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Molecular Probes of Spermatozoan Structures

(fluorescent probes/sperm motility/agglutination/lectins/subcellular fractions)

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ABSTRACT **Several methods have been devised for the isolation and labeling of structural components of spermatozoa. Rodent spermatozoa were cleaved rapidly and specifically at the junction of the heads and tails by treatment with various proteases, and the separate components were isolated by density-gradient centrifugation. Treatment with reducing agents released the mitochondrial membranes from the midpiece, exposing the underlying tail structures.**

Mouse spermatozoa were found to contain about 10^7 sites per cell that bind concanavalin A; most of the sites appear to be on the head, for fluorescein-labeled conjugates of concanavalin A were bound mainly to the acrosomal region. Binding of concanavalin A resulted in rapid agglutination of spermatozoa; mixed agglutinates could be formed with somatic cells, as well as with spermatozoa of other species. Fluorescent probes (naphthalenesulfonic acids) bound to the sperm plasma-membrane and caused an immediate loss of motility. In contrast, ethidium bromide bound to the nuclear structures, but did not cause immediate immobilization. These isolation and probing procedures should facilitate detailed chemical analysis of the major components of mammalian spermatozoa.

Apart from the obvious importance of spermatozoa in the study of fertilization, they are of interest because of their great cellular specialization (1, 2). Acrosomes contain a number of key enzymes necessary for the fertilization reaction (3, 4). Other proteins that may be concerned with motility, such as acetylcholinesterase (EC 3.1.1.7) (5) and Na,K-ATPase (EC 3.6.1.3) (6), are located in the spermatozoan membrane and the tail, which itself contains microtubular and colchicine-binding proteins (7). The mechanism of spermatozoan motility remains a major puzzle, the solution of which may shed light on the role of microtubular proteins in cell motility.

We have explored a number of approaches to the analysis of sperm structure and function. In this paper, we describe (*a*) a proteolytic method for cleaving the heads of rodent spermatozoa from intact midpieces and tails, (*b*) the use of reducing agents for removing mitochondrial components of the midpiece, (*c*) agglutination of mammalian sperm by concanavalin A (Con A), (*d*) comparisons of Con A-receptor sites on the sperm plasma-membranes with those on somatic cells, and (*e*) the labeling of sperm surface structures and DNA by means of fluorescent probes.

Abbreviations: ANS, 8-anilino-1-napthalenesulfonic acid, magnesium salt; Con A, concanavalin A; Fl-Con A, fluorescein-labeled concanavalin A; PBS, phosphate-buffered saline: 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, pH 7.4; TNS, 8-*p*-toluidino-1-naphthalenesulfonic acid.

MATERIALS AND METHODS

Preparation of spermatozoa and treatment with proteases

Mouse, rat, guinea pig, and rabbit spermatozoa were obtained from the *vas deferens* by extruding its contents into PBS (phosphate-buffered saline, pH 7.4). Inbred mouse strains were NCS from Rockefeller University and AKR/J, C58/J, DBA/J, CE/J, BALB/c, and C57BR from Jackson Laboratories. Human spermatozoa were obtained from fresh ejaculates and were suspended and washed in PBS. Freshly prepared suspensions of spermatozoa in PBS were made up to about 10^7 cells/ml, as determined with a hemocytometer. Enzyme solutions (trypsin, B Grade, Calbiochem, Lot no. 001449) were freshly made in PBS and diluted 1:1 with the cell suspensions. The reaction mixture was gently shaken and incubated at 21°C and pH 7.4 with 0.1 mg/ml of trypsin, for 5–30 min, before it was washed with PBS or an inhibiting agent, such as soybean trypsin inhibitor, was added. After resuspension of the treated spermatozoa in 0.5 ml of 0.05 M Tris·HCl (pH 7.4), heads could be isolated from intact midpieces and tails by sucrose density gradient centrifugation (3). 1.0-ml fractions were collected from the top of the gradient and their contents were assayed with a hemocytometer.

Con A binding and agglutination reactions

Spermatozoa in buffer (10^7 cells/ml) were mixed with a solution of Con A (8) in the same buffer (0.001–1.0 mg/ml). Optimal conditions (pH 7.4, 21°C) led to agglutination within minutes. The minimal concentration of lectin needed for complete aggregation of 10^7 sperm/ml within 5–10 min was 0.1 mg/ml, but slight activity could be detected with Con A concentrations as low as 0.01 mg/ml. In mixed-cell preparations, equal volumes of cell suspensions containing 10^7 cells/ml in PBS (pH 7.4) were mixed with Con A (0.1 mg/ml).

