BIOLOGY OF REPRODUCTION 32, 145–153 (1985)

# Albumin-Mediated Changes in Sperm Sterol Content During Capacitation[1]

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## ABSTRACT

The role of albumin in mouse sperm capacitation was studied in relation to its activities as a lipid-solubilizing protein and a sterol acceptor. Two bovine serum albumins (BSA) which supported capacitation, Fraction V and fatty acid-free, both contained cholesterol and phospholipid but were without detectable levels of serum high-density lipoprotein (HDL). The lipid content of BSA could be reduced by trichloroacetic acid (TCA) precipitation; however, removal of all detectable lipids required precipitation with ethanolic acetone and diethyl ether extraction. In medium supplemented with Fraction V, fatty acid-free, or TCA-precipitated BSA, mouse sperm were capacitated as evidenced by their ability to fertilize eggs, concomitant with decreases in total cellular sterol and increases in phospholipid content. Delipidated BSA, fractionated on Sephadex G-100 in guanidine HCl also supported capacitation and mediated a 20% decrease in sperm sterol content, while cellular phospholipid levels remained unchanged. When BSA was modified by cholesterol augmentation, fertilization was inhibited in a cholesterol dose-dependent manner. These findings suggest that modulation of sperm lipid levels comprises an event of capacitation and that albumin mediates this process through its activity as a sterol acceptor.

## INTRODUCTION

Mammalian sperm acquire the ability to fertilize eggs during a period of residence in the lumen of the female reproductive tract (Austin, 1951; Chang, 1951). This process, called capacitation, involves biochemical and physiological changes in the sperm which prepare the cells to undergo the acrosome reaction, a morphologically distinct alteration consisting of a series of point fusions between the outer acrosomal membrane and the overlying plasma membrane (Bedford and Cooper, 1978). The acrosome reaction results in the release of acrosomal contents which facilitate penetration of the egg investments and fusion with the egg plasma membrane (Allison and Hartree, 1970).

Although the physiological site of capacitation is the female reproductive tract, sperm capacitation can be achieved in vitro in balanced salt solutions containing appropriate concentrations of electrolytes, metabolic energy sources, and serum albumin as the sole added protein. The protein requirement in these capacitating media is specific for albumin; ovalbumin, globulins and fibrinogen do not support capacitation (Brackett et al., 1972; Davis, 1976a). Despite the inclusion of albumin in culture media for in vitro capacitation and fertilization, its role in these processes is not well understood. In addition to its binding and transport functions (Kragh-Hansen, 1981), albumin can also mediate the efflux of sterol from cultured cells. This activity has been described in several cell types, including fibroblasts (Chau and Geyer, 1978; Bartholow and Geyer, 1981), macrophages (Werb and Cohn, 1971) and vesicular stomatitis virus (Pal et al., 1981). A consideration of albumin's function must also take into account the contamination by other serum proteins, enzymes, hormones, lipids and the presence of ligands, including large organic and inorganic ions. Variability in

Accepted September 11, 1984.
Received June 1, 1984.

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the nature and quantity of nonalbumin components may provide a basis for the observation that not all albumin preparations are effective in in vitro fertilization systems (Meizel, 1977).

The objectives of this study were dual: *1*) to elucidate the role of albumin in sperm capacitation by assessing if albumin, by virtue of its ability to bind lipids and its sterol-releasing activity, modulates sperm lipid levels during capacitation; and *2*) to determine if the ability to support sperm capacitation is intrinsic to the albumin molecule or is derived from or dependent on albumin-bound ligands or other components in the preparation. Mouse gametes were employed in an in vitro fertilization system in which a requirement for albumin in capacitation has been established (Toyoda et al., 1971; Miyamoto and Chang, 1973; Inoue and Wolf, 1975). Mouse sperm capacitation is dose responsive to albumin (bovine) within the concentration range of 2–20 mg/ml in a modified Krebs Ringer bicarbonate buffer supplemented with metabolic energy substrates (Wolf, 1979).

