Changes in Plasma Membrane Glycoproteins of Rat Spermatozoa during Maturation in the Epididymis

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ABSTRACT Glycoproteins on the plasma membrane of testicular and cauda epididymidal spermatozoa have been labeled with galactose oxidase/NaB $[^3H]_4$ and sodium metaperiodate/NaB$[^3H]_4$, followed by analysis on SDS polyacrylamide gels. The major glycoprotein labeling on testicular spermatozoa has a molecular weight 110,000 whereas on cauda epididymidal spermatozoa >90% of the radio-label is incorporated into proteins of molecular weight 32,000. These 32,000-mol wt proteins are homologous with proteins of similar molecular weight purified from the epididymal secretion and which have been shown previously to be synthesized in the caput epididymidis under hormonal control. Immunofluorescence revealed that the 32,000-mol wt proteins are present on the flagellum of mature but not immature spermatozoa and that they have a patchy distribution suggesting that they are mobile within the plane of the membrane. The membrane-bound 32,000-mol wt proteins possess hydrophobic domains as revealed by charge-shift electrophoresis and they also label with a lipophilic photoaffinity probe suggesting that they are in contact with the lipid bilayer. The evidence indicates that there is a considerable reorganization of the molecular structure of the plasma membrane of spermatozoa during maturation in the epididymis and that some of the changes are brought about by a direct interaction with epididymal secretory proteins.

The acquisition of motility and fertilizing capacity by mammalian spermatozoa as they pass through the epididymis is accompanied by a number of distinct, albeit in some cases subtle, changes in their morphology, composition, and metabolic activity (reviewed in references 2 and 42). That the epididymis plays a significant role in regulating sperm maturation is not in doubt, but relatively little is known about the processes mediating or controlling these changes, the sequence in which they take place, and their relative importance to the ultimate ability of the sperm to fertilize an egg. Recently, interest has focussed on maturation changes in the plasma membrane as the initial events during fertilization involve cellular recognition and membrane fusion. The concept that the plasma membrane of mature spermatozoa is a mosaic of heterogeneous protein and lipid domains has arisen from studies on the binding of lectins (31), distribution of antigens (1, 11, 15, 16, 25, 28, 30, 33), surface charge (21, 37, 50), and the arrangement of intramembrane particles (12, 29, 32). However, it is not altogether clear from these studies whether this heterogeneity is already expressed on spermatozoa in the testis or if it arises by modification of the plasma membrane during maturation in the epididymis.

As a preliminary step towards investigating the role of the epididymis in regulating sperm maturation we have examined the synthesis and secretion of proteins and glycoproteins in the rat epididymis (8, 26). Several androgen-dependent proteins and one testicular fluid-dependent protein were identified and characterized from the luminal secretions. In this communication we have extended these observations by comparing the nature of plasma membrane glycoproteins on testicular and cauda epididymidal spermatozoa. By inference, differences between the two types of spermatozoa must have developed at some stage in the epididymis and are likely to be related, either directly or indirectly, to the acquisition of fertilizing capacity. The results suggest that there is a significant reorganization of the molecular architecture of the sperm plasma membrane during maturation and that some of these changes are brought about by a direct interaction with epididymal secretory proteins.

MATERIALS AND METHODS

Materials: Adult male Wistar rats (300–350 g body weight) were used throughout these experiments. All chemicals and enzymes were of the highest purity available commercially and were purchased from BDH (Poole, Dorset,

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UK) or Sigma Chemical Co. (London, UK). Glass fibre filters (GF/C), CM-23 cellulose and DE-23-cellulose were supplied by Whatman Biochemicals (Maidstone, Kent, UK), and peroxidase- or fluorescein-conjugated antibodies by Uniscience Ltd (Cambridge, UK). DEAE-Sephadex A-25, and *Staphylococcus aureus* cells were obtained from Pharmacia Ltd (Hounslow, Middlesex, UK) as were protein standards of known size. These were α-lactalbumin (14,000 mol wt), soya bean trypsin inhibitor (20,100 mol wt), carbonic anhydrase (30,000 mol wt), ovalbumin (43,000 mol wt), bovine serum albumin (BSA) (65,000 mol wt) and phosphorylase b (94,000 mol wt). $NaB[^3H]_4$ was purchased from the Radiochemical Centre (Amersham, UK), and [^{125}I] hexanoyldiiodo-*N*-(4-azido-2-nitrophenyl) tyramine was a gift from Dr. M. J. Owen, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, UK. Neuraminidase (Behringwerke) from *Cholera vibrio* was supplied by Hoechst Ltd. (Hounslow, Middlesex, UK), galactose oxidase and pancreatic trypsin by Worthington Enzymes (Millipore Ltd, London, UK), and pronase by Cambrian Chemicals Ltd (London, UK), Endoglycosidase H from *Streptomyces griseus* was obtained from Seikagaku Kogyo Ltd (Tokyo, Japan) and a preparation of mixed glycosidases from Sigma Chemical Co., Ltd.

Collection and Washing of Spermatozoa: Testicular spermatozoa were collected by puncturing the extratesticular rete testis of rats which had the efferent ductules ligated (EDL) for 16–18 h previously. Approximately 100–150 μl of testicular fluid per rat was collected with a sperm density of 0.5–1.0 × 10^8/ml. Spermatozoa were flushed from the cauda epididymidis by retrograde injection of buffered isotonic sucrose (0.264 M sucrose-5 mM HEPES pH 7.4) via the vas deferens. All manipulations were performed with the tissue under a × 8 stereomicroscope, and any samples suspected of containing traces of blood or tissue fluid were discarded.

The luminal contents from the cauda epididymidis were diluted in isotonic sucrose (~40 μl in 1 ml), and 1–2 ml of sperm suspension was layered over 3 ml of 0.3 M sucrose-5 mM HEPES pH 7.4. After centrifugation at 200 *g* for 5 min and 500 *g* for 10 min, the supernatant was retained as diluted cauda epididymidal plasma (CEP), and the infranatant was discarded. The sperm pellet was resuspended to ~10^8 cells/ml in sucrose buffer. Testicular spermatozoa were washed directly as described above without dilution and resuspended in sucrose buffer to ~10^8 cells/ml. Rete testis fluid (RTF) was stored frozen pending analysis. Sperm concentration was determined by haemacytometer counts.

