As we approach the three hundredth anniversary of the discovery of the spermatozoon (1677) it seems timely to review what we know of the structure of this fascinating cell. Perhaps no better example could be found of the slow beginnings of biological science and of the rapid recent acceleration in the tempo of discovery resulting from advances in instrumentation.

Using a microscope with a single highly convex lens, Leeuwenhoek was able to observe the general form of spermatozoa and to write a description of the character of their swimming movements that is difficult to improve upon today. But his belief that they were normal constituents of the semen and that they initiated and participated in the development of the egg was not accorded general acceptance until two hundred years later (1875) when Hertwig demonstrated sperm penetration and the union of the egg and sperm nuclei.

The introduction of the compound microscope and the progressive improvement of its resolving power in the 19th century sharpened the images of the external features of the spermatozoon but contributed little to our understanding of its internal structure. Indeed, most of what was learned from 1677 to 1950 can be depicted in a very simple drawing (Fig. 1). The introduction of commercial electron microscopes in the 1950’s and of microtomes capable of cutting ultrathin sections, initiated an exciting and remarkably fruitful period of exploration of biological structure at magnifications up to half a million times and resolutions that have now reached 4 to 5 Å. The sperm tail which previously appeared devoid of substructure was soon found to have a remarkably complex organization (Figs. 2, 3, 4). In the past decade studies of the internal structure of this cell with the transmission electron microscope have provided the structural basis for a sliding filament theory of sperm motility; they have clarified the nature of the acrosome reaction, and revealed details of sperm penetration and gamete fusion that were far beyond the reach of the light microscope. The introduction of the freeze-cleaving technique now makes it possible to split the sperm plasmalemma, and to examine regional specializations within the plane of the membrane—specializations that may be essential to the acrosome reaction and to gamete recognition and membrane fusion which are the cardinal events of fertilization and appropriate targets for efforts to develop safe, effective methods of fertility control.

Apart from the potential societal benefits of research in reproductive biology and population control, investigations of spermatozoa have much to contribute to our fundamental understanding of cell and developmental biology. Highly specialized though they are, spermatozoa have many components and properties shared by all cells. Their availability in bulk, their oc-
In this review it will become evident that the structural analysis of the mammalian spermatozoon has been carried further than has that of almost any other differentiated cell type, yet the molecular mechanisms of motility, sperm activation, zona penetration, syngamy and chromatin decondensation still elude us, and many intriguing problems remain concerning the development of this cell.

THE SPERM HEAD

The Nucleus

In the development of the spermatozoon, its nucleus acquires a shape characteristic of each species. This shaping of the nucleus takes place while its chromatin is undergoing a remarkable condensation that renders it metabolically inert and highly resistant to digestion. Associated with this morphological transformation of the nucleus there is a progressive stabilization of the chromatin through establishment of disulfide bonds (13, 22). This is accompanied by a progressive decrease in the binding of tritiated actinomycin-D to DNA as its binding sites are obliterated by cross linking (27). These changes in the physical state of the chromatin have usually been interpreted as a strategem of Nature to protect the genome from damage on the perilous journey to the site of fertilization and as a means of diminishing nuclear volume in order to streamline the cell and facilitate its motility. In mammals where the egg is surrounded by a thick zona pellucida, the condensation and stiffening of the sperm nucleus and its tapering anteriorly may be advantageous for penetration of the zona.

Microscopists of an earlier generation believed that if higher magnification and improved resolution could be achieved, it would be possible to see in the condensed nucleus, a precise arrangement of closely packed chromosomes. This expectation has not been borne out. The chromatin of the mature mammalian spermatozoon usually appears uniformly dense in electron micro-
Fig. 2. A schematic representation of a typical mammalian spermatozoon as it would appear with the cell membrane removed to reveal the underlying structural components. An acrosomal cap covers the anterior two

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graphs with no indication of chromosomal boundaries and little other resolvable organization. As seen in thin sections the dense homogeneous chromatin is interrupted only by randomly scattered small clear areas that seem to represent defects in the condensation process. Polarization microscopy (65, 66) and X-ray diffraction (132) have revealed a highly ordered substructure in the sperm heads of some invertebrates and this has fostered the belief that a comparable degree of order may exist in mammalian sperm nuclei even though it is obscured in electron micrographs by the close packing and intense staining of the condensed chromatin. Some support for this assumption has come from recent studies with the freeze-cleaving technique which have revealed a lamellar substructure in the flattened nuclei of mature spermatozoa from several different species (50, 69, 70). The observation of multiple lamellae of chromatin parallel to the flat surfaces of bull and rabbit sperm heads has been used to support the speculation that the shape of the mammalian sperm head is determined from within by the configuration of specific DNA-protein complexes (37) and is not the result of externally applied morphogenetic forces during spermiogenesis as has been claimed by some (79). How the birefringence of some sperm heads and their lamellar pattern of internal organization in freeze-cleaving can be made consonant with the concept of the individuality of the chromosomes and their

thirds of the nucleus. The connecting piece is inserted in an implantation fossa in the posterior aspect of the nucleus. The internal structure of the sperm flagellum is best understood from the study of cross sections at various levels. Running through the axis of the sperm tail for its entire length is the axoneme, a longitudinal bundle of microtubules similar to that found in cilia and flagella in general. Outside of the axoneme is a row of nine longitudinally oriented outer dense fibers that are not found in other flagella. Three segments of the sperm tail are defined by the nature of the sheaths that envelope the core complex of microtubules and dense fibers. The middle piece is characterized by a sheath of circumferentially oriented mitochondria. A dense annulus marks the caudal end of the middle piece. In the long principal piece, the core complex is enclosed in a fibrous sheath of circumferential dense fibers. The end piece is the portion beyond the termination of the fibrous sheath, consisting only of the axoneme. The plasma membrane invests all of the structures shown.
Fig. 4. Diagrammatic representation of a segment from the principal piece of a spermatozoan illustrating one of the two longitudinal columns of the fibrous sheath and the associated ribs. Inward prolongations of the longitudinal columns attaching to doublets 3 and 8 divide the tail into two unequal compartments, one containing three outer fibers and the other containing four.

Persistence in the interphase nucleus remains a challenging problem.

In the majority of species the uniformity of the product of spermatogenesis is remarkable—hundreds of millions being produced each day with very little variation in head shape and with a surprisingly small percentage of developmental anomalies. Human spermatozoa are exceptional in this regard, exhibiting considerable variability in head shape, and having a surprising frequency of large irregular cavities visible in the condensed chromatin by both light and electron microscopy. These have usually been called nuclear vacuoles even though they are unlike other vacuoles in not being limited by a membrane. As a rule, they appear empty in electron micrographs or at most contain a sparse granular precipitate. They may be single or multiple and some are large enough to distort the shape of the sperm head. Whether these anomalies of nuclear condensation have an effect upon fertilizing capacity is not known and will be difficult to ascertain without a comparable animal model.

In addition to these relatively gross defects in the human sperm nucleus, electron micrographs disclose a marked variation in the degree of chromatin condensation. Whereas in most other mammalian species this progresses to a dense homogeneous state in nearly all sperm, a significant proportion of the spermatozoa in human ejaculates have nuclei that still display the coarse granular pattern characteristic of the penultimate stage of condensation (14). When exposed to agents that break disulfide bonds, these nuclei decondense more rapidly than do those with dense homogeneous chromatin (14). This heterogeneity in nuclear ultrastructure in the human ejaculate suggests that the process of chromatin condensation does not proceed to completion in all members of the sperm population. Here again, it is not clear whether incomplete condensation is correlated with diminished fertilizing capacity. Comparative studies suggest that this is probably not the case, for in at least one rodent genus (Citellus) the sperm heads never become fully condensed yet there is no evidence of low fertility in these species.

The discovery that the human Y chromosome can be selectively stained with fluorescent quinacrin dyes (97) has made it
possible to identify the male-determining spermatozoa by the presence of a brightly fluorescent spot in the nucleus (7). It was to be expected that selective staining of the Y chromosome would provide evidence for or against a consistent arrangement of the chromosomes within the sperm nucleus. Unfortunately it has not done so. The bright yellow spot is usually situated in the anterior half of the nucleus, but a precisely reproducible localization has not been clearly demonstrated. However, this cannot be interpreted as conclusive evidence, one way or the other, because a consistent pattern of chromosomal arrangement might be considerably distorted by the occurrence of intranuclear vacuoles of the kind described above. Enumeration of sperm with two fluorescent spots, in which diploidy can be ruled out by comparative measurement of DNA content, has made it possible to estimate the frequency of non-disjunction in the second meiotic division—this being the only other way in which a YY sperm could arise (33). Examination of ejaculates by this relatively new technique may yield other useful genetic information.