## MATERIALS AND METHODS

### *Albumins*

Fraction V and fatty acid-free bovine serum albumins (FV-BSA and FAF-BSA) were obtained from Sigma (St. Louis, MO). FV-BSA was precipitated with trichloroacetic acid (TCA) by the method of Schwert (1975). This preparation, TCA-BSA, was reconstituted in a Krebs Ringer salt solution (KRSS; 119.37 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2 \cdot 2H_2O$, 1.19 mM $KH_2PO_4$ and 1.19 mM $MgSO_4 \cdot 7H_2O$) at a concentration of 50 mg/ml.

Delipidated BSA (DL-BSA) was prepared by the method of Rothblat et al. (1976). Ten volumes of chilled acetone: ethanol (1:1, v/v) were added to a solution of FV-BSA with stirring. This mixture was maintained at −20°C with occasional stirring for 4 h. Precipitated protein was collected on Whatman no. 1 filter paper under gentle suction applied to a Buchner funnel and washed with chilled diethyl ether. The protein was dissolved in KRSS, dialyzed against 5 vol of KRSS at 4°C and adjusted to a concentration of 50 mg/ml.

DL-BSA (50 mg/ml) was purified by column chromatography on Sephadex G-100 in 6 M guanidine HCl. The albumin peak was pooled, dialyzed against deionized $H_2O$ at 4°C and lyophilized. This albumin, designated G-BSA, was dissolved in KRSS at a concentration of 30 mg/ml prior to use. TCA-BSA, DL-BSA and G-BSA preparations were sterilized by passage through 0.22-μm Millipore filters, stored at 4°C and used within 2 wk.

Cholesterol-enriched BSA (50 mg/ml) was prepared by adding cholesterol (5 mg/ml in ethanol) to FAF-BSA in KRSS and sonicating (LNR Ultrasonics) for 30 min at 4°C. This preparation was incubated overnight at 37°C and passed through a 0.45-μm Millipore filter prior to use.

### *Procedures*

In vitro fertilization using mouse gametes was carried out as described by Inoue and Wolf (1975). A modified Krebs Ringer bicarbonate buffer (KRB) was prepared by supplementing KRSS with $NaHCO_3$ (25.07 mM), glucose (5.56 mM) and lactate (25.0 mM). BSA (20 mg/ml) was added to KRB for both sperm capacitation and egg insemination. Epididymal sperm ($1 \times 10^7$ cells/ml) were incubated in KRB-BSA for 60 min and ovulated eggs were recovered from excised oviducts. Following dispersal of cumulus cells with hyaluronidase (1 mg/ml), eggs were transferred through several washes of KRB-BSA and introduced into insemination dishes (Nunc Multidish, Vanguard International, Neptune, NJ). Following addition of sperm, (final concentration, $10^6$ sperm/ml) the dishes were aerated with 5% $CO_2$ in air and incubated for 4–4.5 h at 37°C. Eggs were then removed, washed free of adherent sperm and mounted on glass slides. Eggs were fixed in 3% glutaraldehyde followed by 10% formalin, dehydrated and stained with 0.25% acetolacmoid. Eggs were considered fertilized when at least one decondensed sperm head, inner acrosomal membrane and corresponding sperm tail, or male pronucleus were identified within the ooplasm.

In experiments evaluating capacitation with cholesterol-enriched albumin, eggs were inseminated in medium consisting of KRB supplemented with the polymer, polyvinyl alcohol (PVA) as described by Bavister (1981) so that exposure to BSA was confined to sperm.

### *Extraction of Sperm Lipids*

Epididymal sperm suspended in KRB or incubated in KRB or KRB-BSA for 1 h at 37°C were collected by centrifugation (600 × *g* for 15 min) and washed twice with equal volumes of KRB. The final sperm pellets were resuspended in 0.2–0.5 ml KRB and sperm concentrations were determined by hemocytometry. Lipid extraction was carried out using a modified Folch extraction (Tietz, 1976). The organic layer was collected and washed once with theoretical Folch upper-phase reagent (chloroform:methanol:$H_2O$, 3:48:47, v/v/v) (Folch et al., 1975) to remove any residual protein, and then reduced to a volume of 0.2 ml under a stream of $N_2$ in a warm water bath, and stored under $N_2$ at −25°C until assayed.

