

CHEMICAL DISSECTION OF MAMMALIAN SPERMATOZOA

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ABSTRACT

Spermatozoa from several mammalian species have been dissected by chemical methods to yield free heads, tails with attached midpieces, and tails from which the mitochondrial components of the midpiece were removed. Mouse and rat spermatozoa were cleaved by brief treatment with trypsin to yield free heads and tails, while human, guinea pig, and rabbit spermatozoa were cleaved by trypsin only after incubation with 2-mercaptoethanol or dithiothreitol. Spermatozoa were also cleaved at the junction of the head and the tail by treatment with acid and base. Mitochondria were removed from intact spermatozoa or isolated tails by mechanical shear after treatment with 2-mercaptoethanol or dithiothreitol. The dissected components of spermatozoa were fractionated with good yield and high purity by density gradient centrifugation. Ultrastructural analysis indicates that proteolytic cleavage to yield separated heads and tails occurs at a specific location in the neck of the spermatozoon, leaving the basal plate attached to the head of the cell. In contrast, after acid cleavage the basal plate remains with the midpiece. Proteolytic treatment has no apparent effect on any other spermatozoan structures, whereas acid or base treatment results in damage to the plasma membrane, the acrosome, and other structures. The specificity of the proteolytic cleavage suggests that a particular protein or group of proteins may be responsible for the linkage between the sperm head and tail.

INTRODUCTION

Ultrastructural studies have revealed a number of features of the specialized structures of mammalian spermatozoa (1-4). Although these morphological studies have implicated certain regions of the spermatozoon in the fertilization process (5-9), the biochemical roles of many of the subcellular components are not known.

An understanding of the structure and function of mammalian spermatozoa requires the molecular characterization of these components. Several attempts have been made in this direction. Studies on acrosomes released from the head by the use of a cationic detergent (10), have revealed that enzymes resembling those from lysosomes are responsible for the digestion of the zona pellucida surrounding the ovum (11). In addition,

mechanical and enzymatic cleavage techniques have been devised to separate spermatozoan heads from tails (12, 13, 14), although functional roles for the isolated cell fragments have not been described.

We have been attempting to develop several chemical procedures for the dissection of mammalian spermatozoa in a rapid and reproducible manner. In previous experiments, spermatozoa of the mouse and rat were cleaved at the junction of the head and the tail by the application of endopeptidases, particularly trypsin (15). In the present report, we describe: (a) the chemical cleavage of spermatozoa of various mammalian species, including man, by endopeptidases and alterations of pH, (b) the complete removal of the

mitochondrial component of the spermatozoan midpiece by treatment with reducing agents, (c) the ultrastructural characterization of the products of these chemical dissection techniques, and (d) the fractionation of heads, intact tails, tails without mitochondria, and whole sperm without mitochondria in quantities sufficient for biochemical analysis.

MATERIALS AND METHODS

Preparation of Spermatozoa

Mouse, rat, guinea pig, and rabbit spermatozoa were obtained from the vas deferens by excising the tubule and extruding its contents into PBS (phosphate-buffered saline, pH 7.4, 8.00 g NaCl, 1.15 g Na_2HPO_4 , 0.20 g KH_2PO_4 , 0.20 g KCl per liter of H_2O) or into 0.05 M Tris-HCl, pH 7.4. Inbred DBA/2J and Balb/cJ mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Randomly bred NCS mice from The Rockefeller University were also used for some experiments. Randomly bred rabbits, guinea pigs, and rats were obtained from The Rockefeller University. Human spermatozoa were obtained from fresh ejaculates and were suspended and washed three times in PBS before use. Freshly prepared suspensions of spermatozoa were adjusted to 10^7 – 10^8 cells/ml as determined with a hemocytometer. About 80% of the spermatozoa were motile after incubation for 15 min in PBS at 25°C.

Chemical Dissection Procedures

For the proteolytic cleavage of sperm heads from tails, cells were suspended in PBS or Tris buffer containing 0.05–0.1 mg/ml trypsin (EC 3.4.4.4, B grade, lot 001449, Calbiochem, San Diego, Calif.). The final cell concentration was 10^7 cells/ml. The reaction mixture was gently shaken and incubated at 25°C for 5 min before it was washed in fresh buffer or before the addition of a 10-fold molar excess over trypsin of soybean trypsin inhibitor (Type II-S, Sigma Chemical Co., St. Louis, Mo.).

To determine the extent of cleavage of spermatozoa over a wide range of pH, freshly prepared cells were suspended to a concentration of 10^7 – 10^8 cells/ml in a 0.05 M citrate-0.1 M PO_4 buffer for pH values between pH 1.0 and pH 7.2 or 0.1 M Tris-HCl for pH values between pH 7.4 and pH 12.6. For pH values less than pH 2.6, 0.1 N HCl was used to adjust the citrate- PO_4 buffer. For pH values greater than pH 10.0, 0.1 N NaOH was used to adjust the Tris buffer. The reaction mixtures were incubated for 5 min at 25°C with continuous shaking, and the extent of cleavage was quantitated by observation with phase contrast microscopy.

The mitochondrial components of spermatozoa were removed by suspending the cells (10^8 /ml) in 0.05 M Tris-HCl, pH 7.4, 0.1 M in dithiothreitol (DTT) (lot 010164, Calbiochem) or 0.1 M in 2-mercaptoethanol (Matheson, Coleman, and Bell, Cincinnati, Ohio). The spermatozoa were incubated at 37°C for 90 min without shaking, after which the reaction mixture was agitated vigorously using a Vortex Jr. mixer (Scientific Industries, Inc., Queens Village, N.Y.) for 15 s.