The availability of the fluorochrome staining method for identification of the Y chromosome has stimulated renewed interest in the isolation of human X and Y spermatozoa with the goal of enabling parents to choose the sex of a child conceived by artificial insemination (32a, 112a). Preliminary results indicate that when a suspension of spermatozoa is layered over a discontinuous gradient of bovine serum albumin, the Y sperm enter the albumin more rapidly than the X. By repeating the process with increasing concentrations of albumin, yields of up to 85% actively motile Y spermatozoa are reported (32a). Since there is, as yet, no good morphological evidence of significant differences in head size or tail length, it is difficult to account for the greater capacity of Y spermatozoa to penetrate the interface and enter the relatively viscous albumin.

**Acrosome**

Cytologists using the light microscope were not able clearly to resolve the membranous investments of the sperm head or the limits of the acrosome. The frequent artifactual loosening and elevation of the cell membrane over the anterior portion of the head led to descriptions of a *galea capitis* or *head cap* which was envisioned as a separate structure overlying the acrosome. The early electron microscopic studies eliminated confusion on this point by clearly showing that the acrosome is not confined to the tip of the sperm head but is, in fact, a membrane-limited, cap-like structure closely applied to the tapering anterior portion of the nucleus (34, 36). The cell membrane directly invests it (Fig. 5). Thus there is no structure conforming to the traditional descriptions of the galea capitis and this term has now been abandoned. The outer acrosomal membrane immediately beneath the cell membrane is continuous at the posterior margin of the cap with the inner acrosomal membrane which, in turn, is closely applied to the nuclear envelope. The two acrosomal membranes run parallel throughout most of their extent and enclose a narrow cavity occupied by an acrosomal content of low electron density. In the human spermatozoon the acrosome is relatively small and does not extend anteriorly much beyond the leading edge of the nucleus (34, 102). In many other mammalian species, however, a conspicuous thickening of the acrosomal cap extends anteriorly well beyond the nucleus. This region, designated the *apical segment* of the acrosome, often has a shape characteristic of the species (36). The spermatozoa of the guinea pig, chinchilla, and ground squirrel, for example, have a very large apical segment of the acrosome (35, 36). There is nothing unusual in the structure of the egg investments in these species to suggest a
need for a larger acrosome and at present there is no satisfactory explanation for the marked specific differences in acrosomal shape and volume. That portion of the cap that covers the anterior portion of the nucleus is described as the principal segment. It exhibits only slight differences from species to species. A narrower caudal portion of the cap, called the equatorial segment, varies somewhat in its length (36), being unusually long in boar spermatozoa (90). In the human spermatozoon a linear differentiation within this segment gives its limiting membrane a pentalaminar appearance (102). In guinea pig spermatozoa a differentiation of the content of the equatorial segment results in a conspicuous palisade formation of parallel ridges observed in freeze-fractured preparations running obliquely forward from the caudal margin of the cap (50, 71). No functional significance has been assigned to these structural details but interest in this region runs high because its fate during fertilization is different from that of the other segments of the acrosome. It persists after the apical and principal segments have been lost in the acrosome reaction and appears to be unchanged on sperm observed in the perivitelline space (12, 13).

Cytochemical studies of the acrosome suggested that its contents were rich in carbohydrate (73) and this has been verified in chemical analyses which have established the presence of galactose, mannose, fucose, galactosamine, glucosamine, and sialic acid (25, 62).

Penetration of the egg envelopes by the spermatozoon was originally attributed to a mechanical perforating function of the acrosome, but the localization of hyaluronidase activity in the sperm head some thirty years ago (4, 113) directed attention to the possible role of lytic enzymes in the process. This interpretation has gained general acceptance in the period of heightened interest in hydrolytic enzymes, that followed the discovery of lysosomes. An acrosomal protease has been localized cytochemically in the spermatozoa of several mammalian species (141) and the capacity of sperm heads to digest gelatin films has been clearly demonstrated (52). The acrosomal contents have been shown by chemical analysis to contain several acid hydrolases including acid phosphatase, $\beta$ glucuronidase, $N$-acetylglucosaminidase (2, 28) and a trypsin-like protease, called acrosin or acrosomase (120, 123, 124, 145). These findings together with the observation that the acrosome stains supravitally with the fluorescent dye acridine orange (2) have led to the interpretation of the acrosome as a highly specialized lysosome.
In the presence of a recently ovulated egg, capacitated sperm undergo the *acrosome reaction* (8, 49, 137). The membrane limiting the apical and principal segments of the acrosome fuses at multiple sites with the overlying cell membrane creating openings through which the enzyme-rich contents of the acrosome are released (Fig. 6). This process of membrane fusion and vesiculation progresses until the major part of the outer acrosomal membrane and the overlying cell membrane are lost, leaving the anterior half of the sperm head invested only by the inner acrosomal membrane (8, 11, 12). The equatorial segment remains intact and seems not to participate in the acrosome reaction. The release of enzymes during the reaction is believed to disperse the cells of the cumulus and facilitate access of the sperm head to the zona pellucida. A puzzling aspect of the problem, however, is the fact that nearly all of the acrosomal content is released during the early stage of cumulus dispersion, leaving behind little or no acrosomal substance for dissolution of the thick zona pellucida which would seem to be the principal barrier to sperm penetration. The equatorial segment is still present and not visibly altered on spermatozoa in the perivitelline space. It has been concluded therefore that if acrosomal protease is involved in penetration of the zona, the enzyme must reside, at least in part, in the inner acrosomal membrane. The observation that in traversing the zona, the sperm cuts only a very narrow path no larger than the greatest diameter of the head (29) is consistent with the interpretation that the enzyme involved is bound to the inner membrane. Evidence favoring this interpretation has also been adduced by localization of fluorescein-labeled trypsin inhibitors on this membrane (124) and by detection of residual enzymatic activity on sperm experimentally denuded of the outer acrosomal membrane and acrosomal contents (19). On the other hand, some investigators studying sperm penetration with the electron microscope find the narrowness of the opening in the zona and the sharpness of the discontinuity in density along the edge of the path made by the sperm head, difficult to reconcile with a purely enzymatic mechanism. Therefore they entertain the possibility that the firm wedge-shaped sperm nucleus advanced by vigorous motility may also play a significant mechanical role.

There is very little morphological evidence bearing upon the localization of the several enzymes within the acrosome. In the early electron microscopic studies the contents were described as homogeneous, but later studies of the guinea pig (38) and chinchilla (41) revealed distinct areas of differing density. These have since been identified in many species. They are most obvious in the apical segment and especially after glycolmethacrylate embedding (46). There is often a pale outer zone around an inner zone of greater density. In the rabbit spermatozoon, discrete dense bodies with ill-defined boundaries are also described in this region of the acrosome. In
electron micrographs these exhibit a fine periodic structure interpreted as a crystal lattice (46). In the rat a highly ordered substructure with a periodicity of 42 Å has also been observed in a superficial zone on the convex portion of the curved acrosome (107) and a similar periodicity has been described in the acrosome of the human spermatozoon (101, 102). It is not known whether the different enzymatic components are uniformly distributed or segregated in the observed crystalline areas and zones of differing density within the acrosome. It is noteworthy that inhomogeneity of content and crystalline inclusions are also observed in peroxisomes, specific granules of eosinophilic leucocytes and other lysosomes.

The interpretation of the acrosome as a kind of lysosome has led to the suggestion that sperm penetration might be prevented by pharmacological stabilization of the acrosomal membrane to prevent release of its hydrolytic enzymes, or by development of specific inhibitors or antibodies that would inactivate the enzymes (122, 144). There is considerable skepticism about the feasibility of this approach to fertility control for lack of a delivery system, and because lysosomes are so widely distributed in the body and so essential to normal physiological processes that a general inhibition of their hydrolases might have far-reaching and intolerable effects outside of the reproductive system. Recent studies have demonstrated, however, that acrosomal hyaluronidase is a sperm-specific isoenzyme distinct from the common lysosomal hyaluronidase (143). Inhibition of sperm hyaluronidase by antibody has been shown to be highly species specific and capable of inhibiting fertilization in vitro (81). These indications of specificity keep alive the hope of control of fertility by enzyme inhibitors or isoimmunization.

Between the apical segment of the acrosome and the tip of the nucleus is a subacrosomal space (Fig. 5) which, in most mammalian species, is quite small and generally devoid of contents after preparation for electron microscopy. In spermatozoa of rats and mice this space is more capacious and is occupied by a moderately dense, resistant material which is often referred to as the perforatorium. This is an unfortunate term because it implies a mechanical function in sperm penetration that has not been demonstrated.

