

## Sialylglycoconjugates Release During *In Vitro* Capacitation of Human Spermatozoa

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Modifications of the human sperm surface during incubation in capacitating conditions were studied by radiolabeling terminal sialic acid residues of cell surface glycoconjugates using the sodium metaperiodate/sodium tritiated borohydride method. During *in vitro* capacitation, sialylglycoconjugates were released from the human sperm surface according to well reproducible kinetics. This release could be inhibited by the presence of seminal plasma in the capacitation buffer. Two principal size classes of sialylglycoconjugates were detected in the capacitation medium and analyzed by gel filtration chromatography and SDS-PAGE. The smaller class was characterized by glycopeptides less than 5,000 Da, whereas the larger class was characterized by two sialylglycoproteins of approximately 15,000–16,000 and 22,000–23,000 Mr. The role of human albumin, a key component of the capacitation buffer, in the removal of these molecules from the sperm surface was studied in light of its constant association with large amounts of released material.

**Key words:** human sperm, capacitation, glycoconjugates.

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Human sperm, like those of other mammalian species, must undergo capacitation before they are capable of fertilizing an oocyte. Although the

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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 (Rogers, 1978). In this medium, sperm acquire the ability to undergo an acrosome reaction and to penetrate the human zona pellucida (Gould et al, 1985).

According to present knowledge, sperm capacitation in nonprimate mammalian species is a multifaceted process involving removal and/or alteration of surface-bound components (see reviews by O'Rand, 1979; Yanagimachi, 1981; Farooqui, 1983; Clegg, 1983), as well as alterations of surface charges (Vaidya et al, 1971; Rosado et al, 1973), membrane cholesterol levels, and membrane phospholipid structure (see review by Langlais and Roberts, 1985). These modifications lead to an increase in plasma membrane fluidity and in permeability to extracellular calcium. Chang (1957) first noted that capacitation involves alterations of

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the sperm surface and observed that reintroduction of seminal plasma causes a reversible inhibition of capacitated sperm. However, the mechanism by which seminal plasma affects the fertilizing capacity of sperm is unclear at present. Release of epididymal polyactosamynil substrates adsorbed into the sperm surface has been clearly described by Shur and Hall (1982) as one aspect of sperm capacitation in mice. In humans, alterations of sperm surface during the capacitation process have been only indirectly assessed by using lectins (Koehler, 1981; Singer et al, 1985; Cross and Overstreet, 1987). Since it is known that human sperm surface contains sialylglycoconjugates (Wongkome and Chulavatnatol, 1984) and that capacitation results in the decrease of surface negative charges (Rosado et al, 1973), we investigated whether sialylglycoconjugates are released during incubation in capacitation medium. Specifically, we analyzed the kinetics of the release of these components and their biochemical characteristics by gel filtration and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). We also found that the presence of seminal plasma inhibits this release.

## Materials and Methods

### Collection and Labeling of Spermatozoa

Human sperm were obtained from three healthy adult donors after 36–72 h of sexual abstinence. The donors provided 11 samples each over the course of 1 yr. Ejaculates were allowed to liquefy at room temperature for 30 min before being centrifuged at 2,000 rpm for 10 min. The supernatant, mainly consisting of clear seminal plasma, was further centrifuged at 15,000 rpm for 5 min to eliminate particulate components. The resulting supernatant was used for the inhibition experiments. The sperm pellet was resuspended in 50 ml of phosphate buffer saline (PBS), pH 7.0, and centrifuged at 2,000 rpm for 10 min. The packed sperm were resuspended in 1 ml of PBS and the sialoglycoconjugates were radiolabeled according to the method of Gahmberg and Anderson (1977). Briefly, 6.25  $\mu$ l of a 0.08 M solution of sodium metaperiodate were added to the sperm suspension (final concentration 0.5 mM) and incubated in darkness with constant shaking for 10 min at room temperature. The suspension was then washed by centrifugation with 50 ml of PBS. These conditions were essential to maintain sperm motility. The sperm were labeled by reduction of the oxidized sperm pellet resuspended in 1 ml of PBS with 250  $\mu$ Ci of Na B<sup>3</sup>H<sub>4</sub> (100 mCi/mole) followed by incubation at room temperature for 30 min. Reduction was stopped by the addition of 50 ml of cold PBS containing 0.2 mg/ml of unlabeled sodium borohydride. Excess radioactivity was removed by washing twice in the same volume of buffer. This method selectively labels terminal sialic acids residues. Control experiments were done to determine the amount

