EFFECT OF STORAGE AT 5° C ON THE DISC ELECTROPHORETIC PATTERNS OF OVINE AND BOVINE SEMINAL PROTEINS

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Summary. Separation of ovine seminal plasma proteins in the disc electrophoretic system revealed a consistent pattern of sixteen fractions with some variation among six rams in the relative amount of minor components, whereas bovine seminal plasma had twelve fractions. In both species, the major seminal plasma components had electrophoretic mobilities somewhat less than did the albumin band of the corresponding blood plasma. The prealbumin region of the seminal plasma electrophoretograms contained four fractions in the ovine samples and three fractions in the bovine samples. Storage of diluted ovine and bovine semen at 5° C did not alter the total protein concentration in the seminal plasma. Standard disc electrophoretic patterns showed that two of the sixteen ovine seminal plasma protein fractions disappeared during such storage. No changes were evident in either the standard or basic electrophoretograms of bovine seminal plasma during storage of semen. Standard disc electrophoretograms of ovine and bovine sperm extracts showed many lightly staining bands, but no progressive changes were evident in these electrophoretic patterns during storage of semen.

INTRODUCTION

Although the heterogeneity of the protein constituents of bovine seminal plasma is well established (Larson & Salisbury, 1954; Szumowski, 1956; Vesselinovich, 1959; Bennett, 1965), knowledge of the protein constituents of ovine seminal plasma is limited. Bennett (1965), using agar-gel electrophoresis, found six protein fractions in ovine seminal plasma and eight in bovine seminal plasma. The seminal plasma of both species, however, probably contains minute quantities of other proteins since eleven fractions have been detected in bovine seminal plasma using free boundary electrophoretic methods (Larson & Salisbury, 1954).

The successful preservation of mammalian semen *in vitro* requires the inclusion of some proteinaceous material in the diluent. Although ovine and bovine seminal plasma contain 35 to 70 mg protein/ml (Wales, Scott & White,

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1961) and serves as an excellent vehicle for natural insemination of spermatozoa, it is not an ideal medium for sperm survival (Shannon, 1965). Very little information is available as to the effect of storage on ovine and bovine seminal proteins. The seminal plasma proteins of some species, particularly man, undergo rapid proteolysis to proteoses and free amino acids soon after ejaculation (Jacobsson, 1950; Lundquist, 1952). This proteolytic activity results in a breakdown and redistribution of protein fractions during refrigerated storage of human seminal plasma (Schneider, 1955). Although bull and ram semen possess relatively little proteolytic activity (Wales *et al.*, 1961), an increase in amino nitrogen during incubation of bovine semen (Bhargava, 1957; Bhargava, Bishop & Work, 1959) indicates that some proteolysis does occur during storage *in vitro*. Subsequent studies (Srivastava, Adams & Hartree, 1965) have shown that acrosomal preparations from ovine and bovine spermatozoa possess proteolytic activity. The presence of proteolytic enzymes could be detrimental to the survival of the spermatozoa during storage *in vitro*. Degradation of seminal proteins by these enzymes may not be revealed by total protein determinations because the initial products would also give positive protein values. Such changes, however, should be evident from a comparison of the electrophoretic patterns of seminal proteins after various storage intervals. The present experiments were undertaken to determine the disc electrophoretic patterns of ovine and bovine seminal proteins and the effect of semen storage on these patterns.

MATERIALS AND METHODS

All semen used in this study was collected by artificial vagina. The ovine semen was from four mature Columbia rams and two mature Suffolk rams. Analyses of individual ram ejaculates were made on semen collected 2 weeks following that used for storage studies. Portions of the ejaculates of six mature Holstein bulls were withdrawn for individual bull data before pooling the remaining semen for storage studies. Semen from both species was diluted and stored in calcium-free Krebs-Ringer-phosphate fluid (KRPF), pH 7.0, containing 0.46% fructose (Umbreit, Burris & Stauffer, 1957). Sperm concentration was determined turbidimetrically (Salisbury, Beck, Elliot & Willett, 1943).