Lipids were quantitated following saponification of samples with ethanolic KOH. Total cellular cholesterol was determined by the method of Rudel and Morris (1973). Phospholipids were measured by the method of Raheja et al. (1973) using L-α-dipalmitoylphosphatidylcholine as a standard. The lower limits of sensitivity for these lipid assays are 1.0 μg cholesterol and 0.5 μg phospholipid phosphorus, respectively. In the replicate experiments summarized in Tables 1–4, values agreed to within 10% and averages of the data are presented.

Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as a standard. Electrophoretic separation of proteins was

carried out in sodium dodecyl sulfate (SDS)-urea polyacrylamide slab gels according to the method of Laemmli (1970).

Student's *t* test was used to determine statistical significance between paired data.

## RESULTS

### *Characterization of Albumins*

Several different albumin preparations were used: Fraction V BSA (FV-BSA) and fatty acid-free BSA (FAF-BSA) were obtained from a commercial source and analyzed for the presence of protein contaminants by electrophoresis in SDS-urea polyacrylamide gels. In addition to albumin, other Coomassie blue-staining components were detected in both preparations (Fig. 1). Components in the high molecular weight range may represent aggregates of albumin which can self-associate through disulfide bonding (Janatova et al., 1968) or immunoglobulins which can complex with albumin (Sharma et al., 1981). Components of lower molecular weight were also visible in FAF-BSA. The major protein associated with serum high-density lipoprotein (HDL), apolipoprotein A-I (apo A-I), has been detected in commercial albumins (Fainaru et al., 1981). Since apo A-I has lipid-binding properties which could contribute to the sterol-releasing activity of albumin, the presence of this polypeptide in these albumins was assessed. However, no component with a relative mobility equal to that of bovine apo A-I (Rm=0.86) was detected by Coomassie blue in either FV-BSA or FAF-BSA. For comparison, crystallized BSA, generally considered to be of higher purity, exhibited an electrophoretic profile similar to that of FV-BSA. FV-BSA was further modified by precipitation with trichloroacetic acid and delipidation yielding TCA-BSA and DL-BSA, respectively. Both unmodified and modified albumins were evaluated for total cholesterol and phospholipid content (Table 1). FAF-BSA was relatively lipid-rich, containing 10-fold the levels of cholesterol and phospholipid detected in FV-BSA. TCA precipitation reduced the phospholipid content of FV-BSA by 25% while removing all detectable sterol. No lipids could be detected in FV-BSA following delipidation. Crystallized BSA contained levels of cholesterol and phospholipid comparable to those in FV-BSA. The ability of FV-BSA, FAF-BSA, TCA-BSA and DL-BSA to support sperm capacitation were evaluated in an in vitro fertilization assay. At a concentration of 20 mg/ml, all of these preparations supported capacitation and fertilization equally, with 48.1–59.6% of eggs fertilized in 5–10 trials using each preparation. The success of DL-BSA as a capacitating agent suggests that the lipid complement of albumin is not required for this activity.

