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Localization of cholesteryl sulfate in human spermatozoa in support of a hypothesis for the mechanism of capacitation

(epididymis/sterol-sulfatase/phospholipids/lecithin:cholesterol acyltransferase/acrosin)

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ABSTRACT **Cholesteryl sulfate is a normal constituent of human spermatozoa. The *in vitro* uptake of tritiated cholesteryl sulfate resulted in the labeling of all spermatozoa as demonstrated by light-microscope radioautography. The binding of the sterol sulfate was localized mainly in the head and midpiece. Radioautography at the level of the electron microscope revealed that the sterol sulfate is localized on the plasma membrane, mostly in the region of the acrosome. Further proof for this localization was obtained by selective dissolution of the plasma membrane and acrosome of the spermatozoa with low concentrations of Triton X-100. This treatment resulted in the simultaneous removal of tritiated cholesteryl sulfate bound to the spermatozoa. A hypothesis is presented concerning the role of cholesteryl sulfate as a membrane stabilizer and enzyme inhibitor during the maturation of spermatozoa in the epididymis. According to this hypothesis, the cleavage of the sulfate moiety within the female reproductive tract triggers a cascade of events leading to sperm capacitation and fertilization.**

One of the challenges remaining in reproductive biology is the elucidation of the biochemical events involved in the maturation and capacitation of mammalian spermatozoa. A review of the literature (1–5) indicates that the following biochemical events occur during capacitation. Stabilizing factor(s), associated with the spermatozoal membrane during transit or storage within the epididymis inhibit the release of acrosomal enzymes in the male tract, but the factor(s) must be removed during migration in the female tract to allow the contact of the acrosomal enzymes with the investments of the ovum in order to facilitate penetration. Removal of the stabilizing substance(s) is thought to be enzyme-catalyzed and involves changes in membrane conformation and permeability, ultimately leading to the acrosome reaction.

Evidence is accumulating in support of our contention that sterol sulfates play an important role in the biochemistry of sperm maturation and capacitation. Thus, we have reported that cholesteryl sulfate ($CholSO_4$) is an important component of human spermatozoa and is avidly taken up by these cells during *in vitro* incubation (6, 7). In addition, during transit through the epididymis, hamster spermatozoa exhibit a severalfold increase in desmosteryl sulfate concentration from the caput to the cauda regions (8). The finding of sterol sulfotransferase activity in the hamster epididymis (9) demonstrates that the biosynthesis of sterol sulfates can occur in this tissue.

That low concentrations of sterol sulfates can block *in vitro* capacitation by hamster cumulus cells (10) and also inhibit acrosin (11), the sperm acrosomal proteinase involved in the penetration of the zona pellucida of the ovum (12), provides evidence that sterol-sulfatase (sterol-sulfate sulfohydrolase, EC 3.1.6.2) could be involved in the mechanism of sperm capacitation and ovum penetration. Furthermore, sterol-sulfatase is present in the human female reproductive tract (6) and in the hamster reproductive tract, where maximal activity is attained after ovulation (13). In view of the fact that $CholSO_4$ has been implicated in the stabilization of the erythrocyte membrane (14), the enzymatic hydrolysis of sterol sulfates on the sperm membrane by sterol-sulfatase present in the female tract could represent a membrane destabilization event leading to capacitation and the acrosome reaction.

The purpose of the present study was to localize $CholSO_4$ in human spermatozoa at the ultrastructural level in relation to its possible involvement in sperm capacitation.

MATERIALS AND METHODS

[1,2-^{3}H]$CholSO_4$ was synthesized from [1,2-^{3}H]Chol (60 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear) and purified by column chromatography (15); radiochemical homogeneity was verified by thin-layer chromatography (6).

Spermatozoa were obtained from sperm-bank donors of proven fertility and exceptionally good semen characteristics. Within 2 hr after ejaculation, the seminal plasma was separated from the spermatozoa by centrifugation at $600 \times g$ for 10 min. The cells were resuspended and washed in a sperm diluent prepared by the method of Lopata *et al.* (16), except that human serum albumin was deleted from the medium.

