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Posttesticular Surface Modifications and Contributions of Reproductive Tract Fluids to the Surface Polypeptide Composition of Boar Spermatozoa

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ABSTRACT

Caput and cauda epididymal fluids were found to be exceedingly rich in the numbers and kinds of polypeptides when analyzed by two-dimensional (2-D) gel electrophoresis. Only a few of the major (Coomassie-stained) and minor (silver-stained) epididymal fluid polypeptides were identified on epididymal sperm plasma membranes (PM) and even fewer identified in ejaculated sperm. The 2-D electrophoretic patterns of caput sperm PM differed little from those of cauda sperm PM. Thus, epididymal transit resulted in relatively minor quantitative and qualitative modifications in sperm PM composition. Seminal plasma showed a few major polypeptides from the cauda epididymal fluid, but the major constituents were those polypeptides from the seminal vesicle secretions. Sperm appear to acquire one acidic high molecular weight polypeptide from either the bulbourethral gland or prostate gland, and another major acidic polypeptide of high molecular weight from the seminal vesicle gland. Numerous neutral and basic low molecular weight polypeptides, originating from the seminal vesicles, adhered tightly to sperm. These were major polypeptides and constituted a substantial percentage of the total PM protein. Thus, major contributions to the sperm PM polypeptide profile occurred at ejaculation. This study did not address loosely bound polypeptides but is the first to analyze, in a comprehensive way, the origins of tightly bound sperm polypeptides from a single species.

INTRODUCTION

The spermatozoon is covered externally by a plasma membrane (PM) which is specialized in numerous ways to interact with the ovum. PMs undergo changes in the epididymis which provide forward motility (Acott and Hoskins, 1981) and zona binding (Fournier-Delpech et al., 1982; Peterson et al., 1984). PMs are also modified in the female reproductive tract during the process of *capacitation*. This process is poorly understood, but is believed to involve removal of certain components from the cell surface (Johnson and Hunter, 1972; Oliphant and Brackett, 1973; Clegg, 1983). Once capacitated, the sperm PM is involved in binding to the noncellular aspects of the ovum surface (Saling et al., 1979; Russell et al., 1980; Peterson et al., 1981), although this particular role for the PM may not be ascribed to sperm from all species (Yanagimachi, 1981). Subsequent to binding, the capacitated sperm's PM participates in the *acrosome reaction*. It fuses with the underlying acrosomal membrane, liberating the enzymes of the acrosome, presumably to digest the zona pellucida of the oocyte (see Yanagimachi, 1981). Finally, the remaining sperm PM over the postacrosomal region fuses with the oolemma as the initial step of incorporation of the sperm into the egg (see Yanagimachi, 1981).

The PM of a developing cell is subjected to numerous and varied environments prior to ejaculation. Antigenic components are added during spermatogenesis (Millette and Bellvé, 1977; Bechtol et al., 1979). Surface components are added in the sojourn through the epididymis (Johnson and Hunter, 1972; Lea et al., 1978; Voglmayr et al., 1980). At ejaculation, certain components of the accessory sex gland fluids

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are added to the surface (Moore and Hibbitt, 1976; Oliphant and Brackett, 1973).

In this report we have selected a unique approach for studying the surface modifications taking place as sperm traverse the epididymal ducts and, at ejaculation, are presented with fluids from the various accessory sex glands. Our ability to isolate large quantities of highly purified PM from both epididymal and ejaculated sperm (Gillis et al., 1978; Peterson et al., 1980a,b; Russell et al., 1983; Kaplan et al., 1984) allows us to compare PM polypeptide constituents (Russell et al., 1983) with polypeptide maps of epididymal fluids and the accessory sex gland secretions. From these comparisons it is possible to determine which constituents from the epididymis and sex accessory glands are added to the PM.

Two-dimensional (2-D) gel electrophoresis was utilized to study polypeptide profiles of detergent solubilized PMs of epididymal fluids, accessory gland secretions and seminal plasma. This technique allowed a comparison of virtually all polypeptides present (integral and peripheral) in membrane fractions and reproductive tract and glandular secretions.

MATERIALS AND METHODS

Collection of Sperm

Epididymides were collected from freshly slaughtered, sexually mature boars. Within 3 h of slaughter, sperm were obtained for cavitation from the caput and cauda regions of the epididymis. Caput sperm were obtained by incising the capsule of the caput region and several underlying tubules with a razor blade. Sperm and caput fluid oozed from the cut tubules and were collected by a Pasteur pipette. Care was taken to avoid aspirating blood from cut vessels. Two hundred epididymides were used to obtain approximately 1×10^9 caput sperm for cavitation. Examination of smears of caput sperm showed virtually no red blood cells in the preparation.

Cauda sperm were obtained by backflushing the epididymal tubules with phosphate-buffered saline [PBS; 0.14 M containing 0.2 mM phenylmethanesulfonylfluoride (PMSF)]. A syringe with an 18-gauge needle was inserted into the vas deferens and clamped with a hemostat. A surface loop of a cauda tubule, about midway in the cauda, was freed from its connective tissue coverings and surrounding blood vessels. Gentle pressure on the syringe plunger resulted in a slow extrusion of cauda sperm from the cut epididymal tubule, and 1–5 ml of sperm and cauda fluid were obtained from each epididymis. Corpus sperm (not utilized) and fluid were obtained in the same manner as were caput sperm.

Isolation of Reproductive Tract Fluids

Caput, corpus and cauda fluid and seminal plasma were obtained by first separating whole sperm at low centrifugal speeds (3000 × *g* for 15 min at 4°C) and subcellular debris at high speed (100,000 × *g* for 30 min at 4°C). These fluids were initially collected directly into tubes containing PMSF and protein concentrations were determined (Lowry, as modified by Markwell et al., 1978) for 2-D gel electrophoresis. Seminal vesicle fluid was obtained through the duct by exerting gentle pressure on the gland so that fluid was collected in PMSF (>0.2 mM). The fluid was centrifuged at low and high speeds, as described above; protein concentration of the final supernatant was determined to be 80 mg/ml. The prostatic urethra was opened, wiped dry and gentle pressure exerted on the prostate gland. Prostatic fluid was obtained by aspiration with a pipette as the material oozed from the prostatic ducts into the prostatic sinuses. Protein in this fluid (44 mg/ml) was determined as described above. Bulbourethral gland fluid was collected by cutting open the wall of the gland and scooping out the secretory product into a vial containing PMSF (>0.2 mM). Due to its rubbery consistency, the bulbourethral gland secretion was extracted using 3% sodium dodecyl sulfate (SDS), 2 mM EDTA, 40 mM Tris-Cl (pH 8.2), and 0.2 mM PMSF at room temperature for 20 h with occasional vortexing. The buffered detergent solution was treated as described for the other fluids. Protein concentration was determined to be 2.5 mg/ml for 2 ml of bulbourethral secretion extracted into 2.0 ml of SDS buffer. This solution was concentrated to 5 mg/ml on a B15 Amicon concentrator before sample preparation for 2-D electrophoretic analysis. Seminal plasma was obtained at ejaculation by collecting the entire ejaculate into thermos bottles containing PMSF (>0.2 mM). The protein concentration (24 mg/ml) was determined for the liquid portion of the ejaculate which was centrifuged free of particulate matter. All fluids were stored at −70°C until used. Epididymal plasma contains enzymes including glycosidases, neuraminidase (Mann, 1981) and perhaps nonserine proteases. Accessory gland fluids also contain enzymes. Any enzymatic activity, however, had little noticeable effect on the reproducibility of 2-D gel patterns (including the complex patterns observed by silver staining) using fluids obtained on different days.

Membrane Collection

Sperm were washed three times in PBS (pH 7.4; see Gillis et al., 1978) and processed as previously described (Gillis et al., 1978). The gas pressure during cavitation was 650 psi. In all cases, PM vesicles utilized were those sedimenting on top of the 1.0-M sucrose solution (Band I). Transmission electron microscopic analysis of sperm after they were cavitated, and of membrane fractions collected, indicate that the cavitation effect on epididymal sperm was similar to that reported for ejaculated sperm (Peterson et al., 1980a); i.e., the PM is selectively removed (primarily from the sperm head) and this membrane forms smooth vesicles which are collected in Band I and Band II).

Porcine Blood Serum Collection

Porcine blood was obtained from the anterior vena cava of mature boars. The blood was isolated and allowed to clot at room temperature for 1 h. The clot was centrifuged at 2000 × *g* for 15 min at 4°C. The serum was collected and stored at −70°C until utilized.

2-D Gel Electrophoresis

Electrophoretic maps of reproductive tract membranes were performed according to methods used by Peterson et al. (1983) and Russell et al. (1983) which employ a modified O'Farrell (1975) procedure using 300 μg of protein. In some gels, internal standards for isoelectric focusing (IEF) calibration used carbamylated creatine phosphokinase (CPK) from rabbit muscle (Anderson and Hickman, 1979). Calibration of molecular weight in the second dimension used the pattern of rat heart mitochondria where the molecular weights of most proteins have been determined (Giometti et al., 1980).