The Postacrosomal Region and Nuclear Envelope

Much investigative interest has been centered in the region of the sperm head behind the posterior margin of the acrosome, for it is in this region that attachment and fusion of the sperm and egg membranes takes place (9, 125, 137, 138). In electron micrographs of thin sections the plasma membrane of this region has the usual trilaminar appearance but it is underlain by a thin dense layer called the postacrosomal dense lamina or the postacrosomal sheath (36, 39). This corresponds to the structure formerly called the "postnuclear cap." It courses parallel to the membrane at a distance of 150 to 200 Å and regular periodic densities about 120 Å apart project from its outer aspect to the inner surface of the cell membrane (36, 39). Tangential sections indicate that these densities are cross-sectional profiles of circumferentially oriented parallel ridges on the postacrosomal sheath (102). Nothing is known about the chemical nature of this specialized layer. It cannot be isolated for analysis and it is inaccessible to study by freeze-fracturing. A narrow cleft between the sheath and the nucleus is closed behind by the posterior ring (Figs. 5, 28), a narrow circumferential band of fusion of the plasmalemma to the underly ing nuclear envelope (50, 101, 134). The intramembranous specialization of the posterior ring and its probable functional significance will be considered in a later section on the surface membrane.

The nuclear envelope of the mature sperm head is exceptional in several re-
spects. The entire area under the acrosomal cap and in the postacrosomal region is devoid of nuclear pores and the two membranes of the envelope are separated by only 70 to 100 Å. Caudal to the posterior ring, however, the two membranes diverge to the usual 400-600 Å distance. Leaving the surface of the condensed chromatin, the nuclear envelope in most species forms a fold that extends for a variable distance back into the neck region (Fig. 5). In contrast to the rest of the nuclear envelope this redundant portion behind the posterior ring has a large number of typical nuclear pores in close hexagonal array. These are seen to best advantage after the freeze-cleaving technique (Fig. 30). The intranuclear area bounded by this fold of the nuclear envelope has been designated the posterior nuclear space. It is usually devoid of chromatin and appears empty in electron micrographs.

The recurrent limb of the neck fold again comes into close contact with the condensed chromatin on the posterior surface of the nucleus. There again, the pores are absent and the membranes are in very close apposition. This portion of the nuclear envelope lines the implantation fossa, the site of attachment of the tail to the head (Figs. 5 and 7). In the region of the implantation fossa the narrow interspace (60 to 70 Å) between the two nuclear membranes is transversed by regular periodic densities about 60 Å wide and 60 Å apart (Fig. 10). This periodic structure is seen only in favorably oriented thin sections and it may not extend over the entire area of head-to-tail attachment. Freeze-fracturing reveals that the portion of the nuclear envelope lining the implantation fossa is highly specialized within the plane of the membranes (50). A particle-free, relatively smooth region of membrane is found in the central portion of the fossa but on either side of this featureless area there is a very dense population of relatively large (15 nm) intramembranous particles spaced about 20 nm apart (Figs. 11 and 12). It is possible that these particles are shared by both of the closely applied membranes and that they correspond to the periodic densities observed traversing in the interspace between the membranes in thin sections (Fig. 10). This region of the nuclear envelope is covered on its outer surface by a thick layer of very dense material, the basal plate, which lines the fossa and provides attachment for a large number of fine filaments that extend into it from the articular surface of the connecting piece (Fig. 7).

**THE SPERM TAIL**

The principal structural components of the sperm tail were described soon after the introduction of the electron microscope some twenty years ago (Figs. 3 and 4) and the research of the past decade has been devoted to details of their ultrastructure, and to their chemical dissection in an effort to discover the mechanism for generation of the propagated waves of bending that pass along the tail from base to tip. Since there is no fertility without sperm motility, the mechanism of sperm propulsion continues to be a subject of potential importance for population control.

**The Connecting Piece**

Immediately behind the sperm head is the connecting piece. This complex structure has a dense, convex articular region called the capitulum which conforms to the concavity of the basal plate lining the implantation fossa of the nucleus (Figs. 7 and 9). Fine filaments traversing the narrow, electron-lucent space between the capitulum and the basal plate appear to be the structures mainly responsible for attachment of the head to the tail. It is probably these filaments that are dissolved by reagents that separate the tails from heads (30), and it may be the synthesis of this protein that is defective in genetically infertile bulls in which the sperm heads and tails are dissociated in the ejaculate.

Extending backward from the capitulum
are nine segmented columns one to two microns in length. At their caudal end these overlap the tapering anterior ends of the nine dense fibers of the flagellum to which they are firmly united (Figs. 7 and 9). The columns of the connecting piece therefore appear to be continuous with the outer dense fibers, but the two are of different origin (40) and a careful examination of the region at high magnification reveals an oblique line along which they have fused secondarily. In longitudinal sections, the columns of the connecting piece appear to be composed of dense segments alternating with narrower light bands (Fig. 7). The light bands are bisected by a very thin intermediate line. In favorable thin sections eleven fine transverse striations can be resolved within each dark segment. The chemical nature of the cross-banded columns of the connecting piece has not been established but it is believed that they are analogous to the cross-striated rootlets associated with the basal bodies of epithelial cilia.

A transverse or obliquely oriented proximate...
The proximal centriole at this stage has a prolongation called the centriolar adjunct. The distal centriole is beginning to disintegrate concurrently with formation of the connecting piece. (From D. W. Fawcett and D. Phillips Anat. Rec. 165, 1969)

Ultrastructural studies of the neck region of a nearly mature boar spermatozoon from the testis, showing the persistance of the proximal centriole in a niche within the connecting piece. The centriolar adjunct has disappeared, and only traces of the distal centriole remain. The axoneme of the sperm flagellum thus has no basal body comparable to those of cilia.
FIG. 10. Electron micrograph of a thin section through the implantation fossa of a late Chinese hamster spermatid, showing the close apposition of the membranes of the nuclear envelope and the periodic densities.
region of the mammalian spermatozoon have overturned two strongly held beliefs of classical cytologists. One of these was that all motile flagella must have a centriole or basal body to serve as a kinetic center and site of origin of the beat. It is now evident that a centriole is necessary as an organizing center or template during formation of the axoneme, but once the flagellum is formed it is not needed for the initiation or propagation of waves of bending along the tail (136). This conclusion, based upon morphological observations, is borne out by the experimental demonstration that segments of sperm tails dissected away from the head and neck possess the ability to initiate and coordinate waves in the absence of a centriole (77). The second time-honored belief was, that the contribution of a centriole by the spermatozoon was necessary for formation of the first cleavage spindle and initiation of development. Although the proximal centriole does enter the egg with the penetrating spermatozoon in most mammalian species, it has been found that, in the rat, the proximal, as well as the distal, centriole disintegrates late in development (Fig. 7) and neither is present in the mature sperm (136). Thus it is evident that the proximal centriole is not essential for fertilization and cleavage.

The Axoneme

The motor apparatus of the sperm tail is the axoneme or axial filament complex, consisting of two central microtubules surrounded by a row of nine evenly spaced doublet microtubules (Fig. 13). The occurrence of this 9 + 2 pattern in cilia and flagella throughout the plant and animal kingdoms (43, 78) with only rare exceptions (104), remains one of the intriguing generalizations in biology. The reasons for its remarkable phylogenetic stability still elude us, but may become more apparent when the mechanism of flagellar motion is better understood. There have been rapid recent advances in our understanding of the chemical nature of the microtubular components of the axoneme, their mode of assembly, their energy source, and the manner in which they may interact to produce bending. Many of these fundamental studies have been carried out on cilia and on the simple sperm flagella of invertebrates but their inclusion in a review of the mammalian spermatozoon is fully justified by compelling morphological and physiological evidence that the basic mechanisms involved are the same in all motile cell processes possessing a 9 + 2 axoneme.

![Fig. 13. Schematic representation of the current interpretation of the organization of the axoneme of cilia and flagella. (Based upon work of Linck; Stephens; Satir; Warner and others)](image)

that traverse the narrow cleft between them (see at arrows). (From D. W. Fawcett and D. Phillips, Anat. Rec. 165, 1969)

Fig. 11. A freeze-cleave preparation of rat sperm in which the fracture line has passed obliquely across the base of the flagellum. It illustrates a particle poor area in the center of the implantation fossa, surrounded by an area of closely packed particles. The relation of these particles to the periodic densities seen between the leaves of the nuclear envelope in thin sections (see fig. 10) is not clear. (From D. Friend and D. W. Fawcett, J. Cell Biol. 63, 1974)

Fig. 12. Higher magnification of the intramembranous particles in the nuclear envelope lining the implantation fossa. Some of the particles appear to have a central hole or pore (see at arrows).
Fig. 14. Highly schematic three dimensional reconstruction of the axoneme and its associated structures. There is no basis for depicting the arms as rectangular, it is simply intended to indicate that they are periodic and not continuous along the doublets. The details of the attachments of the nexin bridges and radial links remain to be worked out.

