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Studies on the mechanism of capacitation: Albumin-mediated changes in plasma membrane lipids during *in vitro* incubation of rat sperm cells*

(epididymal spermatozoa/cholesterol and phospholipid levels)

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ABSTRACT Plasma membrane isolated from rat sperm cells after incubation *in vitro* had a significantly lower cholesterol/phospholipid mole ratio when the medium contained serum albumin. Transfer of albumin-bound phospholipids to the membrane can largely account for this effect. The result is broadly consistent with a previously proposed model for albumin-induced destabilization of sperm membrane (capacitation) and its reversal by seminal plasma membrane vesicles. Albumin also decreased sialic acid and, more specifically, ganglioside levels, presumably by promoting release of sperm neuraminidase. Cholesteryl ester comprised up to 0.5 mol/mol of cholesterol in these plasma membrane preparations.

Mammalian sperm cells can express fertilizing capacity after incubation *in vitro* for an interval of usually a few hours in a chemically defined medium containing serum albumin (1–6). Although the molecular changes underlying transformation to a capacitated state are unclear, albumin specifically facilitates this process (4). Substantial changes in sperm plasma membrane proteins accompany incubation with albumin (7); however, they seem to involve proteolysis after the acrosome reaction. A clue that lipid binding by albumin might be involved in its ability to promote capacitation was provided by the observation that presaturation of the protein with cholesterol† abolished this capability (4). In view of this finding and the discovery that cholesterol-bearing decapitation factor (DF) vesicles from seminal plasma and synthetic vesicles containing the sterol block capacitation in rabbit and rat spermatozoa (8–12), changes in the lipid bilayer of the sperm plasma membrane seem to be necessary for expression of fertilizing capacity.

One interpretation of these observations is that albumin decreases the cholesterol/phospholipid (Chol/PL) ratio in the sperm plasma membrane (13). Spectroscopic data reveal that cholesterol restricts the thermal motion of fatty acid chains in a phospholipid bilayer above the gel-to-liquid transition temperature (14–16), and this causes a "condensation effect" and decreases permeability (17). An increase in bilayer microviscosity evidently accounts for the inhibitory effect of cholesterol on fusion between plasma membranes of cultured muscle cells (18) and synthetic phospholipid membranes (19). Decreasing the Chol/PL ratio in sperm plasma membrane, therefore, may be anticipated to promote fusion with the outer acrosomal membrane; this intracellular fusion occurring in the acrosome reaction is a precondition for mammalian fertilization (20). A vesicle-induced reversal of this putative change in the plasma membrane Chol/PL ratio might account for the ability of these vesicles to inhibit spermatozoan fertilizing capacity reversibly (11, 12).

To test this idea, plasma membrane preparations isolated from rat spermatozoa after incubation *in vitro* were partially characterized with respect to their lipid composition. It was anticipated bovine serum albumin would decrease the Chol/PL ratio in sperm plasma membrane if the model were correct. The findings are discussed in terms of a possible molecular basis for sperm capacitation. A preliminary report of some of these results has been presented (21).

