PREPARATION OF THE MAMMALIAN SPERMATOZOON FOR FERTILIZATION

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If frog testis is minced up in an appropriately balanced salt solution, spermatozoa are released which if placed with eggs can proceed immediately to penetrate the egg investments and initiate fertilization. The assumption might therefore seem reasonable that the spermatozoa freed from mammalian testis dissected under similar conditions should be capable of the same course of action, but the facts are indeed very different. Mammalian spermatozoa endure a long course of preparation in order eventually to take part in fertilization. After leaving the testis they must first traverse the vasa efferentia and the epididymis (the latter being an essentially mammalian organ), which commonly takes about 12 days, and then in addition a period of some hours residence in the female tract is also needed under normal circumstances. Changes undergone in the epididymis are commonly referred to as "maturation" and those in the female tract as "capacitation", but perhaps we should think rather in terms of an ordered succession of events, beginning when the spermatozoon parts company with the spermatogenic epithelium and ending when the acrosome reaction allows it to penetrate the egg investments. Some of the stages have been shown to proceed *in vitro*, if the proper conditions are arranged, and so the changes involved can be considered endogenous to the spermatozoon, but other steps seem to depend upon specific agents emanating from male or female genital tracts or possibly from the egg itself. Research in this field is still very much in the exploratory stage, and the purpose of this review is to present the more important known facts in such a way as to highlight the areas where information is most needed.

Available data can conveniently be considered under the following heads: (1) Chemical composition of testicular and epididymal fluid; (2) Chemical changes in spermatozoa in the male tract; (3) Surface and membrane properties of testicular and epididymal spermatozoa; (4) Endogenous changes of spermatozoa in the female genital tract or *in vitro*; and (5) the vesiculation of sperm membranes and the possible nature of agents promoting this process.

(1) Chemical composition of testicular and epididymal fluid

The sperm suspension in the testis tubules, in the rete testis and in the vasa efferentia is relatively dilute; great concentration occurs in the caput epididymidis. There-

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after, as the sperm mass moves through the epididymal duct, alterations in chemical composition occur but the concentration of the suspension remains high, and in this state too the suspension enters the vas deferens. The ability of spermatozoa to fertilize eggs when introduced into the female tract by artificial insemination is achieved well before the spermatozoa leave the epididymis–in fact, by the time they reach the more distal part of the corpus epididymidis (see ref. 1); nevertheless the cauda epididymidis and vas deferens may play important roles in the stabilization of sperm membranes which could be crucial for the storage of spermatozoa in these organs.

One of the most striking changes in epididymal fluid composition throughout the epididymis is in the concentration of ions and other osmotically active substances. The osmolarity attributable to sodium, potassium, chloride and bicarbonate ions declines from 296 mOsmole/kg H_2O in seminiferous tubule fluid to 73 mOsmole in vas deferens fluid. The osmolarity due to organic molecules, however, increases from 42 to 256 mOsmole over the same region, so that the total osmolarity remains fairly constant (data assembled by Johnson[2]). The low ionic strength is probably an important factor in the stabilization of sperm membranes and may function also in counteracting the effects of chemical changes in these membranes.

Special features characterize the chemical composition of specific regions of the epididymis—thus, the cells of the caput especially produce remarkably large quantities of glycero-phosphorylcholine; spermatozoa, however, cannot metabolize this substance and so its role (if any) in sperm maturation may depend on its physical properties. (In the female genital tract, esterases split off the glycerol which spermatozoa can metabolize, and the compound could serve as a source of energy under these conditions.) Other special features of epididymal secretions are similarly lacking in known functional significance for sperm maturation. Epididymal fluid also contains high concentrations of sialic acid, glutamic acid, and carnitine, but these derive in part at least from the testis. For references on these topics, see the review by Hamilton[3].

The epididymis is capable of synthesizing androgen from acetate, and the highest activity is in the lower part of the corpus, the region where spermatozoa finally achieve the ability to fertilize when introduced into the female tract by artificial insemination. At the same time, the function of the epididymis in sustaining spermatozoa through maturation is androgen dependent–an unusual example of an organ producing and responding to the same hormone (though presumably the same cells are not involved in both acts).

Epididymal spermatozoa are remarkable for their lack of motility, which is apparently attributable at first to their maturational state but later to the high concentration of cells in the epididymal fluid and the relatively low oxygen partial pressure in that medium. On admixture with accessory secretions or release into suitable artificial media, the motility and metabolism of spermatozoa from the more distal parts of the epididymis are strongly stimulated.