Interpretation of Gels and Display of Gel Information

Each fluid or membrane fraction was processed to obtain four gel profiles. One IEF and one NEPHGE gel was prepared for Coomassie blue staining and the other set for silver-staining (Sammons et al., 1981). Information from silver-stained gels was traced directly on acetate sheets and these sheets were placed on a light box and traced with semitransparent paper. Overlap of similarly migrating polypeptides in IEF and NEPHGE gels allowed the artist to include all polypeptides from both gel patterns in one drawing; however, since NEPHGE polypeptides are not focused (see *Results* section), their relationship to IEF polypeptides is not spacially precise. A vertical line was drawn on the gel map to indicate the separation between the IEF tracing (on the left) and the NEPHGE tracing (on the right). Coomassie blue-stained polypeptides in the drawings of the gel were darkened and thus appear in the drawings as solid spots. These were designated as major polypeptides, since the staining technique to visualize them was less sensitive than the silver-staining technique, and revealed only those present in the highest quantities.

Selected polypeptides which were shared in the epididymal fluid maps were given letter designations. Certain of these letters appear on PM maps of epididymal sperm and designate suspected epididymal polypeptides which are added to the sperm PM. Epididymal PM polypeptides which represent comigrating major polypeptides present in the ejaculated PM reference map have been previously designated with a reference number (Russell et al., 1983) and are here designated likewise. Accessory sex gland polypeptides which appear to contribute to the ejaculated sperm PM map are given the reference number previously assigned to ejaculated sperm PM polypeptides (Russell et al., 1983). Minor polypeptides of accessory gland fluids which appear similar to PM polypeptides are designated *PM* in photographs of silver-stained gels. Finally, seminal vesicle polypeptides which appear in the seminal plasma are numbered, and the number designation is also given a seminal vesicle notation.

RESULTS

General Comments Regarding Interpretation of Gel Patterns

As a general rule the Coomassie technique is quantative for most polypeptides. Polypeptides which appear on Coomassie-stained gels are designated *major* polypeptides. Those additional polypeptides which appear on silver-stained gels are considered in this report as *minor* components of a reproductive tract fluid or of a PM. Caution is recommended in equating spots, as seen on electrophoretograms, with single species of proteins. Globular proteins, especially those in fluids, may be multimeric, hence the number of unique proteins is probably significantly less than the number of polypeptides detected. The reference map of PM designating polypeptides (Russell et al., 1983) is included for comparisons with other gel profiles (Fig. 1).

Epididymal Tract Fluids of the Boar (Figs. 2, 3 and 5)

Caput and corpus epididymal fluids (Figs. 2 and 3) were rich in the numbers and variety of constituent polypeptides. Coomassie-stained gels revealed about 175 polypeptides which were highly variable in both molecular weight [12–300 kilodaltons (kd)] and in isoelectric point (∿pH 3.5 to 8.5). Silver-stained gels revealed over 600 polypeptides. Considering that many of these appeared to show microheterogeneity, there were still about 300 unique species. Figure 3 shows a color illustration of a silver-stained *corpus epididymal fluid* gel (IEF). Its polypeptide composition more closely resembled caput epididymal fluid (Fig. 2) than cauda fluid (Fig. 5), although there were somewhat fewer polypeptides. Approximately 80–90% of the polypeptides in caput and corpus fluid gels could be tentatively identified as similar, based on color staining, polypeptide shape, apparent molecular weight and apparent charge.

Cauda epididymal fluid was also rich in the numbers and variety of its constituents, showing over 300 polypeptides, with about 50 of these also detected by the Coomassie stain. About 150 unique species were present if one considered microheterogeneous groups as a single polypeptide. The designation of apparently similar polypeptides in Figs. 2, 3 and 5 indicates to what degree soluble polypeptides were retained in different regions of the epididymis, reflecting the potential secretory function and resorption capacity of the epididymis (or sperm surface) in these regions. Although numerous major and minor polypeptides of the caput region apparently were absent or not detected in the cauda fluid, there were new polypeptides present (major and minor), not seen in any other region of the epididymis.

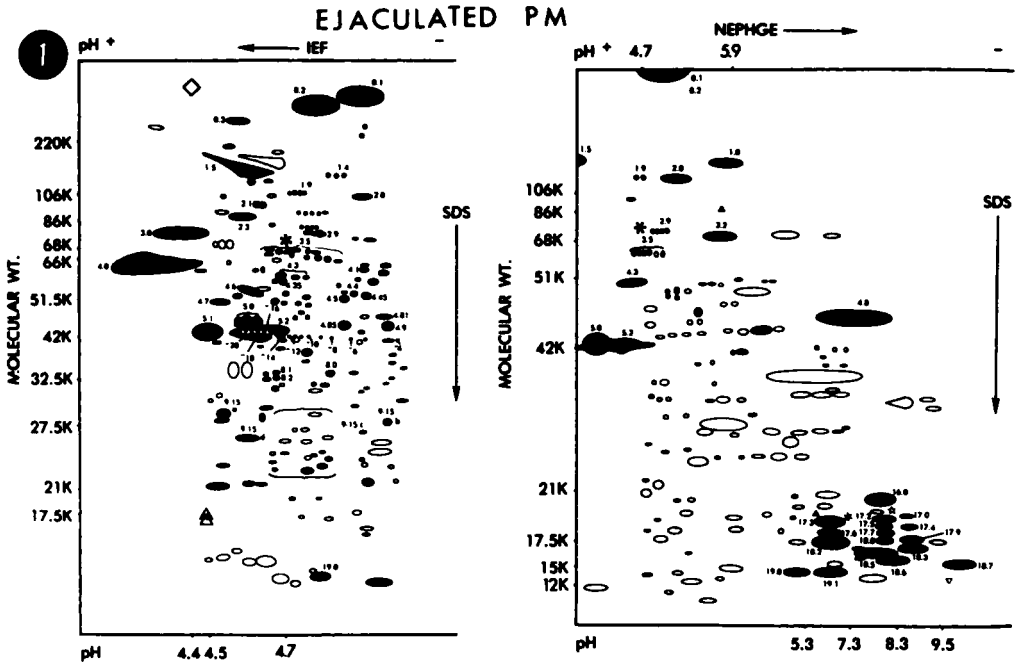


FIG. 1. Reference map for ejaculated PM polypeptides as illustrated in Russell et al. (1983). Polypeptides are numbered according to their banding patterns in single-dimension gels, and this numbering system is used in several subsequent figures to denote similarly migrating polypeptides in fluid and PM preparations. This and all subsequent tracings were taken from silver-stained gels. Polypeptides that also stained with Coomassie blue appear as solid spots.

Accessory Sex Gland Fluids (Figs. 6–9)

Two-dimensional patterns of *seminal vesicle* fluid (Fig. 6) revealed numerous (∿150) acidic-and neutral-range polypeptides spread throughout a spectrum of molecular weights. Most major polypeptides migrated near neutrality or in the highly basic region. Vertical stacking of these neutral and basic polypeptides indicated slight molecular weight differences due to the addition/subtraction of neutral charge moieties of a particular polypeptide. Specific PM polypeptides, which appeared to have been added, were designated utilizing the reference map provided by Russell et al. (1983), and were similarly designated on the seminal vesicle map.

Bulbourethral gland fluid (Fig. 7) shows only about 6 Coomassie-stained polypeptides and about 17 silver-stained polypeptides. Most polypeptides focused in the acidic range and were of moderate to high molecular weight. They have been drawn in the gel map as large spots because they focused poorly, a condition probably due to either molecular interactions with the pH gradient or charge heterogeneity. One particular polypeptide designated as *1.5* in Fig. 7, showed a similar shape, staining affinity and migratory behavior as PM 1.5 (Russell et al., 1983).

Prostatic fluid (Fig. 8) is a very complex accessory gland fluid which displayed about 300 polypeptides encompassing a wide spectrum of molecular weights and isoelectric points. About 70 of these were Coomassie-staining polypeptides.

Coomassie-stained gels of *seminal plasma* (Fig. 9) revealed predominantly low molecular weight polypeptide components, the majority of which focused in the neutral and basic range. Approximately 45 polypeptides were visualized. Silver-stained gels revealed about 200 polypeptides, many of these focusing in the acidic range and having molecular weights above 65 kd. The great majority of these polypeptides migrated with properties that indicated their origin in the vesicular secretion.