Approximately 10 ml of blood were collected from each ram and bull that was used as a semen source. Exsanguination was accomplished through venipuncture of the left jugular vein. Potassium oxalate was used as an anticoagulant and the blood plasma was separated from the cellular components by centrifugation (1250 *g* for 10 min). The blood plasma was stored at -196°C and used as a control for the seminal proteins.

Semen processing procedures

Semen samples for individual animal data were processed in a manner similar to that for 1-hr storage samples, except that a smaller sample size was used.

For semen storage studies, the ovine ejaculates were placed in a waterbath, maintained at 32°C , immediately following collection, and the ejaculates

diluted with one-half volume of KRPF at 32° C. Ejaculates showing at least 50% sperm motility were combined in a 50-ml conical centrifuge tube and the sperm concentration of the pooled semen samples determined. The semen was then diluted to 0.6×10^9 spermatozoa/ml with KRPF and a 5.0-ml sample (after storage for 1 hr at 32° C) containing 3.0×10^9 spermatozoa was withdrawn. The tube containing the remaining semen was transferred to a beaker containing water at 32° C and transferred to a refrigerator maintained at $5 \pm 1^\circ$ C. Subsequent 5.0-ml samples (3.0×10^9 spermatozoa) were withdrawn after 12-, 24-, 36- and 48-hr storage at $5 \pm 1^\circ$ C and immediately evaluated for sperm motility.

After determination of sperm motility, the bovine ejaculates were diluted with KRPF to 0.6×10^9 spermatozoa/ml before withdrawal of individual samples and combining the remaining portions of each in two 50-ml conical centrifuge tubes. The sampling and storage procedures for the bovine semen were the same as described for the ovine semen.

At each of the above mentioned time intervals, the 5.0-ml samples containing 3.0×10^9 spermatozoa were pipetted into 12-ml conical centrifuge tubes and the spermatozoa separated from the seminal plasma by centrifugation (2000 *g* for 7 min). The seminal plasma was removed from the sperm pellet by aspiration and 0.1-ml portions pipetted into 1-ml polyethylene vials. These diluted seminal plasma samples were frozen in liquid nitrogen (-196° C).

In order to examine the effect of storage on some of the proteinaceous materials associated with the spermatozoa, the cells were subjected to ultrasonic treatment with a Biosonick ultrasonic device equipped with a needle probe ($\frac{1}{8}$ in. diameter). In preparation for the sonication treatment, each sperm pellet that remained after aspiration of the seminal plasma was washed twice with 2.5 ml KRPF, resuspended to 2.5 ml with KRPF and transferred to a 5-ml polyethylene vial. The resultant sperm suspension was stored at -196° C and thawed at room temperature (22 to 26° C) immediately before the sonication treatment. When the samples were free of ice crystals, they were transferred into 5-ml beakers that had been packed in crushed ice and subjected to ultrasonic oscillation at full resonance output for 1.0 min. After treatment, each sample was poured into a 12-ml conical tube and centrifuged at 2000 *g* for 7 min. Portions (0.1 ml) of the resultant sonicated sperm supernatant were pipetted into 1-ml polyethylene vials and stored at -196° C for subsequent analyses. All centrifugation procedures used in preparation of the sonicated sperm supernatants were carried out at 5° C.

Analytical procedures

The total protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using purified casein as a standard. Samples containing 200 μ g of diluted seminal plasma proteins or supernatant proteins from twice-washed and ultrasonically-treated sperm cells were separated in a Canaco Model 6 unit using the standard disc electrophoretic system of 7.0% acrylamide separating gel, pH 8.9; 2.5% acrylamide stacking gel, pH 6.9; 25 mM-tris-glycine electrode buffer, pH 8.3 (Davis, 1964; Brewer & Ashworth, 1969) and the basic protein disc electrophoretic system of 7.5%

acrylamide separating gel, pH 4.3; 2.5% acrylamide stacking gel, pH 6.8; and 0.35 M- β -alanine-acetic acid electrode buffer, pH 5.0 (Reisfeld, Lewis & Williams, 1962). Protein fractions migrating in the latter system were termed 'basic proteins' because of their apparently positive charge at pH 4.3. Blood plasma proteins of the corresponding species were used as controls in each analytical system. Following electrophoretic separation of the protein fractions, the gels were stained with 0.5% naphthol blue black (C.I. No. 20470) and de-stained by washing in three to four changes of 7% acetic acid over a 24-hr period. Densitometric scans of the stained gels were made at 660 m μ using a Gilford 2400 spectrophotometer equipped with a linear transport mechanism.