(2) Chemical changes in spermatozoa in the male tract

Testicular spermatozoa differ from those ejaculated by having higher levels of lecithin and cholesterol, and a higher ratio of saturated to unsaturated fatty acids[4]. The

changes seem likely to occur during passage through the epididymis, but the precise location has not yet been identified. Since the substances named are found typically in membranes, associated alterations in sperm membrane properties can be inferred. The principal effect would be an increase in membrane fluidity and thus a decrease in stability. Presumably the implication is that the membranes are brought to (or closer to) a state favouring, under the right conditions, the vesiculation process that takes place in the acrosome reaction (see section 5), but clearly with a decreased stability additional protection is required against environmental stress. We may reasonably infer that such protection is provided by the reduction in the ionic strength of the epididymal fluid already mentioned and also by the deposition of macromolecules on the surface of the spermatozoon (to be described in the next section).

During transit of the epididymis, the sperm nuclear chromatin becomes more intensively cross-linked with disulphide bonds[5]. As a result, the nucleus of the mature spermatozoon is strongly resistant to lysis or phagocytic digestion; this is a feature of placental mammalian spermatozoa and not of marsupial or lower vertebrate spermatozoa.

(3) Surface and membrane properties of epididymal spermatozoa

A number of interesting observations have been made on the nature and distribution of macromolecular receptors on the sperm surface and on the deposition of antigens arising in the epididymis (see ref. 2 for a recent critical review). The principal observations having significance in the present context are the following: (a) Guinea pig spermatocytes and spermatids displayed a bright surface fluorescence when treated with fluorescein-labelled concanavalin A, while testicular and epididymal spermatozoa gave a weaker staining; evidently, receptor sites became obscured, possibly by the acquisition of inert surface material. (b) The total negative charge on the sperm surface increases with passage through the epididymis, whereas the affinity for Sendai virus diminishes; these changes are consistent with the adsorption of protein molecules having polar groups but occluding pre-existing virus receptors. Agglutinability with antibodies also increases. A coating of macromolecules would be very likely to confer protection against environmental stress, and there are numerous reports of sperm-coating antigens (glycoproteins) in epididymal and accessory gland secretions.

Though much more work is clearly needed to support the thesis and fill in the details, the emerging picture is one in which the functions of epididymis involve progressive destabilization of sperm membranes and compensatory protection by coating macromolecules.

(4) Endogenous changes of spermatozoa in the female genital tract and in vitro

There is now a considerable literature supporting the idea that spermatozoa need to undergo some kind of physiological change before they become capable of penetrating the egg investments; the change normally occurs in the female genital tract but is also possible *in vitro* if conditions are appropriate (see ref. 6 for background and references). The change is known as capacitation and the need for it has been demonstrated in several species (mouse, rat, golden hamster, Chinese hamster, guinea pig, rabbit, ferret, cat, sheep,

pig, rhesus monkey and man). Times for capacitation vary greatly, from less than one hour in the mouse to five or more hours in the ferret, monkey and man. It has become clear that capacitation is the necessary preliminary to the acrosome reaction (discussed in section 5) and that this in turn represents the means whereby enzymes are brought into play that make possible the penetration of the spermatozoon into the egg.

Capacitation (at least of the hamster spermatozoon) can take place in a defined medium and so is inferred to assume the form of an endogenous change in the spermatozoon. The nature of the change is still obscure, but it evidently involves initial removal of surface macromolecules, as demonstrated by several investigators, and possibly then the occurrence of other modifications that render the membranes more prone to take part in the vesiculation event that constitutes the acrosome reaction. The first step is aided by enzyme treatment (with protease, amylase, glucuronidase or neuraminidase). The other changes may well include neutralization or reduction of the electrostatic charges on the outer acrosome membrane and the overlying plasma membrane which alone would appear to maintain the two membranes in close yet stable parallel array—electron micrographs plainly show the virtual absence of cytoplasmic material between these membranes[7], which might otherwise act in the manner of "gap substance" in preserving their separation. Alternatively, capacitation may involve the establishment in the plasma membrane of receptors for the acrosome-reaction-inducing agent, or modification of existing receptors.

The nature of capacitation remains a mystery. If it does the things expected of it (as set out in the previous paragraph), an explanation is still needed for the relatively long time usually required for completion. Various possibilities exist. Some kinds of change could be intrinsically time consuming, such as the synthesis of receptor proteins within the cell followed by their insertion in membranes. Again, delay could be attributable in some way to the gradual exhaustion of a substrate, the enzyme utilizing the substrate being responsible for maintenance of membrane charge after the abolition of other stabilizing factors. This would require that the enzyme be "turned on" at an early stage of capacitation, possibly as a result of permeability changes following removal of surface protein. To test all these speculations we need more information; the problem is discussed in some detail by Austin and Bavister[8].