Epididymal and Ejaculated Sperm PM Polypeptides

Figures 4 and 10–13 show Coomassie-stained PM profiles for caput, (Fig. 10) and cauda epididymal sperm (Figs. 4, 11 and 13), and ejaculated sperm (Fig. 12). The IEF maps are shown for caput and ejaculated sperm although NEPHGE profiles were also obtained for these membranes. Cauda PM gels include a NEPHGE pattern (Fig. 13) for comparison with the NEPHGE profile of ejaculated PM (Fig. 1). Selected major polypeptides of ejaculated sperm (Fig. 12) are numerically designated according to the reference system used in our previous publication (Russell et al., 1983) and reprinted herein (Fig. 1). Polypeptides of the epididymal sperm PM which migrated identically to those shown in the map of ejaculated sperm are referenced similarly. The Coomassie-stained map of ejaculated PM showed heavier staining of comigrating polypeptides than did either the caput or cauda maps. Silver-stained gels (IEF and NEPHGE) of these three maps were also obtained, but to reduce the complexity of the patterns, only the major Coomassie-stained polypeptides are shown in these figures. In general, the PM NEPHGE gel map (silver stained) for caput sperm was more complex than that for cauda sperm, showing approximately 10 polypeptides which were not seen in corpus or cauda PM. All siver-stained gels revealed the presence of polypeptides 3.0 and 4.0 (Russell et al., 1983), but for a reason

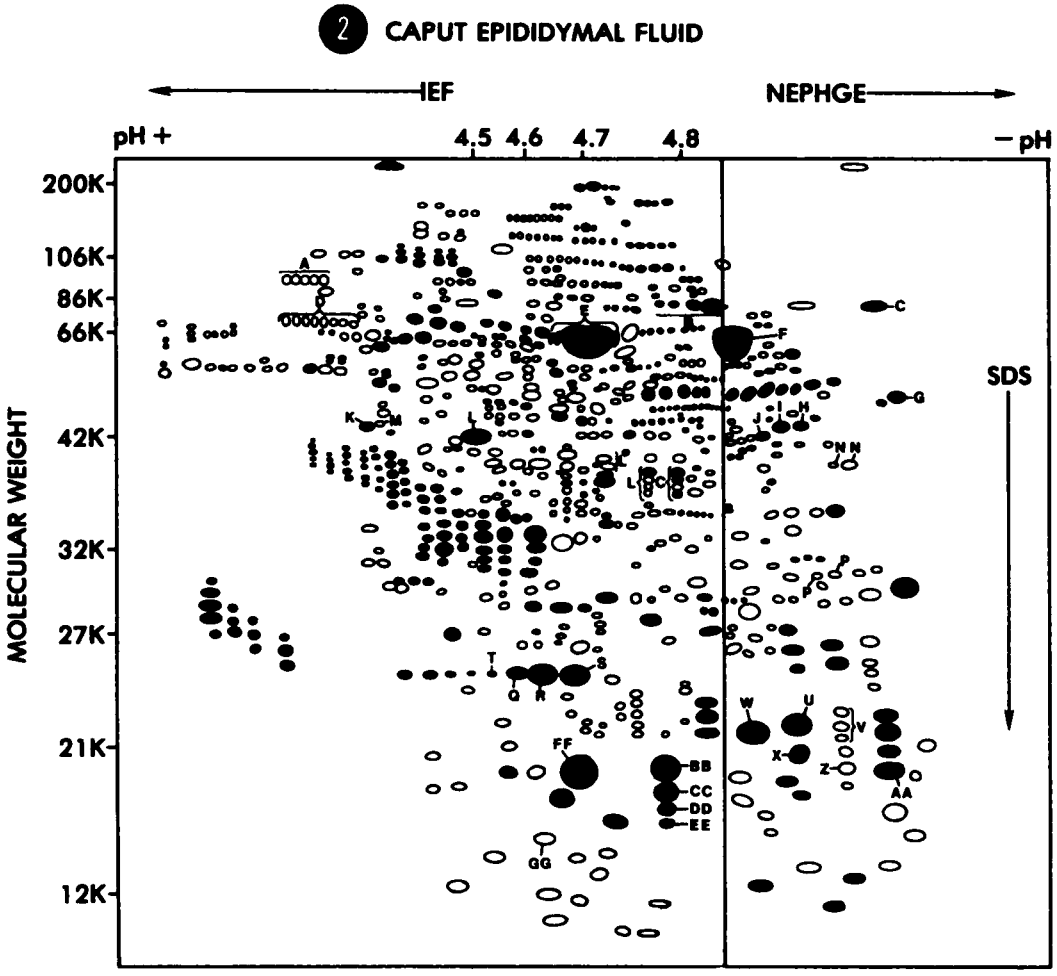
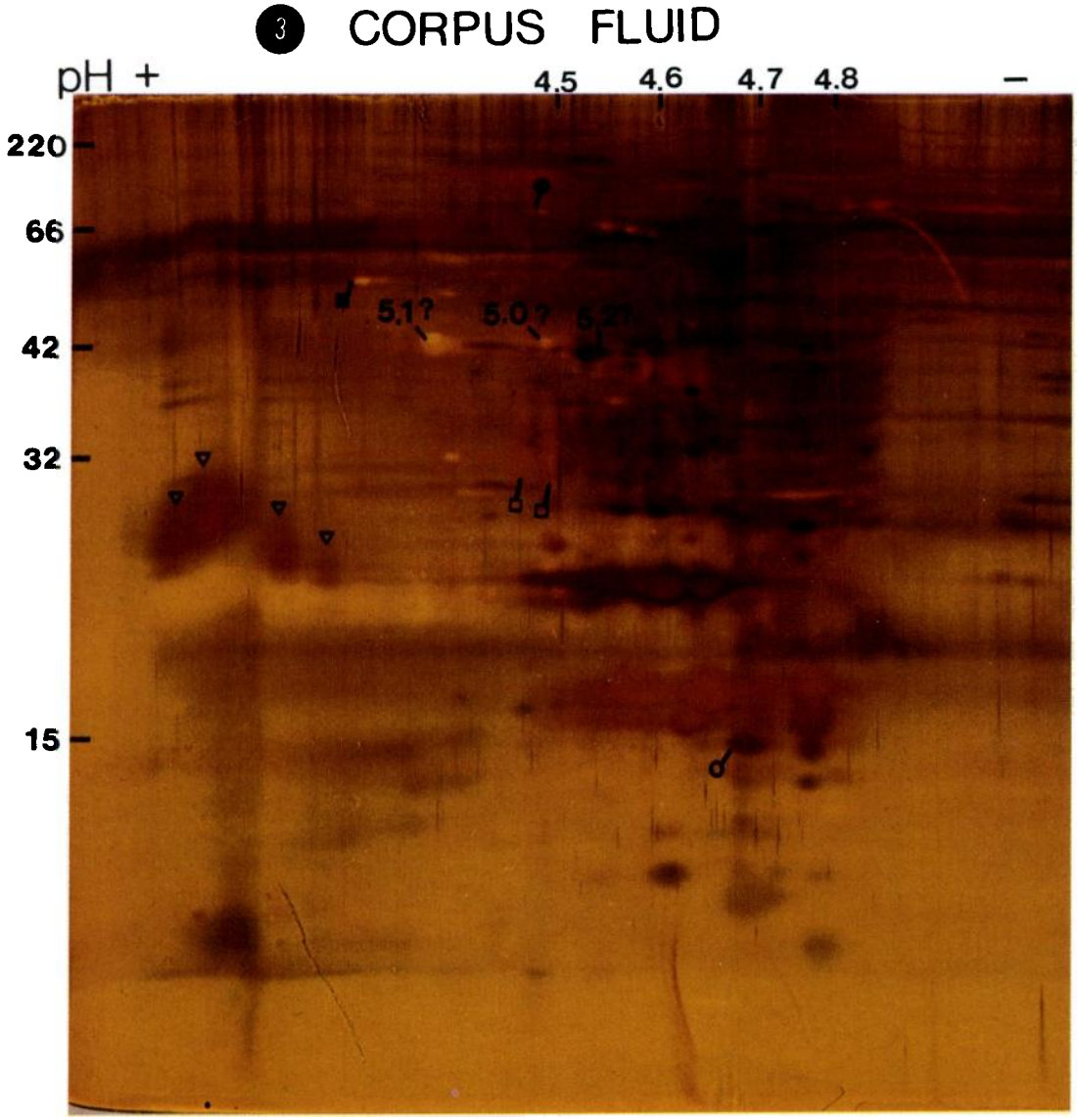


FIG. 2. Caput epididmal fluid tracing. *Letter* designations indicate polypeptides or groups of polypeptides which are similarly designated in cauda epididymal fluid (Fig. 5) and seminal plasma (Fig. 9).

presently unknown to us, these polypeptides were inconsistent in their appearance in 2-D Coomassie-stained gels. Except for relative intensity of staining, major polypeptides in cauda PM gel patterns were very similar to caput PM patterns.