of radioactivity specifically incorporated in the presence of borohydride and in the absence of periodate. The results showed a 10% incorporation of radioactivity compared with that obtained in the presence of periodate.

To confirm that the tritiated residues were sialic acids, released radiolabeled material was desalted on a Sephadex G-50 column, liophylized, and digested with 20  $\mu$ g of neuraminidase from *Clostridium perfringens* (specific activity 1 U/mg; Bohringer, Mannheim) in a total volume of 400  $\mu$ l of PBS for 2 h at 37 C with constant shaking. This material was then loaded on a Sephadex G-50 column and the material eluting at the total volume subjected to thin layer chromatography on silica gel plates using propanol-water (7:3 v/v) as solvent. Mannose and N-acetyl-neuraminic acid (5  $\mu$ g) were used as standards.

### Capacitation of Spermatozoa

Sperm were capacitated by incubation in culture medium (Biggers et al, 1971) containing 3.5% human serum albumin (HSA; fraction V, Sigma Chemical Co., St. Louis, MO). The tubes were loosely capped and incubated for 6 h at 37 C in 5% CO<sub>2</sub> and 95% air. In some experiments, the sperm suspension was divided into two fractions. Ten to 40% cell-free seminal plasma, obtained as previously described, was added to one fraction. The final sperm concentration was adjusted to 10<sup>7</sup> sp/ml. Evaluation of the functional integrity of the membranes of the sperm before and after capacitation was performed by using the hypo-osmotic swelling test described by Jeyendran et al (1984). The maintenance of motility in the sperm samples (more than 60% at the beginning of each experiment) was determined by counting motile cells before, during, and after capacitation in a Burkner's chamber. The labeling protocol was assessed by examining the functional integrity of the sperm membranes and the degree of motility at different concentrations of the sodium metaperiodate. More than 70% of the sperm cells were positive according to the hypo-osmotic swelling test. Motility was not affected when a 0.5 mM concentration of the reagent was used. Higher concentrations caused a marked reduction in the percentage of motile cells and in a strong damage to the integrity of the membranes.

### Time Course of the Release of Sialylglycoconjugates

The time course of the radiolabeled sialylglycoconjugate release during incubation in capacitating conditions was obtained by taking small aliquots (100  $\mu$ l) of the culture medium. The aliquots were centrifuged at 15,000 rpm for 5 min, and the supernatants were counted for radioactivity in a Beckman scintillation counter. At the end of the 6 h capacitation period, the suspensions were centrifuged at 2,000 rpm for 15 min, the supernatants were pooled for further analysis, and the sperm pellet was set aside for SDS-PAGE.

### Gel Filtration Chromatography

The supernatants pooled from several experiments were liophylized, resuspended in 50 mM ammonium acetate, pH 6.0, and loaded on a column (1.5 × 90 cm) of Sephadex