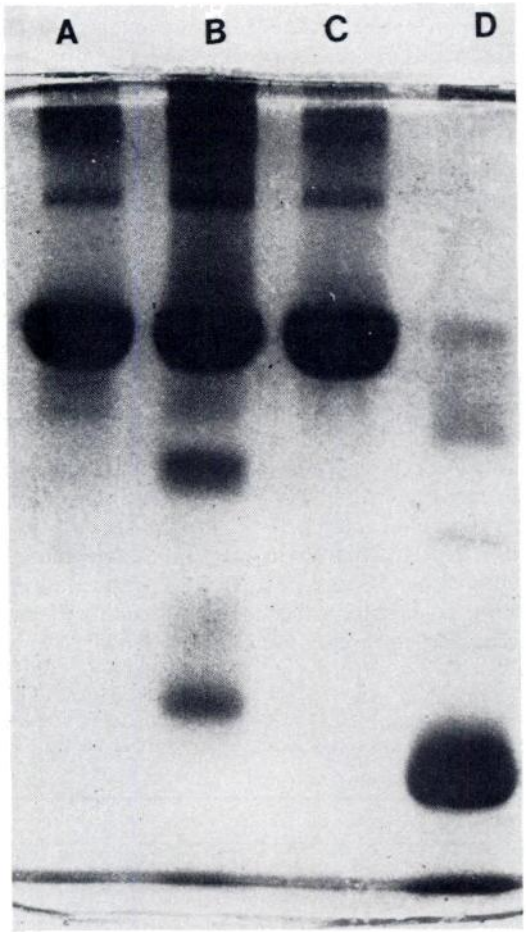


FIG. 1. SDS-polyacrylamide gel electrophoresis of BSAs and bovine HDL. Forty μg protein were applied to each lane: *A*) FV-BSA; *B*) FAF-BSA; *C*) crystallized BSA; and *D*) bovine HDL. The major band represents apolipoprotein A-I.

### *Effects of Albumin on Sperm Lipids*

To assess the effect of FV-BSA, FAF-BSA, TCA-BSA and DL-BSA on cellular lipid levels, epididymal sperm were incubated for 60 min in culture medium supplemented with each of the albumins at a concentration of 20 mg/ml. This time interval was selected to support capacita-

TABLE 1. Lipid content of commercial and modified albumins. Lipids were extracted from 100 mg of each BSA preparation and total (free and esterified) cholesterol and phospholipid contents were determined. Results are the average of duplicate extractions.

| Albumin | Cholesterol (μg/100 mg) | Phospholipid[a] (μg/100 mg) |
|---|---|---|
| Crystallized | 1.2 | 17.0 |
| Fraction V BSA | 2.0 | 22.7 |
| Fatty acid-free BSA | 22.7 | 329.5 |
| TCA-precipitated BSA | ND | 17.0 |
| Delipidated BSA | ND | ND |

[a] μg phospholipid=μg lipid phosphorus X 25.

[b] *ND*=not detected.

tion but to minimize membrane losses from sperm undergoing the acrosome reaction. Compared to washed epididymal sperm or sperm incubated for 60 min in protein-free medium, cells incubated in the presence of albumin contained reduced sterol contents (Table 2). Albumin exposure also affected cellular phospholipid levels (Table 3) in a time-dependent manner over a 60-min incubation period. Concurrent with the diminution of sperm sterol levels, phospholipid levels increased following incubation with FV-BSA, FAF-BSA and TCA-BSA. The maximum increment was observed with FV-BSA (twice control levels), while 57% and 67% increases occurred with FAF-BSA and TCA-BSA, respectively. In contrast, no detectable alteration in sperm phospholipid content was observed following incubation with DL-BSA. The finding that DL-BSA supports in vitro capacitation and modulates sperm sterol levels with no apparent effect on phospholipid levels, suggests that the phospholipid enrichment observed in sperm following treatment with other albumin preparations is not an obligatory event in capacitation.

*Fractionation of DL-BSA*

To further establish that the sperm capacitating activity of albumin is intrinsic to the albumin molecule and does not involve associated lipids or ligands, DL-BSA was submitted to gel filtration under denaturing conditions. Analysis of the fractionated BSA (G-BSA) by SDS-PAGE demonstrated one major protein band corresponding to albumin (Fig. 2), as well as several components in the higher molecular weight range. The latter presumably represent albumin aggregates or nondissociable contaminants. The comigration of G-BSA with unmodified crystallized BSA indicates that the molecular weight of albumin was not altered following fractionation. This albumin was also evaluated for its effects on sperm lipid levels. Sperm incubated in medium supplemented with G-BSA at 20 mg/ml underwent a 20% depletion in sterol content, with no apparent changes in cellular phospholipid level (Table 4). Fertilization levels achieved in medium supplemented with either G-BSA or FAF-BSA were comparable at approximately 48%, indicating that capacitating activity was conserved in G-BSA.