MATERIALS AND METHODS

Mature Sprague–Dawley rats (400–450 g) were used in this work and were housed in a colony kept under constant temperature (21 ± 1°C) and periods of daily light (0700–1900 hr) and fed Purina chow and water ad lib. The spermatozoa released after tubules of the cauda epididymis were sectioned, immediately after sacrifice of the rats, were incubated at a concentration of $\approx 2 \times 10^6$ sperm cells per ml in 50 ml of a modified Krebs–Ringer bicarbonate medium (95.3 mM NaCl/4.8 mM KCl/1.7 mM $CaCl_2$/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/11.5 mM $NaHCO_3$/5.6 mM glucose/0.41 mM sodium pyruvate/27.2 mM sodium lactate/4.2 μg of streptomycin per ml/6.2 μg of penicillin per ml/4 μg of phenol red per ml, adjusted to pH 7.0) sometimes containing crystalline bovine serum albumin (Sigma) at 4 mg/ml. The incubation was performed in a sterile plastic flask (Falcon) in a humidified atmosphere of 5% CO_2/95% air at 37° for 5 hr with the suspension immersed under a layer of mineral oil (Squibb) that had been presaturated with medium (4, 10). To evaluate changes in the amount of plasma membrane phospholipid at various times during the incubation, suspensions containing 0.28×10^8 sperm cells in 14 ml of medium were incubated for indicated intervals up to 5 hr. Sperm cell concentrations were determined with a hemocytometer before incubation. All the spermatozoa preparations used in this investigation showed motility when examined microscopically (×100) after incubation. After incubation, the spermatozoa were sedimented at 1000 × *g* for 20 min, resuspended in 4.5 ml of 10 mM Tris buffer at pH 7.4 at 4°C, and, after equilibration for 2 hr, rapidly frozen. The suspension was subsequently thawed, adjusted to contain 7.5 mM EDTA, sonicated at 2 kHz for 15 sec with a tungsten probe (0.4 cm diameter) attached to a Braunsonic (model 1510) power source, and sedimented at 1000 × *g* for 10 min.

The supernatant, which had a cloudy appearance, was layered on top of a density gradient consisting usually of a linear 20–60% (wt/vol) sucrose gradient in 10 mM Tris (pH 7.4) and

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Abbreviations: Chol/PL cholesterol/phospholipid ratio; DF, decapacitation factor.

* The previous paper in this series is ref. 33.

† Cholesterol refers to the sterol alcohol.

centrifuged at 90,000 × *g* in an SW 27 rotor and Beckman (model L5-75) ultracentrifuge at 4°C. After centrifugation for 2–4 hr, the cellulose nitrate tube containing the gradient was punctured to permit collection of 1-ml fractions. Their absorbance was assessed with a spectrophotometer (Gilford) at 280 nm. The concentration of protein in fractions containing a peak was determined from the absorbance; it had been established with the aid of Lowry's method (22) that 1 absorbance unit at 280 nm corresponded to 0.23 mg of sperm membrane protein per ml. Fraction density was estimated with a refractometer (Bausch and Lomb). Sialic acid levels in the sperm membrane and in lipid extracts were evaluated by the thiobarbituric acid method of Warren (23). The amount of ganglioside was estimated by multiplying by 2.8 the level of sialic acid in lipid extracts. Membrane ATPase activity was assessed from the rate of P_i production [colorimetric assay for P_i (24)] during a 30-min incubation at 36°C in 4 mM ATP (Tris salt)/25 mM $MnCl_2$/5 mM Tris, pH 8.0 (25). Phospholipid levels were obtained by multiplying by 25 the amount of P_i (24) present in an acid hydrolysate of a chloroform/methanol extract (26) of sperm membrane.

Cholesterol, cholesteryl esters, and triglycerides in the extract were separated on thin-layer silica gel plates (Kontes) by development with hexane/ethyl ether/acetic acid, 70:30:1 (vol/vol). After elution with chloroform, the sterol and its esters and triglycerides were quantitated fluorimetrically (27, 28). Lipids remaining in the medium after sedimentation of sperm cells were analyzed in the manner described. Fatty acids in the supernatant were also assayed, after transmethylation, on a gas/liquid chromatograph (Hewlett–Packard) equipped with an integrator for peak quantitation. Statistical significance for observed differences between mean lipid concentrations was established by pairwise comparisons in a *t* test performed with a programmable calculator (Hewlett–Packard).

Thin sections of membrane and postsonication spermatozoa pellets formed by centrifugation for 3 hr at 110,000 × *g* and for 0.5 hr at 12,000 × *g*, respectively, were prepared after glutaraldehyde fixation, staining with OsO_4 and lead citrate, and embedding in Epon (Ladd). Sperm cell surface proteins were labeled with ^{125}I by the lactoperoxidase method (29). The radioactively labeled cells were briefly sonicated, and the membrane-containing fraction obtained was then layered on a discontinuous sucrose density gradient. After centrifugation, the gradient was fractionated and each fraction was assessed for absorbance at 280 nm and, in addition, for radioactivity in a γ-ray counter (Nuclear-Chicago). Analytical grade chemicals and triple-distilled water were used throughout this investigation.

