SOME PHYSICOCHEMICAL PROPERTIES OF SALINE-SOLUBLE PROTEINS IN BOVINE SPERMATOZOA

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Summary. Bombardment of bovine spermatozoa with glass beads and subsequent extraction in phosphate buffered sodium chloride yielded soluble sperm-specific proteins (approximately 10 % yield) sufficient for physicochemical analysis.

Moving boundary electrophoresis revealed three sperm-specific components with mobilities of 2.0, 3.8 and 5.1×10^{-5} cm² volt⁻¹ sec⁻¹ at pH 8.6 in barbital ($\mu = 0.10$). The proportions of these components were 20, 50 and 30 %, respectively. Sedimentation velocity ultracentrifugal analysis revealed at least three sedimentation gradients. The two major gradients had S_{20} values of 1.7 and 12.6. The third exhibited polydispersion. Diffusion coefficients of 4.2 and 10.2×10^{-7} cm² sec⁻¹ were calculated for two sperm-specific antigens by an agar-gel-diffusion method.

Three protein fractions were isolated by chromatographic elution from diethylaminoethyl (DEAE) cellulose with an ionic gradient. The first protein fraction eluted was not bound to the DEAE cellulose. It migrated toward the cathode in agar-gel electrophoresis at pH 8.4, and was firmly bound to carboxymethylcellulose at pH 6.0, 7.6 and 11.0. A second protein fraction from the DEAE cellulose appeared just after the ionic gradient commenced, and a third protein fraction appeared when the ionic gradient approached 0.2 M-sodium chloride.

INTRODUCTION

Miescher (1878) found a substance, presumably a protein, containing more than 4 % sulphur in the heads of bull spermatozoa. Green (1940) discovered that the head membranes of ram spermatozoa were undissolved after 0.01 N-acid and base treatments and that part of each head membrane defied digestion with trypsin or pepsin. The sperm membrane contained 19.3 % nitrogen and was rich in cystine, histidine, and arginine but lacked lysine. Green surmised that the membrane around the sperm head was a keratin-like protein. Zittle &

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O'Dell (1941) treated bovine sperm membranes with thioglycolic acid which made them partially soluble, and made them completely soluble with 4% trimethylbenzylammonium hydroxide, thus providing additional evidence for the hypothesis that the membrane was composed of a keratin-like protein. Thomas & Mayer (1949) treated boar spermatozoa with 1 N-sodium hydroxide and found undissolved spermatozoa which, upon closer examination, appeared to consist of 'ghost' sperm heads resembling the sperm membrane.

Miescher (1897) extracted nucleoprotamine and nucleohistone with distilled water or dilute salt solutions from fish spermatozoa but not from mammalian spermatozoa. Thomas & Meyer (1949) reported that water or salt solutions did not extract proteins or nucleic acid from boar or ram spermatozoa. Although Dallam & Thomas (1953) obtained similar negative results with water or neutral salt extraction of bull, man, dog, boar or ram spermatozoa, homogenization of the spermatozoa and subsequent treatment with 1 N-sodium hydroxide yielded a histone-like protein. This protein precipitated when diluted with four volumes of water, and could not be redissolved in 1 N-sodium hydroxide. Chemical analyses revealed 20.4% nitrogen and 27.8% arginine and nucleic acid in the precipitated protein. Berry & Mayer (1960) homogenized bovine spermatozoa at high speeds with microglass beads, extracted with dilute acid, and precipitated a basic protein by addition of base to pH 11. Chromatography and electrophoresis confirmed the histone-like character of the basic proteins of both mature and immature spermatozoa.