Gradient Fractionation of Spermatozoan Components

Density gradient centrifugation was carried out by a modification of the method of Stambaugh and Buckley (16). After the indicated chemical treatment the spermatozoa were pelleted and resuspended in 0.05 M Tris-HCl, pH 7.4, to a final concentration of 10^7 – 10^8 cells/ml. An aliquot (0.5 ml) of this suspension was loaded on a linear sucrose gradient (30–65%) in 0.05 M Tris-HCl, pH 7.4, with a total volume of 26.5 ml. Centrifugation at 600g for 30 min at 4°C in an International centrifuge, model PR-2 (International Scientific Instruments, Inc., Palo Alto, Calif.) resulted in the fractionation of the spermatozoan components. Heads were found in a sharp band of high density and tails were found in a broader band of lesser density. Yield from the gradients varied from 65 to 75%.

Cytological Techniques

A Zeiss Universal microscope was used for the phase contrast microscopy; photographs were taken using Kodak Panatomic-X film.

Specimens for electron microscopy were fixed for 1 h at 25°C in 1% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) buffered with PBS, pH 7.4. Post-fixation was done with 1% osmium tetroxide (Polysciences) in PBS at 25°C for 1 h. In some experiments, a third fixation was done with 6% uranyl acetate in distilled H_2O (17) for 1 h at 25°C. Samples were dehydrated in a series of ethanols graded from 70 to 100%, after which they were transferred to 100% propylene oxide before embedding in Epon 812.

Sections about 60-nm thick were cut on a Porter-Blum MT-2B ultramicrotome and stained with uranyl acetate followed by lead citrate. Specimens were examined with a Phillips EM-300 electron microscope at 60 kV using a 30- μ objective aperture.

RESULTS

Proteolytic Cleavage of Spermatozoa

As previously observed (15), treatment of mouse or rat spermatozoa with trypsin resulted in the

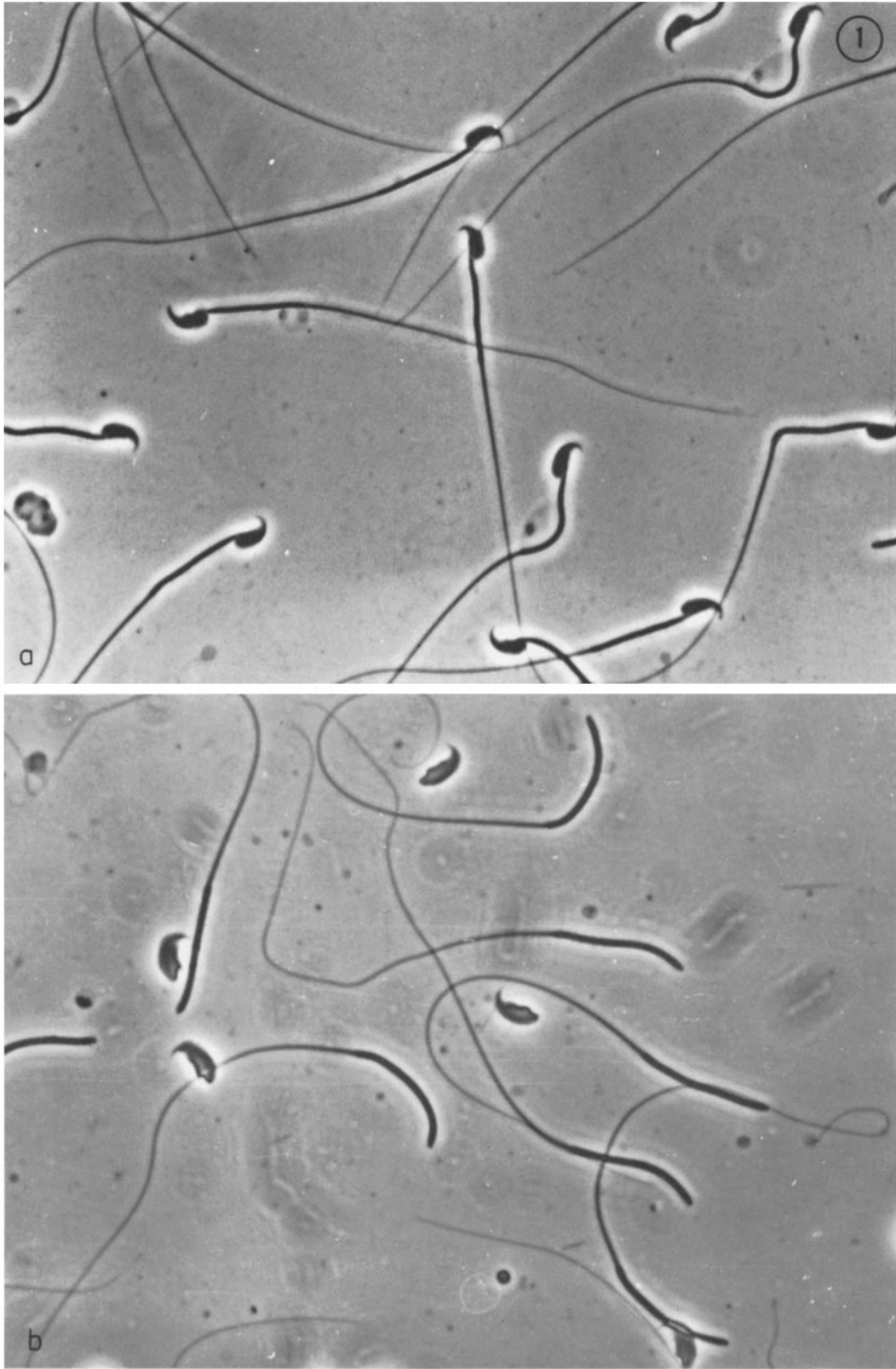


FIGURE 1 (a) NCS mouse spermatozoa in PBS, pH 7.4. $\times 1,540$. (b) NCS mouse spermatozoa after treatment with 0.05 mg/ml trypsin in PBS, pH 7.4, for 10 s at 25°C. $\times 2,120$.