RESULTS

A typical absorbance profile is given in Fig. 1 for a cauda epididymal rat spermatozoa membrane preparation. It shows a peak with a density between 1.13 and 1.16 g/cm^3 after sedimentation for 4 hr on a linear sucrose density gradient. After correction for absorbance by sucrose, which was assessed in a sucrose density gradient containing no sperm fractions, the peak had an absorbance at 280 nm of approximately 0.1. This corresponds to a yield of nearly 23 μg of membrane protein, after sonication of 10^8 sperm cells.

Fig. 2 gives the distribution of ^{125}I on a discontinuous sucrose density gradient after a 2-hr centrifugation of membranes isolated from iodinated rat spermatozoa. The low density peak (fractions 18–20) had been extensively labeled by lactoperoxidase. In contrast, a peak of higher density (fractions 1–6) occurring in this gradient showed only background levels of radioactivity. At the top of this gradient, there was radioactivity from nonsedimenting ^{125}I.

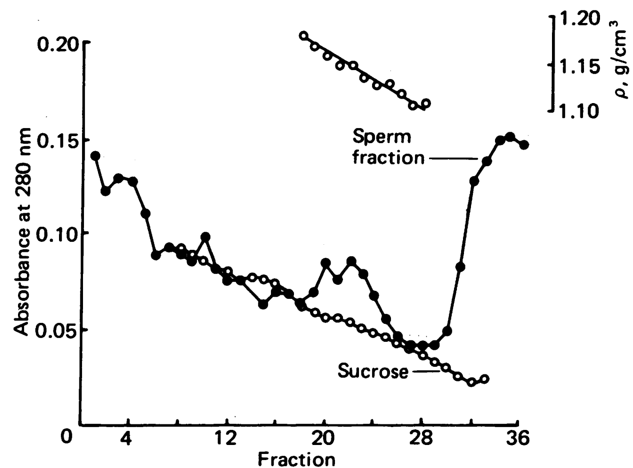

FIG. 1. Absorbance (280 nm) profile of a rat sperm plasma membrane preparation after sedimentation on a linear density gradient [20–60% (wt/vol) sucrose]. Density measurements shown were made with a refractometer. A peak corresponding to plasma membrane is apparent in the density range 1.13–1.16 g/cm^3. A similar gradient, but with no added sperm cell fraction, was also centrifuged and used to determine background sucrose absorbance.

The material in the low-density peak contained membrane with no conspicuous contamination from cell organelles such as mitochondria and acrosomes (Fig. 3). After sonication, sperm cells showed extensive removal of the membrane from the head, midpiece, and tail. Because mitochondria appeared to be intact, the presence of a high-density peak in some gradients tentatively can be attributed to acrosomal membrane, whose density exceeds that of plasma membrane (30).

