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Immunoelectrophoretic Characterization of Fluid and Sperm Entering and Leaving the Bovine Epididymis

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Immunoelectrophoretic analyses were used to compare the antigenic characteristics of sperm and fluids recovered via cannulae implanted in the rete testis and proximal vas deferens of living bulls. Ejaculated semen, seminal vesicle fluid and blood serum also served as antigen sources. Although each reproductive fluid contained some blood antigens, these antigens were most numerous in rete testis fluid. Certain antigens present in rete testis fluid, but not detectable in blood serum, were associated with testicular sperm; others, common to seminal vesicle fluid, may be secretory products or enzymes originating in the seminiferous tubules. Some antigens apparently are selectively resorbed, utilized, or altered within the epididymis since all nonblood antigens entering the epididymis in rete testis fluid were not detected in cauda epididymal plasma. Other antigens in cauda epididymal plasma common to seminal vesicle fluid probably are secretory products of the epididymis.

Studies of saline soluble antigens released by sonication of testicular and epididymal sperm revealed that sperm undergo antigenic modification during their epididymal passage. Some antigens apparently are lost from sperm into the surrounding fluid, while others possibly modified, remain associated with the cells. Furthermore, sperm acquire new antigens presumed to be secreted by the epididymis. Certain antigens available in seminal vesicle fluid at ejaculation already are present on testicular as well as epididymal sperm.

Secretion and absorption of protein, changes in antigenicity of sperm undergoing maturation, and other aspects of epididymal physiology have been elucidated by immunological studies (Hunter and Hafs, 1964; Matousek, 1964; Losos, 1967; Köhl, 1968; Hunter, 1969; Barker and Amann, 1970, 1971; Alumot, Lensky and Schindler, 1971; Johnson and Hunter, 1972; Hunter *et al.*, 1972). However, in each of these investigations it was not possible to determine if antigens detected in cauda epididymal plasma or antigens associated with sperm actually were of epididymal origin; the antigenic composition of sperm and fluid entering the epididymis were unknown. Recent development of surgical techniques for implanting cannulae in the rete testis of living bulls (Voglmayr *et al.*, 1972) has made available for study preparations of testicular effluent presumably identical with that which normally enters the epididymis.

The objective of this investigation was to compare antigenic characteristics of testicular fluid and sperm entering the epididymis with that which emerges so that antigens originating or disappearing within the epididymis might be recognized.

MATERIALS AND METHODS

All washes and dilutions were made with 0.005M sodium-potassium phosphate buffered saline (PBS)[1] at pH 7.4. After initial separation, fluids (filter sterilized) and cells were held for 0–72 hr at 5°C prior to final processing so that they could be pooled with additional homologous

[1] A full list of abbreviations appears in the appendix.

antigen from the same or another bull. After processing, all antigens and immune globulins were stored in 1-ml ampules at −196°C. The antigen preparations and antisera were not the same as used by Barker and Amann (1970).

Fluids and sperm leaving the rete testis or the cauda epididymidis of living dairy bulls were obtained via cannulae previously implanted in the rete testis and proximal vas deferens (Amann *et al.*, 1970; Sexton *et al.*, 1971; Voglmayr *et al.*, 1972) using procedures described elsewhere (Amann *et al.*, 1973). Spermatozoa which were isolated from the testicular effluent by centrifugation (350 or 1000 *g* for 10 min), washed once and combined with other samples were used as testicular sperm antigen (TS). The supernatant rete testis fluid was recentrifuged (10,000 *g* for 10 min), sterilized by filtration and subsequently concentrated six- to ten-fold with an ultrafiltration cell fitted with a Diaflo UM-2 membrane. This concentrated rete testis fluid (RTF) was used for analyses. The cauda epididymal semen was diluted five-fold (occasionally 10-fold) and centrifuged (350 or 1000 *g* for 10 min) to isolate cauda epididymal spermatozoa which were washed once and combined with other samples (CES). The diluted cauda epididymal plasma was recentrifuged at 10,000 *g* for 10 min and pooled (CEP).