(5) The vesiculation of sperm membranes and the possible nature of agents promoting this process

Once capacitation is completed, the spermatozoon is able to respond to certain agents (as yet unidentified) which normally emanate from the egg of its retinue of granulosa cells. The result is the fusion at numerous points between the outer acrosome membrane and the overlying plasma membrane—a phenomenon known as vesiculation (as a cytological process) or as the acrosome reaction (as a step in the mechanism of sperm penetration) (for a recent review see ref. 6). Vesiculation establishes apertures through which the contents of the acrosome can escape to the exterior, and the contents take the form of a variety of hydrolytic enzymes, for the acrosome is a modified lysosome. Of chief importance among these enzymes is hyaluronidase, which is capable of dissociating and liquefying the protein-hyaluronic acid complex that composes the matrix of the cumulus oophorus. Release of hyaluronidase enables the spermatozoon to penetrate the cumulus, an act that

it is incapable of performing otherwise. Having traversed the cumulus, the spermatozoon reaches the surface of the zona pellucida, and it is thought to penetrate this investment with the aid of a zona lysin which appears to exist as an integral part of the inner acrosome membrane. Since this membrane is exposed as a result of the acrosome reaction, the passage of the spermatozoon through both egg investments clearly depends on the occurrence of the reaction.

The agent provoking the acrosome reaction is not limited in its distribution to the egg and granulosa cells, for it (or something else having the same effects on the acrosome) is found also in blood serum. Activity is a feature of several protein fractions[9,10], with a fairly wide range of molecular weights (30,000 to 310,000). The agent itself could possibly be a relatively small molecule which becomes attached to protein carriers of different sizes. The agent could function in several different ways: (a) by cross-linking adjacent protein molecules in the plasma membrane and thus producing intervening areas characterized both by increased fluidity and reduced mutual charge; (b) by becoming incorporated (in the manner of a steroid or lipid) into the phospholipid layers of the membrane and thus increasing both the extent of the membrane and its fluidity (expansion of the acrosome just prior to the acrosome reaction has been described by Yanagimachi and Noda[11], and it is surely of significance also that hen erythrocytes treated with certain fusogenic lipids similarly expand just before fusion, as shown by Ahkong, *et al.*[12]; and (c) by enzymically degrading the polar regions of the membrane protein molecules and thus removing the mutually repulsive charges.

Since the acrosome reaction involves membrane fusion, attention could usefully be given to fusion events among other cells (see ref. 13 for recent review). Somatic cells in tissue culture may fuse spontaneously, and the frequency of fusion can be increased with the aid of viruses, fusogenic chemical substances, or micromanipulation. With these systems, too, membranes often require prior "conditioning", corresponding in some ways to the maturation and capacitation of the spermatozoon. One of the most potent fusogens is lysolecithin which is a product of the action of phospholipase A on lecithin, and interest in this substance is heightened by the fact that phospholipase A has been identified among the acrosomal enzymes (references given by Austin[14]), and occurs in lysosomes as a membrane-bound enzyme[14], while lecithin is a normal component of membranes. Lysolecithin in aqueous solution is prone to induce serious damage to cells, but its action is mitigated by the presence of serum albumin and is further reduced if included in an emulsion; under these conditions, also, its action is slowed so that fusion between cells treated with such an emulsion takes 10 to 30 min. These observations may well suggest the course of further investigations on the sperm acrosome reaction, and also the fact that more than thirty different fat-soluble substances have been found to induce cell fusion, one of the more interesting of which is glyceryl monooleate[15].

Conclusions

(1) The nature of the changes that occur in spermatozoa during passage through the epididymis, during capacitation in the female tract and during the course of the acrosome

reaction–in so far as these changes are understood at the present time–plainly suggest a continuous process, a progressive preparation of the spermatozoon for its task in penetrating the investments enclosing the egg.

(2) Chemical modifications of sperm membranes in the epididymis leave them less stable and more liable to form fusion complexes under appropriate circumstances; in compensation, glycoprotein coating molecules become deposited on the spermatozoon, conferring some protection against external factors that might disrupt the membranes.

(3) Capacitation of spermatozoa certainly removes adsorbed proteins and seems also to increase the instability of the outer acrosomal and overlying plasma membranes; in consequence, the "gun" becomes loaded and awaits only appropriate pressure on the trigger to fire the acrosome reaction.

(4) The acrosome reaction is evoked in the prepared spermatozoon by an agent that normally emanates from the egg or surrounding granulosa cells. This is the culmination of the series of changes that began when newly formed spermatozoon was released from the seminiferous epithelium.

The whole point of the series of events that we have been considering would seem to be that the spermatozoon should be able to bring enzymes to bear for the task of penetrating egg investments but should not release these enzymes until the right moment, namely when it comes into contact with the granulosa cell mass about the egg. In general the system works well, for only as a rare event do spermatozoa evince acrosome reactions precociously.

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