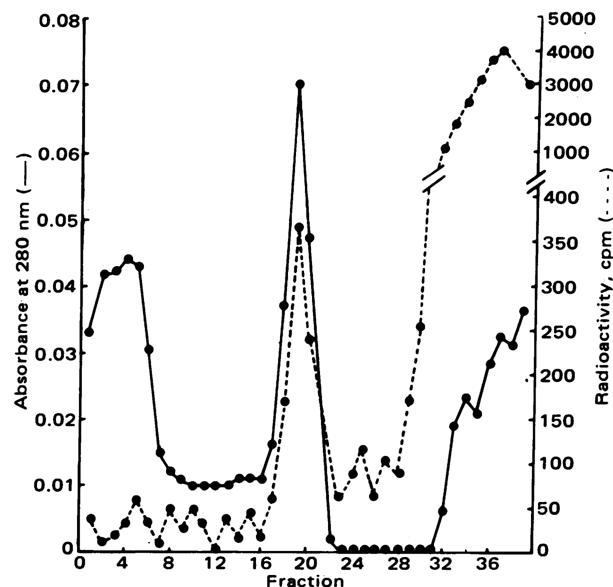

FIG. 2. Distribution of radioactivity and absorbance (280 nm) in a discontinuous density gradient [20%/40%/60% (wt/vol) sucrose] after sedimentation of membrane fractions from sperm cells enzymatically labeled with ^{125}I. The high-density membrane peak (fractions 1–6) had only background levels of radioactivity, whereas the low-density peak (fractions 18–20), which corresponds to plasma membrane, had a high specific activity (2052 cpm/mg of protein). Iodination involved incubation of cauda epididymal rat spermatozoa (14 × 10^6 cells per ml) for 1 hr at 37°C with lactoperoxidase (6.2 purpurogallin units/ml) in 0.1 M phosphate-buffered saline, pH 7.4/1 mM EDTA/40 μM H_2O_2 containing $Na^{125}I$ at 10 μCi/ml (1 Ci = 3.7 × 10^{10} becquerels). To produce the membrane fractions, spermatozoa were sonicated briefly prior to centrifugation.

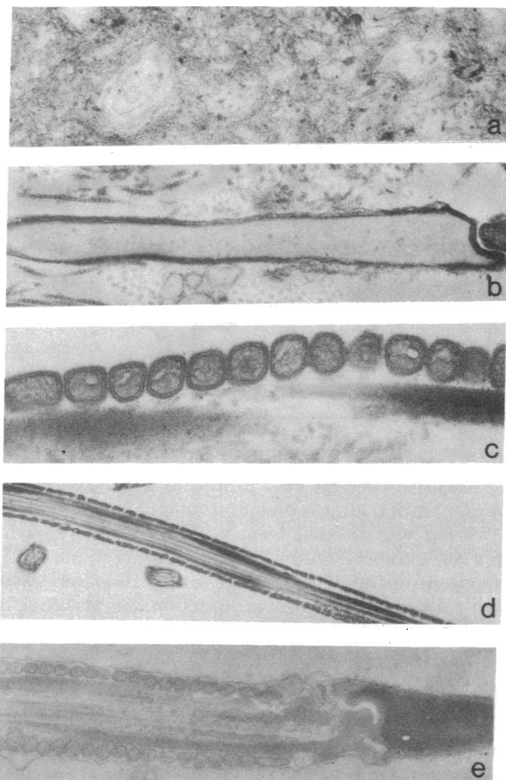

FIG. 3. Electron micrographs of a membrane fraction and head, midpiece, and tail regions of epididymal rat spermatozoa after sonication. (*a*) Plasma membrane isolated by isopycnic sedimentation from sperm cells, after incubation for 5 hr in Krebs–Ringer medium, was subsequently centrifuged at 105,000 × g for 3 hr to form a pellet and then fixed, stained, and sectioned for examination under an electron microscope. (×61,200.) (*b*) Section through the head of a sperm cell showing complete removal of plasma membrane. (×19,200.) (*c*) Mitochondria, at the sperm midpiece region, with intact membranes. The plasma membrane covering them, however, has been completely removed by ultrasonication. (×61,200.) (*d*) Complete removal of the plasma membrane is also apparent in this section through the sperm tail. (×61,200.) (*e*) Plasma membrane covers the head, midpiece, and tail (not shown) of an unsonicated sperm cell. (×19,200.)

Plasma membrane preparations from epididymal rat sperm cells contained an average of 0.37 μmol of sialic acid per mg of protein (Table 1). This is 5-fold higher than in unincubated,

Table 1. Some characteristics of plasma membrane from rat spermatozoa

Feature	Value
Density	1.13–1.16 g/cm^3
Phospholipid + cholesterol*	0.92 mg lipid/mg protein
Sialic acid	0.37 μmol/mg protein†
ATPase	73 μmol P_i/mg protein per hr‡

Plasma membrane used in these determinations was isolated from cauda epididymal spermatozoa of mature rats by ultrasonication for 15 sec and then centrifugation on a 20–60% sucrose gradient for 3 hr at 90,000 × g. The sperm cells had been incubated for 5 hr in Krebs–Ringer bicarbonate medium, except in the ATPase assay which involved unincubated sperm cells.
* Includes free and esterified forms.
† The sperm cells contained 0.08 μmol of sialic acid per mg of protein.
‡ Spermatozoa ATPase produced 2.7 μmol of P_i per mg of protein per hr.