Spermatozoa (4×10^7) were resuspended in 1 ml of the medium containing 4.5 μCi of [^{3}H]$CholSO_4$ and incubated 4 hr at 37°C. They were then processed for light-microscope radioautography. Upon addition of 3 ml of 3% (wt/vol) glutaraldehyde (Meca Laboratories, Montreal, PQ, Canada) in 0.1 M Sorensen's phosphate buffer, the cells were fixed for 2 hr and then washed three times with phosphate-buffered sucrose (5%, wt/vol) to eliminate unbound [^{3}H]$CholSO_4$. The fixed cells were smeared on slides, air dried, dipped in Kodak NTB-3 emulsion, and exposed for 7 days. Developed radioautographs were stained with a solution of 0.5% toluidine blue and 1% sodium borate.

For electron-microscope radioautography, the cells (20×10^7) were suspended in 2 ml of the medium containing 45 μCi of [^{3}H]$CholSO_4$ and incubated for 4 hr at 37°C. Glutaraldehyde fixation was followed by three washes in phosphate-buffered sucrose, postfixation in 2% (wt/vol) osmium tetroxide and three washes at 4°C with phosphate-buffered sucrose. After the last centrifugation ($600 \times g$ for 10 min), the pellet was gently mixed with an equal volume of 2% (wt/vol) agar solution at 45°C. The gel was chilled to 4°C and cut into 1-mm^3 sections for the de-

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Abbreviations: Chol, cholesterol; $CholSO_4$, cholesteryl sulfate; ACAT, acyl-CoA:cholesterol *O*-acyltransferase; LCAT, lecithin:cholesterol acyltransferase.

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hydration steps with 70%, 90%, 95%, and anhydrous ethyl alcohol with 15-min standing periods at 4°C. After two standing periods in propylene oxide, the cells were embedded in EPON 812. Ultrathin (gold) sections of the labeled spermatozoa were prepared with a diamond knife and an LKB ultramicrotome and were placed on slides coated with collodion. The slides were stained with a lead citrate solution, covered with a thin carbon film, and dipped into dilute (1:4) Ilford L_4 emulsion. Radioautographs were developed in Microdol-X (Kodak) after 1- and 6-mo exposures and visualized with a Philips 300 electron microscope. Statistical analysis of silver grain distribution was done with radioautographs obtained at a final magnification of ×13,500.

Detergent treatment of human spermatozoa labeled with [^{3}H]CholSO_4 was as follows. Spermatozoa were incubated with [^{3}H]CholSO_4 as described, washed twice with the medium, and distributed into a series of tubes containing 2×10^7 cells in 0.1 ml of medium. One milliliter of medium containing increasing concentrations of Triton X-100 was added to each tube. Quadruplicate experiments were performed at each concentration of detergent. After 5 min of incubation at 25°C, 3 ml of medium was added to each of the tubes, which were centrifuged at 1000 × *g* for 10 min. Pellets were either processed for electron microscopy to visualize structural changes or dissolved in 100 μl of 1% $NaDodSO_4$ and 100 μl of 5 mM dithiothreitol and centrifuged. The supernatants and sediments were analyzed for their radioactive content in Ready-Solv (Beckman). This allowed the calculation of percentage of [^{3}H]CholSO_4 bound to the cells and the percentage of [^{3}H]CholSO_4 liberated into the supernatant after treatment with Triton X-100.

RESULTS

Spermatozoa incubated with labeled CholSO_4 took up 55% of the radioactivity incubated with the cells. Light-microscope radioautographs showed that all of the spermatozoa were labeled. This uptake of CholSO_4 was mainly concentrated in the region of the head and midpiece, and a weaker silver grain density was found along the principal piece, whereas control spermatozoa showed a random distribution of silver grains corresponding to the background (Fig. 1). These experiments using light-microscope radioautography confirm our previous report (6) concerning the uptake of the sterol sulfate.

The grain count on the electron-microscope radioautographs (Table 1) revealed that the number of grains found over the cellular surface area (50.1 grains per 100 cm^2) was much higher than that obtained on noncellular surface area (2.6 grains per 100 cm^2), indicating that, under our conditions, CholSO_4 was not a diffusible substance, as observed in the case of unconjugated cholesterol. Thus, only 3.5% of sperm-bound CholSO_4 was lost in the alcohol and propylene oxide washes during the dehydration steps.

Analysis of the cellular distribution of grains associated with spermatozoa after a 1-mo exposure (Fig. 2) revealed that the major proportion (47%) of the grains appeared over the acrosome (Table 2). Furthermore, 78% of the [^{3}H]CholSO_4 bound to sperm cells was located at the cell surface. A significant number of grains were also associated with the membrane debris.