Dallam & Thomas (1953) isolated a lipoprotein complex from bull, boar, ram, dog and human spermatozoa. The complex was extracted with 0.1 N-sodium hydroxide, precipitated with 5% acetic acid, and similarly redissolved and reprecipitated thrice. Electrophoresis of the lipoprotein revealed one homogeneous component when the nucleic acid had been previously extracted. The total lipid of the bull sperm head (6.5%) was accounted for, within experimental error, by the lipid content (31.1%) of the lipoprotein which comprised 19.6% of the sperm head. Immunological tests supported the belief that the lipoprotein was on the surface of the sperm head because anti-serum, specific for the lipoprotein, agglutinated intact sperm heads. Mayer, Madera-Orsini & Berry (1957) and Berry & Mayer (1959) isolated a protein from lyophilized bull spermatozoa which was soluble in 0.01 N-sodium hydroxide and precipitated at pH 6 with acetic acid. This protein accounted for about 42% of the dry weight of bull spermatozoa and was shown to be a lipoprotein complex containing phospholipid, protein, cholesterol, glucosamine and glucuronic acid.

Katsh (1960) homogenized guinea-pig testes and spermatozoa and extracted the mash with 1 N-hydrochloric acid. The hydrochloric acid soluble material was treated with 10% trichloroacetic acid and the supernatant fluid was dialysed against tap water and then lyophilized. This extract induced aspermatogenesis when injected into guinea-pigs.

The methods used for extraction of proteins from mammalian spermatozoa have involved rather strong solvents. The present study was undertaken to develop milder methods for obtaining proteins from bovine spermatozoa and to determine some physicochemical criteria for them.

MATERIALS AND METHODS

Spermatozoa preparation

First-ejaculate semen was collected from Holstein bulls in routine service at Michigan Artificial Breeders Co-operative. The semen was centrifuged within 5 min of collection at 6620 *g* for 5 min. The seminal plasma was discarded and the spermatozoa were washed five times with 0.005 M-phosphate-buffered (pH 7.4) 0.85 % sodium chloride, hereafter referred to as buffered saline. After the final wash the spermatozoa were suspended in distilled water and lyophilized or diluted to 50×10^7 per ml with buffered saline and stored at -20°C .

Preparation of sperm proteins

Initially, sperm proteins were obtained from the stock sperm suspension (50×10^7 per ml) which was thawed and either (a) stored in the buffered saline at 5°C for 0, 1 or 3 days, (b) alternately frozen (-79°C) and thawed (30°C) eight times and then stored in the buffered saline at 5°C for 0, 1, 2 or 3 days, or (c) bombarded, while submerged in ice, with glass beads (Scotchlite Superbrite, size 100, Minnesota Mining and Manufacturing Co, Minneapolis, Minn.) for 15 min at maximum speed in a Sorvall Omnimixer (Ivan Sorvall, Inc, Norwalk, Conn.) and then stored in the buffered saline at 5°C for 0, 1, 2 or 3 days. The proteins extracted from the spermatozoa by these treatments were recovered by centrifugation at 10,300 *g* for 15 min. The supernatant fluids were tested for protein content according to Sutherland, Cori, Haynes & Olsen (1949) and stored at -20°C .

Subsequently, sperm proteins used for physicochemical analyses were obtained from 1-g samples of lyophilized spermatozoa which were suspended in 40 ml of buffered saline, bombarded with glass beads as described above, stored at 5°C for 24 hr and centrifuged at 10,300 *g* for 10 min. The supernatant fluid was concentrated to 2 % protein (biuret method of Gornall, Bardawill & David, 1949) by dialysis against 20 % polyethylene glycol in 0.85 % saline, and stored at -20°C .

Ultracentrifugation

The sperm protein solutions (2 % protein) were dialysed against 0.02 M-sodium phosphate buffer (pH 7.0) for 24 hr at 5°C before being centrifuged at 59,780 rev/min at 20°C in a Spinco Model E ultracentrifuge with a synthetic boundary cell. The density gradients were photographed at 4-min intervals for 1 hr. Sedimentation coefficients were determined according to Schachman (1957).

Electrophoresis

The sperm protein solutions (2 % protein) were dialysed against barbital buffer (pH 8.6, $\mu = 0.1$) for 48 hr at 4°C and electrophoresed at 1 to 2°C in a Tiselius apparatus (American Instrument Company, Inc, Silver Spring, Md.) at 15 mA. Electrophoretic mobilities were calculated according to Alberty (1948) and the concentration of each component according to Tiselius & Kabat (1939).