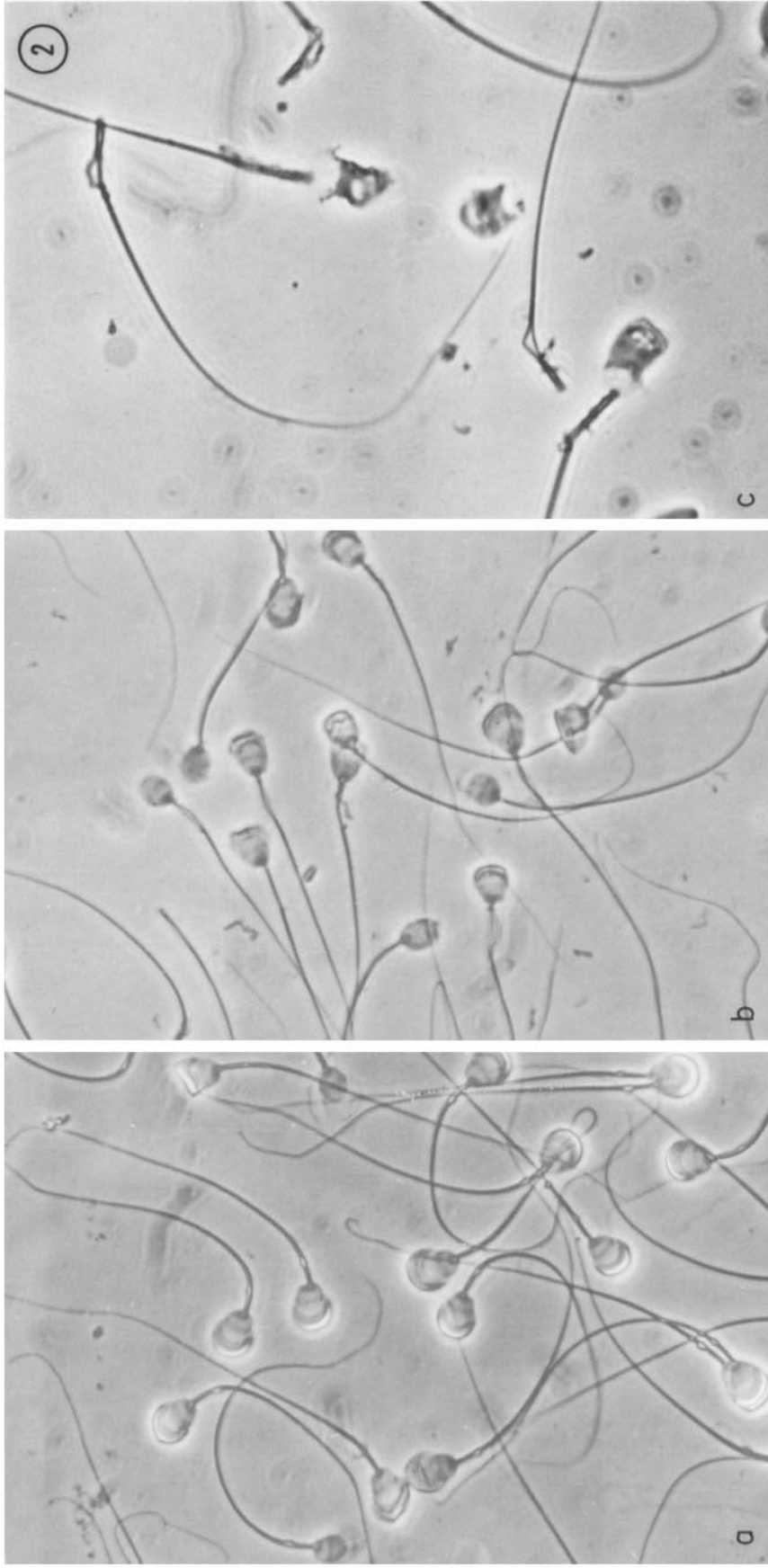


FIGURE 2 (a) Guinea pig spermatozoa in PBS, pH 7.4. $\times 1,400$. (b) Guinea pig spermatozoa treated with 0.1 M DTT in PBS, pH 7.4 for 30 min at 25°C. $\times 1,400$. (c) Guinea pig spermatozoa treated with 0.1 M DTT in PBS, pH 7.4 for 30 min at 25°C followed by 0.05 mg/ml trypsin in PBS, pH 7.4, for 10 s at 25°C. $\times 1,490$.

rapid cleavage of the heads from the tails without apparent damage to the midpiece or the acrosomal regions as viewed by phase contrast microscopy (Fig. 1). In contrast to this result, however, human, rabbit, or guinea pig spermatozoa were not noticeably affected by treatment with as much as 1.0 mg/ml trypsin in PBS for as long as 30 min at 25°C.

Proteolytic cleavage of spermatozoa from these species was achieved, however, by incubation of these cells with 0.1 M DTT or 0.1 M 2-mercaptoethanol in PBS for 30 min before washing in fresh PBS and exposure to trypsin. Under these conditions, a trypsin concentration of 0.05 mg/ml was sufficient for the rapid and quantitative cleavage of the spermatozoan heads from tails (Fig. 2). Cleavage occurred within 30 s at 25°C if the cell suspension was agitated gently during the incubation period. For human, rabbit,

and guinea pig spermatozoa, 90–95% of the cells were cleaved. Incubation of spermatozoa from these species with trypsin (0.05–0.1 mg/ml) followed by thorough washing in fresh PBS and subsequent exposure to either 0.1 M DTT or 0.1 M 2-mercaptoethanol did not result in cleavage of the cells.

Incubation with 0.1 M DTT alone did not result in cleavage of the heads from the tails, but did produce a distinct fraying of the mitochondrial component of the midpiece (Fig. 2 *b*). This reaction was similar for all species tested. If 0.1 M 2-mercaptoethanol was used, only 50–60% of the spermatozoa were visibly affected after 30 min incubation at 25°C.