Ejaculates collected by artificial vagina from three or four dairy bulls were combined and centrifuged at 1000 *g* for 10 min. The supernatant seminal plasma was recentrifuged at 27,000 *g* for 30 min (SP). Ejaculated spermatozoa were washed twice (EJS) and resuspended. Seminal vesicles were obtained at slaughter from four anatomically normal Holstein bulls of unknown history. Fluid expressed gently from the glands was pooled and maintained at 5°C for about 1 hr during transit to the laboratory where it was clarified by centrifugation at 10,000 *g* for 30 min (SVF).

To obtain spermatozoal antigens for gel diffusion analyses, antigens were released into PBS by sonication of sperm using a Biosonic II generator (Bronwill Scientific, Rochester, N. Y.) equipped with the standard probe and set at 90 on the intensity scale. Sonication took place in a 4-ml polyethylene receptacle (1.5 cm i.d.) cooled in an ice bath. The 2-ml suspensions of TS (0.3×10^9 sperm/ml), CES (1.4×10^9/ml) or EJS (0.5×10^9/ml) were given a total of 90 sec of sonication in six passes each of 15 sec duration separated by a 15-sec pause. The PBS-soluble antigens (TS-F, CES-F, and EJS-F) were recovered in the supernatant after centrifugation of the sonicate at 27,000 *g* for 10 min.

Antisera were generated on three occasions against TS, RTF and CEP, twice against bovine blood serum (SER), CES, SP, and EJS, and once against SVF. On each occasion when an antiserum was produced, two or three rabbits were injected per antigen with TS (a total of 1.5, 0.6 or 1.2×10^9 sperm), RTF (45, 12 or 19 mg protein), CES (3.9 or 2.1×10^9), CEP (13, 6 or 6 mg), SP (45 or 40 mg), EJS (3.2 or 2.7×10^9), SER (44 or 53 mg) or SVF (44 mg). Each antigen was combined with an equal volume of Freund's complete adjuvant and injected subcutaneously at 10–20 sites in the scapular region. Two additional injections followed at weekly intervals, but for these the antigen was combined (1:1) with incomplete adjuvant. Ten or fourteen days after the third injection rabbits were bled and the serum harvested. Globulins were precipitated from the serum with an equal volume of saturated ammonium sulfate and dialyzed for 7 days against PBS changed daily. These immune globulins are identified as anti-rete testis fluid (aRTF), etc.

Immunoelectrophoretic analyses were made using LKB6800A equipment as described in the LKB I-6800A-E03 manual. The gel was a 1% solution of Agarose (Nutritional Biochemicals Corp., Cleveland) in barbital buffer (pH 8.6, $I = 0.025$). After a potential of 250 V had been applied for 45 min, slides were incubated with antisera at 20–25°C in a moist chamber for 20–24 hr, fixed, dried and stained with Amido Black 10B. In addition to characterizing each antigen preparation used for generating the immune globulins, similar samples from two or three additional bulls were characterized. Thus, conclusions regarding fluids and sperm entering and leaving the epididymis were based on analyses of samples from four to six bulls.

Immune globulins were combined with various amounts (0.05–0.15 ml/ml) of antigen(s) to perform absorption tests. The mixtures were incubated at 37°C for 30 min, centrifuged (27,000 *g* for 30 min), and the tubes examined for a pellet of precipitate. This procedure was repeated until a precipitate pellet was no longer apparent; the supernatants then were used for immunoelectrophoretic analyses. Unless otherwise stated, each absorbed immune globulin was negative when reacted with the absorbing antigen.

The immunological identity of certain blood proteins suspected to be present in RTF and CEP was confirmed using standardized reagents. Monospecific antisera (Butler and Maxwell, 1972) against bovine IgA, IgG_1, IgG_2, IgM, secretory IgA, and free secretory component were provided by Dr. J. E. Butler and rabbit globulins against bovine albumin and γ-globulin were obtained commercially (Nutritional Biochemicals Corp., Cleveland).