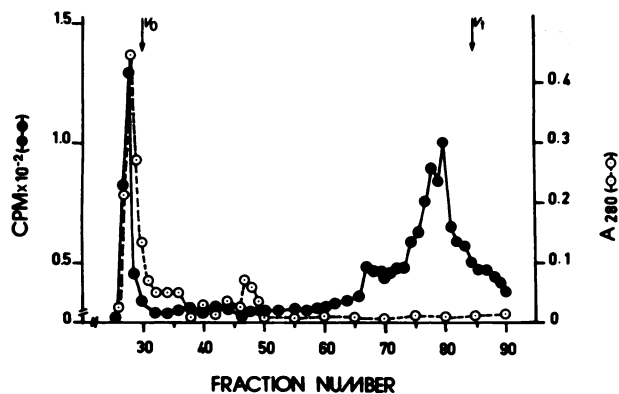


Fig. 1. Sephadex G-100 gel filtration analysis of the material released in the capacitation medium after Affigel blue affinity chromatography. Desalted material obtained from pooled supernatants was loaded on a  $1.5 \times 20$  cm Affigel blue column. The flowthrough was lyophilized, resuspended in 50 mM ammonium acetate, pH 6.0, and chromatographed on Sephadex G-100. 100  $\mu$ l aliquots of each fraction (1.6 ml) were counted for radioactivity ( $\bullet$ — $\bullet$ ). Absorbance readings of each fraction were taken at 280 nm ( $\circ$ — $\circ$ ).

G-50 or G-100. For desalting and other analysis, a column ( $1.5 \times 70$  cm) of Sephadex G-25 was used. Affigel blue (Bio-Rad, Richmond, CA) was packed in a  $1.5 \times 20$  cm column. The columns were equilibrated with 50 mM ammonium acetate, pH 6.0, and eluted with the same solvent. One ml (G-25) or 1.6 ml (G-50 and G-100) fractions were collected. One hundred  $\mu$ l aliquots of each fraction were then counted for the determination of radioactivity. Albumin was determined by reading optical density at 280 nm.

#### Solubilization of Sperm Membrane Proteins

An equivalent number of labeled spermatozoa before and after capacitation were washed once with 50 mM Tris-HCl, pH 7.2, and then incubated in the same buffer containing 1% Triton X-100, 1.25 mM n-amino benzamide, 1 mM phenylmethylsulphonyl fluoride, and 1 mM EDTA for 1 h at 4 C with constant shaking. Suspensions were centrifuged at 15,000 rpm for 5 min, and the clear supernatants were used for electrophoresis.

#### Electrophoresis

Lyophilized released surface components eluted at the void volume of the Sephadex G-100 gel filtration column (Fig. 1) and the solubilized sperm membranes obtained as described above were placed in an appropriate volume of sample buffer and boiled for 2 min. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate was performed according to Laemmli (1970) under reducing conditions using 9–15% polyacrylamide slab gels. The following molecular weight markers were used: Lysozyme, 14,000 Mr; Soybean trypsin inhibitor, 21,000 Mr; Carbonic anhydrase, 31,000 Mr; Ovalbumin, 45,000 Mr; Bovine serum albumin, 66,000 Mr; and Phosphorylase B, 92,500 Mr. After electrophoresis, gels were sliced into

3 mm segments. Each gel section was placed in 10 ml of a 3% solution of Protosol (New England Nuclear; NEN, Boston, MA) in Econofluor (NEN) and incubated at 37 C overnight with a subsequent addition of 10 ml of Econofluor and a further overnight incubation. Samples were then counted in a Packard Scintillation Counter.

## Results

### Kinetics of Sialylglycoconjugates Release

Human sperm were radiolabeled by incubation with sodium periodate/tritiated borohydride. This method is specific for sialic acids (Gahmberg and Anderson, 1977). In fact, in the absence of periodate, little aspecific labeling was found. Furthermore, 50% of the label incorporated in the presence of periodate was released by treatment with *Clostridium perfringens* neuraminidase, and it migrated with a sialic acid standard in thin layer chromatography.

To study the release of cell surface material, radiolabeled sperm were washed and incubated in capacitation buffer for 6 h. The integrity of the sperm during this process was assessed. Aliquots of the sperm-free medium were counted for radioactivity. Radiolabeled material was released progressively during the first 4 h of incubation, after which time a plateau was reached as shown by the two typical release curves of Fig. 2. The kinetics of release were similar in each of the donors examined, although quantitatively variable both between different individuals and in the same subject at different times (not shown). In control experiments in which sperm labeled in absence of periodate were used, the release obtained was about 10% of that obtained from sperm labeled according to the standard procedure.