Cleavage of Spermatozoa by Alteration of pH

In order to define the nature of the linkage between the head and tail, several other methods of cleaving spermatozoa were investigated. It was found that alteration of the pH of the suspension buffer resulted in cleavage without the addition of the proteolytic enzymes. As shown in Fig. 3 *a*, incubation of NCS mouse spermatozoa in citrate-PO₄ buffer at pH 1.8 resulted in the head-tail cleavage of 40% of the cells after 5 min at 25°C; further incubation at this pH for up to 60 min did not appreciably increase the proportion of cleaved spermatozoa. Spermatozoa from the other mammalian species examined were also cleaved at the junction of the head and the tail, but the exact proportion of the cells affected and the optimum pH for the cleavage reaction varied somewhat in each instance (Table I). Incubation of mouse or human spermatozoa with 1 M KCl,

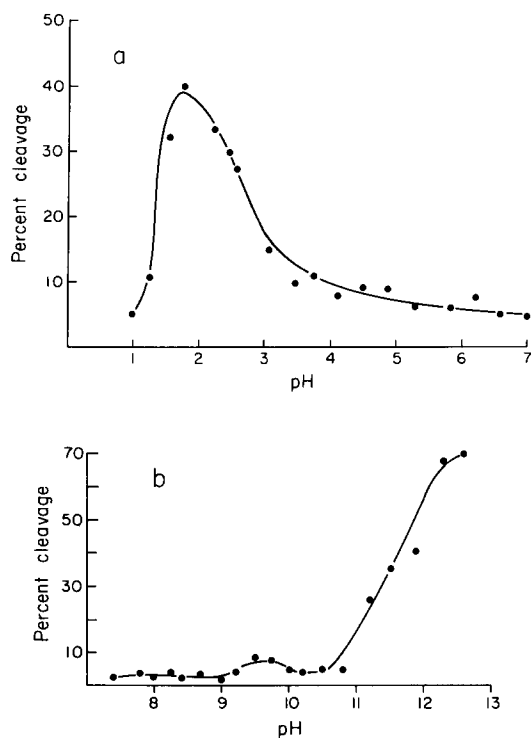


FIGURE 3 (a) Percent NCS mouse spermatozoa cleaved as a function of pH. A citrate-PO₄ buffer was adjusted to values between pH 1.0 and pH 7.2. Spermatozoa were incubated in this medium for 5 min at 25°C. (b) Percent NCS mouse spermatozoa cleaved as a function of pH. A 0.1 M Tris-HCl buffer was adjusted to values between pH 7.4 and pH 12.6. Spermatozoa were incubated in this medium for 5 min at 25°C.

TABLE I
Cleavage of Mammalian Spermatozoa
by pH

Species	% Cleavage under acidic conditions	% Cleavage under basic conditions (pH 12.6)
NCS mouse	40 (pH 1.8)*	70
Balb/c mouse	43 (pH 2.0)	73
Rat	38 (pH 1.7)	69
Human	33 (pH 2.8)	80
Guinea pig	11 (pH 2.3)	95
Rabbit	25 (pH 2.7)	81

* pH value indicates pH of maximal cleavage.

pH 7.4, or 1 M NaCl, pH 7.4, did not result in cleavage of the heads from the tails, and treatment of mouse spermatozoa with NaCl or KCl at 0.2 ionic strength did not cause cleavage.

The effects of alkaline pH on NCS mouse spermatozoa are shown in Fig. 3*b*. Between pH 7.4 and pH 11.0 the spermatozoa of all species tested remained unaffected as determined with phase contrast microscopy. The percentage of cleaved cells from all species increased rapidly between pH 11.0 and pH 12.6; however, the maximum value obtained ranged from 69% for

rat spermatozoa to 95% for guinea pig spermatozoa (Table I). Prolonged incubation of spermatozoa in strongly alkaline solutions resulted in alteration of the tail structure; under these conditions, the flagella were curled and appeared to be partially dissolved. This effect was particularly noticeable with mouse spermatozoa.

Ultrastructural Analysis of Chemically Dissected Spermatozoa

To examine the effects of trypsin on mouse spermatozoa at the ultrastructural level, DBA/