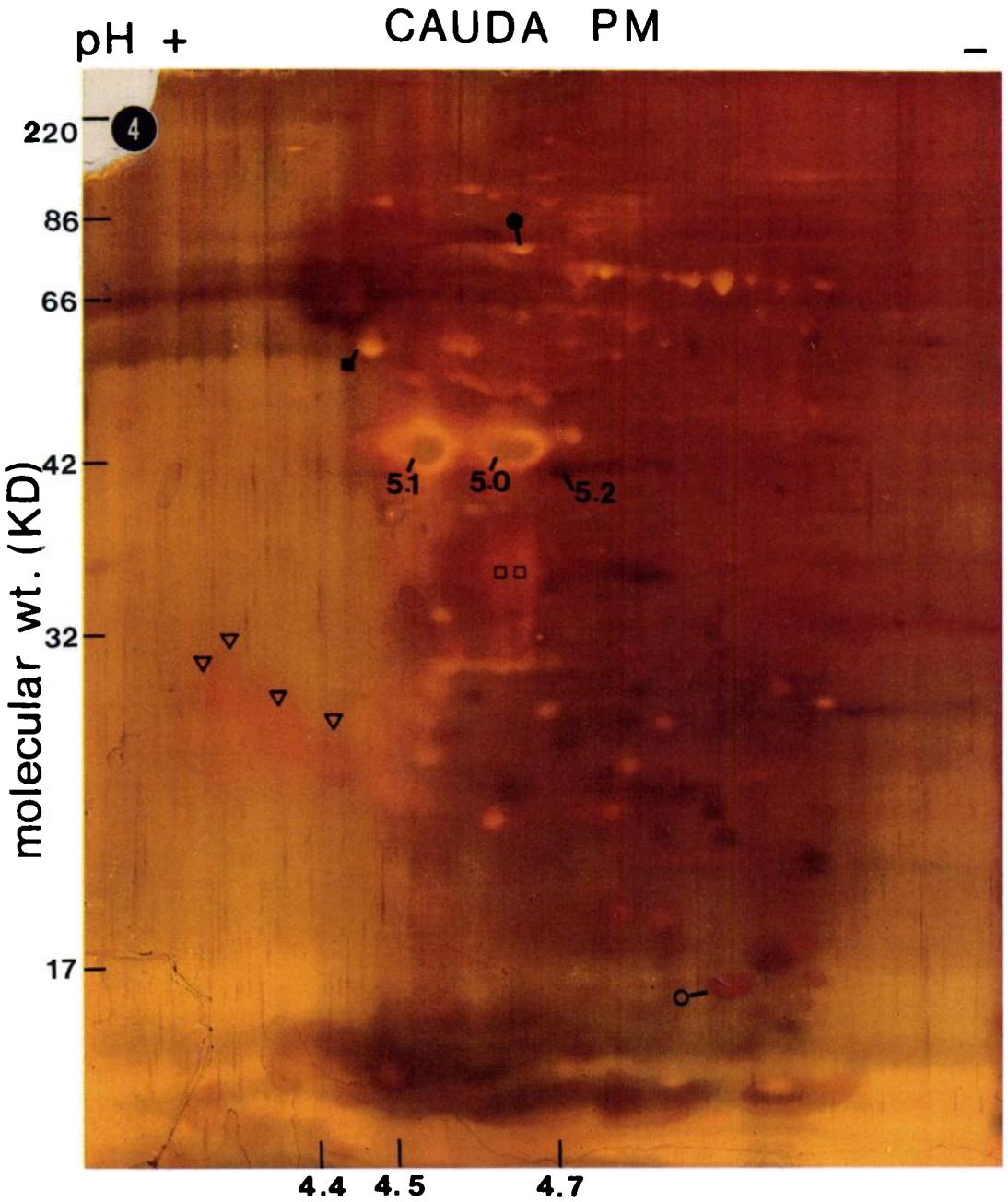
Polypeptides Added to Sperm PM in the Epididymis (Table 1)

Identification of polypeptides present in epididymal fluids and those present in PM of epididymal and ejaculated sperm PM was undertaken by comparing both Coomassie- and silver-stained gels. In relation to the total number of polypeptides of the ejaculated PM which were present in the epididymal fluids. PM polypeptide 5.2 appeared to be one of these polypeptides on both PM and in fluid. It was detectable on caput PM maps, leaving open the possibility that it may have been present prior to epididymal transit. In addition, polypeptide designated EE on the epididymal fluid



FIGS. 3 and 4. Silver-stained gels of corpus epididymal fluid and cauda plasma membranes, respectively. Polypeptides common to both gel maps are indicated by similar *symbols*. Some polypeptides are referenced by the number system of Fig. 1, and were suspected of being common to both maps. The polypeptide doublet

maps comigrated with an unreferenced polypeptide of the epididymal and ejaculated PM maps, suggesting that this polypeptide was added to the PM during epididymal transit. Silver-stained polypeptides were so numerous in gel maps that they potentially confounded the identification of all similarly migrating polypeptides. A color photograph of a silver-stained, cauda sperm PM gel is shown for comparison with a similar gel of corpus epididymidis fluid (Figs. 3 and 4, respectively). The sperm, having recently traversed the corpus region, showed



designated by *open squares* in both figures appeared in ejaculated sperm (Fig. 1) as a similarly appearing doublet to the left and below polypeptide *5.2*.

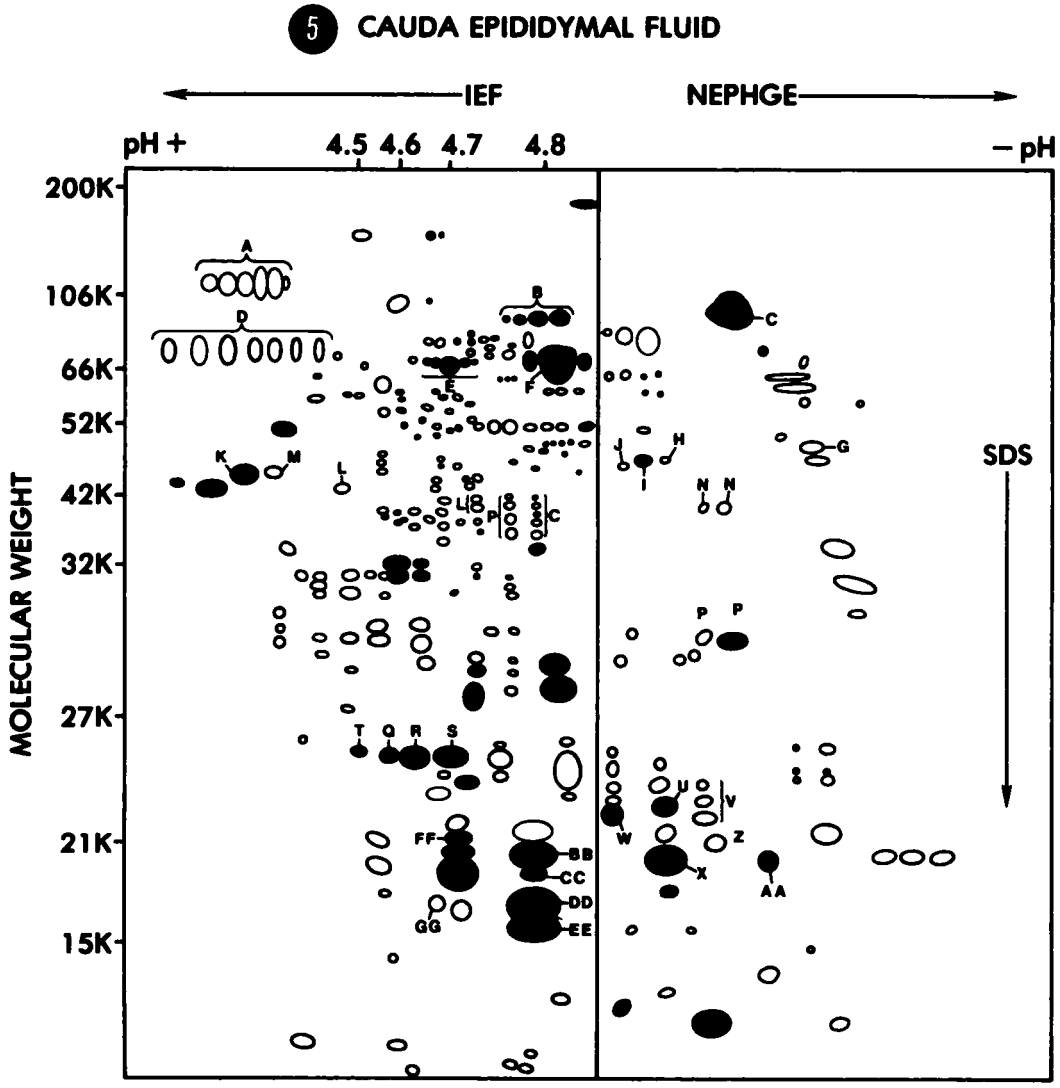


FIG. 5. Cauda epididymal fluid tracing. *Letters* designate common polypeptides in caput fluid (Fig. 2) and seminal plasma (Fig. 9).

several polypeptides (identified in Fig. 3 and 4) which, by their similarities in shape, color staining and electrophoretic migration, suggested that they were common to both corpus fluid and cauda PM gels and may have been acquired in the corpus region of the epididymis. These polypeptides, however, did not all appear on silver-stained maps of ejaculated sperm.

Polypeptides Added to the Sperm PM at Ejaculation (Table 1)

Comparison of cauda epididymal sperm PM polypeptide gel profiles with ejaculated sperm PM polypeptide gel profiles showed that major contributions to the PM were provided by the seminal plasma. These polypeptides were intensely stained and comprised a relatively large number of added polypeptides, as compared to the total number of polypeptides added during epididymal transit. Examination of polypeptide patterns in gels of accessory gland secretions indicated that the seminal vesicles contributed most of the polypeptides added to the ejaculated sperm PM. Reference numbers in Figs. 5–9 indicate polypeptides which we believe were added by these glands, and Table 1 sum-

marizes these data. In general, those added were the clustered groups of low molecular weight polypeptides focusing in the neutral and basic ranges. The high molecular weight (300–400 kd) polypeptides which focused poorly near pH 4.7–4.8 were not seen in epididymal sperm PM, but were seen in ejaculated PM and also in seminal vesicle secretions. Thus, their origin appeared to be from the seminal vesicle glands. We have previously reported (Hunt et al., 1983) that the low molecular weight polypeptides of seminal plasma cross-react with antisera to low molecular weight boar sperm PM polypeptides. Polypeptide 1.5 did not appear in epididymal PM. Similarly shaped, stained and migrating polypeptides appeared in the bulbourethral and prostatic secretions, and are indicated on the bulbourethral gel and prostatic maps as potential contributors to the ejaculated PM polypeptide profile.