TABLE 2. Sterol content of mouse sperm. Lipids were extracted from sperm prior to and following a 60-min incubation in medium supplemented with 20 mg albumin/ml. Data are expressed as μg total sterol per $10^7$ sperm ± SD (number determinations).

| | Albumin | | | |
|---|---|---|---|---|
| | FV | FAF | TCA[a] | DL[a] |
| Control | 12.8 ± 1.1 (7) | 13.2 ± 0.7 (7) | 13.5 | 12.0 |
| Treated | 8.4 ± 1.0[b] | 7.0 ± 0.7[b] | 5.6 | 8.6 |

[a] Average of two experiments.

[b] Significantly different from control values: $P<0.001$.

TABLE 3. Changes in cholesterol and phospholipid levels of mouse sperm as a function of incubation time. Lipids were extracted from sperm and quantitated prior to and following a 30- or 60-min incubation in culture medium supplemented with 20 mg/ml BSA. Data are the average of two experiments.

| Albumin preparation | Duration of incubation (min) | Sperm sterol content per $10^7$ cells | | Sperm lipid phosphorus content per $10^7$ cells | | Sperm cholesterol:lipid phosphorus molar ratio |
|---|---|---|---|---|---|---|
| | | (μg) | (nmol) | (μg) | (nmol) | |
| Fraction V | 0 | 14.3 | 36.8 | 0.9 | 27.3 | 1.3 |
| | 30 | 12.3 | 31.9 | 1.0 | 31.6 | 1.0 |
| | 60 | 10.1 | 26.0 | 1.8 | 57.4 | 0.5 |
| Fatty acid-free | 0 | 12.2 | 31.6 | 0.7 | 20.9 | 1.5 |
| | 30 | 8.3 | 21.3 | 0.8 | 24.5 | 0.9 |
| | 60 | 6.1 | 15.7 | 1.1 | 35.4 | 0.4 |
| TCA-precipitated | 0 | 13.5 | 34.8 | 0.9 | 27.4 | 1.3 |
| | 30 | 8.7 | 22.5 | 1.2 | 38.7 | 0.6 |
| | 60 | 5.6 | 14.4 | 1.5 | 48.1 | 0.3 |
| Delipidated | 0 | 12.0 | 31.0 | 0.8 | 26.6 | 1.2 |
| | 30 | 10.0 | 25.8 | 0.8 | 25.8 | 1.0 |
| | 60 | 8.6 | 22.2 | 0.8 | 25.8 | 0.9 |

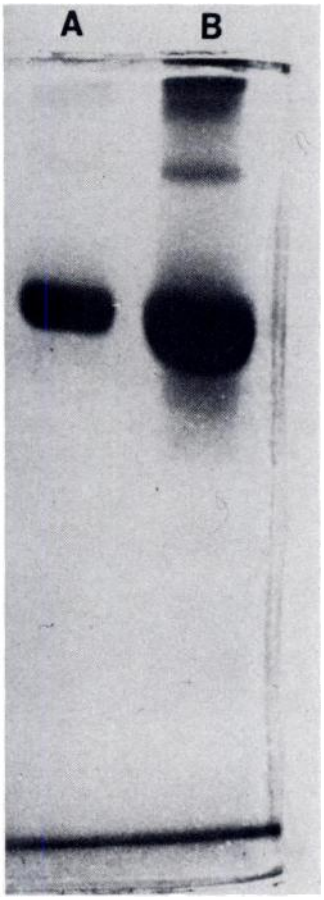


FIG. 2. SDS-polyacrylamide gel electrophoresis of 20 μg crystallized BSA (*A*) and 40 μg of G-BSA (*B*).

*Sterol-Enriched Albumin*

Because the ability of albumin to support capacitation was coincident with a reduction in sperm sterol content, additional studies were undertaken to determine if these two activities were related. A cholesterol-rich albumin was prepared by direct addition of cholesterol to FAF-BSA. This preparation was chosen for cholesterol augmentation because its higher phospholipid content should facilitate solubilization of the added sterol. Cholesterol (in ethanol) was added to FAF-BSA to achieve 2-, 4-, 5- and 10-fold the endogenous sterol content, and these cholesterol-enriched preparations were evaluated for their ability to support sperm capacitation in the in vitro fertilization assay. Without producing obvious detrimental effects on sperm motility, these cholesterol-enriched albumins inhibited fertilization in a sterol concentration-dependent manner (Fig. 3). Thus, it appears that under conditions where sterol efflux from sperm is precluded by a high extracellular cholesterol gradient, or saturation of a sterol-acceptor, capacitation is suppressed. The inhibitory effect of sterol-rich BSA is localized to the sperm since only these cells, and not the eggs, were exposed to albumin.

