrine state of the female. There is little doubt that progesterone depresses capacitation in the rabbit uterus (Chang, 1958; Soupart, 1967; Bedford, 1967b; Hamner *et al.*, 1968), and there is some evidence that this is so in the rat (Hamner and Sojka, 1967). Nonetheless, some initial phase of capacitation can still be achieved in the progesterone-dominated rabbit uterus (Bedford, 1967b). In the absence of ovarian estrogen, the uterus of the rabbit can only partially capacitate sperm in 10–12 hr; for such sperm fertilized eggs in the fallopian tubes of recipients only when inseminated into this site as early as 9½–12 hr after an ovulating injection of HCG (Chang, 1958; Noyes *et al.*, 1958). If these sperm were inseminated tubally later than this, virtually no ova were fertilized, though control-capacitated sperm fertilized eggs in the contralateral oviduct (Soupart, 1967; Hamner *et al.*, 1968; Bedford, 1970b). Experiments in which ova were transferred to the uterus in ovariectomized does make it apparent that sperm in this estrogen-deficient environment first acquire the ability to penetrate eggs only after about 17–20 hr; thus, the time required for complete capacitation in the uterus seems to be doubled in the ovariectomized rabbit (Bedford, 1970b). The results of Soupart (1967) suggest that absence of ovarian estrogen accounts for reduction in capacitation potential after ovariectomy.

In summary, a state of progesterone dominance brings about a greater depression of uterine capacitating activity than does a lack of estrogen; for complete capacitation does not occur in the progesterone-dominated uterus within the lifetime of the sperm.

The rabbit fallopian tube apparently remains competent to capacitate sperm regardless of the ovarian status of the female. After ovariectomy there is a reduction in the level of capacitation activity within the oviduct (Bedford, 1968a, 1970b), but this is nevertheless maintained at a basal level sufficient to capacitate larger numbers of sperm than normally reach this site (Noyes *et al.*, 1958). Contrary to the recent conclusions of Hamner *et al.* (1968), progesterone does not inhibit significantly the process of capacitation in the fallopian tube (Chang, 1958), and an adequate basal level of capacitating activity remains in the oviduct of the ovariectomized female receiving high doses of progesterone (Bedford, 1970b). Thus the rabbit oviduct possesses an innate ability for capacitation which can be enhanced by estrogen, but which cannot be depressed significantly by progesterone, even in the absence of both ovaries.

It has been claimed that the capacitating activity of the rabbit uterus can be regulated in a positive or an inhibitory way by administration of different levels of HCG (0–400 IU) at the time of insemination and that the coital release of luteinizing hormone (LH) stimulates the capacitating activity of the uterus at this time (Soupart, 1966, 1967). Such treatment did not appear to influence capacitation in the fallopian tube. These claims of a sensitive effect of gonadotropin over such a short period require confirmation. Wetteman and Hafs (1969) failed to find any depression of capacitation with up to 2 mg of LH, but they did note some apparent depression of uterine capacitation after the injection of high doses of human chorionic gonadotropin (HCG). The present author has been unable to detect any depressive effect on uterine capacitation after injection of 500–1000 IU HCG, assayed either by tubal insemination of recipient does, or by egg transfer to the uterine capacitation site.

The Decapacitation Factor (DF) in Seminal Plasma

In 1957, Chang reported that seminal plasma from intact or vasectomized male rabbits, from intact bulls and from men could bring about a reversible inhibition of the fertilizing ability of capacitated rabbit sperm,

in concentrations as low as 5% v/v. This initial observation has been the stimulus for subsequent investigation into the nature of the active factor and has perhaps provided a tool which may help in analysis of the nature of capacitation.

The report by Bedford and Chang (1962) that the inhibitory decapacitation factor, or DF activity in rabbit seminal plasma can be removed by ultracentrifugation, is non-dialyzable, and cannot be destroyed by heating to 65 C, indicated that DF is a stable substance associated with a molecule of relatively high molecular weight. These observations were largely confirmed by Williams and coworkers, who have since shown that an inhibitory factor is present also in rabbit epididymal secretions (Weinmann and Williams, 1964), and that DF occurs in stallion, boar, and monkey seminal plasma but not in that of the dog or rooster (Dukelow *et al.*, 1967a). The fact that the DF in rabbit seminal plasma was retained in the precipitate produced by treatment with 60% ethanol at —30 C (Bedford and Chang, 1962) suggested that DF might be associated with a protein; the immunological investigations of Hunter and Nornes (1969) make it seem possible that the conjugated or crude rabbit DF exists as a glycoprotein. Rabbit DF activity can no longer be precipitated by centrifugation following exposure to pronase, after which the decapacitation activity persists in the supernatant fraction (Williams *et al.*, 1967). It is likely, therefore, that centrifugable DF in the rabbit is bound to protein but that the active factor is not protein. The active moiety itself appears to be a small molecule of less than 2000 mol wt, on the basis of molecular sieving experiments (Pinsker and Williams, 1967), and possibly may be carbohydrate. Claims have been made that DF activity is destroyed after treatment with B amylase (Dukelow *et al.*, 1966). The implication here that the active element of DF is carbohydrate must be treated with caution, as these authors have themselves since cast doubt on the purity of the enzyme preparations used. At present, it is fair to say that we do not know the basic nature of the active DF in seminal plasma.

The mode of action of DF, its effect apparently being reversible (Chang, 1957), has remained something of a mystery. Seminal plasma is not normally toxic to and does not affect the motility of capacitated sperm. There is evidence, however, that the potentially competent spermatozoa in one ejaculated sample may not all become fully capacitated within the same minimal period (Dziuk, 1965). Hence, it could be argued that DF may in fact destroy the fertilizing ability of any completely capacitated sperm, and that during the supposed "recapacitation" period other sperm in the sample would then be able to continue and complete their capacitation for the first time. This explanation is rendered unlikely, by the demonstration that "recapacitation" apparently requires approximately as long as initial capacitation of ejaculate or epididymal sperm (Dukelow, Chernoff, and Williams, 1967b). Since the inhibitory effect of DF really does appear reversible, this militates against the idea of structural changes being expressed in sperm as a concomitant of capacitation *per se* (Bishop, 1961; see below, "Changes in Sperm during Capacitation"). There are reasons to believe that DF may act at the sperm surface. This question has been studied by the author in two types of experiment. In the first, an attempt was made to discover whether exposure of capacitated rabbit sperm to DF affects the establishment of sperm/egg contact in the immediate postovulatory period; this seemed possible since epididymal or ejaculate sperm have little ability to reach and contact the surface of the rabbit egg *in vivo* during the major part of its fertile life (Bedford, 1967c; and section on significance of capacitation in development of acrosome reaction and in fertilization, of this paper). In this experiment, rabbit uterine sperm

samples obtained 12 hr after mating were treated with seminal plasma 3:1 v/v, concentrated by mild centrifugation for 3–4 min, and 0.025 ml of the capacitated sperm/DF mixture was then inseminated directly into one fallopian tube of a recipient female about 1 hr postovulation. An equivalent number of untreated uterine sperm in Tyrode's solution were inseminated a few minutes later into the contralateral tube. This experiment was performed in six animals. When collected 5–6 hr later, 82% of the eggs exposed to the untreated uterine sperm were fertilized and showed significantly greater mean numbers of sperm adhering to the zona surface (24.6 sperm/egg) compared to those (unfertilized) in the contralateral tube (4.8 sperm/egg) exposed to an equivalent number of DF-treated sperm. These results suggest that DF acts in some way to hinder the passage of sperm to the zona surface during the fertile life of the egg. This could possibly occur after direct inhibition of some acrosomal enzyme activity, since DF has been claimed to inhibit a "corona cell removing enzyme" (Zaneveld *et al.*, 1968), though this idea does not seem to fit with evidence that the action of DF is reversible. An alternative possibility that DF acts to stabilize, or to mask, active sites on the sperm head surface appears more likely in light of the results in a second type of experiment described here. Observed in the electron microscope, the acrosomes of > 95% of rabbit 12-hr uterine sperm were disrupted and lost after 30-min exposure 1:1 v/v to fresh rabbit follicular fluid; the acrosomes of an equivalent number of epididymal or ejaculate cells were not affected by this treatment. Control samples were examined in both groups. Uterine sperm samples were next treated 3:1 with rabbit seminal plasma; this uterine flush/seminal plasma sperm suspension was centrifuged after 10 min and the supernatant fluid decanted. By contrast, the acrosome and the head plasma membrane remained intact in most DF-treated uterine sperm, after exposure to follicular fluid, and were comparable morphologically to those in untreated control samples. This experiment suggests that seminal plasma DF serves to protect or stabilize the plasma membrane, and probably acts to prevent the normal acrosome reaction at the site of fertilization. Similar observations in the phase-contrast microscope have been made in hamster spermatozoa treated with DF before exposure to follicular fluid (Gwatkin, personal communication).