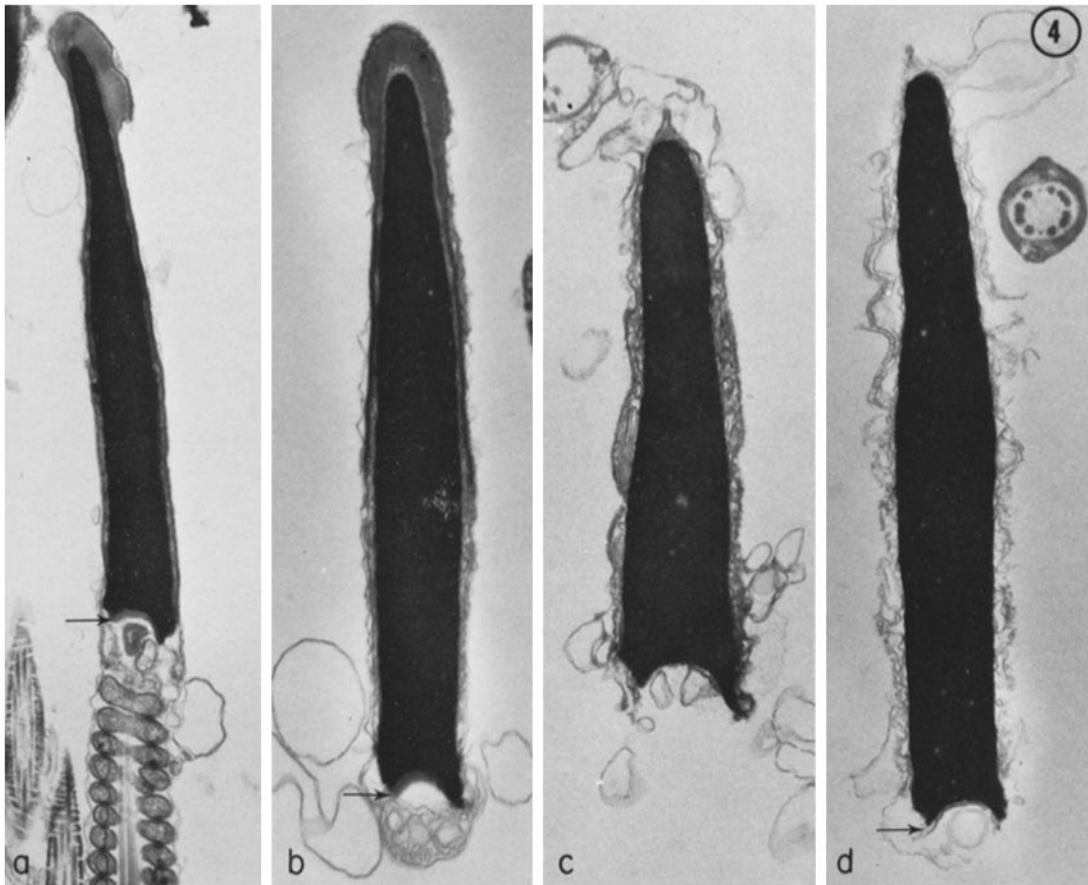


FIGURE 4 (a) Section through the head of a NCS mouse spermatozoon showing the acrosome and the structure of the neck and midpiece. The arrow (\rightarrow) indicates the basal plate. $\times 19,000$. (b) Head of NCS mouse spermatozoon after treatment with 0.05 mg/ml trypsin. The acrosome, the membrane folds along the posterior end of the head, and the basal plate (\rightarrow) are intact. The plasma membrane surrounding the head appears to be relatively undamaged. $\times 23,620$. (c) Head of NCS mouse spermatozoon after treatment with citrate- PO_4 buffer at pH 1.8. The acrosome is destroyed and the plasma membrane is extensively vesiculated. There is no adherent basal plate. $\times 36,100$. (d) Head of NCS mouse spermatozoon after treatment with Tris-HCl buffer at pH 12.6. The acrosome is destroyed and the plasma membrane is severely damaged but the basal plate remains (\rightarrow). $\times 26,350$.

2J, NCS, or Balb/cJ cells were treated with the enzyme and then prepared for electron microscope observation. As shown in Fig. 4, the point of cleavage of the heads from the tails was between the basal plate, which remained attached to the head of the spermatozoon, and the connecting piece of the neck, which remained attached to the midpiece. The membranous folds along the anterior portions of the midpiece were not visibly affected by trypsin. In addition, the acrosome was not noticeably damaged by the proteolytic procedure (Fig. 4 *b*), and the plasma membrane surrounding both the head and the tail portions of the spermatozoon was not removed or vesiculated. Ultrastructural examination of the midpiece of mouse spermatozoa after cleavage by trypsin revealed that the complex structures present in this region of the cell, including the mitochondria, the proximal centriole, and the connecting piece, remained firmly attached to the underlying flagellar filaments. No significant differences were detected in preparations from any of the three mouse strains examined.

In contrast to the results of enzymatic cleavage, treatment of mouse spermatozoa with acid or base resulted in extensive damage to the plasma membranes of both the head and tail portions of the cell (Figs. 4 *c* and *d*). The membranes were vesiculated and often separated from the nuclear membrane of the head and from the filaments of the tail apparatus. After treatment at basic pH (Fig. 4 *d*) the cleavage of heads from tails appeared to occur in the same plane as observed in the case of tryptic separation, although occasional heads without an adherent basal plate were seen. Treatment of spermatozoa with acid, however, resulted in separation of the basal plate from the head in all cases observed. The basal plate remained attached to the neck of the spermatozoon as shown in Fig. 5.

Other differences in the results obtained by cleavage with proteolytic enzymes and by alterations of pH were also detected by ultrastructural examination. Although trypsin treatment did not alter the appearance of the anterior region of the sperm head (compare Figs. 4 *a* and 1 *b*), treatment with acid or base resulted in the dissolution of the acrosome (Figs. 4 *c* and *d*). In addition, acid or base treatment of spermatozoa caused the extraction of electron dense material from mitochondria with extensive disruption of the cristae (Fig. 5).



FIGURE 5 Midpiece and neck region of an NCS mouse spermatozoon after treatment with citrate- PO_4 buffer at pH 1.8. The basal plate adhering to the connecting piece of the neck is evident. $\times 24,170$.

Guinea pig spermatozoa were examined with the electron microscope to compare the effects of DTT plus trypsin on these cells with the effects of proteolytic enzymes alone on mouse spermatozoa. As shown in Fig. 6, treatment of guinea pig spermatozoa with 0.1 M DTT alone caused ex-