The presence of grains on acrosomal membrane fragments still attached to spermatozoa (Fig. 2 *b*, *c*, and *d*) and probably arising from procedural manipulations offered further proof that CholSO_4 is indeed a membrane component. That [^{3}H]CholSO_4 binds predominantly to those membranes overlying the acrosome of spermatozoa was clearly evident on the electron-microscope radioautographs developed after 6 mo of exposure (Fig. 3).

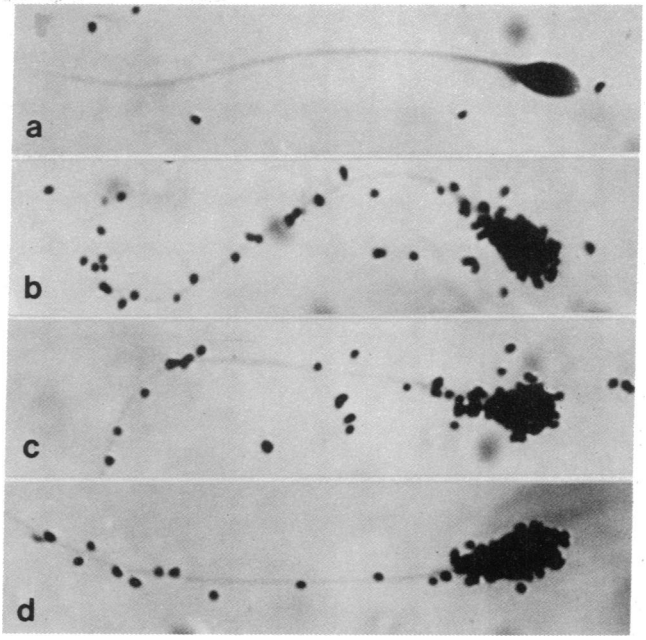

FIG. 1. Light-microscope radioautography of human spermatozoa after 1 wk of exposure. (*a*) Control cell incubated without [^{3}H]CholSO_4. (*b–d*) Experimental cells. Developed slides were stained with toluidine blue. (×1500.)

Treatment of spermatozoa with increasing concentrations of Triton X-100 resulted in the removal of the bound [^{3}H]CholSO_4. At a concentration of 0.04% of the detergent, as much as 85% of the bound CholSO_4 was extracted (Fig. 4). This treatment also resulted in the concomitant removal of the plasma membrane and the acrosome (Fig. 5).

DISCUSSION

The ultrastructural localization of CholSO_4 at the level of the plasma membrane is in accord with our contention that this class of compound is involved in a stabilization process. The interaction of sterol sulfates with biomembranes is supported by the finding that CholSO_4 is as potent as free Chol with regard to its interaction with phospholipids (17); thus, the 3β-hydroxyl group is not an absolute requirement for this interaction. Hydrogen bonding through the water associated with the sulfate and phospholipid head groups and the bulkiness of the sulfate moiety, would favor the localization of CholSO_4 on the external monolayer of the plasma membrane. If this is so, the calculated surface covered by the endogenous sterol sulfate on the human spermatozoon amounts to as much as 20% of the surface of the head (Table 3). This percentage of surface area covered by CholSO_4 would be of even greater magnitude over the acrosomal segment, where most of the conjugate is localized, be-

Table 1. Analysis of electron microscope radioautographs of human spermatozoa labeled with [^{3}H]CholSO_4

	Total	Cellular	Noncellular
Surface* (× 100 cm^2)	142.8	11.3	131.5
Grains obtained	909	566	343
Grains expected†	—	72	837
Grains expected/100 cm^2	6.36	6.36	6.36
Grains obtained/100 cm^2	6.3	50.1	2.6

* Surface areas were estimated using a Gelman planimeter.
† Grains expected if [^{3}H]CholSO_4 is randomly distributed, as in the case of steroid diffusion during dehydration.