RESULTS

Antigens in Reproductive Fluids

Immunoelectrophoretic analyses using aSER revealed that some blood serum antigens were present in each reproductive fluid although they were most numerous in RTF (Figs. 1 and 2). Clearly and consistently some blood serum antigens detected in RTF with aSER were not present in CEP. One component present in both RTF and CEP had a relative mobility during electrophoresis similar to that of serum albumin and was detectable as a single arc following reaction of either antigen with anti-bovine serum albumin globulin. Although not shown, several samples of RTF and CEP were evaluated for the presence of specific immune globulins by double diffusion analyses (Barker and Amann, 1970) and the location of these components in immunoelectrophoretograms also was confirmed. Bovine IgG_1, IgG_2, IgM, and secretory IgA were consistently found in both RTF and CEP. Although IgA was detected in all samples of CEP, only occasionally were trace amounts of IgA found in RTF. Certain unidentified blood serum antigens present in CEP were undetectable in RTF. Furthermore, aCEP absorbed with RTF still contained antibodies which formed precipitin arcs when reacted with blood serum.

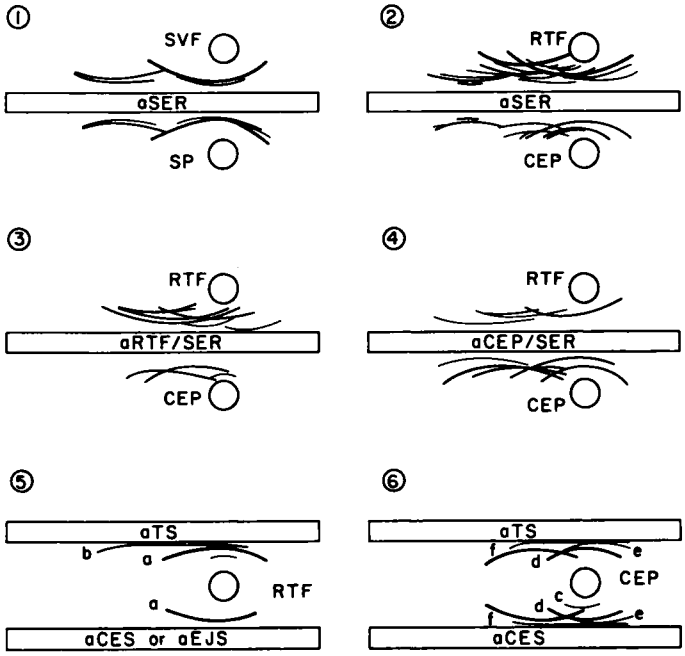
Absorption tests were performed to identify reproductive fluid antigens not common with blood serum. When aRTF, which previously had been absorbed with blood serum, was reacted with RTF seven precipitin arcs formed, but only three antigens were detected in CEP (Fig. 3). Reciprocal studies with absorbed aCEP revealed at least six non-blood antigens in CEP while only four were detected in RTF (Fig. 4). Thus, certain non-blood antigens are common to both RTF and CEP but others are found in only one of the two fluids.

Although not shown, other analyses with aRTF absorbed with blood serum revealed two non-blood antigens common to SVF and three common to SP. Studies using absorbed aCEP revealed three non-blood antigens common to SVF and six common to SP. Using aSVF absorbed with serum, three antigens were found common to RTF and one to CEP. Thus, both RTF and CEP share certain non-blood antigens with SVF. Since aCEP absorbed with RTF still contained antibodies to an antigen present in SVF but not detectable in SER, at least one antigen in CEP which is common to SVF apparently was not present in RTF.

As shown in Fig. 5, three precipitin lines were detected after reaction of RTF with aTS, but only line "a" formed with aCES or aEJS. However, after absorption of aTS with CEP, line "b" was not detected and line "a" was less intense. Four antigens were detected in CEP with aTS but one additional antigen was found with aCES (line "c," Fig. 6). However, only precipitin lines "c," "d" and "e" formed when CEP was reacted with aEJS. Following absorption of aTS with RTF, line "f" was not detected and line "d" was less intense. No reaction occurred when immune globulins produced against sperm were tested against blood serum. Thus, RTF and CEP share certain antigens also common to sperm and not detectable in blood serum, but each fluid contains at least one such antigen not found in the other. Although not shown, one antigen was detectable in SVF with aTS and aCES. However, this was one of the antigens common to several reproductive fluids, since absorption of aTS or aCES with RTF or CEP, but not blood serum, blocked detection of that antigen in SVF.