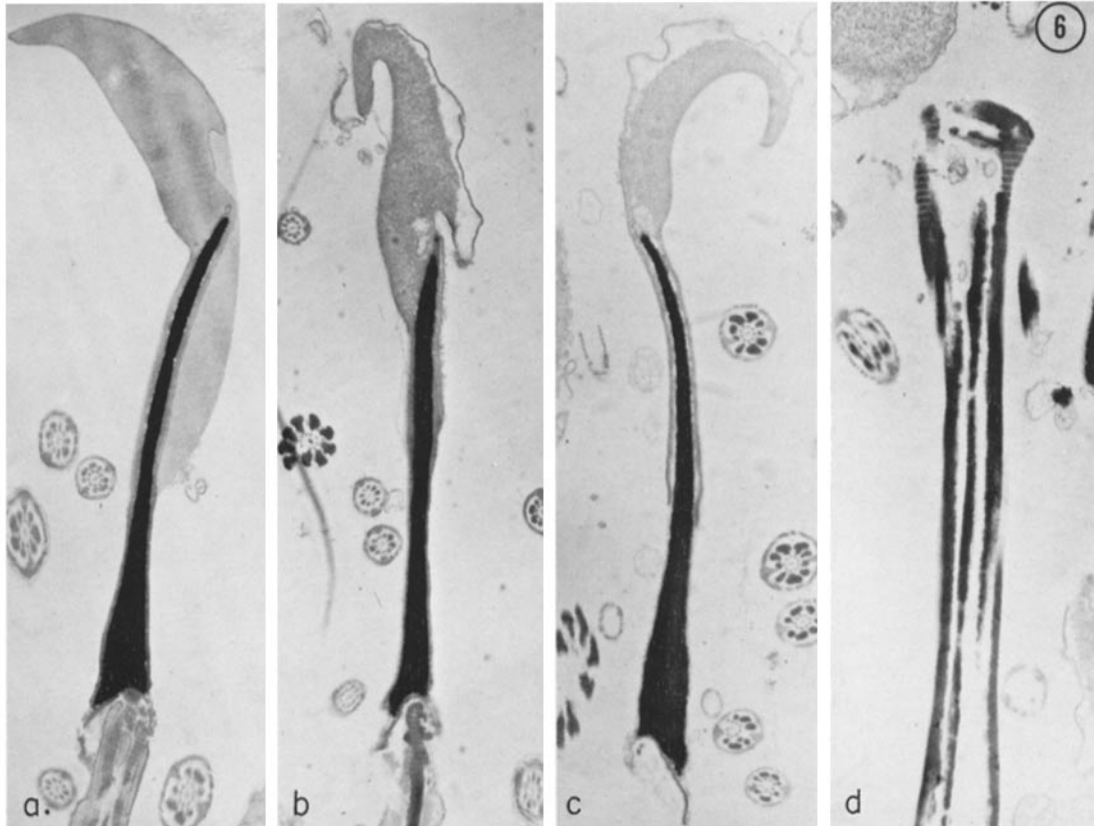


FIGURE 6 (a) Section of a guinea pig spermatozoon showing the acrosome and the structure of the neck and midpiece. $\times 13,600$. (b) Guinea pig spermatozoon after treatment with 0.1 M DTT. The acrosome is partially extracted and the plasma membrane is removed. $\times 13,950$. (c) Guinea pig spermatozoon after treatment with 0.1 M DTT followed by 0.05 mg/ml trypsin. The cell has been cleaved, leaving the basal plate with the head. $\times 14,500$. (d) Neck and midpiece of a guinea pig spermatozoon after treatment with 0.1 M DTT and 0.05 mg/ml trypsin. The mitochondria have been removed revealing the underlying filaments of the flagellum. $\times 21,500$.

tensive damage to the plasma membrane with complete removal of the membrane along large regions of the head, midpiece, and tail segments. Untreated control specimens showed intact plasma membrane along most of the cell surface (Fig. 6 a); in some cases separation from the nuclear membrane was evident. Treatment of guinea pig spermatozoa with DTT also caused the partial extraction of acrosomal components (Fig. 6 b), although the acrosomal membrane itself did not seem to be damaged severely. Only the most anterior, least electron dense, portion of the acrosome was noticeably affected.

The midpieces of some guinea pig spermatozoa were completely stripped of their mitochondria (Fig. 6 d) by treatment with DTT. In general,

however, the effects of reducing agent upon the mitochondria were restricted to extraction of internal material and disruption of the lamellar structure usually seen in these organelles. A small proportion of the mitochondria appeared swollen, with a cross-sectional diameter about 50% greater than that seen in untreated control specimens.

After treatment with both DTT and trypsin, guinea pig spermatozoa were cleaved at the junction of the basal plate and the connecting piece (Fig. 6 c). Thus, the basal plate remained attached to the head of the spermatozoon; this result resembled that found after mouse spermatozoa were treated with trypsin alone.



FIGURE 7 (a) NCS mouse spermatozoa treated with 0.1 M DTT for 90 min at 37°C. The mitochondria have been removed and the filaments of the tail are separated. $\times 3,180$ (b) NCS mouse spermatozoa treated with 0.1 M DTT for 90 min at 37°C, followed by 0.05 mg/ml trypsin for 10 s at 25°C. Cleavage of the heads from the tails allows the tail filaments to separate completely. $\times 11,120$.

Dissection of the Spermatozoan Midpiece

In an attempt to remove the mitochondria completely from the midpiece of mammalian spermatozoa, cells were incubated at 37°C in 0.05 M Tris-HCl, pH 7.4, containing 0.1 M DTT for 90 min. After this incubation, more than 95%

of the spermatozoa had midpieces with swollen and partially detached mitochondria and approximately 10-20% of the cells had no adherent mitochondria. Agitation of the cell suspension for 15 s resulted in the complete removal of the partially detached organelles from more than 96% of the spermatozoa. Although some cleavage