Since it is well known that seminal fluid inhibits capacitation, we investigated the effect of seminal fluid on release. Human sperm were incubated in capacitation medium containing 40% (v/v) cell-free seminal fluid, and the release of the sialylglycoconjugates monitored. The presence of seminal plasma in the capacitation medium was found to reduce the release of radioactivity as shown by the two representative inhibition curves of Fig. 2. However, as already shown for fertilization (Kanwar et al, 1979), the inhibition of glycoconjugate release due to seminal fluid is highly variable both between different individuals and in the same individual at different times (not shown). In one donor, inhibition varied between 0 and 84% with an average of 33%, whereas in another, inhibition varied between 33 and 95% with an average of 55%. Although these

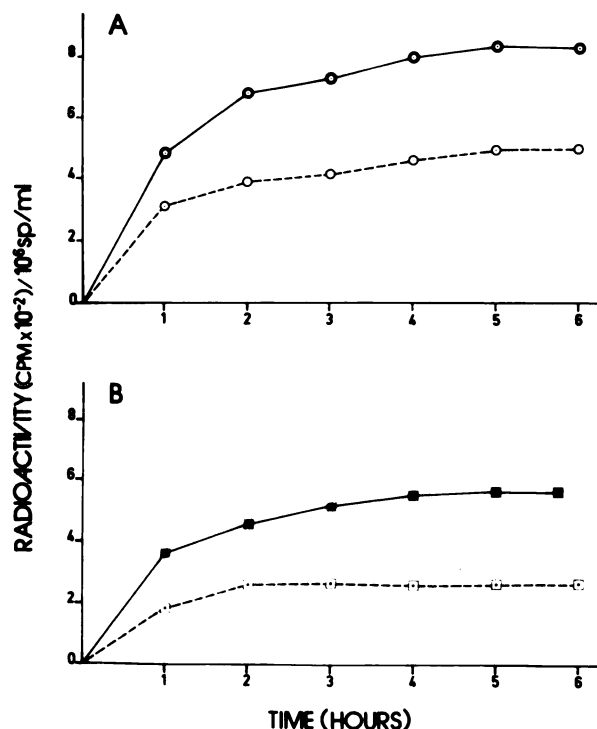


Fig. 2. Time course of sialoglycoconjugate release from spermatozoa of two different donors (A and B) during incubation in capacitation buffer. Sperm were radiolabeled by the sodium metaperiodate-tritiated sodium borohydride method and incubated in BWB containing 3.5% HSA. Aliquots (100  $\mu$ l), corresponding to  $1 \times 10^6$  sperm/ml, were collected at regular intervals and centrifuged. The supernatants were counted to determine radioactivity (—). The dotted lines (-----) represent the kinetics of release in the presence of 40% (v/v) cell-free seminal plasma.

experiments were conducted using 40% seminal fluid (v/v), we noticed that concentrations of seminal fluid was low as 10% did not influence the extent of the inhibition.

#### Gel Filtration Chromatography of the Released Glycoconjugates

Gel filtration chromatography was then used to ascertain the molecular size of the released material. Since it is known that sialoglycoproteins of the sperm surface range between 12 and 38 Kd (Wongkome and Chulavatnatol, 1984; Kallajoki et al, 1986), pooled material from different capacitation experiments was desalted on Sephadex G-25 and analyzed on a Sephadex G-50 column. The elution profile of this column, shown in Fig. 3, revealed that 35% of the material released from the sperm surface eluted as two discrete peaks near the total volume of the column. These peaks were further charac-