Labeling of Plasma Membrane Proteins: D-Galactose and β-*N*-acetylgalactosamine residues on membrane glycoproteins were labeled with galactose oxidase/$NaB[^3H]_4$ by a modification of the method of Steck and Dawson (46). A portion (0.5 ml) of washed testicular or cauda epididymidal spermatozoa was mixed with 5 μl (1 Sigma Unit/μl; 5 μg/μl) of galactose oxidase and 20 μl (0.5-1.0 m Ci) of $NaB[^3H]_4$. Control samples, consisting of sperm + $NaB[^3H]_4$ alone, were included in each experiment to assess nonspecific binding of radiolabel. The samples were incubated for 5 min at 25°C and spermatozoa were separated from enzyme and free label by washing through 3 ml of 0.3 M sucrose-5 mM HEPES, pH 7.4. The sperm pellet was then extracted in detergents as described below. Preliminary experiments showed that under these conditions incorporation of $NaB[^3H]_4$ into acid-insoluble protein reached a plateau after 5-min incubation and that the inclusion of enzyme + $NaB[^3H]_4$ together was not qualitatively nor quantitatively different from pre-incubating spermatozoa with galactose oxidase alone followed by $NaB[^3H]_4$ (46).

For labeling of soluble protein, RTF or CEP was dialyzed overnight against PBS, and a portion containing 5 μg total protein was mixed with 3 U of galactose oxidase, ~0.5–1 mCi $NaB[^3H]_4$ and PBS to a final volume of 120 μl. After incubation for 30 min at 25°C, 10 μl of 0.5% albumin solution was added followed by 9 vol of absolute ethanol. Precipitated protein was solubilized in SDS buffer for electrophoresis on SDS polyacrylamide gels. Preliminary experiments showed that incorporation of radiolabel into protein was linear between 1 and 10 μg of total protein incubated.

Terminal sialic acid residues on membrane glycoproteins were labeled by preincubating 1-ml suspensions of spermatozoa in the presence of 5 mM sodium metaperiodate pH 7.0 for 10 min at 25°C. Five ml of sucrose buffer were added, and the spermatozoa were washed and resuspended to 1.0 ml in sucrose buffer. Approximately 0.5 mCi of $NaB[^3H]_4$ was added, the suspensions were incubated for 5 min at 25°C and then the spermatozoa were washed and extracted with detergents as described below. Preliminary experiments showed that the optimal concentration of metaperiodate was 5 mM and that 5-min incubation was sufficient for maximum incorporation of $[NaB]^3H_4$.

Hydrophobic domains of integral membrane proteins were labeled with the photoactivatable reagent, [^{125}I]hexanoyldiiodo-*N*-(4-azido-2-nitrophenyl)tyramine. This reagent partitions preferentially into the lipid bilayer of membranes and, when activated by light, labels proteins in contact with it (44). Spermatozoa were homogenized with a cell disruptor (Stansted Fluid Power, Stansted, Essex, UK) and centrifuged at 10,000 *g* for 30 min. The supernatant was centrifuged at 100,000 *g* for 60 min and the pellet, consisting of plasma membrane and acrosomal membranes, suspended to 1 mg protein/ml in 10 mM-Tris/HCl pH 7.4. Electron microscopy showed that this suspension consisted only of small membrane-bounded vesicles. The membrane-bound vesicles were labeled with the ^{125}I-nitrene reagent as described (44).

Enzyme Treatment of Spermatozoa: Labeled spermatozoa were washed and resuspended to ~0.5 × 10^8/ml in sucrose buffer. Portions (0.5 ml) were incubated for 60 min at 25°C either without addition, or with 25 μl of pancreatic trypsin (1 mg/ml), or 25 μl of pronase (1 mg/ml) or 100 μl of endoglycosidase H (0.5 U/ml), or 100 μl of a solution of mixed exoglycosidases (3 mg/ml). For neuraminidase, washed spermatozoa were first incubated for 60 min at 25°C with 25 μl of enzyme (500 U/ml: Behringwerke), washed again, and then labeled. $CaCl_2$ was included to a final concentration of 5 mM for neuraminidase and 10 mM for trypsin and pronase.

Solubilization of Membrane Proteins: Washed, labeled spermatozoa were resuspended in 0.2% sodium deoxycholate (DOC) in sucrose buffer, pH 8.0, and incubated for 45 min at 4°C. Suspensions were centrifuged at 1,000 *g* for 10 min, and the supernatants recentrifuged at 10,000 *g* for 10 min. The 1,000 *g* sperm pellets were then reextracted in 1.0% SDS in sucrose buffer, pH 7.4, and centrifuged as before. A time-course of the rate of protein solubilized in each detergent showed that a maximum was reached after 45 min. Protein concentration was measured by the Lowry procedure (36) or by extinction at 280 nm. BSA was used as standard.

Electrophoresis and Detection of Labeled Proteins: One-dimensional electrophoresis of proteins extracted from spermatozoa was performed under reducing conditions on 15% polyacrylamide gels containing 1% SDS as described (26). Proteins were stained with 0.05% Coomassie Brilliant Blue or Amido Black in an aqueous solution of 40% methanol and 7% acetic acid for 60 min at room temperature and destained overnight in the same solvent without dye. Approximate molecular weights were calculated from the mobility of proteins of known size. Relative amounts of protein were quantified by densitometry, using an integrating densitometer (Helena Laboratories) to calculate peak areas. Labeled proteins were detected by fluorography (5) on preflashed film (Kodak-Omat H) and quantified by densitometry.

For electrophoresis on nondenaturing Ornstein-Davis gels (9), proteins were first dialyzed against 10 mM Tris/glycine buffer pH 8.4, freeze-dried, and solubilized in 0.1 of the original volume of distilled water. Samples, containing equal amounts of protein, were electrophoresed on 7% polyacrylamide slab gels, pH 8.9.

For two-dimensional electrophoresis, the appropriate track was excised from nondenaturing gels and the strip was incubated overnight at room temperature in 62.5 mM Tris/HCl pH 7.4 containing 1% SDS, 1% 2-mercaptoethanol. Proteins were then separated in the second dimension by positioning the gel strip on top of an SDS polyacrylamide gel prepared and electrophoresed as described above.

Hydrophobicity of proteins was demonstrated by charge-shift electrophoresis (22). Labeled spermatozoa were extracted with either 0.2% DOC/1% Triton X-100 or 0.2% cetyltrimethylammonium bromide (CTAB)/1% Triton X-100, and proteins were separated on 1% agarose gels (22). BSA and lysosyme were used as markers. After electrophoresis, gels were sliced into 1.5-mm segments and radioactivity was counted in 10 ml of Packard 299 scintillation fluid.

Purification of 32,000-mol wt Proteins from Epididymal Secretions: Two major acidic glycoproteins were purified from dilute CEP by ion-exchange chromatography on DEAE Sephadex A25 at pH 7.9 (26). The purified proteins were >95% homogeneous as judged by electrophoresis on SDS polyacrylamide gels (26) and gave a positive reaction for carbohydrate when stained by the periodic acid-Schiff procedure (51). When analyzed on nondenaturing polyacrylamide gels at pH 8.9 (26), the preparation migrated as two separate bands near the anode. However, when analyzed in the second dimension on SDS polyacrylamide gels, a single diffuse-staining band with an average molecular weight of 32,000 was obtained. A single peak, also corresponding to ~32,000, was obtained by gel filtration on Sephadex G-75 (26). We conclude that our preparation consists of two proteins whose properties are very similar and probably represents a case of microheterogeneity. They co-purify under all chromatographic conditions used so far. Henceforth, we shall refer to them as the 32,000-mol wt proteins.