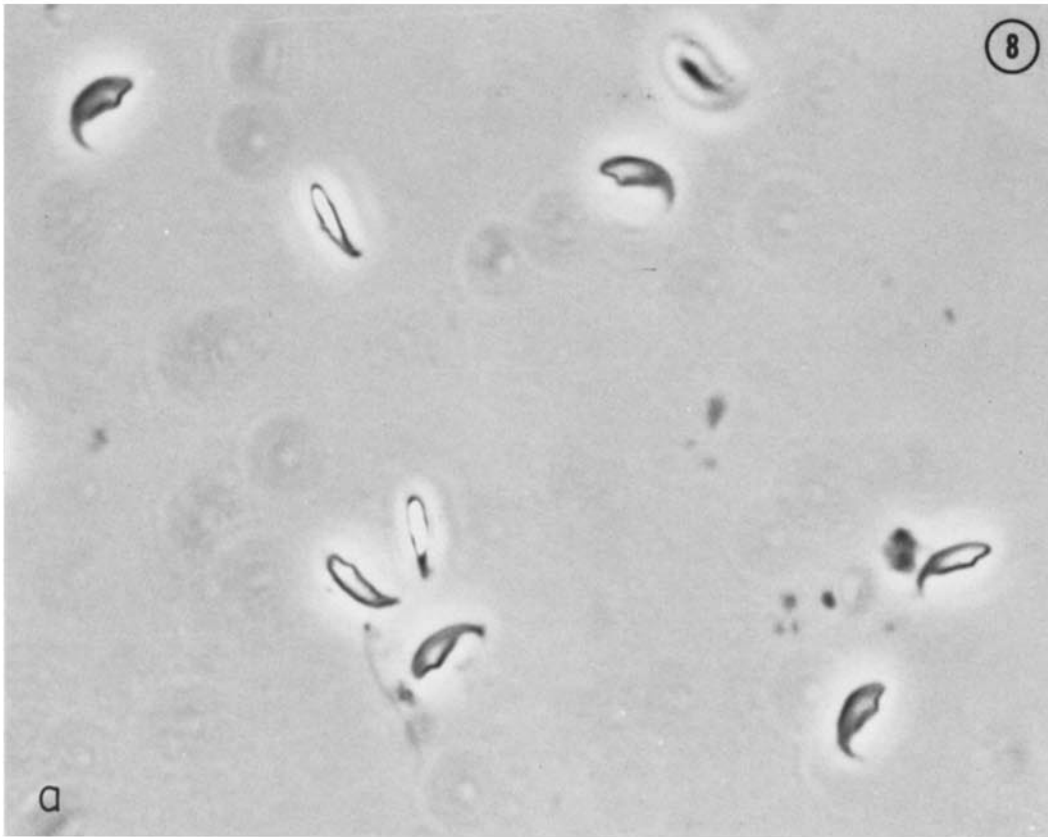


FIGURE 8 (a) Heads of NCS mouse spermatozoa after treatment with trypsin and fractionation by density gradient centrifugation. $\times 3,720$. (b) Tails of NCS mouse spermatozoa after treatment with trypsin and fractionation by density gradient centrifugation. $\times 1,820$.

of heads from tails was caused by the vigorous agitation, the extent of this mechanical cleavage was always less than 5%. Treatment of spermatozoa with 0.1 M DTT caused the separation of the central flagellar filaments in the midpiece region of the tail. The observed separation of filaments occurred only in the area of the midpiece (Fig. 7). The principal piece and end piece segments of the spermatozoan flagella were not noticeably altered.

Fractionation of Dissected Spermatozoan Components

Dissected mammalian spermatozoa were fractionated by density gradient centrifugation in order to obtain homogeneous populations of subcellular components in quantities sufficient for biochemical analysis. A modification of the technique of Stambaugh and Buckley (16) yielded satisfactory results.

Heads and tails were fractionated after treatment of mouse spermatozoa with trypsin. Isolated preparations of heads were contaminated with less than 5% tails, and isolated preparations of tails contained less than 3% heads (Fig. 8) as determined by phase contrast microscopy. The overall yield of material from the sucrose density gradients ranged from 65 to 75%. Electron microscope examination of these preparations indicated that the ultrastructure of the fractionated components was not significantly altered by the isolation procedure.

Purification of spermatozoan components after treatment of mouse cells with either 2-mercaptoethanol or DTT also was accomplished by density gradient centrifugation. Intact spermatozoa without mitochondria were obtained in 70–75% yield. After exposure to both reducing agent and trypsin, free heads and free tails without midpiece mitochondria were isolated in 70% yield. The separated tails were contaminated by less than 2% spermatozoan heads. So far, however, it has not been possible to recover intact mitochondria from the sucrose density gradients.

DISCUSSION

The cleavage of mammalian spermatozoa at the junction of the head and the tail by trypsin appears to occur at a specific location. Our ultrastructural observations indicate that the plane of separation is identical for both mouse and guinea pig cells and that it is located imme-

diately posterior to the basal plate of the head. Mouse spermatozoa are cleaved without alterations in the appearance of the acrosome and without severely affecting the plasma membrane of the head or tail. Furthermore, the structural components of the neck remain intact and attached to the underlying filaments of the tail. In contrast to these findings, treatment of guinea pig spermatozoa with DTT results in the partial extraction of acrosomal material and removes large portions of the plasma membrane although the effects of trypsin treatment itself are qualitatively similar for the two species. Moreover, the membranous folds in the neck region of both mouse and guinea pig spermatozoa are not affected by either DTT or trypsin and remain attached to the head after cleavage.

These results suggest that trypsin acts on a particular structure in the spermatozoan neck. Proteolytic cleavage occurs at the same location in the neck for all species tested. The requirements for DTT treatment before trypsin treatment in order to effect cleavage in some species may be explained by structural variations seen in the neck regions of various mammalian species (18, 19). For example, the membranous folds found in the neck are more pronounced in the spermatozoa of some species than in others (19). Our observations indicate that these structures are more apparent in mouse than in guinea pig spermatozoa. It is possible that a specific protein in the neck of the spermatozoon is responsible for maintaining the physical connection between the head and the tail. The observations presented here imply that the trypsin-sensitive site is normally inaccessible to the enzyme for all the species tested except for the mouse and rat. The effect of DTT treatment on guinea pig spermatozoa suggests that elements of the midpiece sheath of this species may physically inhibit penetration of the enzymes. Bull spermatozoa are also resistant to cleavage by trypsin (14). It remains to be determined whether cleavage can be effected after treatment of bull spermatozoa with a reducing agent.

After proteolytic cleavage *in vitro*, rodent spermatozoa bear a close morphological resemblance to abnormal spermatozoa from certain strains of Guernsey bulls. These gametes are cleaved at the junction of the head and the tail during passage through the epididymus (20). Ultrastructural examination of the naturally occurring fragments has revealed that the basal plate remains attached

to the head while the tail and midpiece structures remain intact (21), as we have observed after the proteolytic cleavage of mouse spermatozoa. Our findings suggest several possible explanations for the fragmentation of the bull spermatozoa. A trypsin-sensitive structure important for the integrity of the head-tail junction may be defective in these strains of bulls. Alternatively, these particular bulls may have active proteases in the epididymus or they may lack specific protease inhibitors.

In contrast to the limited effects on membranes of spermatozoa caused by proteolytic enzyme, treatment with either acid or base causes severe vesiculation of the plasma membranes of the sperm head, destroys the membranous whorls, and disrupts the membrane overlying the midpiece and principal-piece regions of the spermatozoan tail. Mitochondria are also damaged. The acrosome, which is not affected by trypsin, is disrupted by alterations of pH leaving only the apical body covering the nuclear DNA.