Although the spermatozoa of most invertebrates are, in a sense, capacitated when they leave the male, it is possible that the stimulus for the acrosome reaction is basically similar in invertebrates and mammals. The fact that the action of DF is not species-specific among mammals, led us to test the effect of rabbit DF on the acrosome reaction and fertilization in *Arbacia punctulata*. These experiments performed with Mr. Kenneth Gregg in the laboratory of Dr. C. B. Metz showed that rabbit seminal plasma at a concentration of 25% has no inhibitory effect on the induction of the acrosome reaction by a standard "egg water" (fertilisin) preparation. In further experiments, fresh ova and relatively small numbers of sperm were each incubated for several minutes in a medium of > 90% rabbit seminal plasma which had been dialyzed against sea water. A high fertilization rate was observed when these gametes were allowed to mix in the same DF-containing medium, notwithstanding the fact that dialysis against sea water has no effect on DF. It thus appears that DF has no inhibitory effect on fertilization in *Arbacia* at least. If DF does indeed act at the sperm head surface, then this might be construed as evidence of some basic difference in the nature of the receptor sites at this surface in mammals and echinoderms, respectively.

Relationship of Capacitation to the Fertile Life of Spermatozoa

The fertile life of mouse sperm approximately equals their short motile life (McGaughey *et al.* 1968) but sperm in the female

retain their motility for a longer period than their fertility in the rat (Yochem, 1929; Soderwall and Blandau, 1941;), the guinea pig (Yochem, 1929; Soderwall and Young, 1940), the sheep (Dauzier and Wintenberger, 1952) and the rabbit (Hammond and Asdell, 1926; Noyes and Thibault, 1962; Chang and Pincus, 1964). Noyes and Thibault have suggested that some hours after completion of functional capacitation, a deterioration of the capacitated state occurs, with a concomitant loss of fertilizing ability in otherwise motile spermatozoa. Support for this idea came from Soupart and Orgebin-Crist (1966) who reported that in sperm incarcerated in a ligated uterus, capacitation was delayed but the fertile life of these sperm seemed to be prolonged beyond that in a normal uterus. Later, Bedford (1967e) showed that rabbit sperm residing in the rat uterus, in which their capacitation is not completed, retain their fertilizing ability for the whole of their motile life. This, sometimes a period of 50–55 hr, is considerably longer than the fertile life of rabbit sperm in the rabbit uterus.

The reason for apparently earlier failure of fertilizing ability after capacitation in some species is not known. It may be that the increased metabolic activity of sperm in the tract (see below, "Changes in Sperm during Capacitation" cannot be maintained—this would be important if sperm do require extra motive force to penetrate the zona. This point could easily be subjected to test, however, with samples of rabbit sperm incubated in the rat uterus. It is equally possible that another more subtle aspect of the preliminaries to fertilization, perhaps involving hypothetical sperm head surface receptors or some other facet of the acrosome reaction, might undergo a relatively rapid deterioration after activation.

In other species the apparent relationship between capacitation and diminution of the fertile life of sperm is not obvious. Ferret sperm first become capacitated in the oviduct in 3–4 hr (Chang and Yanagimachi, 1963), yet they wait in the female some 30 hr for arrival of the ovum (Hammond and Walton, 1934), and they may remain fertile for more than 100 hr (Chang, 1965). Pig sperm passing from the vagina to oviduct can become capacitated within 2 hr (Hunter and Dziuk, 1968), but tubal eggs may be fertilized 48 hr or more after vaginal insemination (Du Buisson and Dauzier, 1955; Pitkjanen, 1960). Possibly, the sperm of some species are capacitated completely only when they reach the fallopian tube, and for a proportion of the sperm population this may not happen until many hours after vaginal insemination. Certainly, there is suggestive evidence that once sperm enter the fallopian tube, their fertile life thereafter is relatively brief (Quinlan, Maré, and Roux, 1932; Edgar and Asdell, 1960; Dukelow and Williams, 1967), though this, perhaps, needs to be substantiated. Clearly, we now need greater understanding of the respective role of the uterus and fallopian tube for capacitation, in a variety of different species.

CHANGES IN SPERM DURING CAPACITATION

There is little understanding of the exact nature of the changes which occur in sperm as a direct concomitant of capacitation. Any approach to this question must logically involve a consideration of the morphology of the sperm, the properties of the surface, and the metabolic state of the sperm cell.

Morphological Aspects of Capacitation

Since the recognition of the need for capacitation, it has seemed an attractive possibility that structural changes might occur in the sperm as a concomitant of this phenomenon. It is probable, however, that no morphological changes of note accompany the process of capacitation *per se*.

The results in several light- and electron-microscope studies (Bedford, 1963a, 1964a; Austin, 1963; Adams and Chang, 1962b) indicated that no structural change is involved

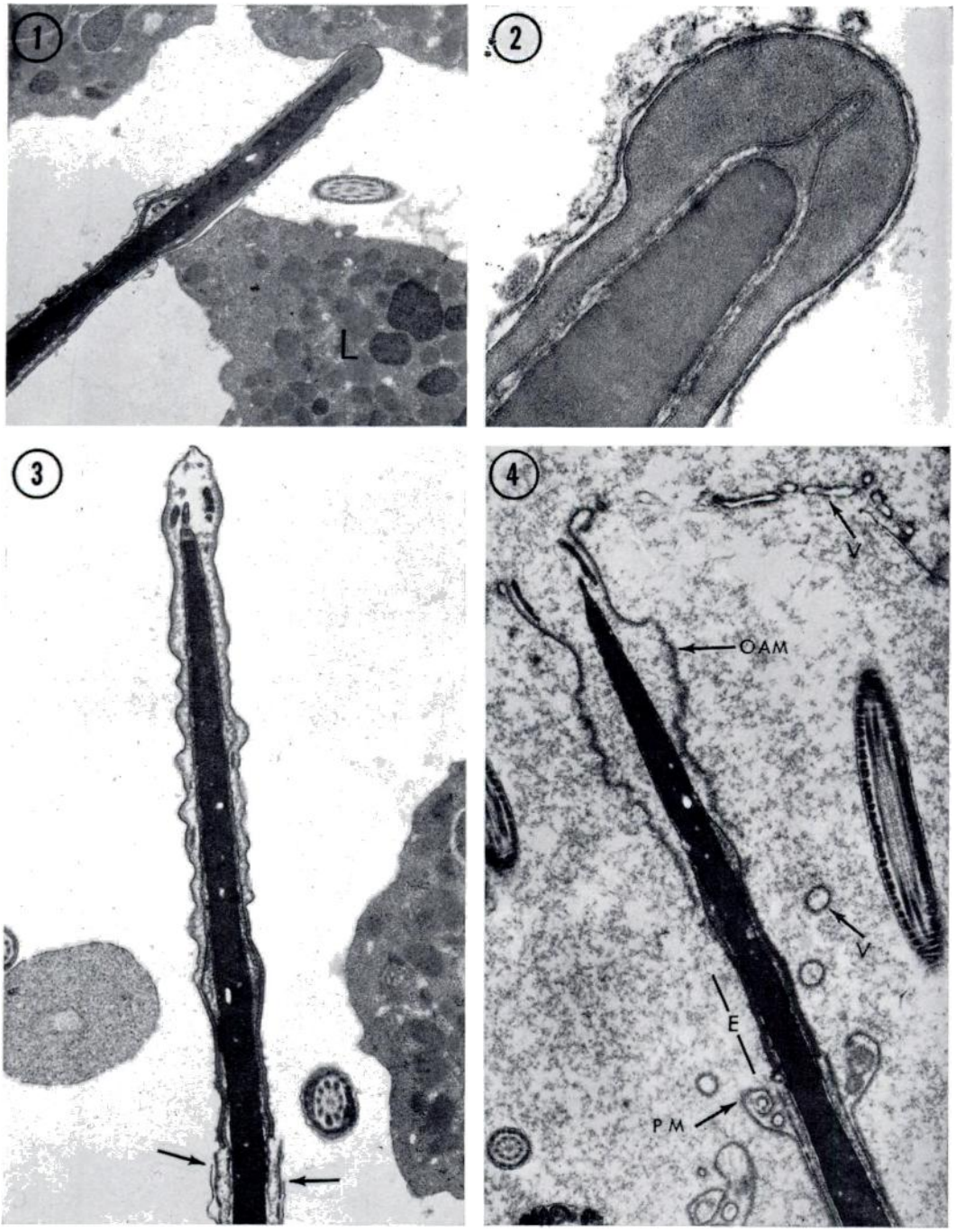

FIG. 1. Typically, no fine structural change is seen in a majority of rabbit sperm recovered from the uterus 13–15 hr after natural mating. This rabbit sperm is sticking to uterine leukocytes (L) by the intact plasma membrane overlying the acrosome. ×16,000.