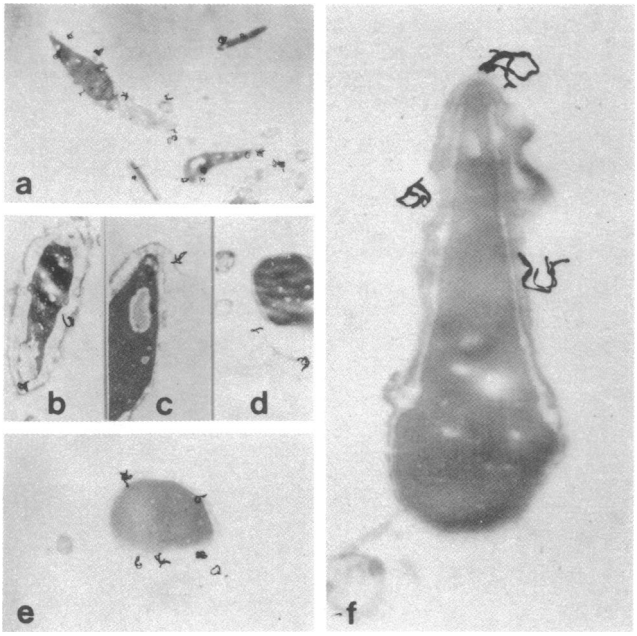

FIG. 2. Radioautographs of spermatozoa after incubation with [^{3}H]CholSO_4 and developed after 3-mo exposure. (*a*, *e*, and *f*) Intact cells. (×3300, ×5600, and ×20,000, respectively.) (*b*, *c*, and *d*) Acrosomal membrane fragments arising from tissue preparation. (×5600.)

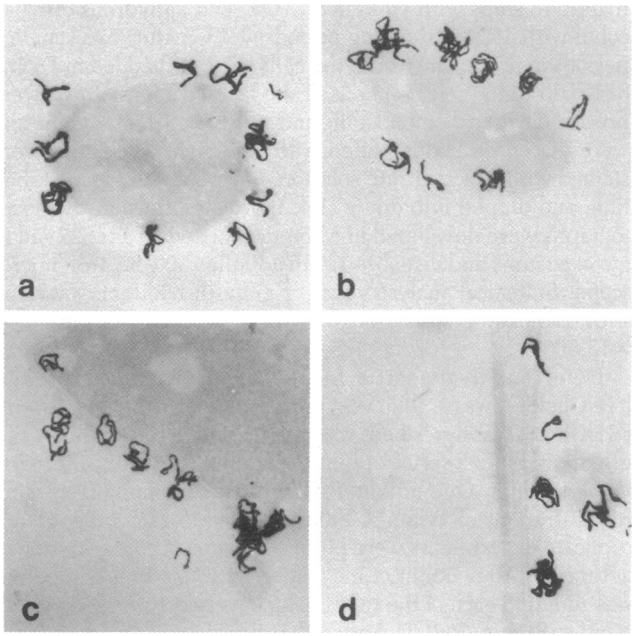

FIG. 3. Radioautographs of human spermatozoa labeled with [^{3}H]CholSO_4 (6-mo exposure). Silver grains are mostly found over the membranes overlying the acrosome (*a*, *b*, and *c*) and over plasma membranes covering the more distal segments of the flagellum (*d*). (×13,000.)

cause the surface area of the acrosome is less than that of the entire head used in the calculation. In view of the heterogeneous density patterns of unconjugated sterol observed in guinea pig sperm plasma membranes (20), it is possible that CholSO_4 may be concentrated in specific areas of the acrosomal segment. The preferential localization of CholSO_4 in the membranes overlying the acrosome provides further support for its involvement in the regulation of sperm capacitation.

Molecular modifications of the plasma membrane of mammalian spermatozoa occur during *in vitro* capacitation, which is characterized by an increase in the transport of calcium (21) and a decrease in the Chol/phospholipid ratio (22, 23). Chol and lecithin are major components of biological membranes, and any alterations in their structure or molar ratio, or both, may result in profound changes in membrane fluidity and permeability. The production of lysolecithin and Chol esters during *in vitro* capacitation of rat sperm cells in the presence of albumin (22, 23) could result from the concerted action of phospholipase A_2, fatty acid thiokinase, and acyl-CoA:cholesterol *O*-acyltransferase (ACAT; EC 2.3.1.26). An alternate pathway would require the presence of lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43), catalyzing the transfer of the acyl group from the β-position of lecithin to Chol, yielding lysolecithin and Chol esters (24). LCAT is present in porcine follicular fluid (25), and because LCAT exhibits a high affinity for high density lipoproteins (26) which are found in human plasma and follicular fluid (27), LCAT also may be a component of human follicular fluid. Capacitation factors are present in this fluid (28), and it is noteworthy that it also contains a high concentration