Cleavage of spermatozoa by acid does not result in the same alterations of spermatozoan structure as does treatment with base, however. Exposure to strong base causes partial disintegration of the sperm tail; acid treatment shows little or no effect in this region of the cell. Moreover, cleavage of spermatozoa by acid appears to involve a process with a pH optimum, as opposed to the results obtained using strong base. The pH optimum noted for acid cleavage of spermatozoa suggests either the unfolding of a specific protein or, alternatively, the possibility that acid proteases may cause the separation of the spermatozoan head and tail.

There are striking similarities in the ultrastructural features of mouse spermatozoa observed after treatment *in vitro* with acid and those observed in the ovum after fertilization. Specifically, electron micrographs of the fertilization process (22) have revealed that the basal plate remains attached to the flagellum after formation of the male pronucleus, that the plasma membrane becomes vesiculated, and that the mitochondria are damaged. These observations suggest that treatment of spermatozoa with acidic solutions may mimic a physiological process that occurs in the cytoplasm of the ovum. Thus it is possible that acidic conditions or acid proteases may play a role in the cleavage of the head from the tail during *in vivo* fertilization of the egg.

Complete removal of the mitochondrial components of the spermatozoan midpiece has not been achieved previously by any of the mechanical methods developed for dissecting mammalian spermatozoa (12, 13). Chemical dissection of spermatozoa with reducing agents such as DTT, however, allows the fractionation of tails that are not fragmented and that appear to consist of material comprising the flagellar structures themselves. The reasons for our inability to obtain fractionated preparations of the spermatozoan mitochondria are not clear, but Neubert and Lehninger (23) have noted that thiol and disulfide reagents cause swelling of isolated rat liver mitochondria at reagent concentrations much less than the 0.1 M DTT or 0.1 M 2-mercaptoethanol used in the experiments described here. It is possible that exposure to high concentrations of reducing agents followed by centrifugation in sucrose results in the complete disruption of the organelles.

DTT treatment of mammalian spermatozoa also results in separation of the flagellar filaments in the tail region immediately underlying the mitochondrial sheath. Although the disruption of organization in this area may be caused by the removal of physical constraints imposed by the mitochondrial covering, the observed separation may also be due to a chemical process. Other workers have presented evidence that the number of disulfide bonds in mammalian spermatozoa increases during maturation of the cell in passage through the epididymus (24), although the location of these bonds remains unknown. Conceivably, some of these bonds could be responsible for maintaining the intricate structure of the spermatozoan flagella; treatment with high concentrations of DTT could reduce such linkages to allow separation of the filaments. Earlier studies by Grigg and Hodge (25) on fowl spermatozoa treated with distilled water also revealed extensive separation of tail filaments, but in this instance only the end-piece and principal-piece regions of the sperm tail were affected. Further chemical studies are necessary to determine the role of disulfide bonds in the architecture of the tail apparatus.

Under the conditions described in this report DTT does not greatly affect the structure of the nucleus of the mammalian spermatozoon. There was no indication of chromatin disaggregation similar to that found by Lung (26) after exposure of human sperm to 0.1 M thioglycolate, pH 9.0.

The inability of DTT or 2-mercaptoethanol to achieve decondensation of the nuclear DNA may be due to the lower concentration of thiol-containing reagent used or to the lower pH at which the chemical dissection described here was done.

Reducing agents, however, do affect other regions of the spermatozoan head. Although no effect of DTT was noted for mouse spermatozoa, guinea pig cells did show partial extraction of acrosomal components. The ultrastructure of the guinea pig acrosome is more complicated than that of the mouse; a subdivision of the acrosome into electron dense and transparent regions is particularly evident. DTT seemed to preferentially affect the material at the anterior, or least electron dense, segment of the guinea pig acrosome.

The membranous whorls, like the acrosome, are another unique architectural feature of gametic cells. The ability to obtain large quantities of fractionated heads with these attached membranes should facilitate the biochemical characterization of any materials present in this membrane system. Some investigators have hypothesized that the membrane whorls have no physiological functions, but are simply the remnants of the spermatid nuclear membrane (27). It has also been suggested that this convoluted membrane system may actually be reincorporated into the membrane of the developing male pronucleus (28) or that the membrane folds, being located at the posterior end of the spermatozoan head, may facilitate the interchange of information between the cytoplasmic droplet of maturing spermatozoa and the nucleus of these cells (29). Both of these hypotheses imply functional roles for the nuclear membrane extensions. It is also possible, considering the proximity of this structure to the site of proteolytic cleavage, that the membranous whorls mediate the process of enzymatic cleavage either because they contain proteolytic enzymes that are released during the processes of fertilization or because they regulate the penetration of exogenous proteolytic enzyme through the pores present in these membranes (29).

Previous attempts to dissect spermatozoa have been somewhat limited. The acrosomes of ram spermatozoa have been obtained by the application of a cationic detergent and the contents of this spermatozoan structure have been identified (11). Bull spermatozoa have been

cleaved by pronase with extensive fragmentation of spermatozoan tails (14). Cleavage of spermatozoan heads from tails has been achieved previously by ultrasonication (13), but this technique does not yield the homogeneous population of cell fragments necessary for precise biochemical analysis. In contrast, the chemical dissection methods presented in this report are rapid, reproducible, quantitative, specific, and may be applied to a variety of mammalian species.

Fractionation of spermatozoan components after chemical dissection provides the opportunity to investigate molecular constituents in particular regions of the cells that are important in the maintenance of cellular architecture and in the physiological mechanism of fertilization. In addition, these methods should facilitate investigations of spermatogenesis. Chemical dissection of spermatozoa at different stages of maturity may, for example, clarify the manner in which transit through the epididymus alters the appearance and potential activity of the male gamete. Combination of this method with the use of antibodies directed against specific proteins isolated from fractionated mature spermatozoa should be particularly valuable for studies of the initial stages of gametic differentiation. These reagents could be used to determine the time of appearance and location of proteins in developing spermatozoa and could also be used to interrupt spermatogenesis at various stages in order to clarify some of the events in this complex process.

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