Porcine Serum and Sperm PM Profiles

To assess potential contamination of epididymal fluids and accessory gland fluids with serum components, we prepared a Coomassie-stained IEF gel of porcine serum. The patterns of epididymal and accessory sex gland fluids, and porcine blood serum (not shown) were largely dissimilar; however, we noted the possible presence of porcine serum albumin (polypeptide designated with the letter *f* in epididymal polypeptide maps and an *arrow* in the prostatic fluid map) in both epididymal and prostatic fluid (Figs. 10–13). The tentative finding of albumin alone in these fluids with-

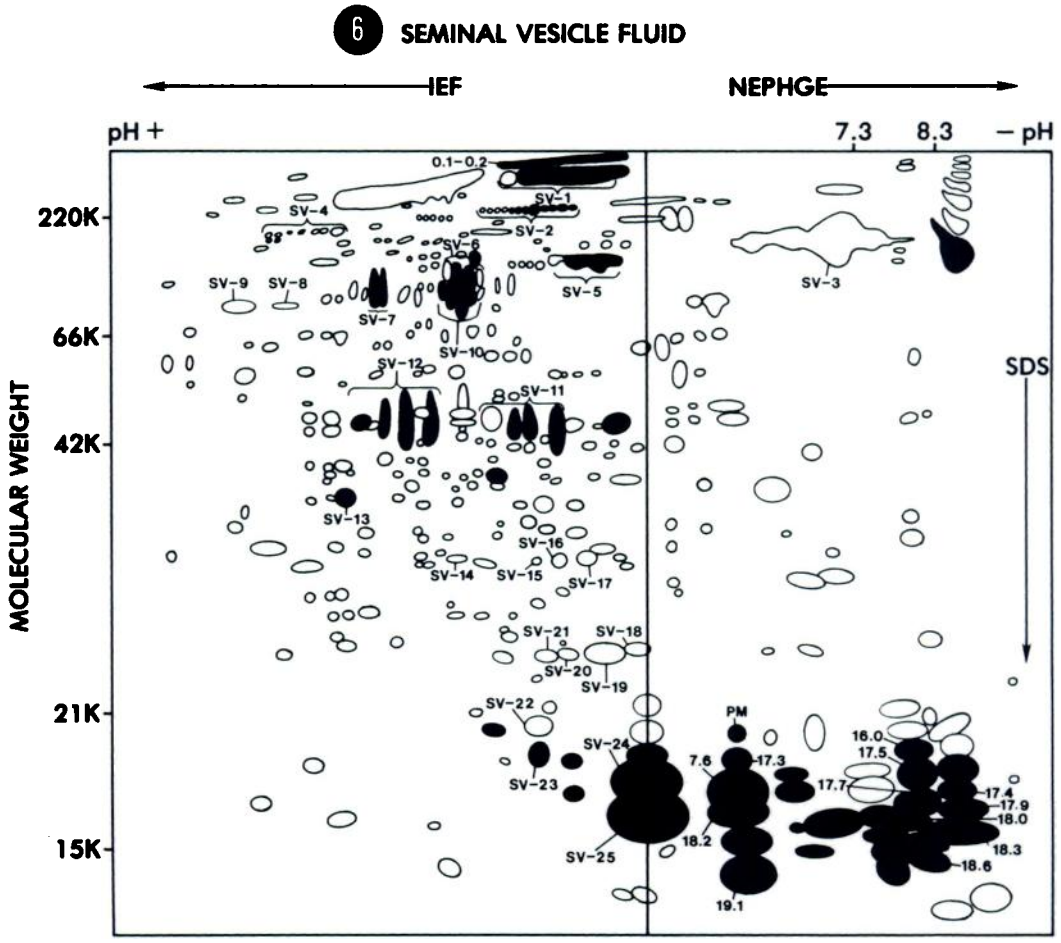
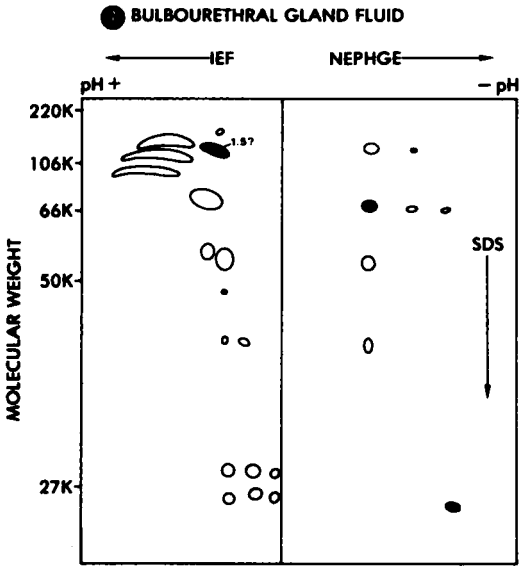
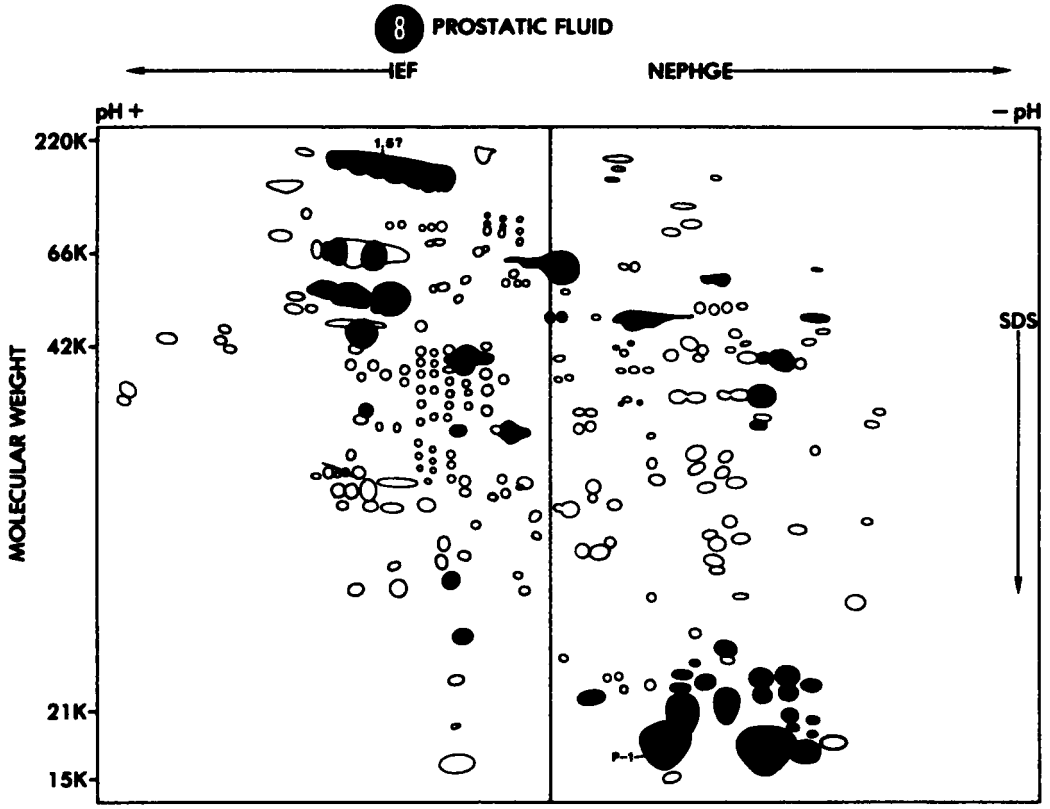


FIG. 6. Seminal vesicle fluid. Polypeptides numbered were those thought to be added to the ejaculated sperm. Polypeptides prefaced by *SV* were those contributing to the seminal plasma (Fig. 9).



FIGS. 7 and 8. Bulbourethral gland and prostatic fluid tracings. One of the polypeptides designated *1.5* in each tracing was suspected of appearing on the PM reference map (see Fig. 1). The polypeptide designated with a *P* in the prostate map was suspected of appearing in seminal plasma (Fig. 9).