RESULTS

Characterization of seminal proteins

Semen characteristics of six rams are given in Table 1. The ejaculate of Ram 6 contained a very high concentration of spermatozoa though only 10% were

TABLE 1
SEMEN CHARACTERISTICS, SEMINAL PLASMA AND BLOOD PLASMA
PROTEIN CONCENTRATIONS OF THE SIX RAMS USED AS A SOURCE OF
OVINE SEMEN

Ram no.	Ejaculate volume (ml)	Sperm. $\times 10^9$ /ml	Sperm motility (%)	Diluted seminal plasma protein* (mg/ml)	Blood plasma protein (mg/ml)
1	0.3	1.77	60	9.7	91
2	1.0	3.06	60	5.3	74
3	0.6	4.92	60	2.6	80
4	0.6	3.78	60	4.7	73
5	0.6	3.42	60	3.6	82
6	0.7	7.92	10	2.7	82
Mean	0.6	4.14	52	4.8	80

* Protein concentration in the diluted seminal plasma after dilution of the ejaculate to 0.6×10^9 spermatozoa/ml with calcium-free KRPF containing 0.46% fructose, pH 7.0.

motile. However, the total sperm count/ejaculate (5.5×10^9 cells) was not considered abnormal. The other five ejaculates had sperm motilities of 60% and contained from 0.53 to 3.06×10^9 spermatozoa/ejaculate. The variation in protein concentration of the diluted ejaculates was, in part, due to the dilution procedures. Electrophoretic separation of the seminal plasma proteins obtained from the six rams revealed a characteristic pattern, but demonstrated relative differences in the amount of certain fractions. These differences were especially evident in the region nearest the anode. Analyses of densitometric scans, however, indicated that the relative electrophoretic mobilities of the sixteen protein fractions were similar for all rams when expressed as percentage of the migration distance of the prealbumin component with the greatest negative charge (Text-fig. 1).

Ovine seminal plasma had relatively little albumin when compared with blood plasma, but seminal plasma did contain four protein fractions having electrophoretic mobilities greater than albumin (Text-fig. 1). In ovine blood plasma, these fractions (Fractions 1 to 4, Text-fig. 1) were either totally absent or present in much smaller quantities. The ovine seminal plasma samples possessed prominent protein bands (Fractions 6, 7 and 8, Text-fig. 1) in the postalbumin (blood serum α -globulin) region of the electrophoretograms

Semen characteristics of the six bulls are given in Table 2. After dilution to 0.6×10^9 spermatozoa/ml, the protein concentration in the seminal plasma of the

TEXT-FIG. 1. Densitometric scan and diagram of ovine seminal plasma proteins separated with the standard disc electrophoretic system. Components are numbered, starting with the prealbumin fraction with the greatest negative charge, and plotted in relation to positions occupied by standard blood plasma proteins (Pre = prealbumin, A = serum albumin, α = α -globulin or postalbumin region, β = β -globulin, γ = γ -globulin).

six bulls ranged from 32 to 87 mg/ml. The dilution procedure accounts for at least some of this variation in protein concentration.

A consistent electrophoretic pattern was obtained for the seminal plasma proteins of all bulls, but slight differences were detected in the amount of some minor components. The major protein constituents of bovine seminal plasma possessed electrophoretic mobilities equivalent to the postalbumin region of the respective blood plasma (Text-fig. 2). Bovine seminal plasma also contained three prealbumin components that were not detected in blood plasma.