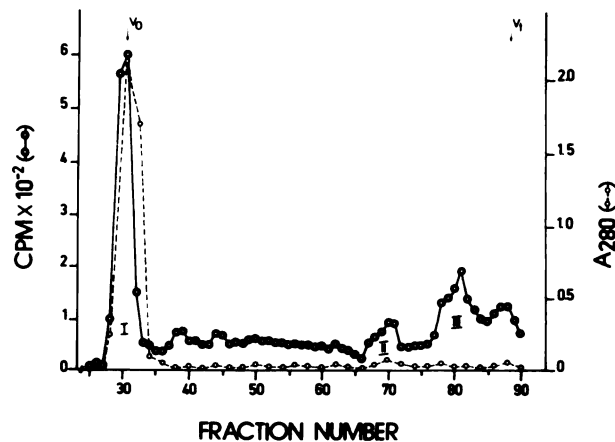


Fig. 3. Sephadex G-50 chromatography of pooled radiolabeled material released from the sperm surface during capacitation. The released material was desalted on a Sephadex G-25 column, lyophilized, resuspended in 50 mM ammonium acetate, pH 6.0, and chromatographed on a Sephadex G-50 column (1.5  $\times$  90 cm) equilibrated with the same buffer. Aliquots of the fractions (100  $\mu$ l) were assayed for radioactivity (○—○) and absorbance readings of each fraction (1.6 ml) were taken at 280 nm (○-----○).

terized on Sephadex G-25. Each of these eluted as a single species, as illustrated in Fig. 4, indicating that they are probably less than 5,000 Da.

Figure 3 also shows that another 35% of the radioactivity eluted in the void volume together with the albumin present in the capacitation medium. This observation and the observations mentioned above about the molecular weight of the sialoglycoproteins of the sperm membrane suggest that albumin may function as a carrier, thus causing the released species to elute as high molecular weight components. To separate albumin from the labeled glycoconjugates and to ascertain the size of these components, the material eluted in the void volume of the G-50 column was chromatographed on Sephadex G-100. As shown in Fig. 5, the elution-profile of albumin was unexpected, since it eluted as two peaks, one corresponding to that expected from its molecular size and one in the excluded volume. The radioactivity profile was also unexpected since no sialoglycoproteins larger than 38,000 Mr have been reported. The fact that the radioactivity profile coincides with that of albumin supports the hypothesis that this protein may function as a carrier of the released glycoconjugates.

To eliminate the possibility that this unexpected behavior could be related to the large amount of albumin present in the capacitation medium, we repeated the analysis of the released surface material

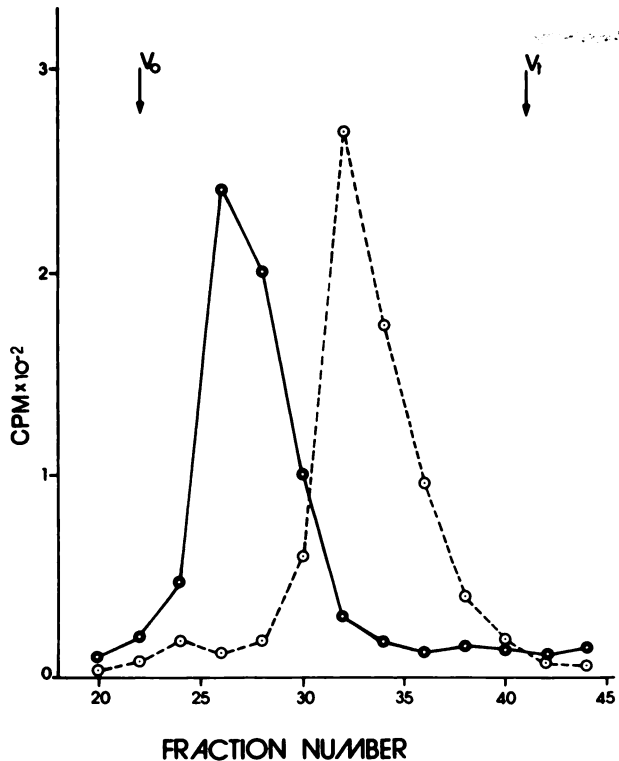


Fig. 4. Sephadex G-25 chromatography of the included peaks II (○—○) and III (○---○) of Fig. 3. Fractions 66-74 and 76-84 from the G-50 column were pooled, lyophilized, and chromatographed on a Sephadex G-25 column (1.5 × 90 cm), and 1 ml fractions were collected. 100 μl aliquots were counted for radioactivity.

after removal of the excess albumin. A fresh preparation of sperm surface released material was desalted on Sephadex G-25 and then chromatographed on Affigel blue, which is known to selectively bind albumin. This column retained about 90% of the original albumin in the sample and 40% of the released material (not shown), again suggesting that at least part of the released glycoconjugates may be bound to albumin. The remaining radioactivity eluting in the flowthrough was then chromatographed on Sephadex G-100.