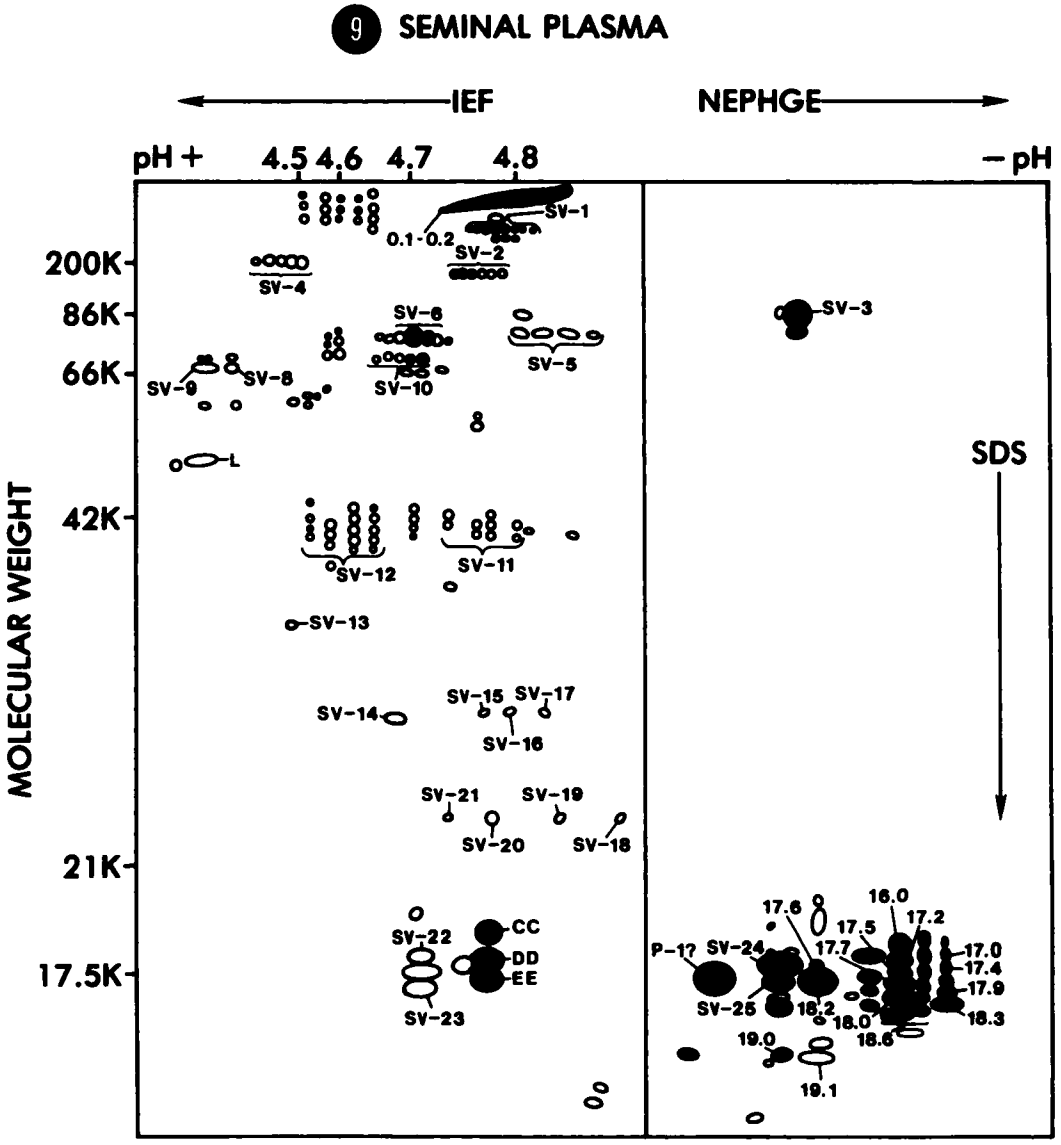
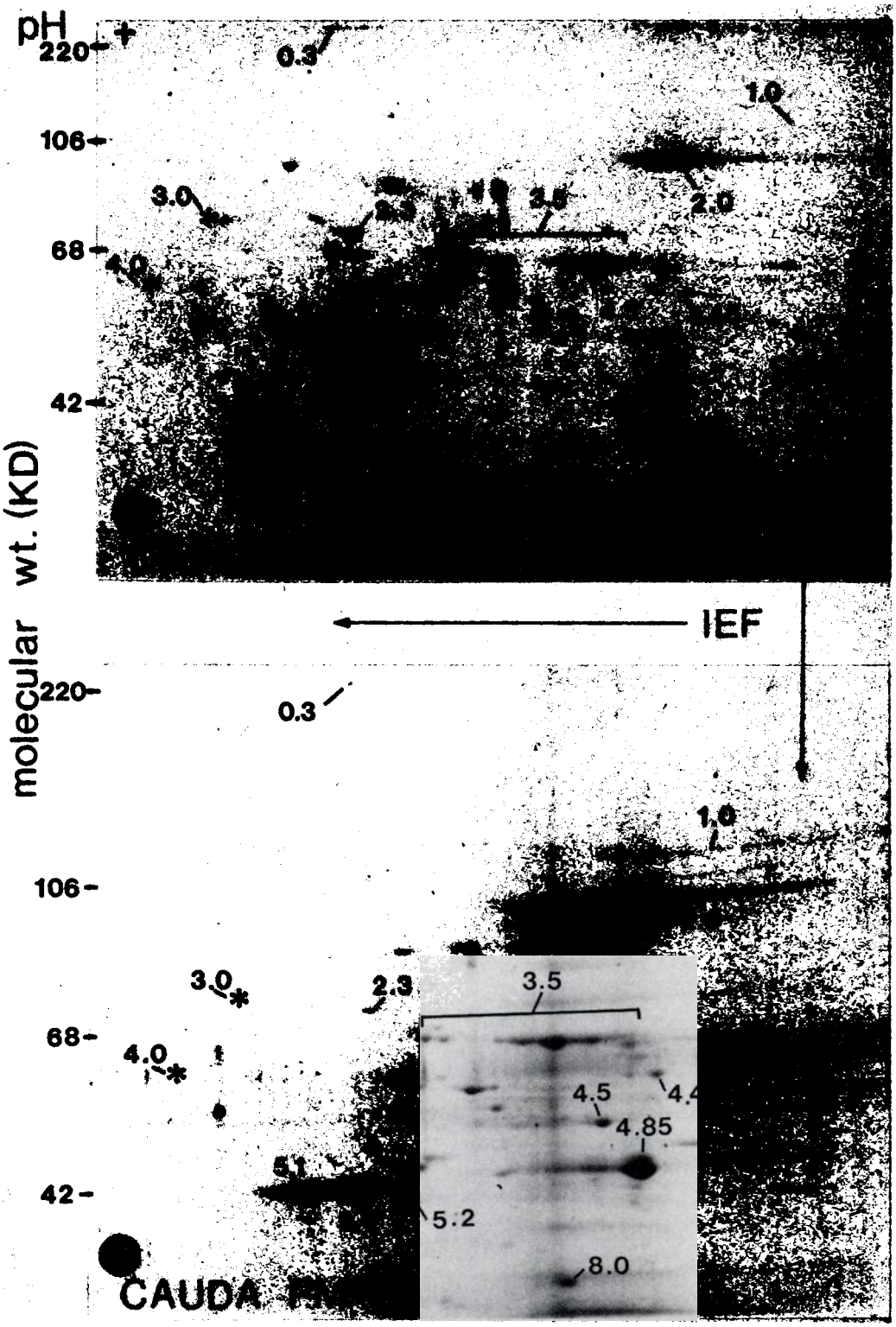


FIG. 9. Seminal plasma gel tracing showing the probable source of its constituents (*SV*=seminal vesicle, *P*=prostate, *alphabetical designations*=epididymal fluid). Polypeptides with numerical designations are referenced similarly to comigrating ejaculated sperm PMP (Fig. 1).

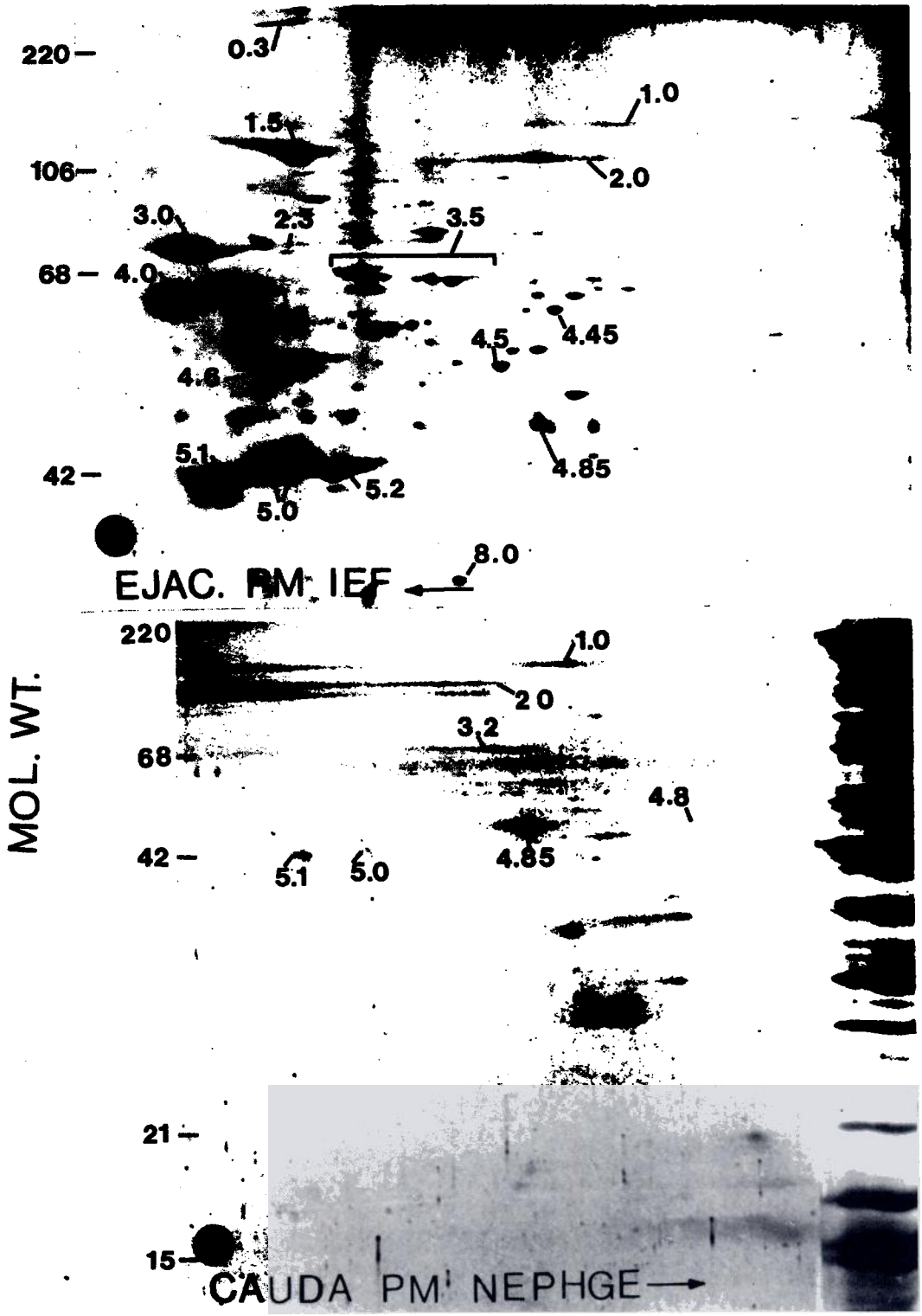
out other accompanying major serum proteins indicated selective transfer of albumin into the epididymal ducts and prostatic glandular cavities, but not contamination with whole serum.