Semen storage and seminal proteins

Seven ejaculates were collected from the six rams and pooled for the storage

TABLE 2

SEMEN CHARACTERISTICS, SEMINAL PLASMA AND BLOOD PLASMA PROTEIN CONCENTRATIONS OF THE SIX BULLS USED AS A SOURCE OF BOVINE SEMEN

Bull no.	Ejaculate volume (ml)	Sperm. $\times 10^9/\text{ml}$	Sperm motility (%)	Diluted seminal plasma protein* (mg/ml)	Blood plasma protein (mg/ml)
1	9.5	2.60	70	32	133
2	10.1	1.66	60	43	131
3	5.4	2.05	70	36	145
4	4.1	1.31	60	43	149
5	4.4	2.05	70	64	126
6	5.9	1.11	50	87	128
Mean	6.6	1.80	63	52	135

* Protein concentration in the diluted seminal plasma after dilution of the ejaculate to 0.6×10^9 spermatozoa/ml with calcium-free KRPF containing 0.46% fructose, pH 7.0.

experiment. One ram contributed two ejaculates of 0.3 and 1.1 ml. The volume of semen contributed by the other rams varied between 0.2 and 1.2 ml. The individual sperm motilities of the seven ejaculates varied from 50 to 70%. After dilution to 0.6×10^9 spermatozoa/ml, the pooled semen had a sperm motility of 60%. Sperm motility and seminal protein concentrations during storage of the diluted semen are given in Table 3.

TEXT-FIG. 2. Densitometric scan and diagram of bovine seminal plasma proteins separated with the standard disc electrophoretic system. Components are numbered and plotted as in Text-fig. 1.

Subtle progressive changes were apparent in at least two of the sixteen ovine seminal plasma protein fractions separated in the standard disc electrophoretic system during storage of diluted semen. Densitometric scanning of the gels indicated that these two fractions gradually decreased between 24 and 36 hr of storage and disappeared by 48 hr (Text-fig. 3, scans A and B). The relative electrophoretic mobilities of the sixteen fractions remained constant during storage. Separation of ovine seminal plasma in the basic electrophoretic system revealed at least two protein fractions possessing electrophoretic mobilities greater than any fractions found in the corresponding blood plasma. An additional seminal plasma fraction became evident only after prolonged storage of ovine semen (Text-fig. 3, scans C and D).

TABLE 3
SPERM MOTILITY AND SEMINAL PROTEIN CONCENTRATIONS DURING
STORAGE OF OVINE AND BOVINE SEMEN DILUTED WITH CALCIUM-FREE
KRPF CONTAINING 0.46% FRUCTOSE

<i>Storage time (hr)</i>	<i>Temperatures (°C)</i>	<i>Sperm motility (%)</i>	<i>Diluted seminal plasma protein* (mg/ml)</i>	<i>Sonicated sperm supernatant protein† (mg/ml)</i>
Ovine semen				
1	32.0	60	3.4	5.1
12	6.0	30	3.2	5.7
24	4.5	20	3.5	5.0
36	5.0	10	3.5	5.2
48	5.0	0	3.3	5.0
Bovine semen				
1	32.0	60	46	4.0
12	5.5	40	47	4.0
24	5.0	20	44	3.7
36	6.0	20	44	3.2
48	5.0	20	56	2.9

* Protein concentration after dilution to 0.6×10^9 spermatozoa/ml.

† Protein concentration in the supernatant resulting from centrifugation to twice-washed and ultrasonically treated sperm cells.

The disc electrophoretograms of the ovine sonicated sperm supernatant proteins separated in the standard system showed many lightly staining bands. Although subtle differences among gels were apparent, no progressive changes were evident in these electrophoretograms of stored sperm proteins. Examination of the basic protein electrophoretograms of ovine sonicated sperm supernatant proteins revealed at least seven fractions. The two components with a greater positive charge possessed electrophoretic mobilities greater than any of the protein fractions of ovine blood plasma.

Each bull contributed between 8.9 and 15.0 ml of diluted semen (0.6×10^9 spermatozoa/ml) for the storage experiment. Sperm motility and seminal protein concentrations during storage of diluted semen are given in Table 3. The protein concentration in the supernatant from the sonicated bovine spermatozoa tended to decrease during storage. Since these apparent protein losses could have occurred during the washing procedures used in preparing the cells

for sonication, the protein concentrations of the wash solution were determined; these showed a similar trend.