The doublets consist of two subunits, subunit A which is a complete microtubule, circular in cross section and about 26 nm in diameter, and subunit B which is C-shaped in section with its ends attached to the wall of subunit A (Fig. 14). The cylindrical wall of subunit A is made up of 13 straight protofilaments 3.5 nm in diameter (Fig. 15) each composed of 80 Å dimers of a protein called tubulin (Figs. 16 and 17). These dimers are associated end-to-end (3, 61) and it is believed that those of adjacent protofilaments are in staggered array. Subunit B is composed of similar dimeric units of tubulin in about 10 protofilaments (130, 131). In cross sections, subunit A is seen to provide attachment for two diverging arms that project toward the next doublet in the row (1). The arms consist mainly of dynein, a protein with ATPase activity (54-56). Also attached to each subunit A are two slender nexin links that connect it to the adjacent doublets (127) and a radial spoke that joins it to a helical sheath around the central pair of microtubules. The arms of the doublets are spaced at regular intervals of 240 Å along subunit A (23, 74, 75) and the radial spokes, studied by negative staining of dissociated axonemes, are grouped into pairs or triplets (64, 114, 131). The members of the central pair of microtubules in the $9 + 2$, like subunit A of the doublets, are each composed of 13 protofilaments. The two central tubules are joined to one another along their length by regularly spaced bridges about 135 Å apart (98, 101) and they are enclosed in a sheath, that is said to be formed of helically wound 60 Å filaments (Fig. 14).

The tubulin which is the principal structural protein of the flagellar microtubules is probably nearly identical to that of microtubules found in the interphase cyto-
plasm of cells generally and in the spindle apparatus of dividing cells. It occurs as a dimer of molecular weight about 110,000 made up of subunits of 55,000 molecular weight, each of which has associated with it one molecule of guanine nucleotide. The dimer of tubulin has the property of binding one molecule of the alkaloid colchicine. Advantage is taken of this affinity in the isolation of tubulin. Two fractions of tubulin can be distinguished electrophoretically—tubulin α and tubulin β. The dimers in the doublets probably have one subunit of each. Evidence is accumulating to suggest additional heterogeneity of tubulins, in that, the A- and B-subunits of flagellar doublets have different solubility properties (16, 74, 126). Heating doublets at 37°C results in selective solubilization of the B-subunit. After treatment with Sarkosyl the walls of the tubules are solubilized except for a more stable unit consisting of three protofilaments (82, 133). This was initially interpreted as the segment comprising the wall between the tubules of the doublet, but this has now been challenged (76). Doublet microtubule preparations contain at least nine minor protein components accounting for 25–35% of the total protein (76). Some of these may be involved in attachment of the B-subunit of the doublet to the A, or the attachment of the arms, radial spokes, or nexin links. It is not clear whether these proteins reside in specific protofilaments or in the associated layer of material which is stained after fixation of cilia and flagella in solutions containing tannic acid (17, 84) (Fig. 18).

The flagellar protein principally concerned with converting chemical energy into mechanical movement is dynein—a large molecule with a molecular weight of about 500,000, possessing ATPase activity. As in the case of tubulin, it is heterogeneous and can be separated electrophoretically into A1, A2, and B fractions (55). The A fractions, comprising two-thirds of the
total, are located in the arms on the doublets; the location of the B fraction is still unclear.

In the reductionist approach to biology, it is always gratifying when one has "taken the alarm clock apart," to be able to begin putting it back together. Conditions have now been defined under which tubulin in solution can be repolymerized into microtubules in vitro (94). Also after dynein is extracted from flagella, subsequent electron micrographs show that the arms have been removed from the doublets. When axonemes so extracted are then exposed to solutions of dynein under the appropriate conditions, electron micrographs reveal that the arms have been restored to the doublets (53, 56).

The details of the mechanism by which the $9 + 2$ complex of microtubules produce flagellar movement still elude us but one feels that a satisfactory explanation is not far off. It is generally agreed that microtubules are incapable of shortening to produce bending and the most reasonable alternative is for localized sliding to take place between neighboring doublets. In favor of this is the morphological observation that the length of the doublets appears to remain constant during bending (115). Compelling evidence for sliding has recently been adduced by ingenious experiments (128). When ATP is added to demembranated flagella that have been subjected to mild trypsin treatment, doublets move out opposite ends of the segments of

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**Fig. 16.** A model of a three dimensional reconstruction of a subfiber A based upon a computer method of image analysis. Four and 8 nm periodicities are shown, and the two types of 4 nm morphological units are labeled $\alpha$ and $\beta$. (From L. A. Amos and A. Klug. *J. Cell Sci.* 14, 523-550, 1974)

**Fig. 17.** A simple model suggesting the most probable arrangement of tubulin dimers in a doublet tubule as deduced from filtered images. (From L. A. Amos and A. Klug, *J. Cell Sci.* 14, 523-550, 1974)
FIG. 18. Axoneme and associated fibers in the principal piece of a spermatozoan after fixation in glutaraldehyde containing tannic acid. After this treatment the protofibrils in the walls of the central microtubules and of the doublets are visible in negative image. The cortex and medulla of the outer fibers are also clearly differentiated. (Electron micrograph courtesy of D. Phillips)

flagella, thus providing direct evidence of sliding (128). Therefore it is now widely accepted that flagellar movement involves a sliding mechanism analogous to the sliding filament mechanism of shortening in skeletal muscle. How the arms on one doublet interact with the neighboring doublet to generate movement remains to be worked out, as well as the mechanism responsible for propagation of the wave of bending along the tail.

That these details of axonemal structure are not solely of theoretical interest is apparent from the recent report (103) of an infertile human with adequate numbers of sperm of normal appearance by light microscopy but with no motility. When examined by electron microscopy the axonemes appeared to lack arms on the doublets, suggesting a genetic inability to synthesize the dynein necessary to convert the chemical energy of ATP to mechanical movement.

**Outer Dense Fibers**

An understanding of the motility of cilia and the simple flagella of invertebrate sperm does not seem far beyond our reach but the additional fibrous components and circumferential sheaths of the mammalian sperm tail would seem to impose restraints to bending and to the sliding of the axonemal microtubules that make the problem of sperm locomotion in higher animals much more complex.

For a major part of the length of the mammalian sperm tail, the axoneme is surrounded by nine *outer dense fibers*, thus creating a $9 + 9 + 2$ cross-sectional pattern (Figs. 2 and 19). Each of the nine dense
fibers is fixed anteriorly to one of the segmented columns of the connecting piece, and it courses longitudinally just peripheral to the corresponding doublet of the axoneme. Unlike the doublets, which are all identical in appearance, the nine associated dense fibers differ from one another in cross-sectional area and shape. Fibers number 1, 5, and 6 are usually distinctly larger than the others and their cross-sectional configuration may be highly characteristic of a given species. There are also marked interspecific differences in the prominence of the outer fibers. In some species, the outer fibers are very thick and extend the full length of the principal piece, while in others they are relatively slender and terminate about halfway along the principal piece. Near its termination each fiber appears to be fixed to the wall of the corresponding doublet (36, 42). Herein lies one of the puzzling problems of explaining mammalian sperm tail motility by a sliding mechanism. Since the outer dense fibers are fixed to the nucleus anteriorly via the connecting piece and are attached to the doublets of the axoneme at their caudal end, these attachments would seem to impose a serious restraint to sliding of axonemal components unless the outer fibers themselves are either contractile or freely distensible.

The fibers are not homogeneous but have a thick medulla, and a thin cortical layer of lower density. The cortex is continuous over the abaxial surface of the fiber but it is
usually absent on the side nearer the axoneme (Figs. 18 and 19). It stains heavily with phosphotungstic acid (60). Surface replicas of isolated outer dense fibers exhibit an oblique striation in the cortex which appears to be composed of globular subunits (Fig. 20) (95, 135). In very thin longitudinal sections, a very fine 40-50 Å periodicity may also be detectable in the medulla (101).

In cross-sections of the middle piece and proximal portion of the principal piece, small punctate or angular profiles are observed in the flagellar matrix between the outer dense fibers (Fig. 19). Some authors have referred to these structures as granules but they can be seen in longitudinal sections as linear profiles coursing parallel to the axoneme and outer fibers (39). Their staining affinities are identical to those of the cortex of the outer dense fibers. Moreover, they appear to arise by exfoliation from the free edge of the cortex on the sides of the outer fibers. These structures have been called satellite fibrils (42). They do not seem to be attached to other components at either end but this is difficult to ascertain for technical reasons. Satellite fibrils are present in limited numbers in most mammalian species but reach their most spectacular development in the ground squirrel (36, 42) and bandicoot (24) which have very thick sperm tails.

The outer fibers and satellite fibrils are resistant to solubilization by any of the methods commonly used for extraction of contractile proteins, but they can be put into solution by treatment with dithiothreitol and sodium dodecyl sulfate. Upon electrophoresis of solubilized outer fibers from rat sperm, four bands are detected, corresponding to polypeptides of molecular weights 40,000, 25,000, 12,000, and 11,000. The 25,000 MW polypeptide accounts for 58% of the total (112). Similar studies on bull sperm from another laboratory yielded somewhat different results, with three major bands corresponding to molecular weights of 55,000, 30,000, and 15,000, of which the 30,000 MW polypeptide was the

Fig. 20. Above, Replica of spermatozoon of the squirrel monkey (Saimiri). Where the mitochondrial sheath has been removed, fine oblique striations can be seen in the outer dense fibers. Below high magnification of outer fibers of rat spermatozoon showing globular subunits within the oblique striations in the fiber cortex. (Micrographs by G. Olsen)
most abundant (6). Amino acid analysis in both studies revealed a high content of cysteine. A surprising finding was an appreciable content of bound triglyceride (103 μg/mg protein). The reports are in disagreement concerning carbohydrates, one recording their presence in the fiber cortex (6) and the other denying their presence (112). There is agreement on the absence of ATPase activity (6, 109, 112). The absence of phosphorus in the fibers would also seem to indicate that energy yielding nucleotides are not a part of these fibers.