DISCUSSION

The present study has been directed toward making four major determinations: *1*) the composition of fluids in various regions of the epididymis, *2*) the numbers and kinds of polypeptides added to the sperm PM during their epididymal transit, *3*) the polypeptide composition of the accessory sex gland secretions and the contribution(s) of these proteins to seminal plasma, and *4*) the polypeptides which are added to the sperm PM by mixture with accessory gland fluids at ejaculation. The methodologies employed allowed a more comprehensive study of reproductive tract



FIGS. 10–13. High and medium molecular weight regions of Coomassie-stained gels of caput (Fig. 10, IEF), cauda (Fig. 11, IEF; Fig. 13 NEPHGE) and ejaculated (FIg. 12, IEF) sperm. Polypeptides designated from the reference tracing (Fig. 1) are indicated for comparison of Figs. 10–12. In Fig. 13 few polypeptides were visu-



alized in the low molecular weight region at basic pH. Comparison with ejaculated sperm PM, seminal vesicle, and seminal plasma NEPHGE patterns indicated that numerous seminal vesicle polypeptides were added in this region.

TABLE 1. Selected major Coomassie-stained ejaculated plasma membrane polypeptides and their source.

Polypeptide[a]	Caput (+ = present; − = absent)	Cauda (+ = present; − = absent)	Source[b]
0.1–0.2	−	−	Seminal vesicle
0.3	+	+	
1.0	+ (Weak)	+ (Weak)	
1.5	−	−	Prostate or bulbourethral gland
1.9	+	+	Seminal vesicle
2.0	+	+	
2.3	+	+	
3.0	+[c]	+[c]	
3.2	+	+	
3.5	+	+	
4.0	+[c]	+[c]	
4.3	+	+	
4.6	+	+ Inconsistent staining	
4.8	+	+	
4.85	+	+	
5.0	+ (Weak)	+	
5.1	+	+	
5.2	+ (Weak)	+ (Weak)	Epididymal fluid (?)
16.0	−	−	Seminal vesicle
EE[d]	+	+	Epididymal fluid
17.0	−	−	Seminal vesicle
17.2	−	−	Seminal vesicle
17.4	−	−	Seminal vesicle
17.5	−	−	Seminal vesicle
17.6	−	−	Seminal vesicle
17.7	−	−	Seminal vesicle
17.9	−	−	Seminal vesicle
18.0	−	−	Seminal vesicle
18.2	−	−	Seminal vesicle
18.3	−	−	Seminal vesicle
18.6	−	−	Seminal vesicle
18.7	−	−	Seminal vesicle
19.0	+ (?)	+ (?)	Seminal vesicle (?)
19.1	−	−	Seminal vesicle

[a] Numerical designation of PM polypeptides according to Russell et al., 1983.

[b] No designation indicates that they are already present in caput PM.

[c] Appears erratically in some Coomassie runs, but always in silver-stained gels.

[d] Polypeptide unreferenced in PM map, but comigrates with polypeptide of epididymal fluid designated *EE*.

fluids and PMs and their relationships than previously recorded. In general, they demonstrated that reproductive tract fluids and accessory sex gland secretions were more complex, in terms of polypeptide composition, than previously described. They provide reference maps of various boar reproductive tract fluids and PM components for use in future studies.

Methodologies

Investigators working with species producing fewer sperm have out of necessity utilized whole sperm or detergent extracts from whole sperm to study surface properties and epididymal or seminal plasma contributions to the sperm. This has raised the methodological question of whether all the isolated components originated from the sperm surface. Coupled with these techniques, most investigators have employed radiolabeling, lectin, or charge affinity procedures to detect surface components. While such studies clearly have been useful to show that components are indeed added to sperm PM, we believe they are limited in scope and to a certain degree biased by the

nature of the labeling process. Most investigators demonstrate less than 10–12 peaks (usually from 4–7) of radioactivity in a variety of species, suggesting similar numbers of labeled polypeptides (Vierula and Rajaniemi, 1980; Voglmayr et al., 1980; Ji et al., 1981; Young and Goodman, 1980, 1982; Brown et al., 1983; Jones et al., 1983). Only certain exposed surface moieties (perhaps less than 10% as recent unpublished data suggests) may be radiolabeled, thus limiting the total number of polypeptides found, and at the same time selecting the population of polypeptides which are most exposed. This selection would be biased toward identifying polypeptides which have recently become associated (either by a specific or a nonspecific association) with the sperm's surface, since they remain as the most external feature of the sperm.

Unmasking of surface components with time, as viewed in surface labeling studies, might allow labeling and thus suggest that a particular polypeptide has suddenly become associated with the sperm PM. Conversely, masking would suggest that a polypeptide is no longer associated with the sperm's surface. Slight changes in conformation of polypeptide-polypeptide association might influence labeling and lead to false interpretation of events.

In the boar, highly purified PM fractions demonstrate over 250 integral and peripheral polypeptides (Russell et al., 1983) and our recent data from human PM (Russell et al., 1984) show slightly fewer numbers of PM polypeptides. Thus, there is reason to believe that PM from other species will show similar complexity. The larger numbers of silver-stained polypeptides in some gels overwhelmed our ability to identify all comigrating polypeptides in separate samples, and only the most apparent examples of comigration are identified. Silver staining revealed the complexity (qualitative) of the membrane fraction while Coomassie blue staining, which is less sensitive, demonstrated its predominant components (quantitative). By using gel tracings, we have consolidated the information for any particular fluid or PM from four gel patterns to one comprehensive map.

The present study employed the complete analysis of both reproductive tract and accessory sex gland fluids, as well as PM preparations. Our ability to demonstrate virtually the entire population of polypeptides, in both a fluid and the membrane exposed to that fluid, allows a comprehensive evaluation of molecular events transpiring in the particular region under consideration.

Composition of Epididymal Fluids

To date a comprehensive evaluation of polypeptides of the epididymis, especially in various epididymal regions, has not been performed, although single-dimension patterns have been obtained from the boar (Einarsson et al., 1970; Lavon and Boursnell, 1971). In the present study, all regions of the epididymis were found to be rich in both the numbers and variety of constituent polypeptides. Caput and corpus fluids were the most complex, each demonstrating about 300 polypeptides. Adsorption of polypeptides to sperm is one possible explanation to account for their absence in the cauda fluid. Sperm PM prepared for nitrogen cavitation have been washed extensively and the resulting wash fluid containing loosely bound material has been discarded. Studies are presently under way to determine which peripheral polypeptides are readily released upon washing of sperm. Also discarded, and consequently not analyzed, was the small particulate matter which sedimented at 100,000 × *g*.

The present study has shown the degree of conservation of polypeptides in the epididymal fluid. Polypeptide maps of caput and corpus fluid show many similar constituents (>70–80%), whereas the cauda map is less complex than the caput or corpus map (<40–60%). Most major polypeptides (Coomassie-stained) are conserved throughout the duct system, suggesting, in a quantitative sense, that there are relatively minor modifications in epididymal fluid polypeptide composition during epididymal transit.