For titration of binding sites on various tissues, testis, kidney, and thymus cells were prepared from NCS mice by forcing the excised organs through a 30-mesh stainless-steel screen after the organs were minced in PBS. Liver cells were prepared by a modification of the method of Anderson (9) using 28 mM EDTA in PBS to perfuse the organ. Peripheral lymphocytes were isolated (10) and freed of erythrocytes (11). ^{125}I-labeled Con A (specific activity 10^7 cpm/mg) (12) was incubated with the cells in buffer for 30 min at 21°C. Increasing aliquots of lectin were used to ensure saturation of receptor sites. As a control, [^{125}I]Con A was first incubated with 0.1 M α-methyl-D-mannoside. Cells were washed in buffer until no further removal of excess Con A was detected and were assayed by liquid scintillation spectrometry. To calculate the number of receptor sites per cell, a subunit molecular weight

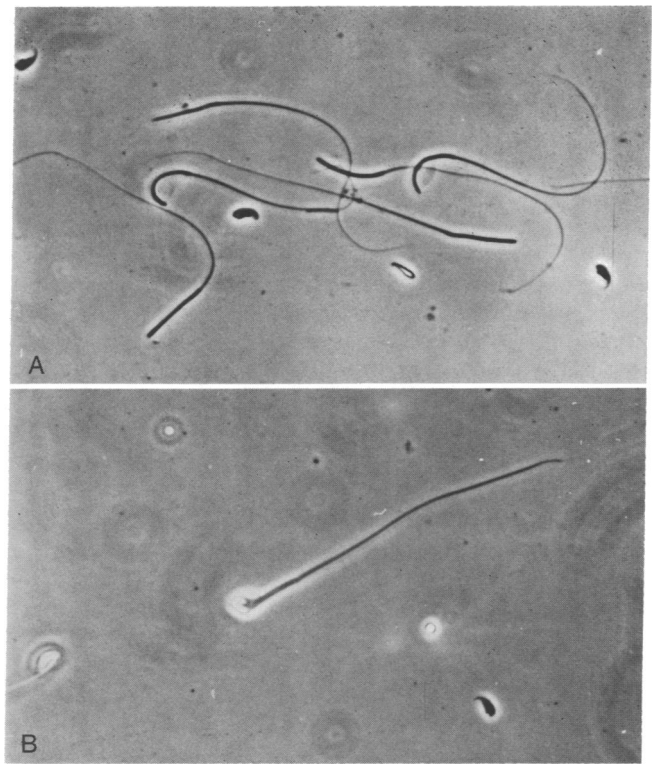

FIG. 1. (*A*) NCS mouse sperm treated with 0.1 mg/ml of trypsin in PBS (pH 7.4), with shaking for 5 min at 21°C. Phase-contrast microscopy, ×146 magnification. (*B*) NCS mouse sperm treated with 0.1 mg/ml of trypsin in PBS in a stationary well slide for 30 min at 21°C that shows the morphological alteration of the spermatozoan head. Phase-contrast microscopy, ×146 magnification.

for Con A of 27,000 was used; at pH 7.4 the lectin probably exists as tetramers (8). Replicate experiments gave values that were reproducible to within 15%.

Labeling with fluorescent Con A and fluorescent probes

Fluorescein isothiocyanate Isomer 1 (Baltimore Biological Laboratories) was used to label Con A (13). Labeled protein solutions were diluted to a final concentration of 1.0–1.5 mg/ml and incubated with cells for 15–30 min at 21°C before they were washed. A Zeiss Universal microscope with incident light fluorescence, BG12 and BG38 exciting filters, and a no. 53 barrier filter was used to observe labeled cells.

Cells were incubated in 1 mM solutions of 8-*p*-toluidino-1-naphthalenesulfonic acid (14) or 8-anilino-1-naphthalenesulfonic acid (Eastman Organic Chemicals) in PBS (pH 7.4) for 15 min at 21°C before washing with this buffer. Fluorescent filters used were the same as those used to examine Fl-Con A. Sperm were also incubated in a 1 mM solution of ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridium bromide; Boots Pure Drug Co. Ltd) in PBS for 15 min at 21°C; exciting filters BG3, BG12, and BG38, barrier filters 41, 44, and 53, and a Wratten 27A filter were used.