As shown in Fig. 1, the majority of radioactivity (75%) eluted as two heterogeneous peaks, probably representing the same material seen as peaks II and III in the Sephadex G-50 (Fig. 4). About 20% of the radioactivity loaded on this column (or 10% of the original) coeluted in the void volume with the albumin not removed by Affigel Bleu. This, combined with the radioactivity remaining on the Affigel column, accounts for roughly 50% of the released radioactivity as comigrating with albumin.

These results suggest that about half of the

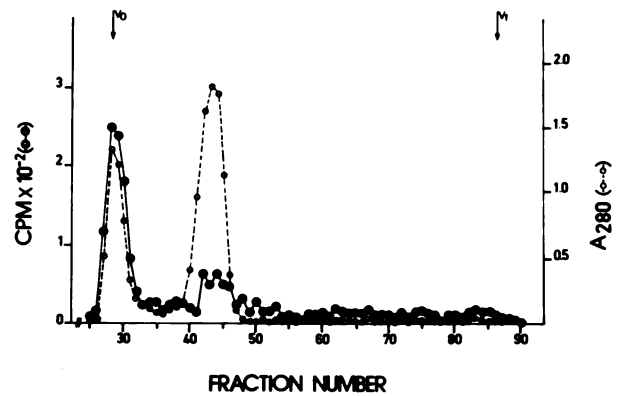


Fig. 5. Sephadex G-100 chromatography of the material eluted at the void volume (peak I) of Sephadex G-50. Fractions 27-33 from the G-50 column were pooled, lyophilized, and chromatographed on a Sephadex G-100 column (1.5 × 90 cm). 1.6 ml fractions were collected and absorbance readings were taken at 280 nm (○---○). 100 μl aliquots were counted for radioactivity (○—○).

sialylglycoconjugates released from sperm surface during capacitation are complexed by albumin.

#### SDS PAGE Analysis of Sperm Surface Sialylglycoconjugates

To determine the molecular weight of the glycoconjugates coeluting with albumin, the material was analyzed on SDS-PAGE. The lane containing our sample was excised from the gel, cut, and counted for radioactivity. The results, illustrated in Fig. 6, indicate that the majority of radioactivity migrates as two major species of approximately 15,500 and 22,500 Mr. Two very minor peaks of 32,000 and 37,000 Mr were observed.

To ascertain whether corresponding molecular weight components are present on the surface of freshly ejaculated sperm and are lost during capacitation, detergent extracts of an equivalent number of tritium labeled sperm before and after capacitation were run on SDS PAGE. Figure 7 shows the radioactivity profile obtained after counting the gel slices. It is evident that the labeling procedure used preferentially labels two major glycopeptides species of 15,700 and 23,000 Mr, respectively. Figure 7 also shows that after capacitation both species are partially lost from the cell surface. Although a 50% reduction was observed for the 23,000 Mr species, a 75% loss was observed for the smaller species.

#### Discussion

The mammalian fertilization process requires that sperm recognize and bind to the zona pellucida of

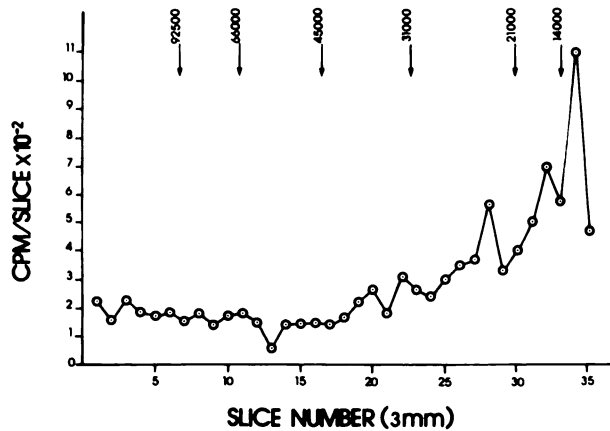


Fig. 6. Electrophoretic analysis of the released radiolabeled material eluted in the void volume of the Sephadex G-100 column. This material was lyophilized, resuspended in sample buffer, and run on a 9-15% SDS-polyacrylamide slab gel. 3 mm slices were cut and counted to determine radioactivity. Two major (15,500 and 22,500 Mr) and two minor (32,000 and 37,000 Mr) peaks were observed. Numbers above the arrows indicate molecular weight markers.