Table 2. Effect of bovine serum albumin on composition of plasma membrane from rat spermatozoa

Component	*n*	μg/10^8 sperm	
		No albumin	With albumin
Cholesterol:			
Alcohol	8	7.6 ± 1.76	5.7 ± 0.90
Ester	8	4.5 ± 0.61	4.0 ± 1.30
Phospholipid	8	15.8 ± 2.62	23.1 ± 3.73*
Triglyceride	3	1.4 ± 0.53	1.0 ± 0.61
Ganglioside	4	0.1 ± 0.03	0
Sialic acid	2	4.7	2.8
Protein	8	31.9 ± 5.56	32.4 ± 4.27

Cauda epididymal rat spermatozoa (approximately 2 × 10^6 sperm per ml) were incubated for 5 hr at 37°C in 50 ml of Krebs–Ringer bicarbonate medium with or without bovine serum albumin(4 mg/ml). After incubation, plasma membrane was isolated by brief sonication of the spermatozoa; after sedimentation of the cells, the supernatant was centrifuged on a sucrose density gradient. These data are the mean ± SEM for the indicated number of determinations.
* Statistically significant difference ($0.05 > P > 0.025$).

intact sperm cells. Prior to incubation, membrane ATPase activity was 27-fold higher than in intact spermatozoa. For comparison, membrane vesicles from rabbit seminal plasma produce 450 μmol of P_i per mg of protein per hr (25) and rat liver plasma membrane forms only about 0.12 μmol of P_i per mg of protein per hr (31).

In plasma membrane preparations isolated from rat spermatozoa, after incubation for 5 hr in Krebs–Ringer bicarbonate medium, phospholipid was significantly increased from 15.8 to 23.1 μg/10^8 spermatozoa in medium with albumin; sialic acid and ganglioside decreased (Table 2). The apparently lower plasma membrane cholesterol concentration in sperm cells from a medium containing albumin was not statistically significant.

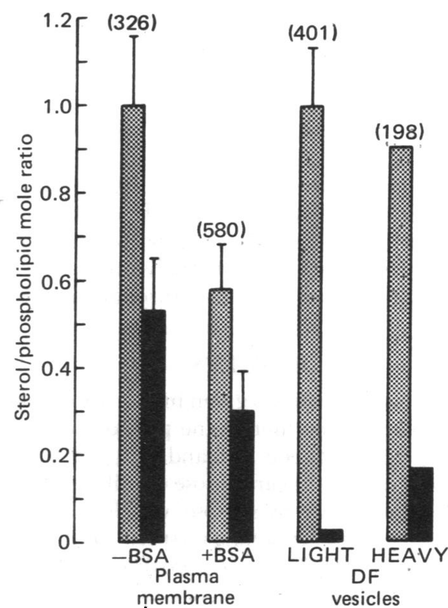

FIG. 4. Cholesterol [free (▩) and ester (■)]-to-phospholipid mole ratios in plasma membrane from rat spermatozoa and in heavy and light DF vesicles from rabbit seminal plasma. After incubation in the presence of bovine serum albumin (BSA), the plasma membrane displayed a statistically significant ($0.05 > P > 0.025$) reduction in the Chol/PL ratio. There were eight determinations for each plasma membrane result and three for light DF vesicle Chol/PL; the remaining data are means of duplicates; the vertical lines signify SEM. Phospholipid levels (μg/mg of protein) for these sources are given in parentheses.