FIG. 2. Higher magnification of the rostral region of a sperm recovered from the uterus 15 hr after mating. The plasma membrane of the sperm displays a typical trilaminar arrangement, and the material which oc-

in capacitation of rabbit spermatozoa. Furthermore, the fact that seminal plasma may reversibly inhibit the capacitated state (discussed below) militates against the possibility that irreversible structural changes take place as a feature of capacitation itself. Nonetheless, since capacitation requires rather longer in the rabbit uterus than had been thought previously (Bedford, 1969a), this question of structural change has been re-examined in the electron microscope. Sperm were recovered from the rabbit uterus and fallopian tubes between 13–15 hr after insemination and fixed in 3% glutaraldehyde in phosphate buffer for 30 min, followed by postosmication and embedding in Epon 812. These recent observations allow essentially the same conclusions as those reported previously, i.e., the great majority of 13- to 15-hr uterine sperm remain quite intact and show no change in the proportions of their organelles. The absence of any ultrastructural change in most rabbit sperm recovered from the uterus or fallopian tube 13–15 hr after insemination argues against the existence of a direct structural concomitant of capacitation itself. In the few cases in which breakdown of the acrosome was evident, or appeared imminent (Fig. 3), the plasma membrane and acrosome were disintegrating haphazardly, as separate structures; this contrasts with the coordinated breakdown and fusion of the outer acrosomal and plasma membranes seen during the true acrosome reaction (Barros *et al.*, 1967 and Figs. 5 & 6). The differences between the "true" and "false" acrosome reaction are considered later in the section devoted to this topic.

Current notions in regard to the morphological aspects of capacitation in other species are somewhat less clearcut. In an important paper which describes phase-microscope observations of sperm penetration in the golden hamster, Chinese hamster, Libyan jird, and guinea pig, Austin and Bishop (1958) concluded (p. 241) that "the acrosome becomes modified in spermatozoa passing through the female genital tract and is detached before the spermatozoon penetrates the zona pellucida. These changes in the acrosome are considered to constitute capacitation." A decade later, Yanagimachi (1969a), studying the capacitation of hamster sperm, has stated (p. 283), "The importance of the alteration (disintegration) of the acrosome as a step of sperm capacitation was thus confirmed . . . such an acrosomal change must represent the final phase of sperm capacitation." Although it might seem that the situation in the rabbit and hamster differ in principle, this question is really a semantic one; i.e., what exactly is meant by or included within the term "capacitation"? On the basis of available evidence it has been suggested (Piko, 1964, 1970; Bedford, 1968a), that the preliminaries to fertilization involve, first, the event of capacitation, which is considered as a discrete physiological change, perhaps the unmasking of surface receptor sites. Second, capacitation, when completed, is thought to render the sperm responsive to "fertilisin-like" stimuli emanating from the granulosa cell/egg mass,

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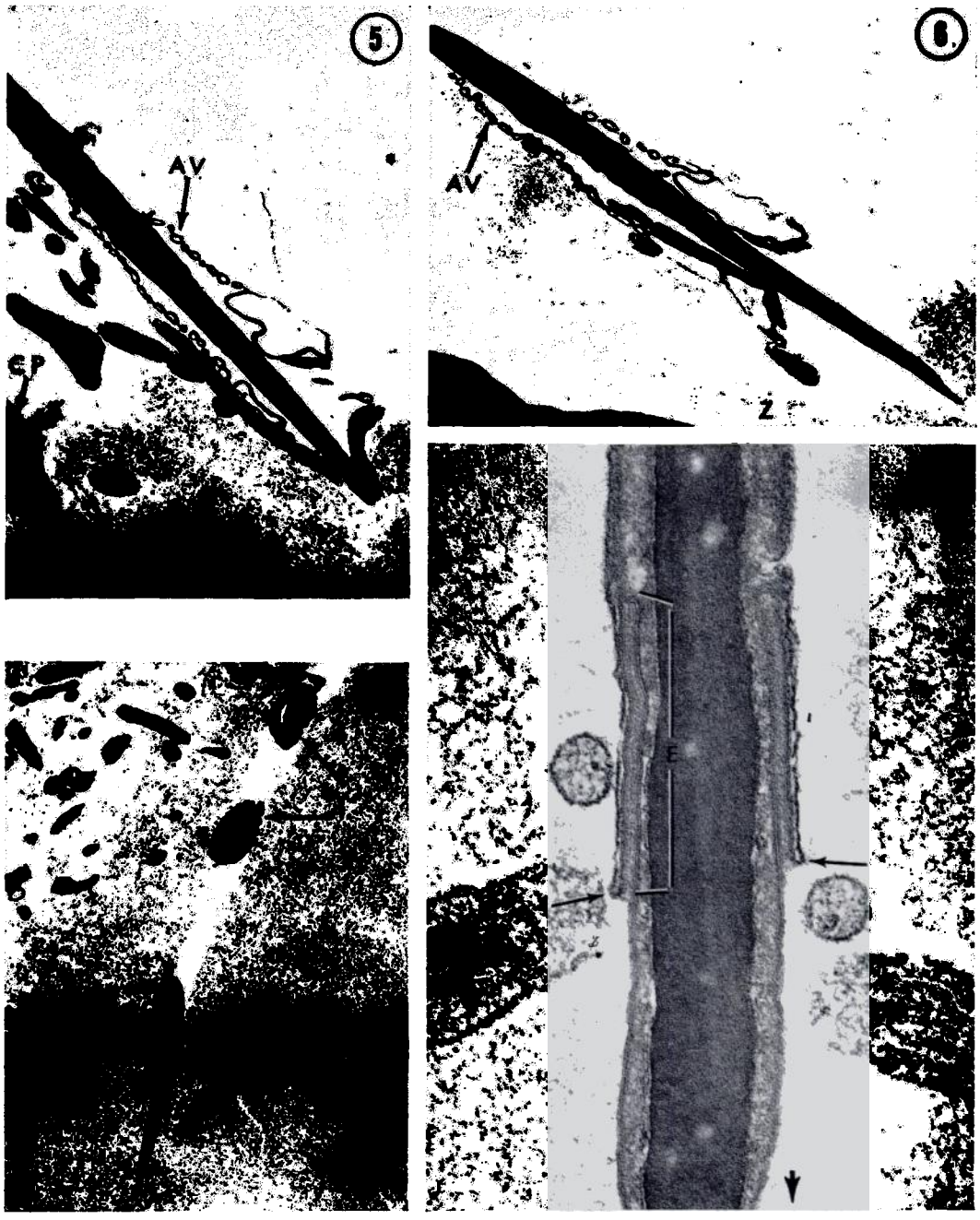
cupies the apical subacrosomal space appears rather more dense in the region immediately beneath the inner acrosomal membrane. ×118,000.

FIG. 3. Uterine sperm recovered 13 hr after mating. This shows the commencement of acrosomal degeneration as a "false" reaction; the plasma membrane overlying the acrosome has been lost and remains only over the postacrosomal region (arrowed). The acrosome is beginning to swell at the apex and much of its content has already been dissipated. ×23,500.

FIG. 4. This ejaculated rabbit sperm has been exposed to fresh rabbit serum, which in most cases induces a false acrosome reaction. In this instance, the plasma membrane around the acrosome has been lost; this discarded membrane often forms a series of vesicles (V) or whorls. The plasma membrane (PM) remains over the postacrosomal region. The outer acrosomal membrane (OAM) is being sloughed, leaving intact only the inner membrane of the acrosome and the posterior "equatorial" segment (E). ×23,500.



FIGS. 5–6. Rabbit sperm on the zona surface (Z) of eggs recovered 11½ hr after natural mating. In both cases the plasma membrane and outer acrosomal membrane have undergone the characteristic fusion and vesicle formation (AV) of the "true" acrosome reaction. As the vesiculated elements fall away from the now bare rostral surface, the sperm begins to enter the substance of the zona. CP = corona cell process. ×21,000.

FIG. 7. Rabbit sperm penetrating the zona pellucida of an egg recovered 11½ hr after mating. Note the

or present in follicular fluids; this interaction then sets in motion the series of events involving membrane breakdown and fusion, which constitute the "acrosome reaction." The distinction between capacitation and onset of the acrosome reaction should be clarified, not merely for semantic reasons. This distinction is important in a broader zoological sense, since present ideas in regard to the nature of the acrosome reaction now favor the concept of a basic similarity in the morphology, and mode of induction of the acrosome reaction, in sperm from such diverse groups as the Enteropneusta, Echinodermata, Annelida, Mollusca and now the class Mammalia (see Colwin and Colwin, 1967a). Ejaculated sperm from species belonging to these other phyla are immediately competent to undergo the acrosome reaction and do not require the prior conditioning of capacitation. Hence it appears inconsistent to consider the structural changes of the acrosome reaction as being a step in the process of capacitation *per se*.