Table 2. Cellular distribution of silver grains associated with spermatozoa on electron-microscope radioautographs

	Silver grains		
Classification	No.	% of total	
Cell surface			
Acrosome	228	46.8	77.8
Midpiece	57	11.7	
Principal piece	94	19.3	
Internal			
Nucleus	70	14.4	22.2
Midpiece	25	5.1	
Principal piece	13	2.7	
	487	100.0	
Membrane debris	79		

Silver grains associated with cell structure were classified according to their distribution in the acrosome, nucleus, flagellum, and membrane debris (membranous material not associated with any recognizable sperm structure). Grains located within the nucleus or flagellum were considered to be internal cellular grains whereas those residing over the acrosome and in plasma membranes overlying the acrosome and flagellum were considered to be located on the cell surface.

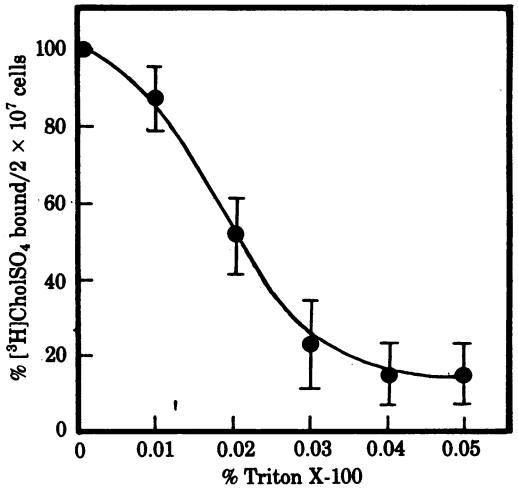

FIG. 4. Detergent treatment of human spermatozoa labeled with [^{3}H]CholSO_4. Cells (2×10^7) were incubated with increasing concentrations of Triton X-100 for 5 min at 25°C. The percentage of [^{3}H]CholSO_4 bound per 2×10^7 cells was determined by assay of the radioactivity in the pellet and supernatant.

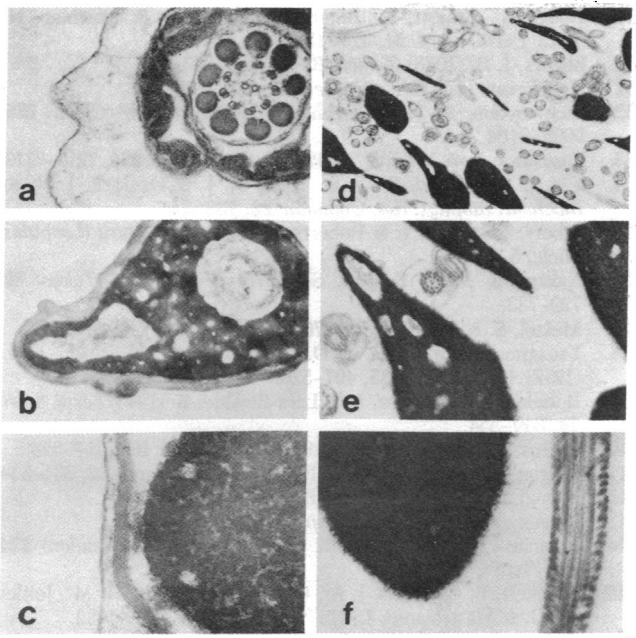

FIG. 5. Electron micrographs of human spermatozoa after treatment with Triton X-100. (*a*, *b*, and *c*) Control cells have intact acrosome and plasma membrane. (×33,000, ×13,000, and ×42,000, respectively.) (*d*, *e*, and *f*) Spermatozoa treated with 0.04% Triton X-100 have lost their acrosome and retain only fragments of their plasma membrane. (×3300, ×10,000, and ×20,000, respectively.)

of albumin (29), a major component of artificial media inducing *in vitro* capacitation (30).