Antigens Associated with Sperm

A preliminary study tested the relationship between the duration of sonication and the release of protein into PBS from twice washed ejaculated sperm suspended at two different concentrations. As seen in Fig. 7, the release of protein during sonica-



FIGS. 1–4. Immunoelectrophoresis of seminal vesicle fluid (SVF), seminal plasma (SP), rete testis fluid (RTF) and cauda epididymal plasma (CEP) reacted with immune globulins against bovine blood serum (aSER) or with absorbed immune globulins against rete testis fluid (aRTF/SER) or cauda epididymal plasma (aCEP/SER); both absorptions were with blood serum.

FIGS. 5 and 6. Immunoelectrophoresis of rete testis fluid (RTF) and cauda epididymal plasma (CEP) reacted with immune globulins against washed testicular sperm (aTS), cauda epididymal sperm (aCES) or ejaculated sperm (aEJS).

tion first reached a plateau after 75–90 sec. Therefore, 90 sec of sonication was used routinely. The ultrastructure of spermatozoa sonicated for 90 sec (Figs. 8 and 9) was dramatically different from that of washed spermatozoa held under identical conditions but not sonicated (Fig. 10). These observations suggested that most of the proteins released as PBS-soluble antigen came from the cell membrane, outer acrosomal membrane and acrosomal contents, and possibly the cytoplasmic droplet, while contributions from the nucleus, equitorial segment, postnuclear cap, mitochondria, fiber bundle and sheath probably were much less.

The antigenicity of testicular sperm was investigated with PBS-soluble extracts prepared by sonication. Five sperm antigens were detected in TS-F with aTS including four also found in CES-F (Fig. 11); of these four antigens only those producing precipitin lines "g," "h" and "i" were present in EJS-F. However, the antibodies in aTS against antigens found in TS-F, but not detected in CES-F or EJS-F, could not be completely isolated by absorption of aTS with CES-F or EJS-F. After repeated attempts, a negative reaction of aTS with the absorbing antigens was not obtained. The testicular sperm antigen not detected in CES-F also could not be demonstrated in CEP with aTS.

Evidence for antigen modification or epididymal secretion also was obtained. Analyses with aCES revealed that the same five antigens were present in CES-F and EJS-F but that only four of these were

detectable in TS-F (Fig. 12). When aCES absorbed with TS-F was reacted with CES-F only one precipitin line formed (line "j," Fig. 12). This antigen, which apparently is not intrinsic to sperm, also was detected in CEP but not RTF or blood serum. Additional analyses using aEJS (Fig. 13) revealed four antigens common to EJS-F and CES-F, but only the precipitin lines "l," "m" and "n" formed with TS-F. The precipitin line "k" which formed with aEJS and CES-F or EJS-F, but not with TS-F (Fig. 13), appeared to correspond with line "j" (Fig. 12) detected in the same extracts with aCES. Thus, each of the PBS-soluble sperm extracts contain at least four antigens in common. However, the TS-F extract contained certain antigens not found in CES-F or EJS-F and both CES-F and EJS-F contain one antigen, apparently of epididymal origin not present in TS-F.

One fast migrating antigen (line "p," Fig. 13) was found in EJS-F with aEJS. This component was not detected in TS-F, CES-F, RTF, CEP, SP or blood serum. However, studies with aSVF revealed two similarly fast migrating components in EJS-F as well as several other antigens (Fig. 14). Four of the latter antigens also were found in CES-F with aSVF, but only precipitin line "q" was detected in TS-F (Fig. 14). The fast migrating antigens detected in EJS-F with aEJS and aSVF apparently are not immunologically identical to serum albumin since they were not detectable with anti-bovine serum albumin. Thus, certain antigens in SVF also detectable in EJS-F with aSVF become associated with sperm at ejaculation, while other antigens contributed by the seminal vesicles are already present in or around epididymal sperm, or even testicular sperm.

Analyses with aRTF and aSER suggested that certain antigens in RTF and also present in blood serum were associated with TS, but were different from those in CES. One antigen (line "r," Fig. 15) detected in TS-F with aRTF was not found in CES-F. Conversely, three antigens detected in CES-F with aRTF were not found in TS-F. Following absorption of aRTF with blood serum only precipitin line "s" was detected. Furthermore, only precipitin lines "r," "t" and "u" (Fig. 15) formed when the same sperm extracts were reacted with aSER.