## DISCUSSION

Capacitation in vitro of sperm from several mammalian species has been achieved in culture media which contain albumin as the sole

TABLE 4. The effect of G-BSA on mouse sperm lipid levels. Lipids were extracted from sperm and quantitated prior to and following a 30- or 60-min incubation in culture medium supplemented with 20 mg/ml albumin. Data are the average of two experiments.

| Duration of incubation (min) | Sperm cholesterol content per $10^7$ cells | | Sperm lipid phosphorus content per $10^7$ cells | | Sperm cholesterol:lipid phosphorus molar ratio |
|---|---|---|---|---|---|
| | (μg) | (nmol) | (μg) | (nmol) | |
| 0 | 12.5 | 32.3 | 0.88 | 28.2 | 1.2 |
| 30 | 11.2 | 28.9 | 0.88 | 28.2 | 1.0 |
| 60 | 9.8 | 25.3 | 0.85 | 27.4 | 0.9 |

protein supplement (Rogers, 1978). The function of albumin in this process is not clearly understood, although several hypotheses concerning its role have been advanced. These include: *1*) the removal or alteration of coating materials at the sperm surface (Yanagimachi, 1981); *2*) the modification of sperm lipid composition through lipid exchange or hydrolysis (Davis et al., 1979a); and *3*) the promotion of plasma membrane protein hydrolysis (Davis and Gergely, 1979). Results from the present study support a mechanism that includes alterations in sperm lipid composition specifically through sterol efflux. Experimental evidence has been obtained showing that: *1*) following incubation with commercially prepared albumins, mouse sperm become depleted of sterol and enriched in phospholipid; *2*) albumin, after delipidation and fractionation, retains both sterol-releasing and capacitating activities but does not effect changes in sperm phospholipid levels; and *3*) incubation of sperm with cholesterol-enriched albumin inhibits fertilization in a cholesterol concentration-dependent manner.

While the ability of albumin to function as a sterol acceptor has been documented in other cells (Chau and Geyer, 1978; Bartholow and Geyer, 1981; Pal et al., 1981), reports of contamination of commercially prepared albumins by HDL suggested that this lipoprotein might be the mediator of sterol efflux. In the present studies, two commercial preparations of albumin which supported capacitation and reduced sperm sterol levels did not contain detectable quantities of the major protein of HDL, apo A-I, as assessed by Coomassie blue binding. Because this method for protein visualization is insensitive to minute quantities of protein, the presence of small amounts of apo A-I in these albumins cannot be ruled out. However, Bartholow and Geyer (1981) have demonstrated that albumin itself can function as a sterol acceptor from cells, and in the present studies, it was observed that delipidated, fractionated albumin retained the ability to deplete sperm of sterol and to support capacitation.

In the rat, Davis et al. (1979a) first described the exchange of lipids between sperm and albumin. This bidirectional flux of cholesterol and phospholipids between cells and medium protein yielded sperm with decreased cholesterol and increased phospholipid levels similar to the mouse sperm observed in the present study. Analysis of the lipid composition of plasma membranes isolated from rat sperm exposed to albumin confirmed that alterations in lipid levels occurred in the plasma membrane fraction, but only the increment in phospho-

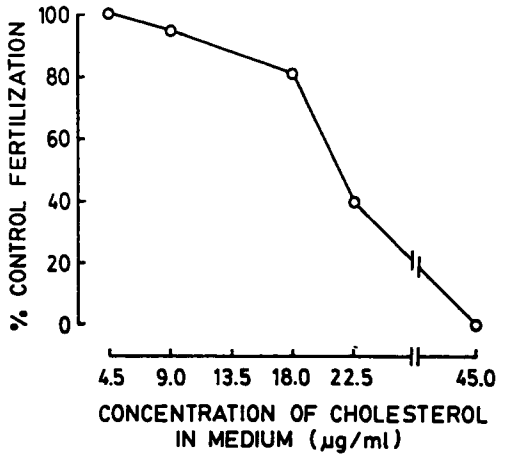


FIG. 3. In vitro fertilization of eggs by sperm which had been incubated with FAF-BSA supplemented with cholesterol to achieve the indicated concentrations.