The interpretation of the function of the outer dense fibers has undergone a major change since their isolation and partial characterization. The early investigators relying mainly upon comparative morphological observations speculated that they were probably contractile. It was noted that the sperm of primitive aquatic mammals have only a simple 9 + 2 flagellum and that the appearance of the outer fibers during phylogeny seems to have coincided with the development of internal fertilization. Associated with the appearance of outer fibers there was a great increase in the number of mitochondria. These observations led to the speculation that the outer fibers were accessory motor elements that evolved to overcome the greater resistance to locomotion in the female reproductive tract (36, 39). The concurrent development of a long mitochondrial sheath closely applied to the outer dense fibers was interpreted as an adaptation to provide the necessary energy for contraction of the outer fibers. Consistent with the belief that these fibers were contractile was the observation that the asymmetry in location of the larger fibers (1, 5, and 6) with respect to the axis of bending, seemed to correlate with observed directional differences in the speed and force of tail movements. Moreover, reports of immunohistochemical studies suggesting that the outer fibers possessed both ATPase activity (86) and antigenic similarities to actomyosin (87) served to further strengthen the belief that the outer fibers were contractile. Logical as these conclusions seemed at the time, their validity is now brought into serious question by the results of the recent chemical analyses of isolated outer fibers (6, 111, 112).

The main thrust of these studies is that there is little chemical resemblance between the outer dense fibers and any known contractile proteins. Therefore it now seems somewhat unlikely that these are active motor elements. Since they are proteins that are stabilized by abundant disulfide cross-linking, they probably have significant passive elastic properties and may serve to stiffen or provide elastic recoil for the sperm tail. Comparative cinemographic studies of sperm motility indicate that sperm tails of those species which have unusually large outer fibers have bending waves of lower amplitude than those with smaller outer fibers. Thus they appear to be stiffer (106).

The current interpretation of the outer fibers as passive stiffening components raises more questions than it answers. How can a sliding mechanism operate when relatively large outer fibers are fixed at their ends to the doublets that are presumed to slide? What possible advantage over a simple 9 + 2 flagellum would these additional stiffening structures provide? In species with very thick sperm tails how can a relatively slender central axoneme overcome the resistance of nine very large peripheral fibers situated some distance from the axis of bending?

Mitochondrial Sheath

The mitochondrial sheath of the middle piece is believed to generate the energy for sperm locomotion. Its topographical relationships would suggest that the mitochondria might be especially important as an energy source for the outer fibers (Figs. 2, 3, 31). But if these are not contractile, as the biochemical evidence now
suggests, then the functional significance of the long mitochondrial sheath of mammalian sperm is far from clear. Very few mitochondria are sufficient to provide energy for the simple 9 + 2 sperm flagella of aquatic invertebrates.

There is a wide range of variation among mammals in the extent of the middle piece. It may be quite short, comprising as few as 15 gyres of mitochondria in man or as many as 300 in some rodents. If one accepts the proposition that the conditions encountered by sperm in the female reproductive tract do not vary greatly from species to species and that the investments of the egg are also quite similar, then there is no satisfactory explanation for the diversity observed in the extent of the energy generating apparatus of the spermatozoa.

In general, the mitochondria of the mid-piece are arranged end-to-end to form a tight helix around the longitudinal fibrous elements of the tail (Fig. 2). The end-on junctions of the mitochondria usually occur at random along the course of the helix, but in some species there is a remarkable regularity in their spacing. These examples of specific arrangement and precise order present fascinating problems of morphogenesis and pose challenging questions concerning the factors that control the assembly of the mitochondrial sheath. In the common brown bat (Myotis) there are two mitochondria of identical size in each turn of the sheath, and their ends always meet on the plane passing through the central pair of microtubules in the axoneme (39). Thus the end-to-end junctions of mitochondria in successive turns of the sheath are aligned in register along the dorsal and ventral aspects of the mid-piece for its entire length. In some rodents, there are two mitochondria in each ring, but their end-to-end contacts in successive turns are offset by 90 degrees so that in surface replicas, the junctions in every other turn are aligned. A similar staggered arrangement is seen in successive rings of four mitochondria in the sperm of certain marsupials (Didelphys, Caluromys) (105). This represents a rotation of 45 degrees from row to row. To maintain the precise alignment of junctions over the whole length of the middle piece in these species, the mitochondria must be nearly identical in size. Such constancy in dimensions of this organelle in other cell types is rare and there is no ready explanation as to how such uniformity is achieved in spermatid differentiation. Nor is it obvious what advantage for motility would be conferred by such a high degree of order and symmetry in the mitochondrial sheath.

The internal structure of mitochondria in the sperm middle piece usually does not differ significantly from that of mitochondria in other cell types, but there are exceptions. In some marsupials (Didelphys; Caluromys) there are remarkable concentric systems of membranes that fill the interior of the mitochondria (36, 105), and in at least one desert rodent (Dipodomys), a granular material of unknown nature accumulates in the space between the inner and outer membranes on the abaxial side of each mitochondrion in the middle piece (108). At present these peculiarities defy physiological interpretation.

The Fibrous Sheath

The fibrous sheath of the principal piece is a structural component peculiar to mammalian spermatozoa. It begins immediately behind the annulus which marks the caudal limit of the middle piece and it consists of a series of circumferentially oriented ribs that pass halfway around the tail to terminate in two longitudinal columns which run along opposite sites of the sheath for its entire length (Fig. 4). The closely spaced ribs of the sheath occasionally branch and anastomose with neighboring ribs. The columns are composed of longitudinally oriented filamentous subunits that appear, in transverse section, as minute punctate densities. The ribs also exhibit a filamentous substructure but
their subunits are thinner than those of the longitudinal columns. In its initial portion, the sheath is fixed to outer dense fibers 3 and 8. These fibers, which are approximately in the plane of the central pair of axonemal tubules, then terminate abruptly leaving the other seven fibers to continue through the principal piece. Posterior to the termination of fibers 3 and 8, the adaxial side of the longitudinal columns tapers to a thin edge which extends inward and appears to attach to a small ridge projecting radially from the wall of doublets 3 and 8 (Figs. 4 and 18). As the sperm tail tapers along its length, the longitudinal columns become smaller and the ribs more slender. Several microns from the tip of the tail, the fibrous sheath ends abruptly, marking the junction of the principal piece and end piece. Attenuated vestiges of the longitudinal columns may continue a short distance into the end piece where they are recognizable as ill-defined densities situated between axonemal doublets 3 and 8 and the flagellar membrane.

Just as the attachment of the ends of the outer fibers to the axonemal doublets would seem to impose some restraint upon their movement, so also the extensive attachment of doublets 3 and 8 to the longitudinal columns of the fibrous sheath would seem to restrict their participation in the sliding movements responsible for flagellar bending. The thick longitudinal columns of the sheath would also seem to limit bending in the plane of the central pair. On the other hand, there should be little restraint to bending perpendicular to this plane because this would only involve lateral bending of the columns and a widening or narrowing of the interspaces between the successive ribs of the sheath. It remains paradoxical that these morphological considerations suggest a specialization of the mammalian sperm tail for two-dimensional bending movements in the plane perpendicular to the central pair of microtubules, while cinematographic studies demonstrate that the propagated waves are in fact three-dimensional in the distal portions of the tail.

The fibrous sheath has not yet been isolated in bulk and characterized chemically but, like the outer fibers of the tail, it seems to consist of proteins that are highly resistant to solubilization. Its basic organization is similar in all mammals but there are significant variations in size and shape of the columns, and in the frequency of anastomosis of the ribs. In sperm of some rodents, the ends of the ribs are bifid so that a triangular electron-lucent interspace is visible between their diverging heads and the longitudinal columns of the sheath. In marsupials the ribs of the fibrous sheath are hollow near their ends and thus, in parasagittal sections, they present open rectangular profiles.

One of the obstacles to an immunological approach to fertility control in the male has been the paucity of antigens specific for sperm. Clearly antibody to tubulin would have far reaching undesirable effects upon cilia and flagella throughout the body. On the other hand, the proteins of the outer fibers and those of the fibrous sheath may prove to be unique to sperm. If this is true, use of these proteins as antigens might conceivably be the basis for selective immunosuppression of flagellum development or motility in mature sperm.