Production and Specificity of Antiserum: An antiserum to the 32,000-mol wt proteins was prepared in New Zealand White rabbits using a protocol described (8). The antiserum was tissue specific and did not cross-react on Ouchterlony double diffusion agar gels (43) with cytosols prepared from rat testis, ventral prostate, seminal vesicles, coagulating glands, liver, or kidney. Furthermore, it did not react against proteins in rat blood serum, testicular fluid, or the other hormonally regulated proteins we have identified and purified from epididymal secretions (26). The antiserum gave a precipitation line only when challenged with epididymal cytosol or with the purified antigen. When a working dilution (1:32) of antiserum was absorbed with 4 mg of washed cauda epididymidal spermatozoa, no detectable activity remained in the supernatant.

Immunoprecipitation: Immunoprecipitation of labeled membrane proteins was carried out by the procedure of Kessler (27). Immune and preimmune sera were decomplemented by heating at 56°C for 30 min and absorbed

three times at 4°C for 45 min each with an equal volume of a mixture of rat spleen, brain, and kidney cells in PBS pH 7.4. DOC extracts were made 150 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl pH 7.4, 1% Nonidet P-40 (NP-40), 1 mg/ml BSA, and 200 μl incubated with 10 μl of immune or preimmune serum for 30 min at 25°C. *Staphylococcus aureus* cells (100 μl of 10% suspension), previously washed three times in NETN buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl pH 7.4, 1% NP-40) containing 1 mg/ml BSA, were added and incubated with shaking for 60 min at 4°C. Before incubation with antisera, DOC extracts were pretreated with 100 μl of *Staphylococcus aureus* cells for 60 min at 4°C. Cells were washed successively with NETN buffer supplemented to 0.5 M NaCl, NETN buffer + 0.1% SDS, and lastly with 10 mM Tris/HCl pH 7.4 + 0.1% NP-40. The final pellet was resuspended in electrophoresis buffer and boiled for 4 min. This procedure gave very low background with ~40% efficiency of precipitation.

Immunofluorescence: Indirect immunofluorescent tracing of the 32,000-mol wt proteins on testicular and cauda epididymidal spermatozoa was done on cells in suspension. Washed spermatozoa were resuspended in immune or preimmune serum diluted 1:32 in PBS/5% BSA and incubated for 30 min at 25°C. The spermatozoa were washed twice in PBS/5% BSA and incubated for a further 30 min at 25°C in FITC-conjugated goat antirabbit IgG. After two more washes in PBS/5% BSA, 5-μl aliquots of spermatozoa were mixed with 5 μl of 1:1 PBS:glycerol, mounted on clean glass slides, and viewed with a Zeiss epifluorescence microscope.

Immunoperoxidase: Detection of the 32,000-mol wt antigens on whole testicular and cauda epididymidal spermatozoa was carried out by the immunoperoxidase procedure as described by Lopo and Vacquier (35).

RESULTS

Comparison of Proteins in RTF and CEP

Since spermatozoa are exposed to a continuously changing milieu during their passage through the epididymis (25), we first compared the protein composition of RTF and CEP. When proteins in RTF and CEP were separated on nondenaturing polyacrylamide gels in the first dimension and denaturing polyacrylamide gels in the second dimension, different patterns were obtained after staining the gels with Coomassie Brilliant Blue (Fig. 1). The major protein in RTF was serum albumin which accounted for ~32% of the total protein. Only one pre-albumin protein, with a molecular weight of 23,000, was detected in RTF, and this accounted for 1.8% of the total protein. By contrast, five major pre-albumin proteins were present in CEP (Fig. 1). These had molecular weights of 18,500 (7.9%), 19,000 (8.1%), 23,000 (5.5%), and 32,000 (9.2%). Values in brackets refer to their proportion of total protein as determined by densitometry. A relatively small amount (5.0%) of albumin is present in CEP from normal rats.

Comparison of Membrane Glycoproteins on Testicular and Cauda Epididymidal Spermatozoa Labeled with Galactose Oxidase and NaB[^{3}H]$_4$

In the presence of galactose oxidase, the incorporation of NaB[^{3}H]$_4$ into protein was stimulated 6- to 10-fold above controls for cauda epididymidal spermatozoa but only 3- to 4-fold for testicular spermatozoa. Furthermore, testicular spermatozoa appear to contain more galactolipid as washing TCA-precipitated material collected on filters with 50 ml of 2:1 chloroform:methanol reduced the amount of NaB[^{3}H]$_4$ on testicular spermatozoa by 15–20% but only by 6–10% on cauda epididymidal spermatozoa.

Separation of labeled proteins extracted from testicular spermatozoa with DOC on SDS polyacrylamide gels followed by fluorography revealed one major labeled protein with a molecular weight of 110,000 (Fig. 2). This protein contained ~90% of the total radioactivity incorporated as determined by densitometry on preflashed film. Minor labeling was also detected on proteins with molecular weights of 42,000, 55,000, 64,000,

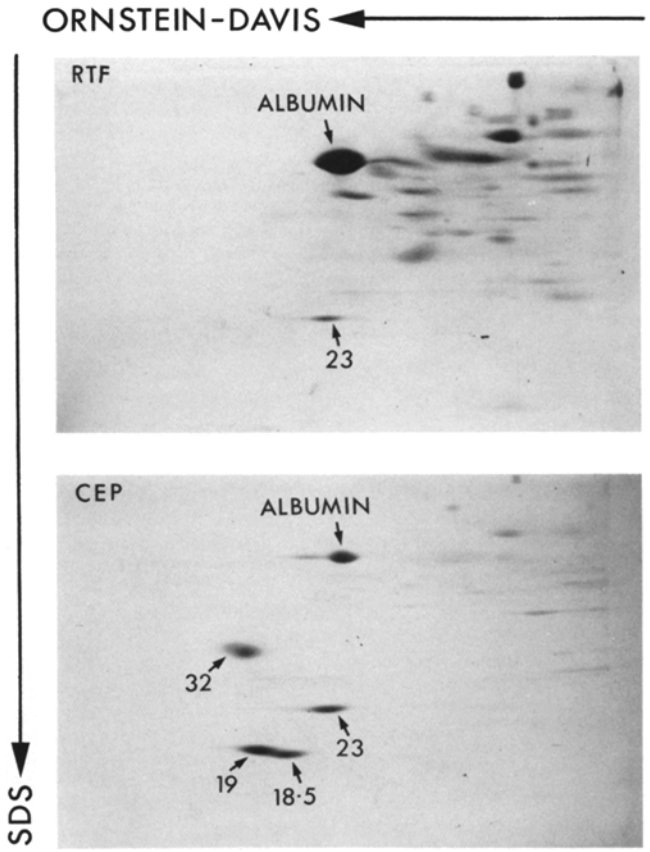


FIGURE 1 Two-dimensional electrophoresis of proteins in RTF and CEP. Proteins were separated in the first dimension on non-denaturing Ornstein-Davis polyacrylamide gels at pH 8.9 and in the second dimension of 15% polyacrylamide gels containing 1% SDS after reduction with 1% 2-mercaptoethanol. Equal amounts of protein (150 μg) were applied to each gel and proteins were stained with 0.05% Coomassie Brilliant Blue. Values given are approximate molecular weights.