RESULTS

Cleavage of heads from tails by proteases

Treatment of mouse and rat spermatozoa with trypsin (EC 3.4.4.4.), chymotrypsin (EC 3.4.4.5), Pronase, or subtilisin (Carlsberg) (EC 3.4.4.16) resulted in the cleavage of the heads from the tails, which remained intact with midpieces

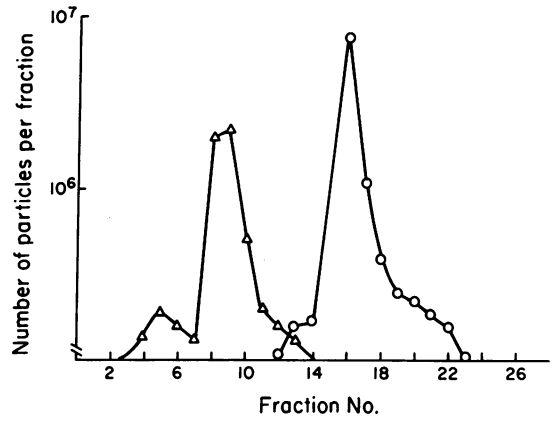

FIG. 2. Sucrose gradient fractionation (3) of trypsinized mouse-sperm heads and tails. Δ—Δ: sperm tails with attached midpieces; O—O: sperm heads.

attached (Fig. 1*A*). The reaction was not dependent upon the presence of motile spermatozoa. Exposure to exopeptidases such as carboxypeptidase-*A* (EC 3.4.2.1) and aminopeptidase (EC 3.4.1.2), as well as RNase (EC 2.7.7.16), DNase (EC 3.1.4.5), lysolecithinase (EC 3.1.1.5), neuraminidase (EC 3.2.1.18) (type *Vibrio cholerae* Z4), α-amylase (EC 3.2.1.1), and muramidase (EC 3.2.1.17) had no effect.

The effect of trypsin and chymotrypsin on various mammalian spermatozoa was examined, but only mouse and rat sperm were cleaved under the conditions used. Spermatozoa from the seven strains of inbred mice tested were all cleaved. In contrast, guinea pig, rabbit, and human spermatozoa were unaffected by exposure to as much as 1.0 mg/ml of trypsin in PBS for as long as 30 min at 21°C.

Detailed conditions for the cleavage by trypsin were determined. An enzyme concentration of at least 0.05 mg/ml was necessary to cleave virtually all sperm within 5 min at 21°C. The reaction was inhibited at 0°C, was effective at 37°C, and could be prevented by prior incubation of the trypsin with a 10-fold molar excess of soybean trypsin inhibitor. Cleavage proceeded most rapidly between pH 7 and 8, but residual activity was still detected over the range from pH 5 to 10.

Gentle shaking of the reaction mixture was essential for rapid cleavage of sperm. When motile spermatozoa were observed in a *stationary* well slide with 1.0 mg/ml of trypsin they underwent cleavage, but the reaction was considerably slowed and the heads of some of the spermatozoa showed marked morphological changes. Separate heads and tails began to appear within 5 min. After 30 min at room temperature, most cells were cleaved; some of the heads had lost their characteristic shape and appeared as birefringent globules (Fig. 1*B*).

Quantities of heads and tails suitable for further study were readily obtained in good purity from sucrose gradients (Fig. 2). The gradients showed a sharp band of high density that contained sperm heads and a broader, less dense band that represented the tails and attached midpieces.

Effects of reducing agents on the midpiece

In attempts to dissociate the spermatozoan midpiece from the tail, various reducing and denaturing agents were used, both before and after treatment of sperm with trypsin. Urea (5 M), guanidine·HCl (2 M), sodium dodecyl sulfate (0.1 mM–