THE SURFACE OF THE SPERMATOZOOON

In recent years the study of cell surfaces has increasingly commanded the attention of cell biologists and there are few cell types in which regional specializations of the surface membrane for specific functions are as clearly defined or as well documented as they are for the spermatozoon. Its anterior portion is concerned with the secretion of acrosomal enzymes; the postacrosomal region is specialized for recognition and fusion with the egg in fertilization, and the tail membrane must permit access of substrates and carry out other functions essential to the maintenance of motility. It was to be expected
therefore that the application lectins and other molecular probes, freeze-fracturing and high resolution electron microscopy, would yield new information about the surface properties and internal organization of the sperm membrane. These findings are of great interest to the membrane biologist as well as to the specialist in reproduction.

Among the first explorations of the properties of the sperm surface were studies in which immobilized bull and rabbit spermatozoa placed in an electrophoretic field, were observed to migrate toward the anode with their tails foremost, thus suggesting that the cells have a net negative charge on their surface, with the tail more strongly charged than the head (89). It was subsequently shown that the electrophoretic properties of rabbit spermatozoa change as they mature, becoming more strongly negative as they pass through the epididymis (10). Recently, there has been a recrudescence of interest in the surface charges on sperm and particularly in morphological methods for their visual demonstration.

In electron micrographs of thin sections of spermatozoa, the plasmalemma has the usual trilaminar unit membrane structure with no readily discernible regional differences in its appearance. By means of various cytochemical techniques, however, it has been possible to demonstrate a carbohydrate rich cell coat or glycolemma (45, 46), which is developed to different degree in the several regions of the cell surface. A relatively crude approach to the determination of surface charge has involved the binding of electron opaque particles of colloidal iron hydroxide to the sperm surface followed by study of their localization in electron micrographs of thin sections (26, 45, 140). Sperm tails are generally found to be more intensely labeled than heads—a finding consistent with the earlier electrophoretic studies. The distribution of iron particles is rather uniform within a particular segment, but abrupt changes in particle concentration are sometimes observed at junctions between segments. The patterns of distribution are reported to be consistent within species but not between species. In the rabbit, for example, the heads are relatively free of label, the middle piece lightly labeled and the remainder of the tail heavily labeled. In the guinea pig, on the other hand, all of the surface is labeled, but the principal and end pieces most heavily. The acrosomal region is said to be more heavily labeled than the postacrosomal segment (140). The abrupt changes observed from one region of the sperm to another have been interpreted as indicating that there are also significant changes in biochemical properties of the membrane at these boundaries. This conclusion may well be justified but, because the binding of colloidal iron is carried out on glutaraldehyde-fixed spermatozoa at pH 1.6–2.0, the validity of the method as an index of charge density and distribution in the living spermatozoon can be seriously questioned.

A more physiological and biochemically interpretable approach has taken advantage of a variety of agglutinins of plant origin (lectins) which bind specifically to particular saccharide residues on the membranes. By coupling fluorescein isothiocyanate or 125I to these lectins, the distribution of specific saccharide binding sites on the cell surface can be demonstrated by fluorescence microscopy or radioautography. If ferritin is coupled to the lectin, the electron density of the iron in the molecule can be used to localize the binding sites with the resolution afforded by the electron microscope. The lectin, concanavalin A, at pH 7.4, consists of a tetramer of which each protomer binds to an α-D-mannose group. When it is added to a suspension of living, motile sperm, it results in their immediate agglutination, indicating that exposed α-D-mannose residues are present on the glycoproteins of the sperm membrane. The distribution of these residues on mouse spermatozoa, as revealed by use of labeled lectins, is not uniform over the whole cell,
but concentrated over the acrosomal region of the sperm head (30). \( \nu \)-galactose residues are also more abundant on the head. From the head-to-head sperm agglutination induced with Sendai or influenza virus it has also been inferred that \( N' \)-acetylneuraminic acid is abundant on the heads of rabbit and hamster spermatozoa but is less prevalent or absent on the tail (32, 92).

The development of the method of freeze-fracturing for electron microscopy has now made it possible to examine the internal organization of cell membranes. In this procedure, cells briefly fixed in glutaraldehyde solution and subsequently treated with glycerol as a cryoprotectant, are instantaneously frozen in liquid Freon 22 (\(-160^\circ C\)) and fractured in the frozen state. The fracture path through the tissue is not random but passes preferentially through the lipid bilayer of cell membranes (19) thus splitting the membranes in half. By evaporating carbon onto the frozen-fractured surface, a coherent replica of the fracture face in the interior of the membrane is produced. Metal is then evaporated on its surface at an angle to increase contrast and to produce a three-dimensional image of the small irregularities in surface contour. When freed of tissue by digestion in acid or chlorox, the replica is ready for examination in the electron microscope. Replicas of unspecialized areas of cell membranes present two kinds of images after freeze-cleaving. On the outwardly directed, inner half of a typical cell membrane (the A-face) one sees a large number of 60-90 A particles randomly distributed on a featureless background. These intramembranous particles are believed to represent the protein constituents of the membrane. The inwardly directed, outer half-membrane (the B-face) has very few associated particles and thus appears relatively smooth, but in high fidelity replicas one may observe shallow depressions or pits corresponding in their distribution to the particles on the opposing face. There is strong evidence that the intramembranous particles can move about within the lipid bilayer which is believed to be fluid at body temperature (119). When it is desirable to visualize the outer surface of membranes unglycerinated specimens are frozen at \(-90^\circ C\) and the ice is allowed to sublime for 1 min—a procedure called freeze-etching.

Electron microscopy of thin sections, surface replicas, freeze-cleaving and freeze-etching all reveal significant species differences when corresponding regions of the sperm membranes are compared. This is consistent with the reported diversity in distribution of negative charges (26), and lectin binding sites (30). In deep-etched samples of mouse sperm heads the external surface of the plasma membrane appears smooth and featureless (120a). In freeze-fractured specimens revealing the interior of the membrane, 90 A particles are distributed randomly over the A-face of the entire membrane of the head. The acrosomal membrane, on the other hand, displays a periodic arrangement of tightly packed 100 A particles (120a).

In guinea pig and rat spermatozoa exposed to concanavalin A or ruthenium red a carbohydrate rich glycocalyx is revealed on the cell membrane over the acrosome. Guinea pig sperm heads associate in stacks or rouleaux in the epididymis with the

**Fig. 21.** Scanning micrograph of guinea pig spermatozoa associated in a stack or rouleau as they commonly occur in the epididymis. This involves a conformity of shape of the acrosomes and a cohesion of the surface membranes.

**Fig. 22.** A thin sagittal section through a rouleau of sperm heads such as that in figure 21 shows the inhomogeneity in density of the acrosomal contents and the close contact of the convexity of one sperm head with the concavity in the next. An area similar to that in the rectangle is shown at high magnification in figure 23.

**Fig. 23.** Electron micrograph of portions of three cohering guinea pig acrosomes. The apposed membranes are quite close and the narrow interspace between these is traversed by very regularly spaced densities. These are interpreted as components of a highly ordered carbohydrate-rich cell coat or glycolemma. (Micrograph by D. Friend)
convexity of each acrosome fitting into the concavity of the next. In regions of contact between the successive acrosomes in such rouleaux (Figs. 21 and 22), the opposing cell membranes are precisely parallel and separated by an interspace of about 10 nm. This space is traversed by very evenly spaced linear densities that have been likened to those in septate junctions of invertebrate tissues (20). At high magnifications (Fig. 23) the regularly repeating structures that extend between the unit membranes are double-contoured and of distinctly lower density than the membranes. Sections tangential to the membrane surface display a geometric array of polygonal profiles. This lattice between the adhering membranes appears to be a special configuration of the glycocalyx in which the glycoproteins are more highly ordered than they are on the glycocalyx elsewhere on the cell surface.

When the cell membrane overlying the guinea pig acrosome is examined by freeze-fracturing, the A-face exhibits areas of irregular outline having a quilted pattern (Fig. 25). These are separated from one another by narrow strips of particle-studded membrane of more orthodox appearance. These highly ordered plaques or crystalline domains within the membrane do not appear to be the result of a close packing and an ordering of the ordinary membrane intercalated particles and no explanation of their molecular organization or significance can be offered at present (45). It seems likely, however, that the order observed in the periodic structures traversing the interspace between the opposed membranes in thin sections, is a reflection of the crystalline lattice seen within the underlying membrane by freeze-cleaving. This configuration of the membrane and cell coat cannot be interpreted as a specialization for adhesion, peculiar to guinea pig sperm, since very similar crystalline areas are observed in the cell membrane over the acrosome in rat spermatozoa which do not associate in rouleaux (Fig. 24). In both rat and guinea pig the acrosomal membrane also exhibits a periodic internal structure but with a pattern slightly different from that of the plasma membrane. If this were a feature common to both membranes in the acrosomal region of all mammalian species one might postulate that this unique structure of the membranes is related in some way to their interaction in the acrosome reaction. The reported absence of such lattices in the plasma membrane of the mouse (120a) and rabbit (47) sperm head makes this speculation less attractive.