Metabolic Changes in Sperm in the Female Tract

An initial report of changes in the energy metabolism of rabbit spermatozoa during the first hours of residence in the uterus (Hamner and Williams, 1963) has been confirmed in principle in the rabbit and other species (Iritani *et al.*, 1969).

The basis for this response by sperm has not been fully investigated, but the most recent evidence indicates that the total stimulatory effect of the female secretions depends on at least two separate factors, one being a dialyzable element which is heat-stable (possibly-HCO_3 or a simple substrate?), while the other appears as a nondialyzable heat-labile substance. This latter could be an enzyme, although there is no other evidence for this speculation as yet (Iritani *et al.*, 1969). During experiments concerned with capacitation of hamster sperm *in vitro*, Yanagimachi (1969b) noted that detoxified bovine follicular fluid contains a sperm-stimulating factor, the presence of which *in vitro* is correlated with the appearance of "extremely active" sperm. This factor has the basic characteristics (nondialyzable, heat-stable) of one of the factors identified by Iritani *et al.* (1969).

Notwithstanding the obvious possibility that metabolic changes in sperm may be of functional significance in capacitation, we have no direct evidence that this phenomenon is of importance in the economy of the fertilizing sperm. It is possible, for instance, that this merely reflects permeability changes in the plasma membrane, perhaps associated with capacitation, which thereby allow substrate and ions to pass more freely. Evidence as to the state of sperm in the progesterone-dominated uterus might help to resolve this point, since capacitation is almost completely inhibited in this milieu (Chang, 1958). Unfortunately, the studies reported so far are conflicting. Brackett (1968) has declared that the uterus of the pseudopregnant rabbit can no longer stimulate sperm metabolism. Murdoch and White (1967), on the other hand, found no significant difference between the estrous and pseudopregnant uterus in this respect, but they had treated

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oblique approach through the zona (arrow is at 90° to zona and vitelline surfaces) and the close apposition of the leading surface of the head to the substance of the zona. T = sections of tail midpiece, with mitochondria. ×8200.

FIG. 8. Midsagittal section of a sperm head which is beginning to penetrate the zona of an egg recovered 12 hr after mating. This shows clearly the point of fusion (arrowed) of the plasma membrane with the outer acrosomal membrane, at the anterior border of the equatorial segment (E) (cf. Fig. 11). Note the diffuse perinuclear material lying beneath the plasma membrane in the postacrosomal space and more rostrally in the subacrosomal space. Arrowhead denotes direction of sperm entry. ×88,000.

their test animals with progesterone for only 3 days before use. It is hoped that this question will be quickly settled.

The observation of "extremely motile" sperm, *in vitro*, at a time approximately coincident with the appearance of capacitated sperm (Yanagimachi, 1969a), is interesting and raises the question of whether changes occur in the motility pattern of the sperm of other species during their residence in the female.

Evidence for Changes in the Sperm Surface During Capacitation

There is, as yet, no definite evidence that capacitation involves changes in the sperm head (and tail?) plasma membrane. This is, perhaps, not surprising in view of the absence of any real understanding of the organization of membranes in general. Nevertheless, the particular character of the acrosome reaction, which can apparently be stimulated only after capacitation, seems to provide circumstantial evidence for inferring the occurrence of changes in the plasma membrane of sperm during their residence in the female. Moreover, the finding that only after capacitation will hamster sperm fuse with the zonaless ovum (Yanagimachi and Noda, 1969), implies that in this species [though perhaps not in the rat (Toyoda and Chang, 1969)], changes occur in the plasma membrane of the postacrosomal region of the head during capacitation. Further supporting evidence lies in the susceptibility of the capacitated state to seminal DF, which, if covalently linked to a large molecule, must probably act at the sperm cell surface. We do not know if capacitation involves loss of some element from the sperm surface, the binding of a substance from the female tract, or both. The demonstration that hamster sperm can be capacitated in an almost-defined medium (Bavister, 1969), perhaps tends to militate against the idea of opsonization of the sperm surface in the female, and although Symons (1967) noted that the heads of some immotile uterine rabbit sperm had bound uterine globulin, he concluded that this does not happen to viable cells during capacitation.

Weinmann and Williams (1964) proposed that capacitation constitutes the removal of a coat, possibly seminal DF, from the sperm surface. This may well be so, but the fact that partial capacitation can occur in the rabbit, and evidence of synergism between capacitating activity in the uterus and fallopian tube (see section on role of the female tract and follicular secretion), implies the involvement of more than one such simple step before the functional state is achieved.

Ericsson (1967a,b) and Ericsson and Baker (1967) noted that fluorescent tetracycline HCl binds to the sperm head and is removed in the estrous uterus, but not in the pseudopregnant, immature, or ovariectomized uterus. This apparent correlation between the removal of surface tetracycline and the environmental conditions which will support capacitation suggested that loss of this fluorescent indicator might be used as a test for capacitation. Unfortunately, this technique is not acceptable as an assay for capacitation in the rabbit, as it has been pointed out by Vaidya *et al.* (1969) that: (1) tetracycline is removed from sperm in the rabbit uterus or fallopian tube after about 2 hr, or in approximately one-quarter or less of the time required for completion of capacitation in either of these sites; and (2) that tetracycline removal does not indicate partial capacitation because a significant degree of capacitation occurs in 12–14 hr in both the immature uterus and the uterus of ovariectomized females, in neither of which is tetracycline removed from sperm. It may be noted that some initial phase of capacitation can occur even in the progesterone-dominated uterus (Bedford, 1967b). It is probable, therefore, that removal of tetracycline from the sperm surface involves other enzymes in the female tract which are affected by the endocrine state of the female, but which have little bearing on capacitation.

In company with the lack of any structural change, the plasma membrane of the rabbit sperm head recovered from the uterus 13–15 hr postcoitum retains the classical trilaminar appearance when seen at high magnification in the electron microscope (Bedford, 1969b and Fig. 2). The question of whether there are modifications in the appearance of membrane laminae after capacitation is currently being studied in the author's laboratory, using different methods of visualization of the membrane and its surface components. Experimental studies with the electron microscope suggest that the permeability or osmotic properties of the plasma membrane may change after capacitation (Bedford, 1964b). Subtle surface membrane changes may also be detectable by uterine leukocytes which readily engulf intact uterine spermatozoa, but which apparently can ingest only the damaged cells in ejaculate or epididymal sperm samples (Bedford, 1965). Although Symons (1967) has concluded from immunofluorescence studies that such phagocytosis may be facilitated by opsonization with uterine globulin which seemed to bind only to immotile sperm, it has been often observed that the heads of highly active uterine sperm may also be ingested without impedance of the tail movement. The possibility of a change in the response of sperm to surface-active agents, after capacitation, is raised by the finding that a potent substance in the seminal plasma of a fertile rabbit, which caused only head agglutination of rabbit ejaculated or epididymal sperm, immediately immobilized rabbit uterine and most foreign sperm to which it was exposed (Bedford, 1970a). Thus, rabbit sperm undergo a change in some of their susceptibilities as well as in their functional state, during capacitation.

There are several methods available for objective study of the character of the cell surface. It is possible, however, that immunological investigation of the fate of sperm surface antigens derived from the accessory secretions, e.g., the sperm-coating antigen, studied by Weil and colleagues (Weil, 1967) and recently identified as an iron-binding protein (Roberts and Boettcher, 1969), may be complicated by the existence of antigenic counterparts in the female secretions (Menzoian and Ketchel, 1966). Direct measurement of the character of charged groups at the sperm surface can be performed using electrophoretic methods which, after initial application as a method for separation of X and Y sperm, have been adapted for objective study of the sperm surface (Bangham, 1961; Nevo *et al.*, 1961) Using this technique, the distribution and intensity of the net negative charge at the sperm surface has been shown to change markedly during the passage of sperm through the rabbit epididymis (Bedford, 1963b). This approach is being applied in several laboratories to the question of whether measurable changes occur in the sperm surface as a consequence of capacitation. The sperm head plasma membrane does not appear to lose its adhesive character after residence in the uterus, as some uterine sperm recovered 12–15 hr after mating still are able to adhere quite readily to leukocytes and to each other. It is an interesting fact that the sperm of almost all eutherian mammals show a distinct tendency for head autoagglutination, with the exception of anthropoid monkey and human sperm (Bedford, unpublished). This would seem to imply the existence of some specific difference in the surface of primate sperm (excluding the sperm of the prosimiae, which agglutinate readily), and this may have some bearing on capacitation in these species.