the egg before undergoing an acrosomal reaction and entering the egg (Saling and Storey, 1979; Wasseaman, 1987). For this process to occur, the receptors for the egg must be exposed and the sperm plasma membrane must be ready to fuse with the acrosomal membrane. Freshly ejaculated sperm are not endowed with these properties, but acquire them while in the female genital tract. This process, called capacitation, can be induced *in vitro* using appropriate buffers (Rogers, 1978). One of the events occurring during this process is the loss from the sperm surface of the coat acquired by the testicular spermatozoon during the journey through the male genital tract (Oliphant and Brackett, 1973; Koehler, 1981; Fraser, 1984). Although little is known about the cell surface and the molecular events occurring during capacitation of human sperm, studies have suggested that surface glycoconjugates may be lost or modified during *in vitro* capacitation of human sperm (Rosado et al, 1973; Singer et al, 1985; Cross and Overstreet, 1987).

In the present study, we investigated the cell surface modifications occurring during *in vitro* capacitation of human sperm by radiolabeling the sialic acid residues of the cell surface; in particular, we studied the kinetics of release and the size of sperm surface sialylglycoconjugates.

The kinetics of release show that the shedding of sialylglycoconjugates reaches a maximum after about 4 h, which is also the time required for *in*

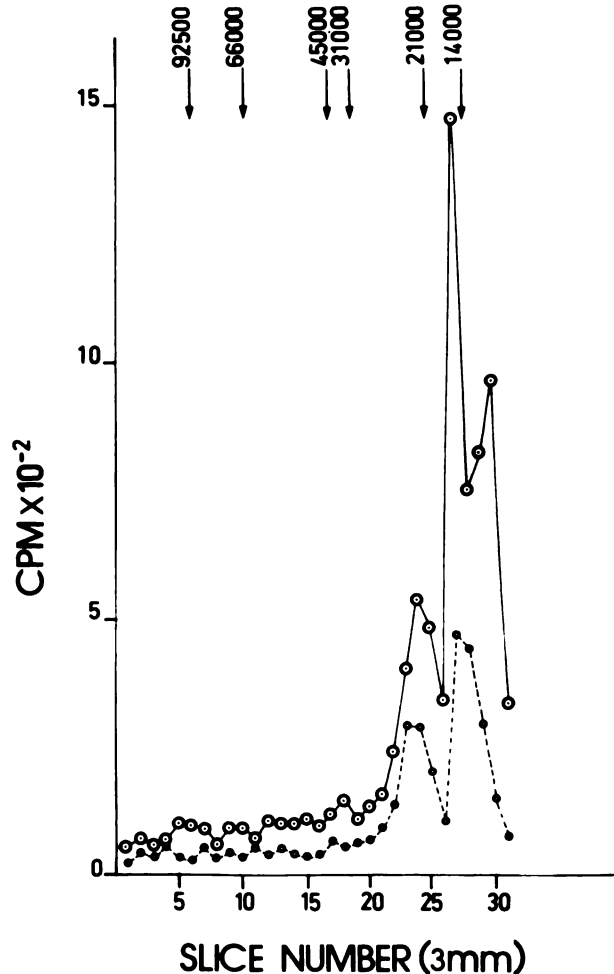


Fig. 7. SDS polyacrylamide gel electrophoresis of Triton X-100 extracts from labeled human sperm membranes. Samples of equivalent numbers of sperm before (⊙—⊙) and after (○- - -○) capacitation were extracted and run on a 9-15% slab gel. The gel was then cut into 3 mm slices and counted for radioactivity. Two major peaks of approximately 15,700 and 23,000 Mr are present in both samples. Note the marked reduction of the two peaks in the capacitated sperm membranes. Numbers above the arrows indicate molecular weight markers.