Because albumin stimulates phospholipase A_2 (31), ACAT (32), and LCAT (33, 34), the role of albumin in sperm capacitation may be that of regulating the Chol/phospholipid ratio of the plasma membrane. Although albumin probably stimulates LCAT through entrapment of Chol esters (35), the sterol esterification activity of LCAT is inhibited by $CholSO_4$ (36). If this inhibition is due to the blockage of the 3β-hydroxyl groups by the sulfate moiety, then $CholSO_4$ may possibly inhibit ACAT by a similar mechanism. Thus, membrane $CholSO_4$ may not be esterified unless sterol-sulfatase cleaves the sulfate groups to yield free Chol. In this manner, $CholSO_4$ would stabilize the membranes overlying the acrosome by ensuring a high Chol/phospholipid ratio, whereas sterol-sulfatase would stimulate a decrease in the Chol/phospholipid ratio regulated by albumin. Ultimately, the formation of Chol esters and their withdrawal from the membrane would favor a decrease in the Chol/phospholipid ratio.

Table 3. Data for the calculation of the surface area covered by $CholSO_4$ on the head of human spermatozoa

Endogenous $CholSO_4$ concentration (6)	15 $\mu g/10^9$ cells
No. of $CholSO_4$ molecules/spermatozoa	19×10^6
% of $CholSO_4$ residing on the head (Table 2)	47%
Calculated no. $CholSO_4$ molecules on the head	9×10^6
Surface area of the $CholSO_4$ molecule*	38 Å^2
Surface covered by $CholSO_4$ on the head	342×10^6 Å^2
Surface area of the head†	16×10^8 Å^2
% of the head surface covered by $CholSO_4$	20%

* Ref. 18 assumes that $CholSO_4$ and unconjugated Chol occupy the same molecular surface area (obviously a minimal value).

† Calculated according to spermatozoa dimensions published in Documenta Geigy (19) by assuming a spherical shape. If the surface area is calculated by assuming a conical shape with a spherical base, this value becomes 25×10^8 Å^2.

Analysis of the foregoing data has led us to propose the following biochemical events in an attempt to explain sperm capacitation and the acrosome reaction *in vivo*. Sterol-sulfatase ensures the enzymatic hydrolysis of the sulfate moiety of membrane-bound $CholSO_4$ and, thereby, increases the levels of free Chol available for esterification. Activation of phospholipase A_2 and ACAT or LCAT (or both) by albumin would alter the Chol/phospholipid ratio with the formation of Chol esters and lysolecithin. Accumulation of lysolecithin would increase membrane permeability towards Ca^{2+} and initiate the acrosome reaction. Sterol-sulfatase could also activate the proteolytic activity of acrosin (11), which is reported to be involved in the acrosome reaction (37) and in the penetration of the zona pellucida (12). Thus, the enzymatic cleavage of the sulfate moiety of $CholSO_4$ at the sperm surface may represent a natural event taking place prior to the destabilization of the plasma membrane leading to the acrosome reaction and fertilization.

Much of the data in the literature can be interpreted to lend support to this proposed mechanism. The uptake of sterol sulfates by the spermatozoal plasma membrane during epididymal transit (8) would explain, at least in part, the negative charge that accumulates on the sperm surface during maturation. Indeed, it has been suggested that sulfate groups may contribute to this negative charge (38). Furthermore, sterol-sulfatase, a key enzyme in our hypothesis, is present in human endometrium, Fallopian tube, and Graffian follicle (6) and could account for the diminution in the negative charge at the surface of spermatozoa during their capacitation (39). In addition, *in vitro* capacitation may be induced with follicular fluid (28, 39, 40) or serum (41, 42), both of which contain LCAT (24, 25), an enzyme that is inhibited by $CholSO_4$ (36). After heat treatment, follicular fluid (28, 40–44) and serum (41, 42) retain their ability to capacitate sperm *in vitro*. It is submitted that this property is due to their content of lysolecithin, a heat-stable fusogenic substance. Penetration of rabbit ova may be achieved *in vitro* by rabbit sperm pretreated with uterine fluid or with lysolecithin (45), which results in ultrastructural changes similar to those described in capacitated spermatozoa undergoing the acrosome reaction (46). *In vitro* capacitation of human spermatozoa leads to an accumulation of lysolecithin (47). Ultimately, increased membrane concentrations of lysolecithin create conditions for cell fusion (48–50).

The activity of capacitation factors would seem to be under hormonal regulation as maximal uterine levels of albumin (51) and capacitation activity (52) are observed in the estrogen-stimulated situation, whereas these levels are significantly diminished under the control of progesterone. The latter hormone also inhibits sterol-sulfatase (53, 54). Thus, the hormonal control of capacitation may be effected through changes induced in the concentration of the key components of the proposed hypothesis.

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