The pattern of bovine seminal plasma proteins resulting from separation in

TEXT-FIG. 3. Densitometric scans of ovine seminal plasma protein fractions after 1- and 48-hr storage of diluted semen at 5° C. A and B are scans of seminal plasma proteins after separation in the standard disc electrophoretic system (7% acrylamide separating gel, pH 8.9; 2.5% acrylamide stacking gel, pH 6.9; 25 mM-tris-glycine buffer, pH 8.3). C and D are scans of identical samples after separation in the basic electrophoretic system with reversed polarity (7.5% acrylamide separating gel, pH 4.3; 2.5% acrylamide stacking gel, pH 6.8; 0.35 M- β -alanine-acetic acid electrode buffer, pH 5.0). Arrows indicate absence of fractions.

the standard disc electrophoretic system did not change during storage of diluted semen at $5 \pm 1^\circ$ C. The relative electrophoretic mobilities of the twelve seminal plasma fractions remained constant during storage. Separation of the

bovine seminal plasma proteins in the basic disc electrophoretic system revealed at least two fractions having electrophoretic mobilities greater than corresponding blood plasma protein fractions. The electrophoretic pattern of bovine seminal plasma basic proteins did not change during storage of diluted semen at $5 \pm 1^\circ \text{C}$.

The standard disc electrophoretic patterns of the proteins in the supernatant from the sonicated bovine spermatozoa showed many lightly staining bands. Comparison of the electrophoretograms of stored bovine sperm proteins revealed several subtle differences though no progressive changes were evident in these electrophoretograms. Examination of the electrophoretograms of the basic proteins in the supernatant from sonicated bovine spermatozoa revealed at least seven bands. An additional fraction became evident in the basic protein pattern after prolonged storage of bovine semen.

DISCUSSION

As previously noted (Weil, 1961; Bennett, 1965), the electrophoretic patterns of seminal plasma proteins show a species-specific character. Comparison of ovine and bovine seminal plasma protein patterns did, however, reveal some similarities. Both species possessed prominent protein bands in the postalbumin region of the seminal plasma electrophoretograms (ovine Fractions 6, 7 and 8, Text-fig. 1 and bovine Fractions 5, 6 and 7, Text-fig. 2). Although differing in the number of fractions detected, the presence of multiple protein bands in the pre-albumin region was also noted in both species.

Wales *et al.* (1961) also found little change in ovine and bovine seminal plasma and whole semen protein concentration even when incubated for 3 hr at 37°C . The redistribution of seminal proteins during storage as noted from some species (Schneider, 1955) does not appear to be characteristic of ovine and bovine semen. The disappearance of some seminal plasma protein fractions during storage of diluted ovine semen may be due to the fact that spermatozoa take up and bind antigenic material from the seminal plasma (Weil, 1960, 1961; Weil & Rodenburg, 1962). The protein patterns from the supernatant of sonicated ovine spermatozoa did not have fractions possessing relative mobilities similar to those seminal plasma components that disappeared during storage. These seminal plasma protein fractions could have been lost because washing procedures were used in preparing the spermatozoa for sonication. Bedford & Chang (1962), however, reported that washing did not remove sperm-coating antigens.

The basic protein fraction with the greatest positive charge in the supernatant from the sonicated ovine spermatozoa possessed an electrophoretic mobility similar to the seminal plasma component that appeared after prolonged storage (Text-fig. 3, scan D). This basic protein fraction apparently originated in the spermatozoa and was lost to the seminal plasma during storage. The presence of basic proteins in mammalian spermatozoa (Berry & Mayer, 1960) and the high permeability of the sperm membrane (Mann, 1951) support this concept. The apparent stability of bovine seminal plasma proteins and the high degree of success achieved with storage of bovine semen *in vitro* may be

related. It should be noted, however, that in diluting to uniform sperm numbers, the concentration of seminal plasma protein present in the media during storage was approximately tenfold greater in bovine semen than in ovine semen.

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