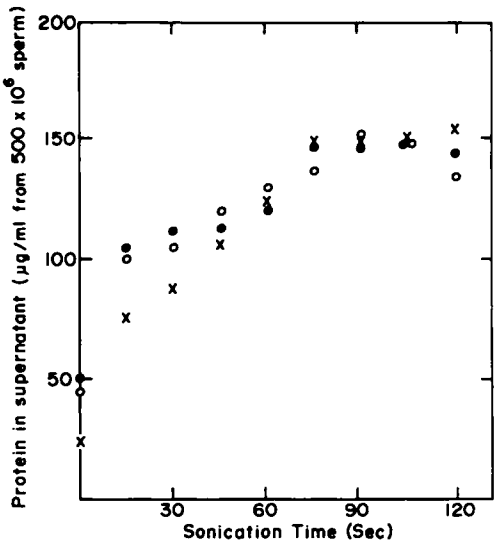
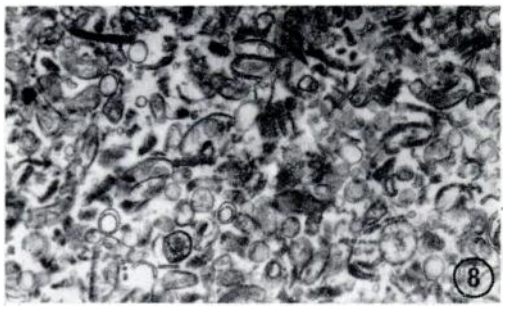


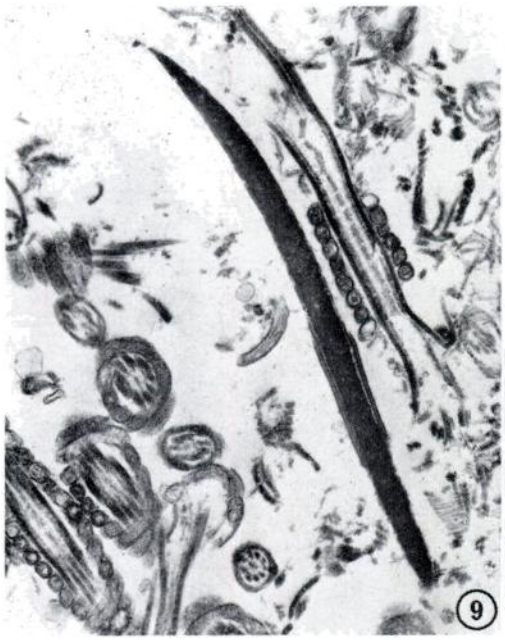
FIG. 7. Protein content of the 27,000 *g* supernatant after sonication of aliquots of washed ejaculated sperm. For one replicate (×) this suspension contained 1.00×10^9 sperm/ml while for two others (○,●) it contained 0.50×10^9 sperm/ml. Protein levels were determined (Lowry, Rosebrough, Farr and Randall, 1951) in duplicate.

Although two antigens were detected in both TS-F and CES-F with aCEP (precipitin lines "v" and "w," Fig. 16), each sperm extract also contained several antigens common to CEP not found in the other sperm extract.

DISCUSSION AND CONCLUSIONS

In studies with ram rete testis fluid concentrated thirty- to seventy-fold, Johnson and Setchell (1968) were unable to detect antigens that were not present in blood serum. However, in our analyses of bovine rete testis fluid concentrated only six- to ten-fold, and using three different batches of RTF to produce antisera, we repeatedly found several nonblood serum antigens.