EVENTS INVOLVED IN FERTILIZATION

The Acrosome Reaction

It has been known for some years that the acrosome may be discharged during the degeneration of mammalian sperm (Blom, 1945; Hancock, 1952) and Bedford (1963a) has concluded that the loss of the acrosome from some rabbit sperm in the uterus is prob-

ably a manifestation of a similar process, rather than of capacitation. While recognizing the possibility that acrosomal detachment may occur as a postmortem change, Austin and Bishop (1958) nevertheless considered that the changes they observed in the heads of rodent sperm in the fallopian tube, or passing through the cumulus, represented a coordinated physiological breakdown of the acrosome and that loss of these elements is a normal preliminary to penetration of the zona.

It is now clear from studies with the electron microscope that the acrosome can be discharged from the sperm head in the course of either a "true" or a "false" acrosome reaction. The "true" or physiological acrosome reaction, described for the hamster and rabbit by Barros *et al.* (1967), involves progressive breakdown and fusion between the plasma membrane and outer acrosome membrane, which together come to form a series of vesicles. These appear as a loose shroud around the anterior half of the sperm head (Figs. 5 & 6). A series of fenestrae between these points of fusion are presumed to provide ports through which a progressive loss of acrosomal content probably occurs after the onset of the vesiculation. The vesiculated plasma membrane/acrosome complex is always lost from the sperm head before penetration of the zona begins (Fig. 7). In the hamster, electron-microscope studies have shown that detachment of the acrosome after membrane fusion and vesiculation need not involve destruction of its basic form; the ultrastructural organization of the hamster acrosome is consistent with phase-microscope observations of elevation of the acrosome as a whole intact unit (Franklin *et al.*, 1968).

The "false" reaction describes the changes in the acrosome which are usually seen in testicular, epididymal, ejaculated, and capacitated sperm as a feature of degenerative breakdown (Figs. 3 and 4). Recently, Saacke and Marshall (1968) have attempted to equate degenerative acrosomal changes seen in fresh bull sperm using differential interference microscopy, with those observed in the electron microscope. The tendency for this type of breakdown varies in different species; for instance, the acrosome of guinea pig and hamster sperm is less stable and tends rather easily to undergo the breakdown seen in the false reaction, compared with that of the rabbit or monkey. In contrast to the true reaction, the false reaction is seen as a random loss of the plasma membrane and outer membrane of the acrosome, as separate entities (Figs. 3 & 4). In some degenerating rabbit sperm the plasma membrane is shed, but the acrosome remains intact, or shows the beginnings of degenerative swelling and dissipation of the contents (Fig. 3). A good example of the false reaction is seen after the treatment of uterine or ejaculated rabbit sperm with fresh homologous serum (Bedford, 1969b), which is known to be spermicidal (Chang, 1947). In about 98% of sperm exposed to fresh serum, the plasma membrane and outer membrane of the acrosome undergo rupture (Fig. 4) and often these elements are lost, leaving only the exposed inner membrane of the acrosome. In nearly all of these cases, the plasma membrane alone forms separate vesicles or membranous whorls, and the acrosome ruptures in a reaction which often first develops at its apex (Fig. 4).

The true and false reactions can almost always be distinguished at the ultrastructural level according to the features described above. Nevertheless, the plasma membrane/acrosome fusion and vesiculation (Figs. 5 & 6) is not an absolute prerogative of the true acrosome reaction, since this type of reaction has been noted on rare occasions in testicular, epididymal, ejaculated, and uterine sperm, and in ejaculated rabbit sperm exposed to fresh serum. However, this mode of acrosomal breakdown is seen in less than 1% of degenerating sperm, in contrast to capacitated sperm in the vicinity of the egg in which the reaction involving fusion between the plasma and acrosomal membranes appears to be the rule.

Significance of Capacitation in Development of the Acrosome Reaction and in Fertilization

It is likely that capacitation prepares or sensitizes mammalian sperm in some way, enabling them to undergo the acrosome reaction in response to a specific stimulus. There is some evidence to support this idea in the hamster and in the rabbit. In the hamster, fresh follicular fluid will induce structural changes in the acrosome only after 2–3 hr incubation in this medium, but no visible acrosomal changes in motile epididymal spermatozoa occur before this time (Yanagimachi, 1969a). Essentially similar results have been obtained in experiments using hamster tubal fluids [presumably a mixture of follicular and tubal fluids (Barros and Austin, 1967)]. The ability to induce the acrosome reaction is minimal in fluid from the immature follicle and increases with maturation of the follicle (Barros and Austin, 1967; Yanagimachi, 1969a). Analysis of the active agents in (bovine) follicular fluid suggests that the acrosome reaction-inducing factor is a nondialyzable heat-labile substance and that this works synergistically with another dialyzable sperm-stimulating factor (Yanagimachi, 1969b).

In the rabbit, no gross structural changes are seen in sperm as a concomitant of capacitation in the uterus or fallopian tube. However, fresh follicular fluid from superovulated rabbits will induce rupture and discharge of the acrosome in these sperm within 30 min, though this medium has no visible effect on ejaculated or epididymal sperm in this time (Bedford, 1969a). Since many tubal rabbit sperm have been observed (in the electron microscope) to undergo acrosomal breakdown in the close vicinity of, or within the cumulus/corona cell mass, it has seemed possible that the reaction might be caused by substances emanating from this mass. This idea that capacitation might involve some change in the sperm surface which allows the rabbit acrosome to respond to hypothetical "fertilisin-like" substances has been tested in the following way. Sperm samples collected from the uterus 13–14 hr after mating were concentrated by centrifugation for 3–5 min at 1000 × *g*. Aliquots of these sperm samples in 0.025 ml were then inseminated into the fallopian tubes of HCG-injected rabbits, about ½–1 hr after ovulation. The tubal ova were recovered 1¼–1½ hr later and were prepared for electron microscopy. This procedure was repeated in a second group using epididymal or ejaculated sperm incubated for 10–12 hr *in vitro* in Tyrode's + 5% heated rabbit serum, before their tubal insemination. In a third group, epididymal or ejaculated sperm were introduced into the fallopian tube 2–3 hr before administration of an ovulation injection (i.e., about 12–13 hr before ovulation) and ova were flushed from the tubes about 14–15 hr after insemination, thus allowing about 1–1½ hr for the sperm to interact with eggs before their collection. The results of these manipulations, assessed in the electron microscope, showed clearly: (1) that rabbit sperm first incubated in the uterus for 13–15 hr will readily undergo the typical vesiculation acrosome reaction within 1½ hr of being placed in the vicinity of tubal eggs *in vivo;* some of these sperm had penetrated deeply into the zona, having shed the outer membrane of the acrosome; (2) that motile sperm incubated *in vitro* for 10–12 hr will not undergo the acrosome reaction within 2–3 hr of *in vivo* exposure to tubal ova. In this group, some of the intact sperm had burrowed into the loose material at the periphery of the zona, but in no case had they penetrated more deeply into the dense zona; and (3) that after residence in the fallopian tube for 12–13 hr before ovulation, many sperm will then undergo the normal acrosome reaction within 1½ hr of ovulation, when close to or adhering to the zona pellucida, and, as in group 1, will begin to penetrate deeply into the substance of the zona within this time.

These experiments seem to indicate that rabbit sperm in the vicinity of the egg undergo

the true acrosome reaction only when they have been exposed for some hours to the uterus or oviduct, before arrival of the ovum. A most striking additional point which arises from this experiment was the finding that in group 2 there were relatively few sperm associated with the granulosa cell mass, or sticking to the zona—and then only to its denuded areas. The extreme difficulty experienced in finding sperm on the zona surface, within 1½ hr after tubal insemination of relatively large numbers of noncapacitated sperm, bears out the conclusions from phase-contrast microscope observation that noncapacitated sperm have little ability, *in vivo,* to traverse the granulosa cells which surround the fresh ovum (Austin, 1960, 1961; Bedford, 1967c). (See below).

The Passage of Sperm Through the Granulosa Cell Investment and Zona Pellucida

Under normal circumstances, sperm present in the ampulla of the oviduct at the time of ovulation must traverse the barriers presented by the cumulus oophorus/corona radiata and the zona pellucida, and it is at this point that capacitation first becomes relevant in a functional sense. A good account of this preliminary phase of fertilization has been given recently (Piko, 1970) and, for this reason, only the essential details and most recent findings are presented here.