78,000, and 180,000. Reextraction of sperm pellets with SDS revealed no additional labeled proteins. However, when DOC extracts of labeled proteins from cauda epididymidal spermatozoa were analyzed on SDS polyacrylamide gels, no labeling was detected in the region of 110,000 mol wt. Instead, >90% of the radioactivity was incorporated into proteins of ~32,000 mol wt with a small amount (~5%) in proteins with molecular weights of 13,500, 58,000, 84,000, and 150,000 (Fig. 2). Reextraction of the sperm pellets with SDS revealed additional labeling of a protein of 47,000 mol wt. It was also apparent from analysis of these SDS extracts that all of the labeled 32,000-mol wt proteins had not been removed in the first place by DOC. As mentioned previously, we consider this area of the gel to contain two acidic glycoproteins which migrate as a diffuse band and whose molecular weights differ by <1,000. We interpret our fluorographs to mean that DOC extracted all of the lower molecular weight protein but <100% of the higher molecular weight protein, the remainder of which was subsequently solubilized with SDS. Fluorographs of control spermatozoa (i.e. spermatozoa incubated with NaB[^{3}H]$_4$ alone) revealed no labeled proteins.

Preincubation of spermatozoa with neuraminidase followed by labeling with galactose oxidase/NaB[^{3}H]$_4$ stimulated incorporation of radiolabel by 7–10% for testicular spermatozoa but 15–20% for cauda epididymidal spermatozoa. However, analysis of detergent-extracted proteins on SDS polyacrylamide

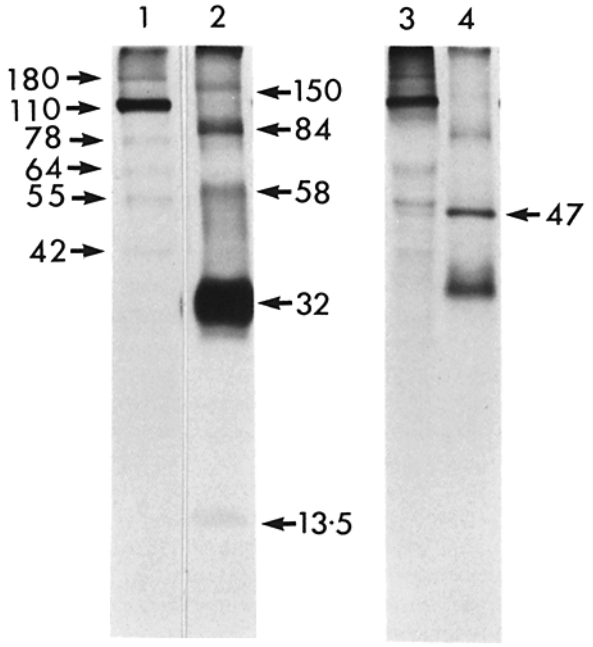


FIGURE 2 Fluorographs of labeled proteins on testicular (lane 1) and cauda epididymidal (lane 2) spermatozoa incubated with galactose oxidase and NaB[^{3}H]$_4$ and extracted with 0.2% DOC as described in Materials and Methods. Lanes 3 and 4 are fluorographs of proteins solubilized with 1% SDS from testicular and cauda epididymidal sperm pellets, respectively, that had first been extracted with DOC. The same amount of radioactivity (40,000 cpm) was applied to each lane, and values indicated are approximate molecular weights × 10^{-3}. No radioactivity was detected on fluorographs when detergent extracts of spermatozoa incubated with NaB[^{3}H]$_4$ alone were analysed.

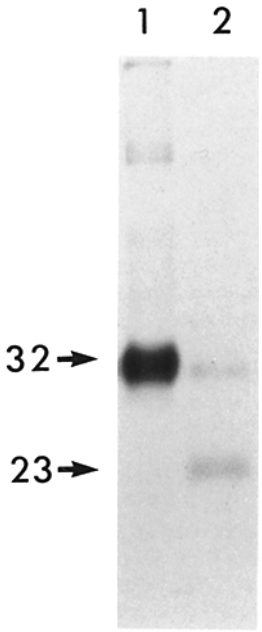


FIGURE 3 Fluorograph of proteins extracted from galactose oxidase/NaB[^{3}H]$_4$-labeled cauda epididymidal spermatozoa which had been incubated without (lane 1) or with (lane 2) 1 mg/ml pancreatic trypsin for 60 min at 25°C in the presence of 10 mM $CaCl_2$. Values indicated are the approximate molecular weight × 10^{-3}.

gels did not reveal labeling of any glycoproteins in addition to those listed above. Incubation of labeled spermatozoa with pronase or mixed exoglycosidases reduced labeling of the 110,000- and 32,000-mol wt proteins to <5% of controls. Endoglycosidase H had a similar effect indicating that both glycoproteins have carbohydrate chains *N*-glycosidically linked to asparagine residues. Digestion with trypsin also caused a reduction in the amount of label associated with the 32,000-mol wt proteins but with the concomitant appearance of a labeled protein with a molecular weight of 23,000–24,000 (Fig. 3). A time-course of the reaction showed that ~80% of the radiolabel associated with the 32,000-mol wt proteins was removed within 10 min and that longer incubation times (up to 5 h) did not reduce it further nor did it alter labeling of the 24,000-mol wt protein. We interpret these results to mean that a portion of the 32,000-mol wt proteins was removed leaving behind some of the original molecule and a small molecular weight fragment still attached to the membrane.

As a corollary to this work we incubated washed testicular spermatozoa in CEP diluted with PBS pH 7.4 to 1 mg/ml and washed cauda epididymidal spermatozoa in undiluted RTF (1 mg/ml) at 34°C for 60 min followed by further washing and labeling with galactose oxidase/NaB[^{3}H]$_4$. However, this treatment did not cause reciprocal labeling of either types of spermatozoa nor did it alter the amount of radiolabel present on the 110,000- and 32,000-mol wt proteins; fluorographs were similar to those of untreated controls.