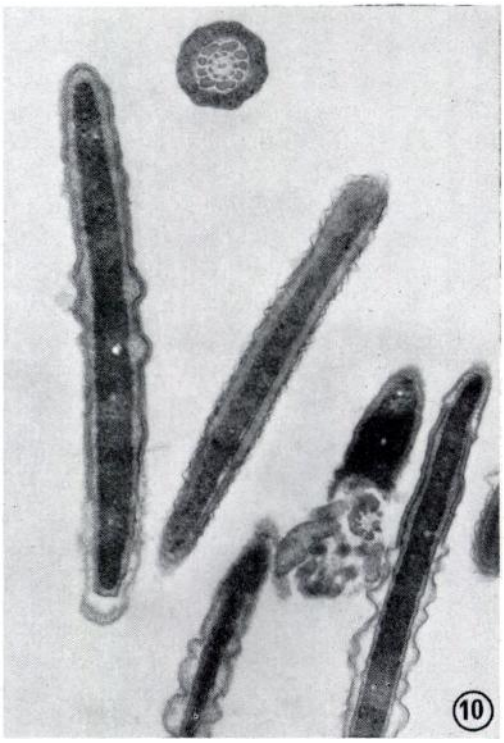


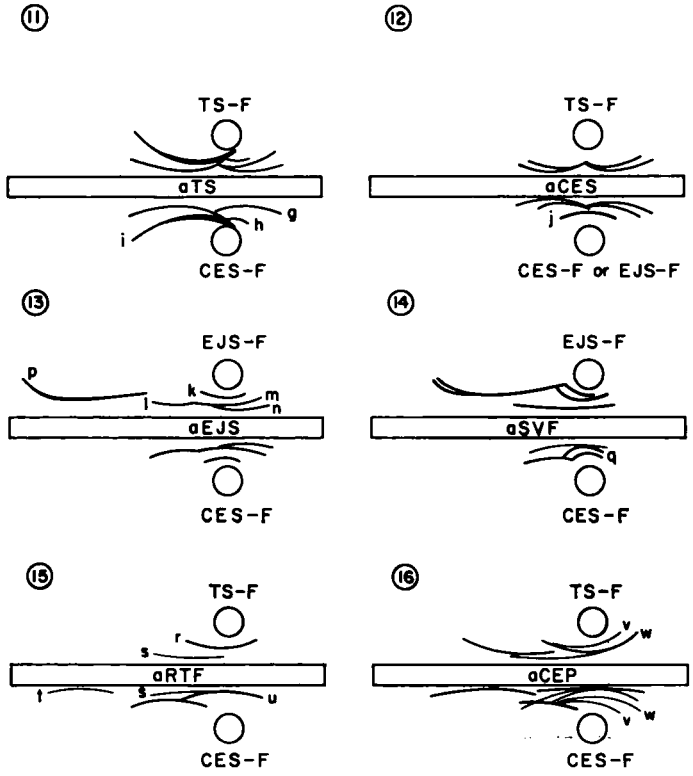
FIG. 8. Electronmicrograph of lighter elements contained in the 27,000 *g* pellet recovered from a suspension of washed ejaculated sperm sonicated for 90 sec. Pellet fragments were fixed in 5% gluteraldehyde in 0.2 M sodium cacodylate buffer at pH 7.4 and post-fixed in 1% osmium tetroxide. Sections were stained with alcoholic 1% uranyl acetate followed by aqueous 0.3% lead citrate. ×19,400.

FIG. 9. Electronmicrograph of heavier elements contained in the same 27,000 *g* pellet sectioned for Fig. 8. ×18,200.

FIG. 10. Electronmicrograph of control washed ejaculated sperm fixed as above and then recovered by centrifugation (1000 *g*, 15 min) before post-fixation. ×18,200.

Some of the nonblood serum antigens present in RTF were associated with TS and others were common to SVF. Nonblood antigens detected in RTF with aTS may be by-products of spermiogenesis such as the residual body (Johnson and Hunter, 1972), although it is possible they are secreted separately into testicular fluid and then acquired by the sperm.