It was first pointed out by Austin (1960, 1961) that only sperm which have resided for some hours in the female tract are able to pass easily among the cumulus and corona cells to reach the zona surface; highly motile ejaculated or epididymal sperm could not pass into the cumulus mass and remained at the surface. The inference that capacitation endows sperm with an increased ability to traverse the cumulus oophorus (see also section V, B) was supported by another study in which rabbit eggs collected 6 hr after exposure to capacitated uterine sperm in the tubal ampulla showed significantly greater numbers of sperm on the egg surface (31.6 sperm/egg), compared with eggs exposed to greater numbers of noncapacitated sperm (1.9 sperm/egg) in the contralateral tube (Bedford, 1967c). Presumably the advantage conferred on the capacitated spermatozoa rests in their ability to undergo the acrosome reaction, with the concomitant release of the acrosomal enzymes, since noncapacitated sperm will adhere readily to the exposed zona surface.

In the electron microscope, the great majority of rabbit sperm in the fallopian tube, 13–15 hr after mating, appear structurally intact. It is probable that in this species the acrosome reaction does not begin before the sperm gains the close vicinity of the granulosa mass. In some instances intact rabbit sperm have been observed in the interstices of the granulosa cells. However, study of rabbit sperm among cumulus and corona cells showed that most sperm cells near the egg had begun or had completed the acrosome reaction, whereas a majority of the intact sperm were located at the periphery of the cell mass (Bedford, 1968a). In various rodents, the acrosome reaction occasionally occurs in motile sperm before their involvement with the granulosa cell mass (Austin and Bishop, 1958), and observations of hamster sperm *in vitro* in the phase-contrast microscope indicate that some which have shed the outer acrosomal remnants, and thus presumably the bulk of the acrosome content, are still able to pass through the cumulus and enter the zona pellucida (Yanagimachi, 1969a). In general, however, it is believed that most capacitated sperm begin to undergo the acrosome reaction, with consequent liberation of enzymes, in close proximity to or at initial contact with the cumulus/egg mass. The initial stages of the acrosome reaction probably ensure a gradual, rather than an abrupt emptying of the acrosomal material, which is presumed to dissolve the matrix between the granulosa cells, thereby facilitating passage of the sperm toward the zona pellucida. Here it may be noted, in passing, that these cells do also

achieve points of close contact bridged either by desmosomes (Bjorkman, 1962) or occasionally by true tight junctions (unpublished). Virtually all sperm found on the zona surface covered by dense corona radiata have undergone the vesiculation reaction or have already lost the outer vesiculated elements of the acrosome. Sperm adhering to the zona surface often retain the vesiculated acrosomal sheath (Figs. 5 & 6) and this sometimes remains at the surface when the sperm have passed into the substance of the zona. Intact sperm occasionally adhere to the zona *in vivo* and may burrow for some distance into its looser surface material. However, these have been noted only in those regions of the egg surface which have been divested of most or all of the corona cell investment.

It is clear from phase-contrast and electron-microscope studies, that the remnants of the outer acrosome/plasma membrane vesicular complex and the bulk of the acrosome content are discharged from the anterior region of the sperm head before true penetration of the zona pellucida begins (Fig. 7). As has been described in consideration of the acrosome reaction, the exposed inner membrane of the acrosome becomes the limiting membrane of the rostral region of the sperm head and thus it is this membrane which is immediately exposed to the substance of the zona.

Mammalian spermatozoa characteristically pass at an oblique angle through the zona, at a tangent to the circumference of the egg (Fig. 7). The penetration slit sometimes left by penetrating sperm may be straight or may curve gradually toward the vitellus. No satisfactory explanation is at hand to explain the consistently oblique angle of sperm entry.

Electron-microscope studies do not lend support to the idea that sperm in transit through the zona pellucida possess a rostrally placed acrosomal or "penetration" filament (Hadek, 1963; Piko, 1969; Bedford, 1968a), as is suggested by some phase-contrast microscope views of sperm penetrating the zona pellucida. (Dickmann, 1964; Dziuk and Dickmann, 1965). Observation of this situation at the fine structural level suggests rather that such filament images are, in fact, clefts which appear in front of sperm within the zona in aging eggs (Bedford, 1968a).

The nature and exact location of the factor(s) which facilitate the passage of the sperm head through the resilient zona pellucida are still not clearly established. While it is acknowledged that the tail provides the motive force, the rather precise species-specificity of sperm passage across this barrier implies the existence of other factors which are currently embodied in the concept of a "zona lysin." Purified hyaluronidase has no action on the zona substance, but since this coat is dissolved by adequate concentrations of trypsin (Smithberg, 1953; Chang and Hunt, 1956), and by pronase (Mintz, 1962), one might expect the hypothetical zona lysin to be a proteolytic enzyme. It is interesting that "acrosome extracts" have been found to possess proteolytic activity as well as hyaluronidase; one of these preparations extracted from sheep sperm, characterized as a lipoglycoprotein, was sometimes effective in dissolving the rabbit zona (Srivastava *et al.*, 1965). A trypsin-like enzyme preparation extracted from rabbit sperm by Stambaugh and Buckley (1968) completely removed the zona from rabbit eggs within about 90 min. Notwithstanding these important findings, the inner acrosomal membrane in penetrating sperm shows no residual acrosomal content around its exposed surface (Fig. 8). It is this surface which first makes contact with the zona and is in close apposition to the substance of the zona in a majority of sperm penetrating fresh ova. If it is assumed that a lytic enzyme is indeed responsible for passage of sperm through the zona, then this enzyme must exist in close association with the inner acrosomal membrane. It is conceivable that the extraction procedures used in isolation of acrosomal enzymes might also remove the perinuclear material located in the subacrosomal space; the possibility re-

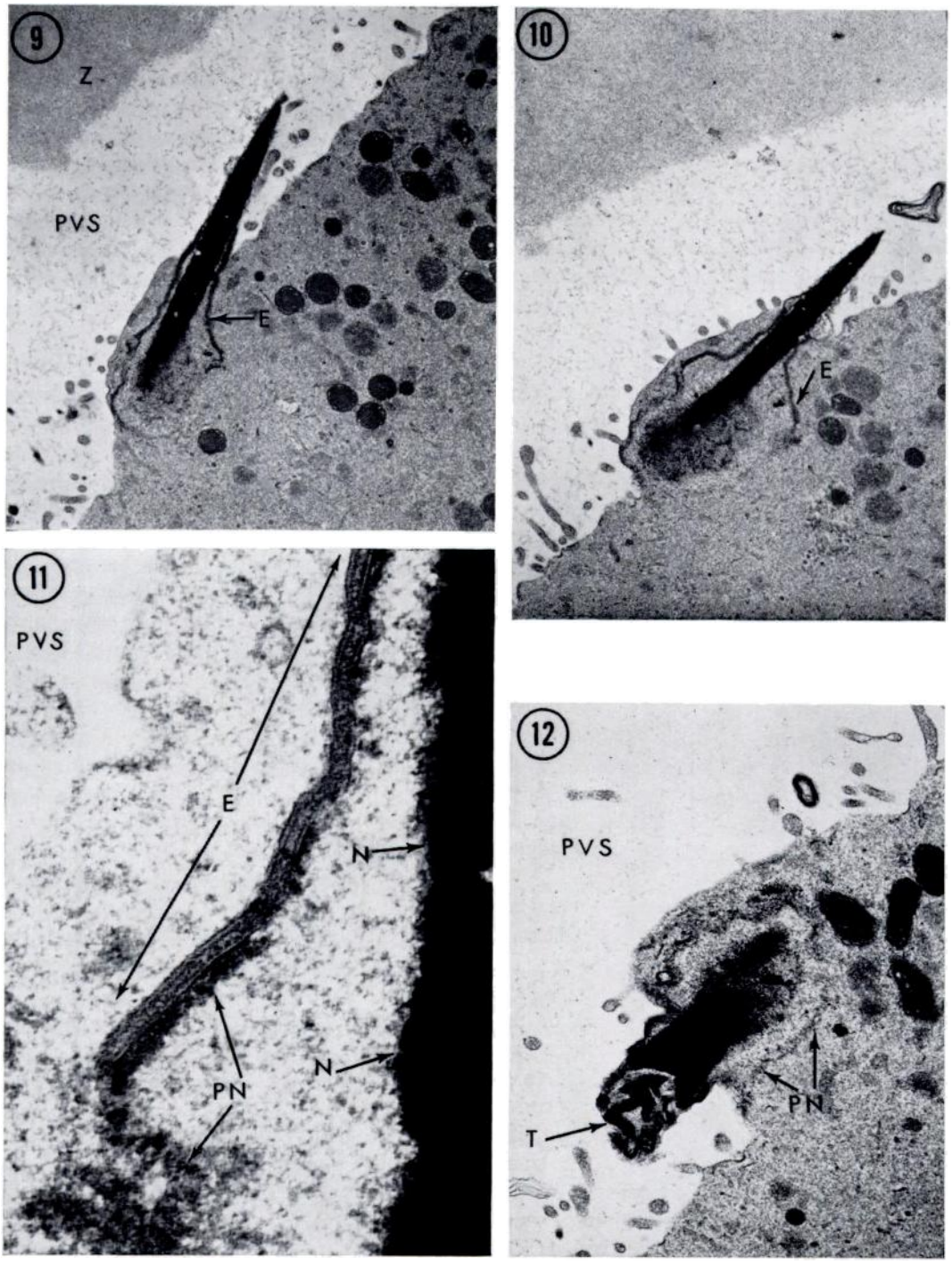

FIG. 9–10. Sections through the fertilizing sperm head in the early stage of fusion with the egg. Note that the sperm interacts with the egg over the posterior region of the head, in the initial stage of egg/sperm fusion. The nucleus within the egg is disintegrating though a major part still remains outside the egg, projecting into