lipid content reached statistical significance (Davis et al., 1979b). In contrast, as demonstrated here in the mouse, phospholipid enrichment during incubation with albumin was not required in sperm capacitation. Delipidated albumin, in which phospholipid levels were greatly reduced, thereby precluding substantial transfer of lipids to cells, retained capacitating activity. In addition, the conservation of both capacitating and sterol-depleting activities in delipidated albumin further modified by fractionation in denaturing solvents suggested that reduction or removal of other albumin-associated lipids or contaminants did not significantly affect these functions.

The inability of cholesterol-enriched albumin to support mouse sperm capacitation suggests that the sterol acceptor activity of this protein is critical. An inhibitory effect of cholesterol, whether provided in conjunction with albumin or in phospholipid liposomes, has been demonstrated in the capacitation of rabbit sperm (Davis, 1976b) and the induction of the acrosome reaction in both rat (Davis, 1980) and guinea pig (Fleming and Yanagimachi, 1981) sperm. At least two possibilities exist for the mechanism by which the capacitating activity of cholesterol-enriched albumin is diminished: *1*) inhibition of sterol desorption from sperm, by either saturation of sterol binding sites on albumin, or provision of a high extracellular sterol gradient; or *2*) enrichment of sperm cellular membrane sterols with attendant changes in membrane properties. Clarification of these possibilities could be made through an analysis of sperm sterol content following incubation in a cholesterol-rich medium. A role for cholesterol in cellular function is certainly not unique to sperm capacitation, as demonstrated by extensive studies conducted with the mammalian erythrocyte. The depletion or enrichment of cholesterol in the plasma membrane of these cells results in measurable changes in transport properties, permeability, enzymic activities, cell shape, and fluid characteristics of the membrane (Cooper, 1978).

The conclusions drawn in the present study regarding cholesterol desorption and alteration in sperm membrane function during capacitation are limited by several considerations. Because total sperm lipid levels were measured, the site(s) of sterol depletion is unknown. Plasma membrane cholesterol is readily exchangeable with that in the extracellular medium, and represents a pool susceptible to sterol acceptors and donors. Further studies should be conducted on purified plasma membranes from sperm so the content and distribution of sterol in this fraction can be ascertained. The utilization of specific markers for sterol such as the polyene antibiotics, filipin or amphotericin B, may circumvent the problems of cell fractionation and provide an approach to the analysis of sperm sterol in situ.

While attention has been focused primarily on cellular sterols in this study, a pivotal role in capacitation for the other major membrane lipid class, phospholipids, has been suggested (reviewed by Clegg, 1983). As substrates for sperm-associated phospholipases, these molecules could give rise to lysophospholipids which may influence the potential of spermatozoal membranes to undergo fusion (Lucy, 1975). In this regard, phospholipase activity has been implicated in the induction of the acrosome reaction in hamster sperm (Lui and Meizel, 1979; Llanos et al., 1982) and has recently been described in mouse sperm (Thakkar et al., 1983). Thus, in addition to its sterol-accepting activity towards sperm, albumin could also be involved in aspects of cellular phospholipid modulation, e.g., serving as a sink for fatty acids, the products of phospholipid hydrolysis.

The modulation of sperm lipid content under capacitating conditions may represent one of the molecular events in capacitation. The findings that albumin mediates changes in sperm sterol levels during capacitation and that cholesterol inhibits fertilization, suggests that the activity of this protein as a sterol acceptor is important in its function as a capacitating agent. Given the regulatory role of the cholesterol level in membrane function, its modulation would be consistent with the pervasive changes in sperm membrane properties envisioned in our current conceptions of capacitation.

## ACKNOWLEDGMENTS

The authors express appreciation to Ms. Gail Alexander for excellent secretarial assistance.

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