The detection of nonblood antigens in RTF is in agreement with disc-gel electrophoretic analyses of rete testis fluid and cauda epididymal plasma (Amann *et al.*, 1973). Similarly, Koskimies *et al.* (1971) reported that the free-flow, primary secretion of the rat seminiferous tubule contained a number of proteins not detected electrophoretically in blood serum or intratesticular lymph. In fluid aspirated from the rete testis 24 hr after ligation of the



FIGS. 11–16. Immunoelectrophoresis of antigens in PBS-soluble extracts of sonicated testicular sperm (TS-F), cauda epididymal sperm (CES-F) and ejaculated sperm (EJS-F) reacted with immune globulins against washed testicular sperm (aTS), washed cauda epididymal sperm (aCES), washed ejaculated sperm (aEJS) seminal vesicle fluid (aSVF), rete testis fluid (aRTF) or cauda epididymal plasma (aCEP).

efferent ducts, however, more serum proteins were detected. Koskimies *et al.* (1971) concluded that nonblood proteins probably originated in the seminiferous tubule, while most blood serum proteins entered the testicular effluent in the rete testis. Tuck *et al.* (1970) pointed out that rete testis fluid may be pumped through the seminiferous tubules and assist in transporting sperm out from the testis. Thus, precise localization of antigen sources within the testis may be impossible.

Immunoelectrophoretic analyses of ram rete testis fluid (Johnson and Setchell, 1968) and ram epididymal fluid (Alumot *et al.*, 1971) have established that immune globulins are present in both fluids. Our analyses show the presence of immune globulins in similar bovine fluids. Bovine CEP contained several proteins found in SER including albumin, IgA, IgG_1, IgG_2, IgM, and secretory IgA. With the possible exception of IgA, each of these proteins were found in RTF. Although these findings might lead to the inference that RTF is the primary source of the blood serum antigens present in CEP, it cannot be considered their exclusive source. Immune globulins against CEP contained antibodies to unidentified blood serum antigens which were not detectable in RTF. Possibly some blood antigens are not transported into the epididymis with the testicular effluent, but enter CEP through the epididymal epithelium. All antigens entering the epididymis in RTF were not detected in CEP.

Apparently some RTF antigens are resorbed, utilized or altered within the epididymis. The fact that only certain of the nonblood antigens present in RTF were detected in CEP suggest that the absorption or alteration process is quite selective.

Some RTF antigens may be involved with spermatogenic regulation. Setchell and Waites (1971) postulated that ram testicular fluid contained a feedback messenger in the form of a sperm substance which was absorbed in the caput epididymidis. This compound was suggested to serve as the first link in transmission to the pituitary of feedback messages which indicate the rate of spermatogenesis. Our detection of TS antigens in bull RTF which were absent in CEP might support this concept. Other RTF antigens may serve to modulate epididymal sperm maturation. The finding (Figs. 5 and 15) that cauda epididymal sperm are associated with different RTF antigens than are present with testicular sperm may reflect changes occurring in the antigen-binding ability of sperm as they traverse the epididymis. Possibly one of these antigens is an androgen-binding protein which brings the appropriate androgen in close association with maturing sperm.

Although certain antigens present in testicular sperm remain detectable in CES-F and EJS-F with aTS, several lines of evidence suggest that spermatozoa lose some antigens during their passage through the epididymis. Studies with aTS revealed testicular sperm antigens in CEP which were not found in RTF or blood serum. Furthermore, studies with aCEP detected certain antigens in the PBS-soluble extracted TS-F that were not detectable in CES-F. An accounting of antigens found with aTS in extracts CES-F and EJS-F showed that these preparations contained fewer antigens than similar extracts (TS-F) of testicular sperm. Although the TS-F antigen absent in CES-F was not detected in CEP, this antigenic component may have been modified within the epididymis rather than being completely removed from the sperm and surrounding fluid. This concept is supported by the finding (Lavon *et al.*, 1971) that the composition and electrophoretic mobility of proteins extracted from bovine spermatozoa are altered during the migration of sperm from the caput to cauda epididymidis. Loss of sperm antigens within the epididymis may result from alteration (Dickey, 1965) or shedding of the enzyme-containing cytoplasmic droplet (Dott and Dingle, 1968) or reflect changes in acrosomal proteinase (Garner *et al.*, 1972), sperm morphology (Rao, 1971) or ultrastructure (Dickey, 1965). However, the alternative possibilities that the TS antigens found in CEP are products of sperm dissolution or are similar antigens which are secreted by the epididymis (Barker and Amann, 1970) cannot be excluded.