mains, therefore, that activity diffusing through the membrane from the subacrosomal material (Fig. 8), especially that of the apical concentration often designated in the past as the "perforatorium," (Fig. 2), might affect the zona pellucida. Since we know almost nothing of the nature of this material, which does not seem to diminish in its density during passage through the zona, this suggestion should be received with the caution it deserves. Other suggestions as to the possible role of this subacrosomal material are offered in the following section.

Ultrastructural Aspects of Sperm Entry into the Vitellus

Investigation of this stage of mammalian fertilization presents obvious technical difficulties. The few observations made so far in the rat, hamster, and rabbit raise several interesting points and indicate that the process of sperm attachment to the egg plasma membrane, and its entry into the vitellus, is similar in all three species and differs from that in invertebrates. Nonetheless, further information is needed before the whole process can be described with any confidence.

In accord with the views of some earlier workers, Tyler (1959) proposed a phagocytic mechanism for sperm entry into the vitellus. It is now reasonably well established in several invertebrate species, however, that sperm fuse with the egg plasma membrane, by the membrane of the acrosome tubule which bounds the apex of the head, and which was originally the inner membrane of the intact acrosome (Franklin, 1965; Colwin and Colwin, 1967a). This mode of sperm entry into the vitellus is then accompanied by further fusion of the sperm and egg plasma membranes, so that the sperm head lying immediately within the vitellus no longer has a limiting membrane (Colwin and Colwin, 1967b). It has been proposed by these latter authors that this scheme of events probably represents the mode of sperm entry into the mammal egg (Colwin and Colwin, 1965). In agreement with this latter view, Szollosi and Ris (1961) concluded from brief observation of sperm penetration in the rat that the sperm head membranes rupture and fuse with the plasma membrane of the egg to form a continuous membrane over the egg and outer surface of the sperm. However, the few observations of this stage of fertilization in the hamster (Barros and Franklin, 1968; Franklin, unpublished), the rat (Piko and Tyler, 1964; Piko, 1964, 1967, 1969) and the rabbit (Figs. 9–15) indicate, that this membrane fusion occurs only over the post acrosomal region of the sperm head, and over the tail. It appears, on the contrary, that entry of the mammal sperm into the vitellus may well be a composite process involving both membrane fusion and a phagocytic phase. Initial contact, which occurs at a series of fusion points between egg microvilli and the sperm plasma membrane, according to Piko (1970), is established over the postacrosomal region; the sperm and egg plasma membranes undergo

the perivitelline space (PVS). The "equatorial" segment of the acrosome is carried into the egg and is seen (E) as an oblique projection from the sperm on both sides of the nucleus. ×10,500.

FIG. 11. Higher magnification of the persistent equatorial segment (E) of a fertilizing sperm, soon after its entry into an egg. The diffuse darker material (PN), which lies inside and below to this posterior acrosomal remnant, is probably the material which occupies the sub- and postacrosomal spaces in the intact sperm head. The relatively great length of the equatorial segment seen here (cf. Fig. 8), is a reflection of the oblique angle of section of the fertilizing sperm head. ×156,000.

FIG. 12. Posterior part of the fertilizing sperm head (the same sperm as in Figs. 9 and 10), in the early stages of entry into the vitellus. Note again that the nucleus within the egg is undergoing active disintegration and that the surface of the sperm appears continuous with that of the egg. The perinuclear material, which normally occupies the postacrosomal space, can be seen as a faint halo (PN) around the disintegrating nucleus. T = tail components. ×34,000.

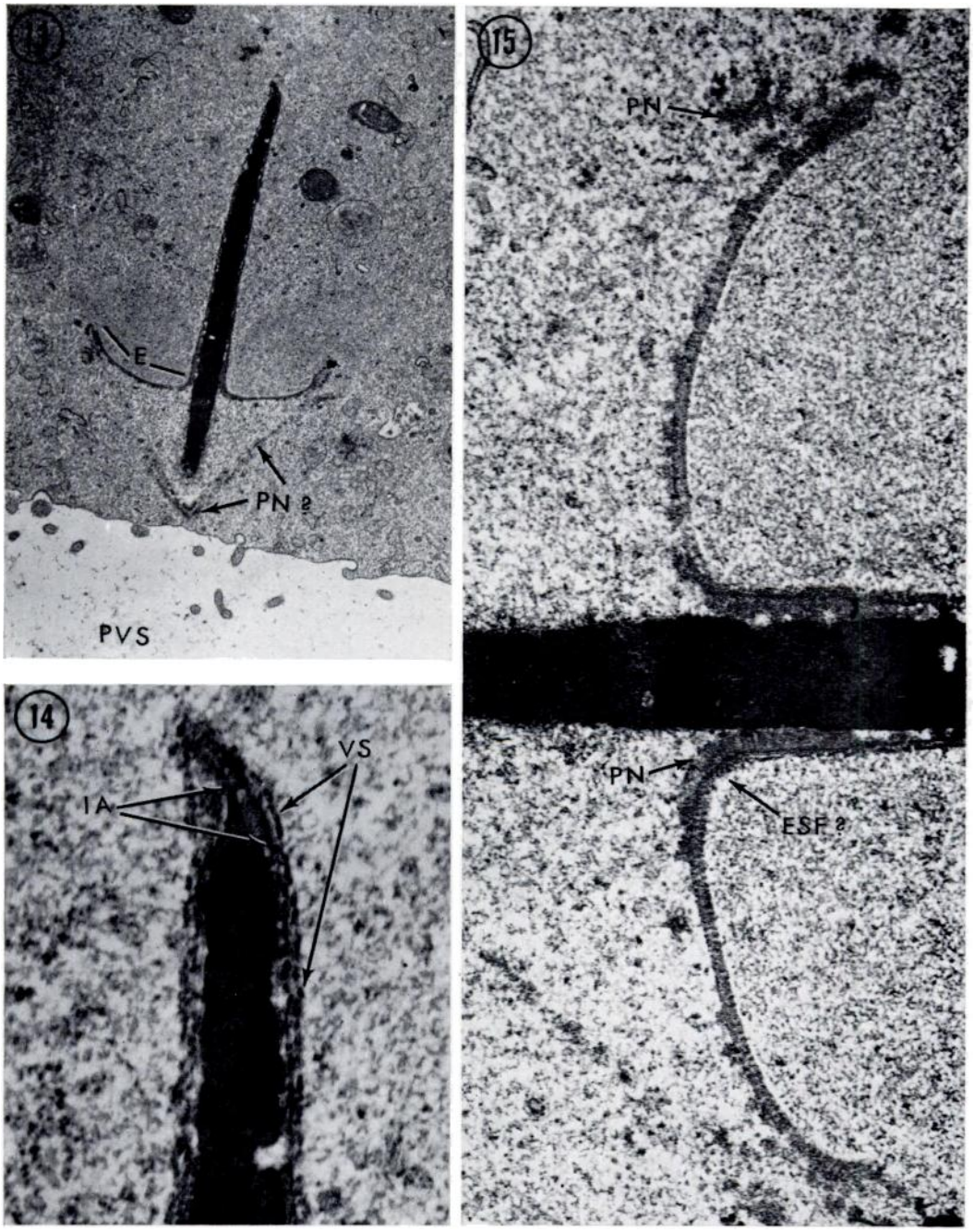

FIG. 13. Fertilizing rabbit sperm head. Note: (a) the disintegration of the posterior part of the nucleus; (b) the faint residual appearance of the perinuclear material (PN) of the postacrosomal space; and (c) the persistent equatorial segment (E) reflected away from the nuclear surface. PVS = perivitelline space. ×11,200.