Diffusion

Rabbit antisera to bovine spermatozoa were produced and absorbed with seminal plasma and with blood serum as previously described (Hunter & Hafs, 1964). The diffusion coefficients of the soluble sperm-specific proteins were determined by the diffusion-in-gel method of Allison & Humphrey (1960). Precipitin lines were stained for glycoprotein as described by Crowle (1961).

Chromatography

The lyophilized soluble sperm proteins were chromatographed on 29×2 cm columns of diethylaminoethyl cellulose (Cellex-D; Calbiochem, Los Angeles, California) suspended in 0.02 M-sodium phosphate buffer (pH 7.0) or on carboxymethylcellulose (Cellex-CM; Calbiochem, Los Angeles, California) suspended in 0.005 M-sodium phosphate buffer (pH 6.0). The sperm proteins (100 to 200 mg) applied to the column were eluted with a linear ionic gradient from 0.0 to 0.7 M-sodium chloride. The protein content of each 5-ml fraction of effluent was determined by measuring the optical density at 280 m μ . The linear ionic gradient was followed by determining the conductivity of each twentieth 5-ml fraction.

RESULTS

Efficiency of protein extraction

Glass-bead bombardment yielded at least 100 mg of protein from each 1-g sample of lyophilized spermatozoa. Microscopy of bombarded spermatozoa revealed complete separation of heads and tails. Irregular holes were visible in about 1 % of the sperm heads.

TABLE 1
EFFECT OF EXTRACTION PROCEDURE ON QUANTITY OF PROTEIN
(MG/ML) IN SUPERNATANT FLUIDS OF SPERM SUSPENSIONS
(50×10^7 /ML)

Extraction treatment	Time after treatment			
	0 hr	24 hr	48 hr	72 hr
Saline solution	1.8	1.9	—	2.0
Freeze-thawing	1.5	1.8	1.6	1.7
Bombardment	1.8	2.7	2.7	2.9

The data (Table 1) indicated that alternate freeze-thawing did not appreciably increase the amount of soluble protein released from the spermatozoa beyond that obtained from spermatozoa stored in buffered saline solution at 5° C. Glass-bead bombardment, however, resulted in 45 % additional protein in the cell-free supernatant fluid after 72 hr at 5° C. Diffusion in agar gel indicated that no additional antigens had been released by glass-bead bombardment. Since procurement of sufficient quantities of protein was prerequisite to the planned studies of sperm proteins, mechanical rupture of spermatozoa

with glass beads was selected for preparing sperm protein for the following physicochemical analyses.

Ultracentrifugation

A sedimentation velocity ultracentrifugal analysis of the saline-soluble proteins of bovine spermatozoa (Plate 1) revealed two major sedimentation gradients with S_{20} values of 1.7 and 12.6, respectively. The lower value (1.7) was indicative of a low molecular weight or a rod-shaped molecule, whereas the larger value (12.6) was indicative of a larger molecular weight or a spherical-shaped molecule such as a globulin. A third component sedimented most rapidly and exhibited polydispersion. This was manifested in the pattern as saw-tooth spikes which were interpreted as sharp concentration gradients within the cell. Jackson, Coulson & Clark (1962) described similar polydispersion for a mucoprotein and attributed it to an aggregation phenomenon.

Electrophoresis

Moving-boundary electrophoresis of sperm proteins (Text-fig. 1) demonstrated three components. The electrophoretic mobilities were 2.0, 3.8 and

TEXT-FIG. 1. Electrophoretic analysis of sperm-specific proteins.
Field strength = 6.6 volt cm^{-2} .

$5.1 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$ and the relative proportions of the components were 20, 50 and 30 %, respectively.