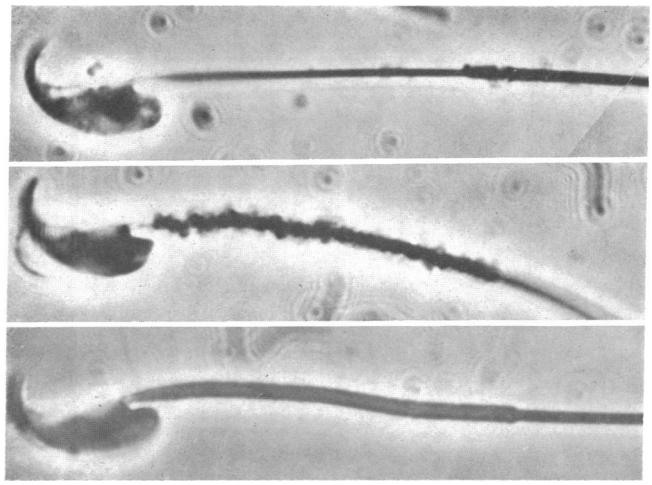

FIG. 3. *Upper*: NCS mouse sperm after treatment with 0.1 M dithiothreitol in PBS for 30 min at 21°C. *Middle:* NCS mouse sperm after treatment with 0.1 M 2-mercaptoethanol in PBS for 30 min at 21°C. *Lower*: Spermatozoan from an untreated control preparation. Spermatozoa were washed with PBS and observed by phase-contrast microscopy at ×850 magnification.

1.0 M), Triton X-100 (0.1–10.0%), and 2-mercaptoethanol (0.1–10 mM) had no visible effect upon the morphology of spermatozoa. However, when 0.1 M or 1.0 M dithiothreitol in PBS was incubated with mouse spermatozoa for 30 min at 21°C, the mitochondrial components of the midpiece were removed completely to reveal the underlying fibers of the tail. 2-mercaptoethanol, at the same concentrations, caused a distinct fraying of the cell membranes in the regions of the midpiece (Fig. 3), but the mitochondria remained attached to the spermatozoa.

Agglutination with Con A and quantitation of Con A-binding sites

As shown in Fig. 4*A*, the addition of Con A to fresh motile sperm resulted in their immediate agglutination. This reaction clearly involved both heads and tails, and at appropriate lectin concentrations large rafts of agglutinated spermatozoa were observed. Mouse, rat, guinea pig, rabbit, and human sperm also gave the reaction, as did all of the inbred mouse strains examined. Mixed agglutinates could readily be formed among spermatozoa from any of these species, as well as between spermatozoa and mouse somatic cells (Fig. 4*B*). When motile spermatozoa were used, the heads of the sperm were often found attached to the surfaces of the somatic cells.

Agglutination could be inhibited by prior incubation of the sperm preparation or the Con A with α-methyl-D-mannoside or glucose; D-galactose, a sugar known not to bind to Con A (15), had no effect. Moreover, spermatozoa, upon incubation with solutions of 11 different proteins, showed no agglutination. Prior incubation of Con A with rabbit antibodies to Con A inhibited agglutination, and the addition of the antibodies to a sperm suspension that had been exposed to a subagglutinating concentration of Con A caused agglutination.

A quantitative comparison of the number of Con A-binding sites on mouse sperm and mouse somatic cells is given in Table 1. Although single-cell suspensions were not obtained from mouse brain and skeletal tissue, when cells were labeled with Con A, it was revealed that striated muscle did bind lectin, but brain tissue remained unlabeled. The results obtained with radiolabeled-Con A were confirmed qualitatively by fluorescence microscopy using Fl-Con A.

Labeling of specific structures with fluorescein-labeled Con A and with fluorescent probes

Con A coupled to fluorescin isothiocyanate (Fl-Con A) also agglutinated spermatozoa. Fluorescence microscopy revealed that the major site of labeling was the convex surface of the head in the region of the acrosome (Fig. 5*A*). Faint and spotty labeling could be detected along the length of the tails, as well as on the midpieces and cytoplasmic droplets. The labeling by Fl-Con A was prevented by the prior addition of the unconjugated lectin, D-glucose, or α-methyl-D-mannoside. Addition of α-methyl-D-mannoside after the cells were labeled diminished the extent of the fluorescence. Although receptors were present along the sperm tail, by far the majority of Fl-Con A molecules were bound to the region of the acrosome.