Comparison of Membrane Glycoproteins on Testicular and Cauda Epididymidal Spermatozoa Labeled with Sodium Metaperiodate and NaB[^{3}H]$_4$

Oxidation of sialic acid groups on membrane glycoproteins with sodium metaperiodate followed by reduction with NaB[^{3}H]$_4$ stimulated the incorporation of radiolabel into both types of spermatozoa. For testicular spermatozoa the degree of stimulation above controls was similar to that observed with galactose oxidase, but with cauda epididymidal spermatozoa it was only 22–25% of that found with galactose oxidase. Subsequent analysis of detergent extracts from labeled spermatozoa on SDS polyacrylamide gels revealed that the major labeled protein on testicular spermatozoa had a molecular weight of 110,000 with minor labeling of 68,000- and 180,000-mol wt proteins (Fig. 4). Cauda epididymidal spermatozoa on the

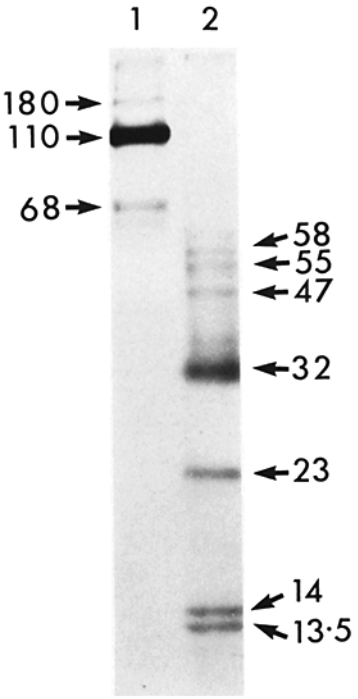


FIGURE 4 Fluorograph of detergent-extracted proteins from testicular (lane 1) and cauda epididymidal spermatozoa (lane 2) labeled with sodium metaperiodate and NaB[^{3}H]$_4$. Because of the lower specific activity of the labeling, DOC and SDS extracted proteins were combined before separation on SDS polyacrylamide gels. Values given are approximate molecular weight × 10^{-3}. Each track received approximately 4,000 cpm.

other hand gave a more complex pattern. Major labeling was detected on proteins of 13,500, 14,000, 23,000, and 32,000 mol wt, with minor labeling on proteins of 47,000, 55,000, and 58,000 mol wt (Fig. 4). Preincubation of spermatozoa with neuraminidase abolished labeling of all the proteins listed above.

Evidence for Homology between Membrane-bound and Secreted 32,000-mol wt Proteins

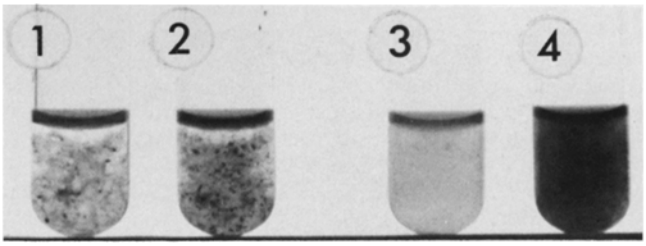
Since the 32,000-mol wt glycoproteins were the major 'maturation antigens' which labeled on cauda epididymidal spermatozoa with galactose oxidase/NaB[^{3}H]$_4$ and sodium metaperiodate/NaB[^{3}H]$_4$, we have focussed our attention on their origin and their disposition relative to the sperm plasma membrane.

We have shown previously (26) that androgen-dependent glycoproteins with molecular weights of 32,000 are synthesized in the caput epididymidis and that they constitute ~10% of the total secreted protein. Evidence that the secreted and membrane-bound 32,000-mol wt proteins are homologous has now been shown by: (*a*) when CEP was incubated with galactose/NaB[^{3}H]$_4$, >90% of radiolabel incorporated into protein was associated with proteins of 32,000 mol wt (Fig. 5 *A*); (*b*) the antiserum raised against the purified secreted proteins gave an intense reaction with the immunoperoxidase technique against cauda epididymidal spermatozoa (Fig. 6). Testicular spermatozoa, on the other hand, showed only a very small increase in the deposition of dye reagent between preimmune and immune sera. Furthermore, a precipitation line was obtained with the antiserum on Ouchterlony gels against DOC extracts of cauda epididymidal but not testicular spermatozoa; (*c*) when DOC extracts prepared from galactose oxidase/NaB[^{3}H]$_4$-labeled cauda epididymidal spermatozoa were treated with antiserum, and the antibody-antigen complex was precipitated with *Staphylococcus aureus*, a single band with of 32,000 mol wt was detected on fluorographs (Fig. 5 *B*). However, treatment of DOC extracts from labeled testicular spermatozoa with antibody followed by incubation with *Staphylococcus aureus* did not precipitate any labeled protein.

Distribution and Disposition of 32,000-mol wt Proteins in the Plasma Membrane of Cauda Epididymidal Spermatozoa

Indirect immunofluorescence using the antibody raised against the 32,000-mol wt proteins and FITC-conjugated goat anti-rabbit IgG revealed a strong reaction over the entire surface of cauda epididymidal spermatozoa (Fig. 7). Fluorescence over the head region was noticeably less than over the remainder of the cell but this could have been due to loss of acrosome and overlying plasma membrane caused by the washing and labeling procedure. More noticeable was the patchy or interrupted distribution of the line of fluorescence over the plasma membrane. The size and frequency of these patches was irregular and not confined to a specific region of the cell. Testicular spermatozoa, on the other hand, showed no photographable fluorescence. Spermatozoa incubated with preimmune serum were also negative.

To investigate whether the patchy distribution of the 32,000-mol wt proteins was indicative of the ability of these proteins to move laterally in the plane of the membrane, we first fixed cauda epididymidal spermatozoa in 2% glutaraldehyde in PBS for 20 min, washed, and then labeled then with fluorescent



FIGURES 6 Immunoperoxidase reaction on suspensions of testicular and cauda epididymidal spermatozoa incubated with pre-immune (tubes *1* and *3*) or immune (tubes *2* and *4*) rabbit antiserum against the 32,000-mol wt proteins followed by horseradish peroxidase-conjugated goat anti-rabbit antiserum and Hanker-Yates reagent as described in Materials and Methods. No deposition of dye reagent was obtained if H_2O_2 or Hanker-Yates reagent were omitted.