FIG. 14. Higher magnification of the rostral part of the fertilizing sperm in Fig. 13. The sperm head is enveloped by two membranous layers; the outer membrane (VS) is considered to be the invaginated vitelline surface membrane and this overlies the inner acrosomal membrane (IA), which is the limiting membrane of

fusion in this localized region of the sperm, in the hamster (Barros and Franklin, 1968) and in the rabbit (Figs. 9 and 10). In contradistinction to the situation in invertebrates (Colwin and Colwin, 1967a), the anterior portion of the mammal sperm head, covered still by the persistent inner membrane of the acrosome is then engulfed, during which the plasma membrane of the egg invaginates around the anterior half of the nucleus. The rabbit sperm head lying completely within the vitellus in Figs. 13–15 shows two distinct membranes around the anterior half of the nucleus—the outer membrane (VS) is considered to have been derived from the egg and the inner (IA) to be the inner membrane of the acrosome (see Fig. 14).

An impressive aspect of this early phase of fertilization is the rapidity with which disintegration of the sperm nucleus occurs—a process which appears almost as the reverse of nuclear condensation in the spermatid. Although the dense sperm nucleus resists many types of treatment *in vitro,* exposure to the ooplasm brings about an immediate dehiscence of its composite nucleoprotein fibers. It is particularly striking in Figs. 9 and 10 and 12 and 13, that significant disintegration of the central part of the nucleus is already well underway, while the anterior half and the most posterior portion of the nucleus still project into the perivitelline space. Nothing is known of the factors in the ooplasm which bring about such decondensation of the fertilizing sperm nucleus; we have found, however, that the accomplishment of this process in ejaculated or capacitated rabbit sperm, *in vitro,* requires the disruption of disulfide crosslinkages (Calvin and Bedford, unpublished). The emergence of further details of sperm–egg fusion may hopefully shed some light on the functional significance of certain sperm head organelles, such as the persistent "equatorial segment" of the acrosome which accompanies the nucleus into the vitellus (Fig. 11), and for which no function has yet been established. Equally obscure is the role of the subacrosomal and postacrosomal material lying immediately superficial to the nuclear membrane (Fig. 8), and which becomes concentrated at the apex as the "apical ridge" or "perforatorium" (Fig. 2). In the posterior part of the head this material appears as the substance of the postacrosomal space ("postnuclear cap") which is limited internally by the nuclear membrane and externally by the sperm plasma membrane (Fig. 8). This perinuclear material appears to possess acid phosphatase activity which is evident particularly in the postacrosomal region, and in the subacrosomal bleb located immediately anterior to the equatorial region in the rabbit (Teichmann and Bernstein, 1969). The fact that this material displays enzymic activity is particularly interesting, since it must be this substance to which the ooplasm is first exposed after initial fusion/breakdown of the sperm and egg plasma membranes in the posterior region of the sperm head. As the incorporation of the sperm proceeds, the posterior (equatorial) acrosomal remnant becomes elevated away from the nucleus, serving to expose further the material situated more rostrally (see Figs. 2 and 4, Barros and Franklin, 1968; and Figs. 11 and 15 of this paper). On a speculative note, it is possible that this enzymic material might be responsible for initial metabolic activation of the ooplasm; this process could be similar to that achieved by Monroy *et al.*,

the rostral half of the sperm head. Note the persistence of the content of the "apical ridge" (sometimes referred to as the "perforatorium"). ×84,000.

FIG. 15. Higher magnification of the posterior region of the fertilizing sperm head in Fig. 13. Note how the lateral reflection of the equatorial segment, which here has a septate appearance, serves to reveal the diffuse subacrosomal material (PN). It is likely that fusion of the egg surface with the outer membrane of the posterior acrosomal (equatorial) segment occurred in the region designated here as ESF. ×51,000.

(1965), who reported the activation of sea urchin egg ribosomes after their exposure to trypsin. Alternatively, it may be that the substance in the postacrosomal and subacrosomal spaces serves to activate an element within the egg which brings about rapid disintegration of the compact sperm nucleus. The possibility that there is in the egg some particular element which affects the nucleus in this way is supported by the observation that when many sperm enter the (pig) egg under conditions which allow polyspermy, only the first few sperm develop into pronuclei; a majority of the sperm heads which enter subsequently undergo little swelling, suggesting the exhaustion of some essential ooplasmic component (Hunter, 1967). The proposition that these hypothetical "nucleolytic" elements might require activation by a specific stimulus at fertilization is, perhaps, supported by experiments which showed that sperm injected into Paracentrotus eggs did not swell or change in any way until the egg was later fertilized normally (Hiramoto, 1962). Neither of these speculative suggestions can easily explain the significance of the subacrosomal accumulation seen in most mammals at the apex of the nucleus (Figs. 2 and 14). Interpretation of this structure is made more difficult by its extreme variability of form in different species, being minimal in the cat and horse (Nicander and Bane, 1966) and apparently nonexistent as a special accumulation in human spermatozoa (Bedford, 1967d).

GENERAL CONCLUSIONS

There is now evidence in several species of mammals that spermatozoa undergo some functional change (capacitation) during their passage through the female tract and that this confers upon them the ability to penetrate the cumulus oophorus and zona pellucida. Both the uterus and fallopian tube play a role in capacitation; however, their relative importance probably varies considerably in different species, and in rodents in particular it is possible that follicular fluids may contribute to *in vivo* capacitation. Sperm of different absolute age are present in the ejaculate and some may require longer for capacitation than do others. There is some species variation in the time required for capacitation, and in the rabbit with a fixed interval of 10 hr between mating and ovulation, sperm passing from uterus to oviduct first become capacitated in about 6 hr; thus the fertilizing ability of rabbit sperm reaches a peak only a short while before arrival of the egg. By contrast, the ferret has a coitus/ovulation interval of about 30 hr, yet these sperm can be capacitated in the oviduct in only 3–4 hr. Clearly, further investigation of capacitation and sperm transport in this and other species is needed to bring a more exact understanding of the relationship between sperm and the female tract.

Capacitation is not entirely a species-specific phenomenon and this potential barrier to hybridization cannot account for the differences which have been maintained between related species. In the rabbit, the only animal studied in detail, hormones may modify the capacitation potential of particular regions of the female tract, the uterus more so than the fallopian tube. Nevertheless, present evidence indicates that capacitation control as a contraceptive measure is unlikely to be achieved with steroid treatment alone. The capacitated state may be inhibited by decapacitation factor(s) (DF) present in seminal plasma of some species. Several physical characteristics of DF have been elucidated, but we do not know its chemical nature. DF preparations are evidently able to inhibit certain sperm enzymes, but experimental evidence and the apparently reversible nature of such decapacitation suggest to the author that DF may act at the sperm surface to mask receptor sites or in some other way to stabilize the sperm surface, so preventing the acrosome reaction.

The changes which occur in sperm as a concomitant of capacitation are uncertain. Modifications seem to occur in the pattern of

sperm metabolism after their residence in the uterus, but the functional significance of this type of change has yet to be elucidated. No morphological changes occur before the onset of the acrosome reaction, which is not considered as a facet of capacitation itself. After capacitation, sperm become competent to undergo the acrosome reaction in response to stimuli which seem to exist in the vicinity of the egg, and in follicular fluid. This and other indirect evidence makes it possible to speculate that capacitation involves changes in the sperm surface, perhaps the unmasking of "receptor sites."

The acrosome reaction involves breakdown of the outer membrane of the acrosome during its fusion and vesiculation with the overlying plasma membrane. This reaction can generally be distinguished, at the fine structural level, from the acrosomal breakdown which occurs during degeneration. Release of the acrosomal enzymes, a consequence of the acrosome reaction, probably facilitates sperm passage through the granulosa cell investment to the surface of the zona pellucida. The outer vesiculating elements of the acrosome are apparently discarded during this passage, or at the surface of the zona. At all events, these remnants are always lost before true penetration of the zona begins. The penetrating sperm, now bounded rostrally by the inner acrosomal membrane, is considered to traverse the zona pellucida with the aid of a "zona lysin." Although proteolytic enzymes have been extracted from spermatozoa, we do not know definitely the nature and disposition of the zona lysin in the sperm head. Once through the zona, the fertilizing sperm becomes associated with the vitelline surface over the postacrosomal region of the head by a process of fusion between the egg and sperm plasma membranes. After this, the posterior part of the sperm head sinks into the vitellus, but the rostral region, which is incorporated later, is engulfed by the egg plasma membrane within a "phagocytic vesicle." A remarkable feature of this phase is the rapidity with which the dense sperm nucleus begins to disintegrate within the egg. Speculation is offered as to the role of postacrosomal and subacrosomal material which overlies the sperm nuclear membrane.

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