Freeze-cleaving studies of the post-acrosomal region of the spermatozoa have not yet been especially rewarding. There are no “crystalline” areas. There does, however, seem to be a greater number of membrane intercalated particles per unit area than in the membrane over the acrosome or the neck region (Figs. 26, 27 and 28). If the generalization is true that the number of particles is correlated with the degree of metabolic activity of the membrane (19), then this finding is consistent with the importance usually assigned to the postacrosomal region in sperm-egg interaction.

In the caudal part of the postacrosomal segment of rabbit spermatozoa there are conspicuous strands or rods on the A-face, made up of closely packed rows of particles...
FIG. 26. Freeze-cleaving preparation showing the intramembranous differentiations of the postacrosomal region in a rabbit spermatozoon. The great abundance of particles in the postacrosomal region can be compared with the relatively sparse population in the neck region. The posterior ring appears here as a distinct groove at the boundary between head and neck. Rods or linear aggregations of particles are characteristic of the postacrosomal membrane near the posterior ring in this species. (Micrograph courtesy of J. E. Flechon)

FIG. 27. Another example of the postacrosomal region of rabbit sperm. This appears to show the B-face of the membranes since the posterior ring is seen as a ridge instead of a groove. The coarse projections behind the ring correspond to pores in this region of the nuclear envelope. (From J. E. Flechon, J. de Microscopie, 1974)
(Figs. 26 and 27). These run obliquely forward from the posterior ring (47, 70). In other species there are in this same region well-ordered geometric arrays of very small particles (45). There are, as yet no clues to the significance of these unusual differentiations in relation to the specific role of this region in gamete fusion.

The unique properties of the postacrosomal region essential for gamete attachment and fusion may not reside exclusively within the membrane proper but possibly in the underlying, dense, postacrosomal sheath, or, more likely, in the outer surface of the membrane which is not seen in freeze-cleaving. The isozyme of lactic dehydrogenase which is specific for spermatozoa (LDH-X) is reported to be localized by immuno-electron microscopy, in higher concentration on the postacrosomal membrane of mouse sperm than elsewhere on the surface (31). In view of the report that female animals immunized against this isozyme exhibit defective fertilization (58) the localization of the enzyme in the postacrosomal region may be significant. An antigen peculiar to a primitive teratocarcinoma and to other early embryonic cells has also been localized in the postacrosomal region of human spermatozoa (44). Particular saccharide residues of the surface membrane may prove to be important in gamete recognition and attachment and specific enzymes may be involved in gamete fusion. Much more work is needed on this region of the spermatozoon to determine how its special properties are related to the postacrosomal sheath and to the membrane intercalated particles and other specializations revealed by freeze-fracturing.

Of particular interest is a circumferential groove in the plasmalemma, called the striated band (109a) or posterior ring (70a, 135), which marks the boundary between the head and the neck of the spermatozoon. In electron micrographs of thin sections, it is evident that this is a line of fusion of three membranes, the cell membrane and the outer and inner nuclear membranes (Fig. 29 and 30). In freeze-fracture replicas the bottom of this circumferential groove shows a very fine striation with a periodicity of about 100 Å. This feature has been reported in spermatozoa of guinea pig, rat (50), rabbit (47), mouse (120a) and man (100) and is probably of general occurrence in mammals. It is thought of as being analogous to a tight junction (47) in that fusion of the membranes along this line effectively isolates the perinuclear compartment from the remainder of the cell. It is well known that experimental damage to the cell membrane causes immediate immobilization and death of the spermatozoon. Yet in the acrosome reaction, the membrane over the entire anterior half of the sperm head is lost without any ill effect upon sperm motility. The explanation for this paradox seems to reside in the posterior ring. The naturally occurring, extensive disruption of the cell surface during the acrosome reaction probably could not be tolerated were it not for the fact that the perinuclear compartment is sealed off from the tail by membrane fusion along the posterior ring.

The plasma membrane of the neck region caudal to the posterior ring contains many fewer particles than that of the postacrosomal region (Fig. 26). The membrane of the middle piece in some species is reported to contain the usual randomly distributed particles, while in the mouse, paracrystalline arrays of particles are described (120a). And in the guinea pig the A-face of the membrane displays extensive linear arrays of 60–80 Å particles generally oriented circumferentially (Figs. 31 and 32) (50, 71). Corresponding rows of shallow pits are seen on the B-face. These beaded strands are not uniformly distributed but are more abundant and more closely aggregated where the membrane overlies the gyres of the mitochondrial helix (Fig. 32). The membrane overlying the grooves or interstices between mitochondria contains only scattered 80 Å single particles or short
rows. This is an interesting example of differentiation within the plane of a cell membrane which has a clear topographical relationship to an organelle in the subjacent cytoplasm. That the alignment of particles and their circumferential orientation depends upon proximity to the mitochondrion is indicated by the fact that where the membrane diverges from the mitochondrial helix to enclose the cytoplasmic droplet, the beaded strands within the membrane become disoriented and largely dispersed into single particles (45).

The linear arrays of intramembranous particles in the guinea pig mid-piece terminate abruptly at the annulus which is marked by a moderate number of larger intramembranous particles scattered in a background which has a stippled or roughened texture reminiscent of that associated with desmosomes (Fig. 33). The membrane over the principal piece has a random pattern of particles (90 Å) on its A-face not unlike that of other cell membranes (Fig. 33). A distinctive feature, however, is a double row of staggered particles that runs longitudinally in the membrane over outer dense fiber number one (Fig. 34). This zipper-like differentiation is always found in the same location and extends throughout the anterior half of the principal piece and possibly farther. In thin cross sections of guinea pig sperm tails, a slight thickening of the membrane can be detected in this position and a local density is sometimes seen between the membrane and the underlying ribs of the fibrous sheath (Fig. 34). In freeze-cleave preparations of rat spermatozoa, a single row of less closely-spaced 80–90 Å particles is seen in the same location (50). A localized thickening of the membrane over outer fiber number one has now been observed in thin sections of sperm tails in other species and it seems likely that a longitudinally oriented linear array of intramembranous particles will prove to be a common feature of the principal piece of all mammalian spermatozoa studied by freeze-cleaving. In preparations that have been deeply etched to reveal the surface of the membrane on the principal piece a longitudinal zipper-like particle array is observed which is comparable to that seen in cleaved preparations (72, 120a). Thus it seems that this longitudinal differentiation is not confined to the interior of the membrane but either projects above the external surface or has a distinct external component in register with the deeper lying double row of particles within the membrane.

The significance of this linear longitudinal differentiation is by no means clear. The internal component bears some resemblance to the so-called “necklaces” of intramembranous particles that course circumferentially around the base of epithelial cilia (57) and around the base of the flagellum of certain invertebrate spermatozoa (18). Comparable longitudinally oriented particle arrays in a flagellar membrane have been described in the undulating membrane of trypanosomes, along the line of attachment of the flagellar membrane to the cell membrane (96). There are associated densities in the matrix of the flagellum and the cytoplasm. In this case, the aligned intramembranous particles are

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Fig. 28. Freeze cleave preparation of the A-face of the plasma membrane over the junction of the acrosomal and postacrosomal regions of a guinea-pig spermatozoon. A palisade of rod-like structures associated with the equatorial segment of the acrosome leave their impression in the overlying membrane. Of particular interest is the higher concentration of intramembranous particles in the membrane of the postacrosomal region.

Fig. 29. Thin section of the posterior part of a monkey sperm head showing the location and appearance of the posterior ring—a circumferential groove where the plasma membrane is fused to the underlying nuclear membranes.

Fig. 30. Freeze-cleave preparation of the posterior region of a guinea pig sperm head. The B-face of the plasmalemma is shown and the posterior ring therefore appears as a ridge instead of a groove. Immediately behind the ring are very regularly arranged pores in the redundant portion of the nuclear envelope. (Micrograph by D. Friend)
clearly associated with a line of membrane-to-membrane attachment. This suggests that the row of particles over fiber one in the mammalian sperm tail, may be a specialization for attachment of the membrane to the ribs of the underlying fibrous sheath. The unrestrained bending of the axoneme and its associated sheaths would seem to require some slack in the membrane and some degree of freedom of movement over the underlying structures of the tail. On the other hand, it could be argued that the tail probably functions more efficiently as a locomotor organ with its membrane fixed to the underlying structures than it would if the bending movements were taking place in a loose unattached sleeve. Why the attachment is situated over the ribs instead of over one of the continuous columns of the fibrous sheath, where it would be subject to the least tension in bending, defies explanation.

Longitudinal arrays of intramembranous particles have recently been reported in certain rows of the cilia in the oral region of *Tetrahymena* (116). The particles are arranged in several longitudinal rows spaced 800–1000 Å apart along one side of the cilium. In thin sections, there is some evidence of linkage of these particle rows to underlying doublet microtubules and it has been suggested that this intramembranous differentiation could modify the form of ciliary beat by restricting the sliding of microtubules on one side of the shaft (116). In the case of the sperm flagellum, such a mechanical basis for modification of beat is less likely because the intramembranous zipper is separated from the corresponding microtubule by the fibrous sheath and outer dense fiber.