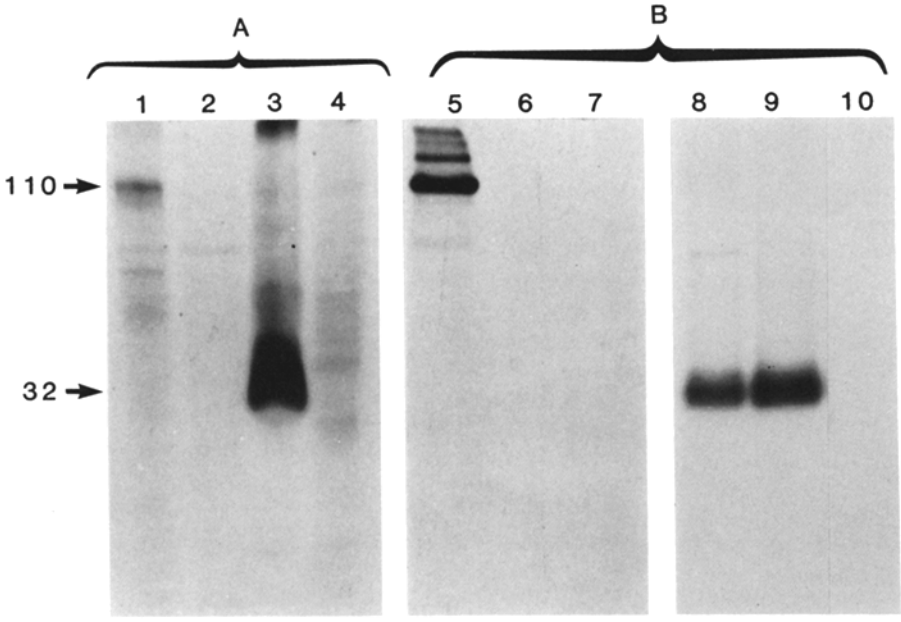


FIGURE 5 (*A*) Fluorograph of labeled proteins in RTF (lane *1*) or CEP (lane *3*) after incubation with galactose oxidase/NaB[^{3}H]$_4$. Approximately 5 μg of protein was incubated with NaB[^{3}H]$_4$ ± galactose oxidase (see Materials and Methods), precipitated with 9 vol of 100% ethanol, and analysed on SDS polyacrylamide gels. Lanes *2* and *4* represent RTF and CEP, respectively, incubated in presence of NaB[^{3}H]$_4$ but without enzyme. (*B*) Fluorograph of labeled proteins immunoprecipitated from DOC extracts of testicular (lanes *5*, *6*, and *7*) or cauda epididymidal (lanes *8*, *9*, and *10*) spermatozoa labeled with galactose oxidase/NaB[^{3}H]$_4$. Extracts were either untreated (lanes *5* and *8*) or incubated with immune (lanes *6* and *9*) or pre-immune (lanes *7* and *10*) serum against the 32,000-mol wt proteins followed by precipitation with *Staphylococcus aureus* cells. Lanes *5*, *8*, and *9* received ~30,000 cpm and lanes *7* and *10* all the radioactivity (<1,000 cpm) precipitated. Values given are approximate × 10^{-3}.

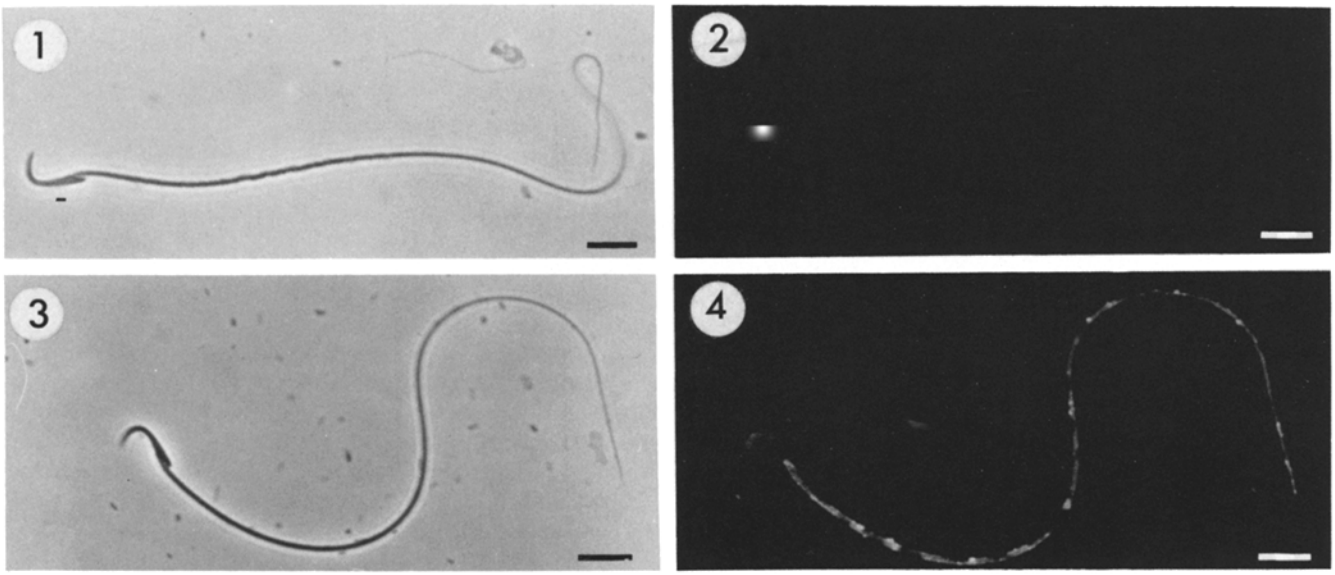


FIGURE 7 Indirect immunofluorescence of the distribution of the 32,000 mol wt antigens on testicular (*1* and *2*) and cauda epididymidal (*3* and *4*) spermatozoa. Spermatozoa were incubated with immune rabbit antiserum followed by FITC-labeled goat anti-rabbit IgG. Preimmune rabbit serum gave no fluorescence. *Left hand side*: phase contrast. *Right-hand side*: UV illumination. Bar, 5 μm. × 1,400.

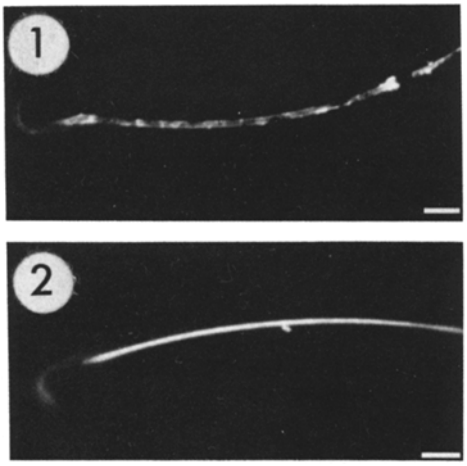


FIGURE 8 Immunofluorescence of the 32,000-mol wt antigens on cauda epididymidal spermatozoa. (*1*) Midpiece region of spermatozoon incubated with immune rabbit antiserum and FITC-labeled goat anti-rabbit IgG at 25°C. (*2*) Midpiece region of spermatozoon fixed in 2% glutaraldehyde followed by incubation with immune rabbit antiserum and FITC-labeled goat anti-rabbit IgG at 25°C. Bar, 5 μm. × 1,000.