*vitro* capacitation to occur (Rogers, 1978; Gould et al, 1983). Furthermore, our observations that seminal fluid, which is known to inhibit capacitation (Van der Ven et al, 1982), also reduces the release of these glycoconjugates, lends support to the hypothesis that removal of these molecules from the sperm surface may play a role in achieving capacitation. A similar study reported that the loss of a surface antigen from mouse sperm was also inhibited by epididymal fluid (Eddy et al, 1987).

Gel filtration analysis of the radiolabeled surface material released during *in vitro* capacitation shows

that at least three glycopeptide species are shed from the sperm surface. Two of the components, accounting for about 50% of the total radioactivity, are less than 5,000 Da in size, whereas one or more species, accounting for the remaining radioactivity, eluted at the void volume of both the Sephadex G-50 and G-100 columns. At present, we have no information about the nature or origin of the smaller components. Since the species eluting at the void volume could not be separated from albumin, it was impossible to determine their size and number by gel filtration chromatography. Consequently, we resorted to the use of SDS-gel electrophoresis. This analysis showed that the species coeluting with albumin contain two major components of 15,500 and 22,500 Mr. The electrophoretic analysis of sperm extracts before and after capacitation confirmed that similar size molecules are present on the human sperm surface and lost during capacitation. This is in agreement with the previously reported size range of human sperm surface sialylglycoproteins (Wongkome and Chulavatnatol, 1984; Kallajoki et al, 1986; Dacheux et al, 1987).

Studies are now in progress to address the temporal sequence of the removal and the functional significance of the three species of sialylglycoconjugates.

It is well known that albumin, a key component of the capacitation buffer (Aonuma et al, 1982), is involved in the removal of cholesterol (Davis et al, 1980; Go and Wolf, 1985) and of a sperm maturation antigen (SMA-4; Vernon et al, 1985) from the plasma membrane. Our chromatography data, showing that the two larger of the released species were bound to this protein, suggest that albumin forms a complex with the larger of these molecules. Although our data do not allow us to establish the mechanism by which albumin is involved in the removal of surface molecules, two possibilities can be entertained. This protein could either function as a sink of the released molecules or directly bind and strip these glycoconjugates from the sperm surface.

As stated in the introduction, removal and/or alteration of surface components is one of the principal modifications associated with the capacitation process (O'Rand, 1979; Yanagimachi, 1981; Farooqui, 1983; Clegg, 1983). The data reported in our study represent the first direct evidence that defined molecular components are shed from the surface of human sperm during incubation in a

capacitation medium. Furthermore, the loss of sialic acid residues in the form of sialylglycoconjugates is consistent with the described decrease of the net negative charge occurring in the sperm in capacitating conditions. Indeed, our data do not allow us to exclude the possibility that part of the sialic acid residues present in the freshly ejaculated sperm is lost as monosaccharides, as suggested by Rosado (1973). Our data also show that greater than 60% of sialylglycoconjugate release occurs within the first hour, reaching about 80% in the second hour. Since 3 h is the least time identified for human sperm capacitation (Lee et al, 1987; Wolf et al, 1985) while the optimum selected time is 6 h (Byrd and Wolf, 1986), we suggest that the shedding could be one of the first events occurring on the sperm surface during *in vitro* capacitation, probably prior to the other alterations. Until data regarding the timing of other modifications, such as sterol and phospholipid alterations, are accumulated, however, suggestions about the causal relationships between shedding and other events leading sperm to undergo an acrosomal reaction are tentative.

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### Postdoctoral Position

A postdoctoral position to study the biochemistry and physiology of ABP is currently available. A suitable person would have a background in current biochemical and immunological techniques. Purified proteins, monoclonal and polyclonal antibodies, and the reagents needed for these studies are already on hand. Contact: Benjamin J. Danzo, PhD, Department of OB/GYN, Vanderbilt University, Nashville, TN 37232. Telephone: (615) 322-4433.