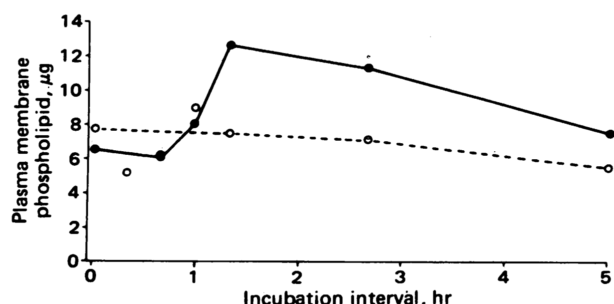

FIG. 5. Effect of bovine serum albumin on the amount of plasma membrane phospholipid obtained from rat spermatozoa at various intervals during incubation *in vitro*. Each determination involved isolation of plasma membrane from a suspension (14 ml) containing 0.28×10^8 sperm cells. ●, Medium with 4 mg of albumin/ml; ○, medium without the albumin.

In addition, the mole ratio of cholesteryl ester to alcohol was approximately 1:3 in these membranes. Triglyceride was also present in both the presence and absence of albumin. Protein levels were comparable for both media, although substantial qualitative differences exist between polypeptide electrophoretic profiles of albumin-treated and untreated sperm plasma membrane (7).

Fig. 4 compares Chol/PL and cholesteryl ester/phospholipid mole ratios in rat sperm plasma membrane and DF vesicles from rabbit seminal plasma. Mean Chol/PL values between 0.90 and 1.00 were obtained for sperm plasma membrane in the absence of albumin and for heavy and light DF vesicles. In plasma membrane with albumin, the mean ratio was 0.58, and this represents a statistically significant decrease. The difference arose largely from an increase in phospholipid in the presence of albumin (580 vs. 326 μg of phospholipid per mg of membrane protein). Likewise, there was a lower cholesteryl ester/phospholipid ratio in plasma membrane with albumin. Heavy DF vesicles had about 5-fold more cholesteryl ester than did light DF vesicles, when normalized with respect to phospholipid. This is consistent with the suggestion (13) that heavy vesicles form through vesiculation of sperm membranes.

Table 3. Lipids in cell-free medium after incubation

Lipid	Lipid content, μg	
	No albumin	With albumin
Cholesterol:		
Alcohol	31.3 ± 4.12	39.3 ± 1.76*
Ester	31.8 ± 21.1	29.0 ± 3.56
Phospholipid	5.1 ± 1.44	32.9 ± 11.5†
Triglyceride	18.5 ± 1.69	0†
Fatty acids:		
C14:0	0	0.3 ± 0.12†
C16:0	0.6 ± 0.04	4.8 ± 1.12†
C18:0	1.0 ± 0.12	4.8 ± 0.80†
C18:1	3.5 ± 0.40	10.6 ± 2.12†
C18:2	0	1.3 ± 0.28†
Total	5.1 ± 0.37	21.9 ± 4.90†

After incubation of cauda epididymal rat spermatozoa in Krebs-Ringer bicarbonate medium containing approximately 10^8 sperm cells, with or without bovine serum albumin at 200 mg/50 ml, for 5 hr at 37°C, the medium was centrifuged at $10,000 \times g$ for 30 min. The cell-free supernatant was extracted with chloroform/methanol and analyzed. Data presented for post-incubation medium containing albumin have been corrected by subtracting lipids added with the protein. The results are the mean ± SEM for four determinations.
* Difference almost significant ($P = 0.058$).
† Significant difference ($0.05 > P > 0.025$).

After a lag of 45 min, phospholipid levels in plasma membrane virtually doubled during incubation with albumin (Fig. 5). The size of this increase agrees with the alterations in membrane Chol/PL ratios indicated by Fig. 4. At intervals longer than 2 hr the difference in phospholipid levels decreased. To test the possibility that this involved loss of sperm phospholipids—e.g., by vesiculation—the lipid content of cell-free medium was analyzed after incubation for 5 hr. Apart from an increase in fatty acid levels, especially of oleic, stearic, and palmitic acid, there was significantly more phospholipid in albumin-containing medium (Table 3).