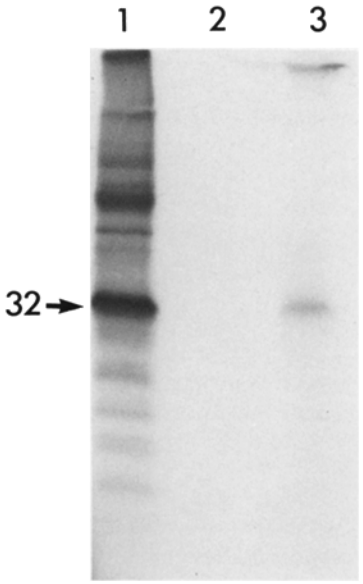


FIGURE 9 Autoradiograph of ^{125}I-nitrene-labeled membrane proteins from cauda epididymidal spermatozoa. A membrane fraction prepared from spermatozoa (see Materials and Methods) was incubated with 50 μCi ^{125}I-nitrene, centrifuged at 100,000 *g* for 45 min, and the membrane pellet solubilized in 0.2% DOC. A portion was analysed directly on SDS polyacrylamide gels (lane *1*) whilst the remainder was treated with preimmune serum (lane *2*) or immune antiserum (lane *3*) against the 32,000-mol wt proteins followed by precipitation with *Staphylococcus aureus* cells. Values indicated are the approximate molecular weight × 10^{-3}.

antibody as before. The result was a uniform distribution of fluorescence over the surface of the flagellum (Fig. 8). Similarly, if the entire labeling procedure was done at 4°C, then the flagellum was uniformly fluorescent. This argues against the possibility that the patchy distribution of antigen was due to damage and loss of areas of plasma membrane.

Incubation of galactose oxidase/NaB[^{3}H]$_4$-labeled cauda epididymidal spermatozoa in distilled water or 3 M KCl solubilized <1% of the radioactivity associated with the 32,000-mol wt proteins. Only detergents were fully effective in this respect, suggesting that the proteins are intrinsic to the membrane. Further evidence that the membrane-bound 32,000-mol wt proteins have hydrophobic domains and make structural contact with the lipid bilayer was shown using the photoaffinity probe and charge-shift electrophoresis. When a membrane fraction prepared from cauda epididymidal spermatozoa was labeled with ^{125}I-nitrene reagent, ~70% of the total radioactivity added was recovered in the membrane pellet. Between 10 and 15% of the added radioactivity was incorporated into protein as assessed by precipitation of membranes with 10% TCA followed by extensive washing with ethanol:diethyl ether (3:1). Analysis of these membrane proteins on SDS polyacrylamide gels revealed a range of labeled proteins, with the major polypeptide of 32,000 mol wt (Fig. 9). This protein could be immunoprecipitated with the antiserum raised against the secreted 32,000-mol wt proteins. Although not shown in Fig. 9, shorter exposure of the autoradiograph revealed that the 32,000-mol wt band was, in fact, a doublet. A further indication

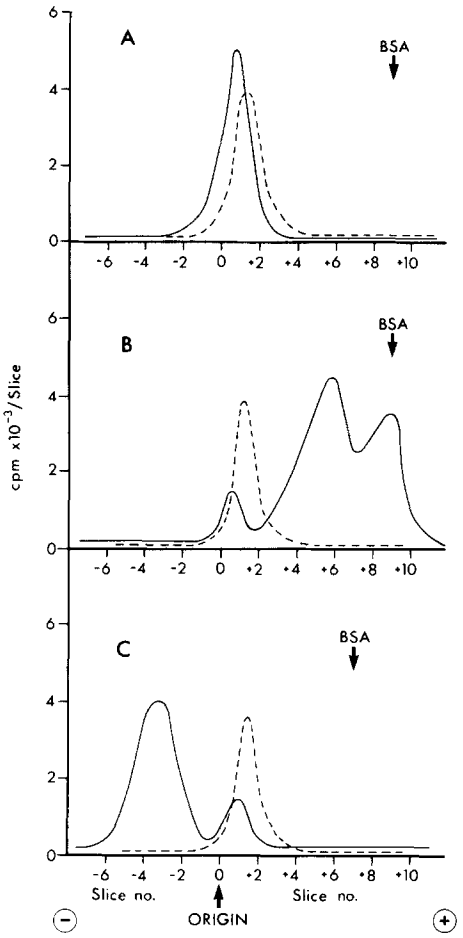


FIGURE 10 Charge-shift electrophoresis of secreted and membrane-bound 32,000 mol wt proteins. CEP and cauda epididymidal spermatozoa were labeled with galactose oxidase/NaB[^{3}H]$_4$, incubated with either 1% Triton X-100 (*A*), 0.2% DOC/1% Triton X-100 (*B*) or 0.2% CTAB/1% Triton X-100 (*C*), and the extracted proteins were analysed on 1% agarose gels as described in Materials and Methods. After electrophoresis, gels were sliced into 1.5-mm segments, dispersed in 10 ml of Packard Scintillator 299, and radioactivity was counted. (——) Membrane-bound proteins. (----) Secreted proteins.

of the hydrophobic nature of the membrane-bound 32,000-mol wt proteins was shown by charge-shift electrophoresis. In the presence of a charged detergent such as DOC, the mobility of the 32,000-mol wt proteins was shifted towards the anode relative to its mobility in the presence of a nonionic detergent such as Triton X-100 (Fig. 10). Binding of DOC consistently produced two peaks of radioactivity, indicative of two proteins. In the presence of the cationic detergent, CTAB, the charge on the 32,000-mol wt proteins was reversed and they migrated towards the cathode. However, the migration of the secreted 32,000-mol wt proteins was unaltered in the presence of charged detergents (Fig. 10).

DISCUSSION

The results of this investigation suggest that there is a considerable reconstruction of membrane glycoproteins on spermatozoa during their maturation in the epididymis and that some of these changes are caused by a direct interaction between spermatozoa and epididymal secretory proteins. Since different glycoproteins are synthesized in different regions of the epididymis (6, 26) it is likely that these changes are coordinated into a sequential series of events which culminate in the conferment of fertilizing capacity on the spermatozoa.