The finding of consistent regional differences in the patterns of internal organization of the sperm plasma membrane, has interesting implications for the currently popular fluid mosaic model of membrane structure. Studies of somatic cells have clearly shown that the glycoprotein surface antigens and lectin binding sites are capable of moving to one pole of the cell in the phenomenon called “capping” (67, 129). These and other observations have given rise to the concept that the plasmalemma of cells in general is a dynamic fluid structure (119). Experiments on spermatozoa with ferritin-conjugated lectins have recently provided evidence for regional differences in the mobility of binding sites, with greater mobility being demonstrated in the postacrosomal, than in the acrosomal or tail regions of the plasma membrane (93). The circumferential strands of intramembranous particles in the guinea pig sperm mid-piece dissociate during prolonged incubation in physiological media which are known to promote "capacitation." The particles obviously move within the membrane in the course of this dissociation and may subsequently reaggregate in new patterns of association (49a, 72). The particles in the membrane of the principal piece may also change their distribution, but the zipper-like double row of particles over fiber number one remains in its usual location under these conditions. Thus although the fluid mosaic model of the membrane is confirmed by the observation of these changes within the membrane it is also evident that regional differences persist and that there are restraints to movement of certain elements such as the longitudinal row of particles in the principal piece. In the case of the circumferentially oriented linear arrays of

**Fig. 31.** A longitudinal thin section of the middle piece of a mammalian spermatozoon. The circumferentially oriented mitochondria are cut transversely. Note how closely the cell membrane is apposed to the underlying mitochondria.

**Fig. 32.** Freeze-cleaving preparation of the membrane of the middle piece from a guinea pig spermatozoon reveals linear arrays of intramembranous particles oriented circumferentially and concentrated over the mitochondria. The membrane over interstices in the mitochondrial sheath is relatively free of particles. These highly ordered arrays of particles are not found in the corresponding region of sperm from other species.
Fig. 33. Freeze cleave preparation of the A-face of the plasma membrane from the junctional region of middle piece and principal piece of a guinea-pig spermatozoan. The beaded strands of small particles in the middle piece end abruptly at the annulus. The membrane of the principal piece contains a population of randomly distributed larger particles. (Micrograph by D. Friend)
FIG. 34. (A) A longitudinal section through the principal piece of a sperm tail, approximately in the plane passing through fibers 2 and 7 or 9 and 4 of the cross-section shown above. The section includes two doublets and one of the central pair of axonemal microtubules, two outer fibers and groups of partially fused ribs of the fibrous sheath. (B) A freeze-cleaving preparation of the membrane on the side of the principal piece over the major compartment containing outer fibers 4 to 7. There is a high concentration of randomly distributed membrane intercalated particles. (C) A freeze-cleave preparation of the membrane on the side of the minor compartment containing fibers 9, 1 and 2. A double row of large particles runs longitudinally within the membrane overlying fiber number one. A slight thickening of the membrane at this site is evident (at the arrow) in the cross-section shown above. (From D. W. Fawcett, D. S. Friend, M. Price and R. W. Linck, Proc. 8th Internat. Congr. Electron Microscopy, Canberra, 1974)
particles in the middle piece of guinea pig spermatozoa, there is strong evidence of a transmembrane effect in that proximity of the membrane to the mitochondria appears to determine the degree of aggregation and the direction of orientation of the beaded strands of particles.

It is especially intriguing that the amplitude of the tail waves increases during incubation in capacitating media and this change in the pattern of flagellar beat is temporally correlated with the redistribution of particles within the membrane (72). These findings focus new attention upon the possibility that internal specializations of the tail membrane may have important functions in coordination of the beat, control of the ionic environment of the motor elements, or access of substrate to the energy generating enzyme systems of the mitochondria (50, 72, 120a).

CONCLUDING COMMENT

The spermatozoon is certainly one of the most highly specialized of all mammalian cells. It may also be true that our knowledge of its structure is more detailed than is that for any other cell type. Yet there remain important gaps in our understanding of its function at all levels.

At the level of clinical evaluation of semen quality, we noted an unusual prevalence of large nuclear vacuoles in human spermatozoa, and very numerous examples of incompletely condensed chromatin. However, since sperm of this kind represent only a fraction of the millions normally present in an ejaculate, we do not know whether these structural or maturational defects affect the fertilizing capacity of those sperm.

The use of quinacrine dyes to stain the human Y chromosome now makes it possible to identify the male determining spermatozoa. Although it has been suggested that there are also differences in head size, shape and tail length there are no valid data to establish whether there is a continuous random variation or a true bimodal distribution that can be correlated with the presence or absence of the Y chromosome. Such correlations should now be technically feasible by sequential application of a quinacrine dye and morphometry on scanning electron micrographs of the same preparation of spermatozoa. Now that preliminary success in isolation of fractions rich in human Y spermatozoa has been reported, and the prospect of choosing the sex of offspring is suggested, it becomes important to carry out such morphometric studies in an effort to determine the basis for preferential penetration of discontinuous gradients by the Y spermatozoa.

The process by which nucleohistones are removed from spermatids in the course of nuclear condensation and replaced by species specific protamine-like proteins is still poorly understood. There is need for more insight into the biochemical mechanisms of chromatin condensation, its biological significance and its rapid reversal in the egg, which is surely one of the most remarkable phenomena in developmental biology.

Much has been learned about the formation of the acrosome, the nature of its enzymes and their mode of release. Their importance in insuring access of the spermatozoon to the egg surface for fertilization is generally accepted. But essentially nothing is known about the factors that normally trigger the acrosome reaction when sperm reach the vicinity of the egg. The enzymes released are credited with dispersing the loosely organized cumulus cells. In this process nearly the entire content of the acrosomal cap is lost before the spermatozoon reaches the principal barrier in its path—the zona pellucida. It remains unclear why a relatively large volume of enzyme-rich acrosomal content is released for cumulus dispersion leaving only a small amount of enzyme bound to the inner acrosomal membrane for lysis of the zona. The relative importance of zona
agnosis and mechanical penetration by the actively motile spermatozoon needs further study.

The selective participation of the post-acrosomal region of the sperm head in recognition and fusion of the sperm and egg membranes at fertilization has now been thoroughly documented. Although some of its structural and enzymatic properties have been defined, the molecular basis for the specificity of this segment of the surface for gamete fusion is still unknown.

There has been rapid progress in the chemical dissection of the axoneme in cilia and in relatively simple sperm flagella of invertebrates. This fundamental research has led to the sliding-microtubule hypothesis for the generation of flagellar waves. Tubulin, the principal molecular species of the axoneme, has been isolated and characterized. The energy for flagellar motion has been shown to come from the breakdown of ATP. The protein, dynein, which forms the arms of the doublets has been identified as containing the adenosine triphosphatase that accomplishes this energy-yielding hydrolysis. These have been landmark discoveries but there remain the more difficult tasks of characterizing the nexin links, radial spokes and other minor components of the axoneme, as well as the chemical fractionation of the doublets to identify the basis for specific binding of dynein, and spoke protein to particular protofilaments in the wall of the doublets.

The isolation and analysis of those fibrous components peculiar to mammalian sperm tails has just begun. With the data available to date it is still not possible to exclude completely the possibility that the outer fibers may be contractile, though this now seems unlikely. If they are not, then further work must be done to explain their universal occurrence in mammals, their effect upon the pattern of motility, and the relation between them and the long mitochondrial sheath, which appeared in phylogeny concurrently with their evolution.

The chemical nature and functional role of the fibrous sheath have yet to be explored.

Our concept of the cell membrane has undergone a remarkable change in the past decade. The former emphasis on its structural uniformity and its passive role as a semipermeable cell boundary has given way to a dynamic view of membranes that recognizes their turnover, their structural and chemical diversity, and their capacity for change. There is a growing appreciation of the great number of important physiological processes that take place at membranes, whether within cells or at their interface with the environment. This is as true of spermatozoa as it is of cells organized into tissues and organs. There is already abundant evidence for a central role of membranes in capacitation, in the acrosome reaction and in the recognition and attachment of the gametes. We need now to know the significance of the patterns revealed by freeze-cleaving within the lipid bilayer in different regions of the sperm membrane, and how the membrane-intercalated particles are related to those superficial oligosaccharides which seem to be the basis for the regional specificity of lectin binding by the sperm membrane.

There has been more progress in our understanding of the spermatozoon in the past 20 years than there was in the first two hundred and eighty years after Leeuwenhoek. If we are to sustain our present rate of progress, developmental and reproductive biologists will have to draw heavily upon the methods and concepts of the membrane biologists, the biochemist, the immunologist, and the molecular pharmacologist. Progress can be made in these new directions from an unusually detailed base of structural information.

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