DISCUSSION

An increase of the plasma membrane phospholipid content in rat epididymal sperm cells during incubation with bovine serum albumin is consistent with capacitation resulting from a transformation in the membrane bilayer, as suggested previously (32). Evidence has recently been obtained of an exchange, involving phospholipids, between rat sperm cells and albumin (33). Our present observations show a decrease in the sperm plasma membrane Chol/PL ratio associated with phospholipid transfer. As already noted, lowering this ratio hastens spontaneous fusion (18) and suggests a molecular mechanism for capacitation and fusion between the plasma membrane and outer acrosomal membrane.

Concordant with the proposed model, albumin loses its capacitation-inducing ability when presaturated with cholesterol (4). Effects of other modifications of albumin-bound lipids implicate hydrophobic membrane-protein interactions. Use of lipid-free albumin, for example, strongly facilitates induction of the acrosome reaction among hamster spermatozoa (34) and may improve, albeit more weakly, the fertilizing ability of rat sperm cells (4). On the other hand, masking hydrophobic sites on albumin, through saturation with palmitic acid, causes an inhibition of fertilization by rat epididymal spermatozoa (unpublished results) and occurrence of the acrosome reaction in hamster sperm (34). Although these observations may suggest that bovine serum albumin acts by depleting fatty acids from plasma membrane (34), cell membranes contain little free fatty acid (35).

Bovine serum albumin binds rapidly to sperm cells under hypertonic conditions (36) and adheres to plasma membrane in aqueous solution (7). When phospholipid bilayers interact with the protein, they become more permeable (37). A similar increase in sperm permeability, especially to Ca^{2+}, could initiate the acrosome reaction (38, 39). Ca^{2+} ionophore A23187 has this effect on guinea pig spermatozoa (40, 41). The significance of an increase in membrane fluidity induced by A23187 (42) on the acrosome reaction, however, has not been evaluated.

An albumin-induced transformation in the sperm plasma membrane possibly causes capacitation in physiological circumstances. Both uterine and follicular fluid have a high concentration of albumin (43, 44), and the protein appears to be responsible for capacitation by ovarian follicular fluid (45). Furthermore, albumin is virtually excluded from seminal plasma (46). Also suggestive from the present viewpoint is the presence of membrane vesicles with DF activity in this fluid. By increasing the sperm plasma membrane Chol/PL ratio, DF vesicles could reversibly inhibit expression of fertilizing capacity. When rat epididymal spermatozoa were incubated in albumin-containing medium with DF vesicles, the acrosome reaction was inhibited (10) and sperm Chol/PL values were higher (13). The transfer of cholesterol from synthetic phospholipid vesicles with DF-like activity (12) to rabbit sperm plasma membrane has also been reported (47).

Unexpectedly high concentrations of cholesteryl ester (sterol fatty acid esters) were found in these plasma membrane preparations. One molecule of cholesterol and between one-third and one-half of a cholesteryl ester molecule occurred for each phospholipid molecule in these membranes. At these concentrations, the membrane should contain separate sterol phases (48), thereby causing an unlikely modification of the fluid mosaic bilayer model of membrane structure. It is of some interest, therefore, to know how cholesteryl ester is distributed in these preparations of plasma membrane.

Total sialic acid and ganglioside levels were noticeably lower in plasma membrane from spermatozoa incubated with albumin. This probably reflects digestion by sperm neuraminidase after the acrosome reaction. Extensive proteolysis under these conditions was demonstrated to coincide with an increase in acrosin activity (7).

In apparent disagreement with the results of this investigation, virus-mediated cell fusion is unaffected by wide changes in Chol/PL ratio (49). In contrast with both results, Hope *et al.* (50) found that high Chol/PL levels stimulate hen erythrocyte fusion after treatment with fusigenic agents. The reason for these discrepancies between model systems with induced fusion, and between spontaneous and induced membrane fusion is unknown at present.

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