The limitations of the galactose oxidase/NaB[^{3}H]$_4$ technique for labeling cell membranes have been reviewed (23). Membrane glycoproteins which do not contain accessible D-galactose or *N*-acetyl-D-galactosamine sugars will not label since steric hindrance is a problem with this technique (17). Surface labeling of sperm membrane proteins with galactose oxidase was first reported by Olson and Hamilton (40), but by optimizing the reaction conditions we have detected labeling of a wide range of glycoproteins on the sperm surface. Assuming that only plasma membrane proteins are labeled with the galactose oxidase/NaB[^{3}H]$_4$ technique, then proteins of 42,000, 55,000, 64,000, 78,000, 110,000, and 180,000 mol wt have an external orientation on testicular spermatozoa, and proteins of 13,500, 32,000, 47,000, 84,000, and 150,000 mol wt are located on the plasma membrane of cauda epididymidal spermatozoa. The 110,000- and 32,000-mol wt proteins are also the most heavily labeled after periodate oxidation, suggesting that they are sialoglycoproteins. It is noteworthy that the 13,500-mol wt protein appears to have more sialic acid than galactose residues, since it labels more heavily after periodate oxidation. However, both metaperiodate and NaB[^{3}H]$_4$ are permeant reagents and some of the proteins shown in Fig. 2*B* may be intracellular, e.g. in the acrosome. In addition, certain membrane lipids also incorporate tritium after periodate oxidation (45). More radiolabel was incorporated into lipids in testicular than cauda epididymidal spermatozoa but no further attempt was made to identify the chloroform:methanol soluble material.

Changes in the presence or distribution of glycoproteins on cell membranes as detected by labeling of carbohydrate moieties may reflect a variety of processes none of which are mutually exclusive. The decrease in labeling of the 110,000-mol wt protein on testicular spermatozoa as they pass through the epididymis may be caused by (*a*) removal of the whole glycopeptide from the membrane, e.g. by proteinases; (*b*) masking by the addition of other surface proteins; or (*c*) removal of the carbohydrate chain by the action of exo- and endoglycosidases. In the latter context is should be recalled that the epididymis is a very rich source of glycosidases and that the secretion of these enzymes is androgen-dependent (7, 24). The fact that labeled carbohydrate moieties on testicular spermatozoa can be removed during incubation in vitro with endo- or exoglycosidases also supports this possibility, although it is difficult to say whether the same mechanisms are operative in vivo or not.

Similarly, the appearance of glycoproteins on the plasma membrane of cauda epididymidal spermatozoa that are not labeled on testicular spermatozoa may be explained either by the glycosylation of preexisting membrane proteins, by the analogy to the major blood group antigens ABH (10, 11), or by uptake of new proteins from the epididymal secretions. There is evidence for both these possibilities. Glycosyl transferases have been reported in testicular and epididymal fluids (3, 20) and could act in concert with glycosidases to modify preexisting membrane components. On the other hand, several of the proteins on cauda epididymidal spermatozoa that label after galactose oxidase or periodate oxidation are present in the epididymal secretions. Those proteins with 32,000 and 47,000 mol wts have been shown to be synthesized in the caput and cauda epididymidis, respectively, and to be androgen-dependent (26). Together with the fact that an antiserum raised against the secreted 32,000-mol wt proteins precipitates proteins with a similar molecular weight extracted from cauda epididymidal

spermatozoa but not testicular spermatozoa, the evidence in this particular case strongly suggests that they are of epididymal origin. We consider it likely that a similar situation may hold for the 47,000-mol wt protein since spermatozoa do not encounter this protein until they enter the cauda epididymidis. Therefore, the 110,000, 47,000, and 32,000-mol wt proteins are potentially good markers for assessing the degree of maturation of spermatozoa in the epididymis.

Since the 32,000-mol wt proteins are the major 'maturation antigens' which label on cauda epididymidal spermatozoa, we have focussed our attention on the distribution and structure of these proteins in the plasma membrane. Many cells show a specific localization of surface antigens (14, 18) but our immunofluorescence data suggest that the 32,000-mol wt proteins are distributed over the surface of the flagellum and midpiece of mature spermatozoa, with possibly fewer sites on the head region. The presence of intensely fluorescent patches at irregular intervals along the membrane and the finding that the patching can be prevented by fixation or low temperature (Fig. 7) suggests that they possess a degree of lateral mobility within the plane of the membrane. Unlike the situation in B lymphocytes, however, there was no evidence that the patches 'capped'. O'Rand (41) has shown that patching of a membrane glycoprotein over the acrosomal region of uncapacitated rabbit spermatozoa occurs and that it disappears when the spermatozoa are capacitated. The irregular distribution of ferritin-conjugated lectins on spermatozoa (39) has also alluded to a class of mobile proteins in the plasma membrane. However, some membrane antigens appear to be restricted in their distribution as they are found only in specific regions, e.g. over the acrosome or midpiece (11, 38). How these proteins are anchored in one place is not known, but the evidence to date would indicate two types of proteins in the sperm plasma membrane: those that are demonstrably mobile, and those that are not.

The results of experiments with the ^{125}I-nitrene reagent indicate that the sperm-associated 32,000-mol wt proteins are within or connected to the lipid bilayer, prompting the question of how secreted proteins are coupled to the plasma membrane. Hydrophobic domains do not seem to be present on the surface of the secreted proteins as assessed by charge-shift electrophoresis. In addition, calculations of the average hydrophobicity (Hϕ[ave]) (4) from their amino acid composition (26) give a value of 956 which does not indicate the existence of masked or internal hydrophobic areas. However, this situation is not without precedent. It is known that when cells are infected with cholera toxin, the B subunit binds to a GM_1 monosialylganglioside receptor on the plasma membrane, forming a channel through which the A_1 subunit interacts with the membrane. Only the A_1 subunit labels with lipophilic photoaffinity probes, leading to the proposal that it alone partitions into the bilayer (49). However, calculations of the Hϕ(ave) of A_1 (48) do not support this contention, nor does the observation that its migration on agarose gels is unaltered by charge-shift electrophoresis (48), that is, it behaves like a typical hydrophilic protein. The mechanism of its insertion, therefore, remains uncertain but it is conceivable that A_1 enters the bilayer after interaction with an integral membrane protein as there is some evidence that it has a requirement for certain types of proteins (19). At present, a similar situation applies to the interaction of epididymal secretory proteins and the sperm plasma membrane. Other workers (13, 34, 47) have also reported coupling of antigens to the sperm surface during epididymal maturation but the mechanisms involved are not known. It is possible that secretory proteins bind to specific receptors sequestered into the membrane during spermiogenesis but to date such receptors have not been identified. Much remains to be learned, therefore, about the structure of the secreted v membrane-bound forms of these proteins.

The physiological effects of the acquisition of epididymal secretory proteins on spermatozoa remain unclear but many proteins are involved in cell-cell recognition, e.g. band III protein on erythrocytes. It is conceivable that by controlling cell contact, the entry and exit of ions, and low molecular weight compounds, or by acting as receptors, plasma membrane glycoproteins on spermatozoa may mediate a diverse range of functions such as maturation, capacitation, and ultimately fertilization.

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