Several studies have indicated that antigens are secreted by the epididymis into the luminal fluid and that some of these antigens become associated with sperm (Hunter and Hafs, 1964; Matousek, 1964; Hunter, 1969; Barker and Amann, 1970, 1971; Johnson and Hunter, 1972; Hunter *et al.*, 1972). However, since the antigens present in the testicular outflow were unknown in these earlier studies, conclusive recognition of sperm antigens which actually originated within the epididymis was not possible. Our analyses revealed that in addition to blood and RTF antigens, bovine CEP contained other antigens including some that were common to testicular and epididymal sperm, and SVF. Nonblood antigens detected only in CEP or CES-F with aCEP may represent epididymal secretion, but the possibility that they are altered forms of pre-existing antigens cannot be excluded. Nevertheless, those nonblood antigens present in both CEP and SVF, but not RTF or TS-F, may truly represent enzymes or other products secreted by the epididymis.

Studies with aCES revealed that CES-F, EJS-F and CEP contain at least one antigen not found in either TS-F or RTF. Al-

though this antigen was not found in SVF, it seems likely that it was secreted by the epididymis into CEP where it became associated with sperm. Furthermore, studies with aCEP detected certain antigens in the PBS-soluble extract CES-F that were not found in TS-F. Indirect evidence that the epididymis secretes sperm antigens also was revealed in studies with aSVF. Four antigens were detected in CES-F with aSVF, but only one of these was found in TS-F. Thus, at least three of the antigens detected in CES-F probably represent secretory products of the epididymis which also are found in seminal vesicle fluid. Immunofluorescent cross reactions (Barker and Amann, 1971) between epididymal secretory cells and cells in the seminal vesicles support this concept.

The present data are the first to enable discrimination of specific antigens originating in the testicular outflow from those originating within the epididymis. The results reveal that the bovine epididymis is capable of selectively resorbing or altering certain antigens entering in the testicular effluent while secreting other antigens into the luminal fluid. Furthermore, some antigens probably are lost from sperm into the surrounding epididymal fluid, while other antigens may be modified but remain associated with sperm during their epididymal transit. Apparently sperm acquire new antigens which are secreted by the epididymis. Although the immunoelectrophoretic analyses reported herein have established certain facts concerning antigenic changes occurring within the epididymis, the roles of these changes in reproduction or the process of sperm maturation must remain speculative until more detailed investigations are possible.

APPENDIX

Abbreviations Used in Preceding Article

PBS = Phosphate-buffered saline (0.005 M) at pH 7.4.

TS = Testicular spermatozoa isolated by centrifugation (350 or 1000 *g* for 10 min) from the effluent obtained by cannulation of the rete testis of living bulls. The testicular spermatozoa were washed once and pooled.

TS-F = Cell-free supernatant prepared (27,000 *g* for 10 min) from TS sonicated for a total of 90 sec.

CES = Cauda epididymal spermatozoa isolated from diluted semen obtained from a vas deferens cannula. The cauda epididymal spermatozoa were washed once and pooled.

CES-F = Cell free supernatant prepared after sonication of CES.

EJS = Ejaculated spermatozoa isolated by centrifugation (1000 *g* for 10 min) and washed twice.

EJS-F = Cell free supernatant prepared after sonication of EJS.

RTF = Rete testis fluid isolated from the testicular effluent by centrifugation (350 or 1000 *g* for 10 min). The rete testis fluid was recentrifuged (10,000 *g* for 10 min), sterilized by filtration, pooled and concentrated six- to tenfold.

CEP = Cauda epididymal plasma isolated by centrifugation (350 or 1000 *g*) after fivefold dilution of semen recovered from a vas deferens cannula.

SP = Seminal plasma separated from pooled ejaculates by centrifugation (1000 *g* for 10 min). The seminal plasma was recentrifuged at 27,000 *g* for 30 min.

SVF = Seminal vesicle fluid pooled from four bulls and centrifuged (10,000 *g* for 30 min).

SER = Bull blood serum.

APPENDIX (*Continued*)

aTS, aCES, aEJS, aRTF, aCEP, aSP, aSVF = Antisera harvested from rabbits injected with corresponding bull seminal antigens and adjuvant.

aSER = Antisera harvested from rabbits injected with bull blood serum and adjuvant.

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