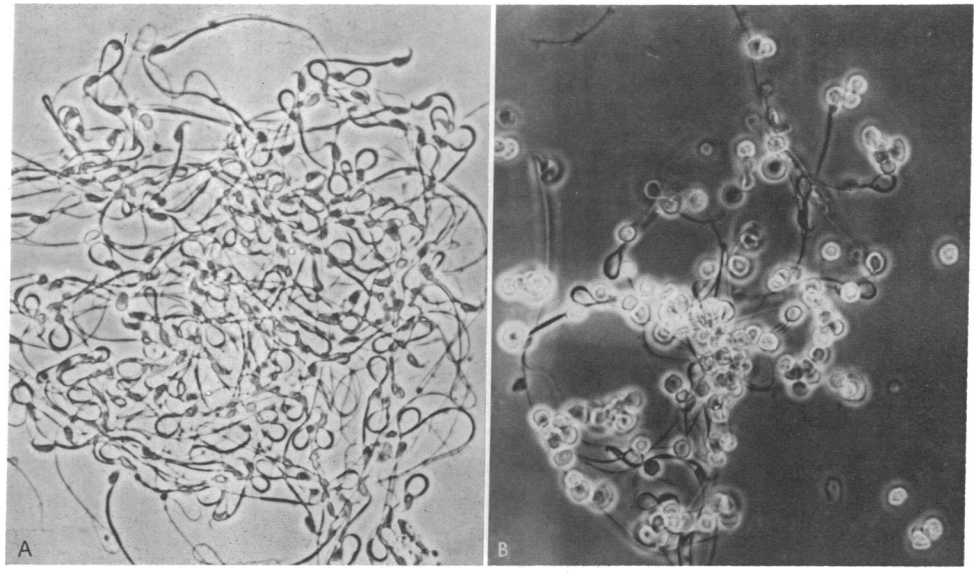

FIG. 4. (*A*) NCS mouse sperm treated with 0.1 mg/ml of Con A in PBS. Phase-contrast microscopy, ×130 magnification. (*B*) Mixed agglutinate of NCS spermatozoa and NCS thymocytes. Phase-contrast microscopy, ×130 magnification.

The fluorescent probes TNS and ANS had a dramatic effect on sperm. Motile mouse sperm were immediately immobilized when exposed to 1 mM TNS in PBS (pH 7.4), and the sperm plasma-membrane exhibited the bright green fluorescence characteristic of the bound hydrophobic probes. As shown in Fig. 5*B*, the membrane of the entire sperm was labeled; rat, guinea pig, rabbit, and human sperm gave similar results.

Because ethidium bromide is known to interact with double-stranded DNA (16), we examined its binding to mammalian cells. Incubation of sperm in 1 mM ethidium bromide, in PBS resulted in intense fluorescence of the head region (Fig. 5*C*), whereas midpieces and tails were unlabeled. There was no evidence of binding of the dye to the acrosomal region as was observed in studies of the binding of acridine orange (17). In contrast to the membrane probes TNS and ANS, selective binding of ethidium bromide to the DNA of the sperm nucleus had little immediate effect upon cellular motility, for motile sperm were detected as long as 15 min after labeling.

DISCUSSION

Rapid chemical methods for preparing sperm heads and tails in large quantities from a genetically well-studied species, such as the mouse, should be particularly valuable in the biochemical and electron microscopic analysis of spermatozoan structures. The use of the molecular probes described here can be extended to studies of various receptors and to the analysis of the interaction of sperm with ova and somatic cells. In addition, agglutination of sperm by lectins and their inmobilization by hydrophobic probes may have practical applications in the development of contraceptive methods and fertility control.

Perhaps the most striking features of the proteolysis of spermatozoa by endopeptidases was the cleavage at a single structural region of rat and mouse spermatozoa. This suggests that, in these spermatozoa, there are proteins that differ from proteins in the neck region of other mammalian spermatozoa, either in type or in accessibility to proteases. An analysis of peptides released by proteases and immunochemical studies using antisera to heads and tails may help identify the susceptible proteins. In contrast to sonication and other mechanical methods (18, 19) that often cause extensive fragmentation of sperm tails, protease treatment does not appear to affect the cell morphology except at the point of cleavage, provided that optimal protein concentrations and gentle shaking are used. The proteolytic reaction is also easy to regulate by the use of inhibitors. Obviously, useful fragments of spermatozoa can be prepared by the use of both the enzymatic and the mechanical procedures in a complementary fashion.

When proteases were used in stationary preparations, the cleavage reaction was slowed and some heads were converted to globular masses with loss of shape. This alteration of the spermatozoan head may result from general digestion of the plasma membrane by trypsin, although it is also possible that trypsin activates the acrosomes of some mature sperm, leading to the release of lipases and other enzymes with consequent formation of the globules.

It is probable that the disruption of the midpiece membrane by 2-mercaptoethanol and dithrothreitol is caused in part by swelling of the mitochondria located in this region, for thiols such as mercaptoethanol and glutathione have been found to cause isolated rat liver mitochondria to swell (20). A reaction of the reducing agents with disulfide bonds of the surface membrane or the underlying tail may also account for the release of the mitochondria.