Diffusion

The soluble sperm proteins resulted in two precipitin lines when diffused against rabbit antisera that had been absorbed with blood sera and seminal plasma. The tangents of the angles between the antigen well and the precipitin lines were calculated to be 1.0566 and 1.6364. Using these values and the reported diffusion coefficient of $3.8 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ for rabbit γ -globulin (Allison & Humphrey, 1960), diffusion coefficients of 4.2 and $10.2 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ were calculated for the two sperm-specific antigens. The precipitin line with the 4.2 diffusion coefficient stained as a glycoprotein.

Chromatography

The protein content of the effluent from diethylaminoethyl cellulose chromatography is illustrated in Text-fig. 2. The first peak (A) represented material which was not bound to the anion-exchanger, indicating a net positive charge at pH 7.0. This material migrated toward the cathode when electrophoresed in agar-gel at pH 8.4. The second peak (B) appeared just after the ionic elution gradient was started, and the third peak (C) appeared maximally at a point representing approximately 0.2 M-sodium chloride. Little, if any, protein was

eluted from the column when the concentration of sodium chloride was increased from 0.35 to 0.70 M. Another sample of sperm protein behaved similarly, though a faster rate of change in ionic gradient was used.

TEXT-FIG. 2. Gradient elution of sperm-specific proteins from a DEAE cellulose column. Five-millilitre fractions were collected at the rate of nine per hour. A linear gradient of sodium chloride was begun at 250 ml. Effluent conductivity (dotted line) was measured at 1° C with a Wheatstone Bridge.

An effort was made to further characterize the material in the first peak (A) by chromatography on carboxymethyl cellulose. A small initial breakthrough peak representing material not adsorbed on the cation-exchanger was observed. A linear gradient of sodium chloride from 0 to 0.7 M in 0.005 M-sodium phosphate solution at pH 6.0 eluted no protein from the column, indicating that the protein was firmly bound to the column. Stepwise addition of sodium phosphate buffer (0.1 M) at pH 7.6 resulted in a second small quantity of protein being released from the column. A third small quantity of protein was eluted (or hydrolyzed) from the column with the step-wise addition of 0.5 M-potassium phosphate at pH 11, but by far the greatest proportion remained bound to the carboxymethyl cellulose even after these rather drastic treatments.

DISCUSSION

The mechanical breakage of bull spermatozoa with glass beads and subsequent extraction of the sperm proteins with buffered saline solution allowed isolation of protein in quantities sufficient for physicochemical analyses. Bombardment of spermatozoa with glass beads did not measurably alter the antigenic or electrophoretic characteristics of the soluble sperm proteins. This method may prove useful for the preparation of active sperm enzymes and antigens.

A comparison of the electrophoretic properties of bull sperm proteins obtained in the present experiment with those of bull seminal plasma proteins obtained by Larson & Salisbury (1954) revealed that only one sperm component (mobility = 5.1) was electrophoretically similar to any seminal plasma protein. The majority of the sperm proteins obtained in the present experiment exhibited mobilities similar to those of the beta and gamma globulins of bovine blood,

PLATE 1

Sedimentation velocity ultracentrifugal analysis of sperm-specific proteins. The patterns were photographed at (a) 4, (b) 12 and (c) 20 min. Phase plate angle = 70° .

(Facing p. 248)

while the majority of the seminal plasma proteins were similar to the alpha globulins of blood. The protein portion of the anti-spermatogenic factor extracted from guinea-pig spermatozoa and testes by Katsh & Katsh (1961) was electrophoretically similar to alpha globulins of blood. Hence, it was probably not similar to any protein isolated in this study.

Larson, Gray & Salisbury (1954) reported that bovine seminal plasma contained at least five ultracentrifugal components. Those possessing S_{20} values of about 3, 5 and 7 accounted for over 95 % of the protein material. By comparison, the three ultracentrifugal components found for bull sperm proteins in the present study were each considerably different from those reported for bull seminal plasma.

The fact that the first protein eluted from diethylaminoethyl cellulose bound firmly to carboxymethyl cellulose indicated a strongly basic protein at pH 7.0. This result and the agar-gel-electrophoresis results established that the isoelectric point of this protein was above 7.0. The nature of this protein fraction and whether it contains nucleoprotein are currently under study.

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