TABLE 1. *Binding of Con A to sperm and somatic cells**

Cell type	Con A sites per cell
Sperm	4.9×10^7
Sperm heads†	7.9×10^6
Sperm tails†	2.5×10^6
Thymus	1.6×10^7
Spleen	1.4×10^6
Lymphocyte	1.3×10^7
Erythrocyte	6.8×10^6
Kidney	3.4×10^7
Liver	3.0×10^7
Testis	5.1×10^7

* Values represent the average of duplicate determinations.
† Prepared after trypsin treatment.

Agglutination of spermatozoa by Con A is a rapid process. At pH 7.4, Con A consists (8) of a tetramer (molecular weight 106,000), and agglutination is undoubtedly a result of the fact that each protomer contains a binding site (21, 8). The finding that Con A agglutinates spermatozoa indicates that carbohydrate receptors, probably in the form of glycoproteins (22, 23), are present on the cell surface. Mouse spermatozoa had about the same number of Con A-receptor sites as mouse somatic cells, but the distribution of these sites on the spermatozoan surface was highly asymmetric. Treatment with trypsin appeared to remove many of the Con A sites, inasmuch as the trypsinized sperm heads and tails together had only one-fifth the number of receptors as whole spermatozoa (Table 1). Our experiments do not rule out the possbility that

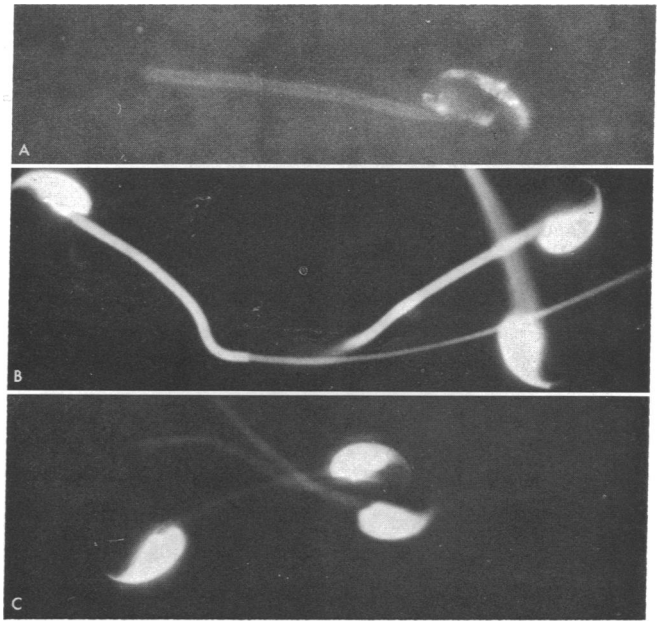

FIG. 5. (*A*) NCS spermatozoa labeled with Fl-Con A, ×638 magnification. (*B*) NCS spermatozoa labeled with ANS, ×638 magnification. (*C*) NCS spermatozoa labeled with ethidium bromide, ×638 magnification. Detailed observation by phase-contrast and fluorescence microscopy showed that the acrosome was unlabeled.

glycoproteins from the fluids of the reproductive tract are adsorbed to the surface of the spermatozoa. Because of the asymmetric distribution of the sites, however, we favor the view that the receptors are a specific feature of the cell surface.

The Con A-binding sites on the head appear to be localized in the acrosomal region. This is in accord with the observations of Srivastava *et al* (24), who have found lipoglycoproteins in this stucture. Con A and similar lectin probes may be useful in examining isolated acrosomal preparations (4) and the role of acrosomal enzymes and membranes in fertilization. The carbohydrate-containing receptors on the sperm cell surface may play a role in the capacitation reaction (25) or in interactions of sperm and ova; it would therefore be of interest to determine whether ova have the capacity to bind Con A. It should be noted, however, that β-glucuronidase (EC 3.2.1.31), which has been reported to capacitate hamster spermatozoa (26), had no effect on the binding of Con A to sperm.

The plasma membrane of the sperm must be particularly important for the regulation and initiation of motility (27). Both TNS and ANS cause immediate immobilization of spermatozoa. It is therefore possible that the membrane-associated Na,K-ATPase (6) is inhibited by these probes; alternatively, the membrane structure may itself be disrupted by binding of these molecules (28–33), which are highly hydrophobic (14, 34, 35). In contrast to TNS and ANS, ethidium bromide had no immediate effects on motility and did not bind to tail structures. This probe may be useful as a tag for sperm heads in studies of sperm–egg interactions. Because it does not appear to bind to the acrosome, it may also be useful in comparing the DNA of spermatozoa from various species.

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