Polypeptides Added to the Sperm Surface During Epididymal Transit

Numerous studies have indicated that the surface of sperm is modified by addition of moieties from the epididymis, the suggestion being that these moieties in some way are part of the maturation process that sperm must undergo during epididymal transit. Voglmayr (1979, 1980) has indicated that epididymal adsorption of proteins is specific, whereas that adsorption in the rete testis is nonspecific. Generally speaking, the studies on epididymal sperm have implied that a relatively large number of polypeptides, at least in terms of the total population of PM polypeptides labeled,

are added to the sperm PM during epididymal transit. The present study has shown that the numbers of polypeptides added to sperm PM are not large, nor do they represent a major contribution in a quantitative sense. Coomassie maps of various regions of the epididymis indicate that only one or two major (Coomassie blue-staining) polypeptides appear to be added in epididymal transit as well as several minor (silver-staining) polypeptides. We do note that in colored silver-stained gels several minor polypeptides, not appearing on our original reference map (Russell et al., 1983), are present in gels of epididymal sperm, suggesting a transient appearance of several polypeptides on the PM. Polypeptides could be secreted by the epididymal epithelium and be adsorbed by sperm at such a rate that they would not be seen in epididymal fluid, even with the highly sensitive, silver-staining technique. Polypeptides added in the caput epididymidis, in the fashion just described, may have been missed since we have not been able to collect PM from testicular sperm to determine whether or not they are present prior to epididymal transit. In addition, this study has not addressed polypeptides which may be loosely bound to the sperm's surface. A compilation of loosely bound polypeptides, which appear in supernatants after washing of sperm, may in the future indicate that there are more polypeptides associated with sperm during epididymal transit. A possible function for loosely associated polypeptides cannot be ruled out.

The gel profiles of caput, cauda and ejaculated sperm show relative differences in staining affinity of specific polypeptides (Figs. 10–12). This may be interpreted in a quantitative sense as addition or deletion of specific components. Alternatively, staining differences might be the result of addition or modification of carbohydrate residues that have little effect on molecular weight or isoelectric point (Sturgess et al., 1978). Mann (1981) has indicated that glycosidases abound in boar epididymal fluid and may be responsible for carbohydrate modification.

Although the present study has shown only minor quantitative and qualitative additions of epididymal fluid polypeptides to sperm, it should not be inferred that major alterations in sperm morphology and/or surface organization do not take place in the epididymis. Jones (1971) has shown changes in acrosome adherence, acrosome shape, and plasma membrane lability during epididymal passage. These changes may take the form of molecular rearrangements or domain reorganization which are influenced by the epididymal environment. In the boar, Suzuki (1981) has elegantly demonstrated distinct changes in intramembranous particle distribution in boar spermatozoa in five different regions of the epididymis.

By identifying the major polypeptides which comprise the integral and peripheral proteins of epididymal PM, we have established the groundwork for future studies. It now becomes possible to isolate polypeptides, raise antisera against them and determine which are involved in surface reorganization occurring during epididiymal transit.

Polypeptide Composition of Accessory Sex Glands and the Polypeptide Composition of Seminal Plasma

All glandular secretions were collected individually except those from the urethral glands. These were not obtained because they are apparently produced with rapidity at the time of ejaculation (Mann, 1981). The amount of the total ejaculate produced by the uretheral glands is large (up to 50% of the ejaculate, McKenzie et al., 1938), but this secretion is thought to be very watery and low in protein (Mann, 1981).

The 2-D gels of seminal plasma were less complex, in terms of the number and variety of polypeptides, than most of its constituent fluids (seminal vesicle secretions, prostate fluid and epididymal fluid) with the exception of that from the bulbourethral glands. The electrophoretic data show that most of the major and minor polypeptide constituents of seminal plasma are derived from the seminal vesicles with small contributions from epididymal fluids. This is an expected finding in view of the relatively large contribution of seminal vesicle fluid to the entire ejaculate and the protein concentration of seminal vesicle fluid. Estimates vary as to the volumetric proportion of the entire ejaculate contributed by the seminal vesicles (from 20% to 57%; Mann, 1964, 1981 and Davies et al., 1975, respectively). The protein concentration of seminal vesicle fluid was approximately twice that of any of the other contributing fluids surveyed. Seminal plasma thus appears as a highly diluted form of seminal vesicle secretion: diluted to the extent that most polypeptides from other glands and the epididymis are not in sufficient concentrations

to be noted (Lavon and Boursnell, 1971), even with the highly sensitive silver-staining technique. Epididymal fluid comprises 2–5% of the volume of the ejaculate and the polypeptide-poor bulbourethral gland from 10–25% (McKenzie et al., 1938). To our knowledge, the volumetric contribution of the prostate is not known but is probably under 5% of the total volume.

There is considerable evidence in the literature that the seminal vesicles make the major polypeptide contribution to the seminal plasma (Glover and Mann, 1954; Mann and Glover, 1954; Boursnell et al., 1962; Lavon and Boursnell, 1971). Lavon and Boursnell (1971), utilizing IEF, estimated that 80–90% of the protein in seminal plasma was derived from the seminal vesicles, with the remaining detectable protein from the epididymal fluid. The present study has, in general, verified these findings and extended them by demonstrating precisely which polypeptides are from seminal vesicle secretions and which are derived from the epididymal fluid. The major polypeptides found were those of low molecular weight with isoelectric points near neutrality and extending well into the basic range. Approximately 30 polypeptides were identified in this low molecular weight cluster, with many of those displaying microheterogeneity as evidenced by regularly spaced vertical stacking and horizontal arrangement of electrophoetic spots.

Polypeptides Added to Sperm in the Seminal Plasma

In the boar seminal plasma, contributions are thought to arise from the seminal vesicles by the addition of basic polypeptides (Moore and Hibbit, 1976), thus changing the isolectric point of whole epididymal sperm at ejaculation from 4.5 to 6.5 (Moore and Hibbitt, 1975). When the seminal vesicles of boars are removed and their sperm are exposed to seminal plasma, the PM fraction isolated after exposure binds twice as much seminal vesicle protein as PM from ejauclated sperm from normal littermates (Moore and Hibbitt, 1976). These authors found that extensive washing did not remove all the labeled protein, suggesting to them an irreversible attachment of the seminal vesicle basic protein to the membrane. The present report demonstrates that numerous polypeptides are indeed added to the PM at ejaculation and that the majority of them are derived from the seminal vesicles, with specific contributions from other accessory glands. These polypeptides do not appear to be loosely bound since they remain within PM vesicles, which have been isolated by first washing intact sperm three times and then by exposing PM vesicles to numerous fresh fluid environments during their isolation. We have not demonstrated loosely bound PM polypeptides derived from seminal plasma, although this remains a major objective for future work.

That specific additions of polypeptides are occurring in seminal plasma has been determined first, by showing that in cauda epididymal sperm certain polypeptides normally found in ejaculated sperm are lacking, and secondly, by showing their presence in PM gels after exposure to seminal plasma (at ejaculation). In many cases, the source of the added polypeptide has been determined from examination of fluids contributing to the seminal plasma. Specific polypeptides added from seminal plasma are the high molecular weight (300–400 kd) polypeptides numbered 0.1 and 0.2 and a large cluster of low molecular weight polypeptides numbered 16.0 through 19.1 (reference map of Russell et al., 1983). Polypeptides 0.1 and 0.2 focus poorly, and previous data (Russell et al., 1983) suggest they are preferentially located in the head region of the sperm, are glycoproteins, and show tissue specificity. Most striking are the additions of low molecular weight, neutral-range and basic polypeptides in seminal plasma which are derived from the seminal vesicles. About 18 of these polypeptides have been tentatively identified in seminal plasma and seminal vesicle secretions and designated with PM reference numbers. One or two of these polypeptides may have their origin from the prostatic secretion, but due to the tight clustering of polypeptides in this region of the gel and the lack of landmarks for prostatic polypeptides, it was difficult to determine the source of one or two of these polypeptides.

The source of polypeptide 1.5 (Fig. 1) is somewhat ambiguous. Lacking in PM gels of cauda epididymal sperm, it is a prominent feature of ejaculated sperm. Gel maps of both prostate and bulbourethral gland fluids suggest similar-appearing "wing-shaped" polypeptides which demonstrate a molecular weight of about 200 kd and which are very acidic, as is polypeptide 1.5 of the PM reference map. Because the bulbourethral gland makes a sizeable volumetric contribution to the seminal

plasma, we favor the origin of polypeptide 1.5 from this source rather than from the prostate gland. In instances where the origin of specific polypeptides is ambiguous, further proof of identity may be obtained by using immunologic techniques such as the Western blot method and/or selective addition of appropriately diluted fluids to epididymal sperm with subsequent isolation of PM.

We estimate that the major polypeptides added to epididymal sperm at ejaculation comprise about 35% of the total protein of the PM and are about 20–25 in number. The actual number of proteins added is not known since many proteins may be structured with more than one polypeptide chain. In comparison with contributions from the epididymis during sperm transit, the seminal vesicle secretion provides a major contribution in both a qualitative and quantitative sense. Recently, research has focused largely on changes occurring in the epididymis in light of the documented importance of the epididymis in sperm maturation (for reviews see Bedford, 1975; Orgebin-Crist et al., 1967). Many investigators feel that contributions from the seminal plasma are not important in view of the observations that cauda epididymal spermatozoa of the boar and other species are capable of fertilization. Some researchers hold the view that seminal plasma functions only as a buffer, a diluent, and a vehicle for sperm transport in both the male and female reproductive tracts, while others hold the opinion that seminal plasma has additional functional properties (see discussions in Mann, 1981; Polakoski and Kopta, 1982). The argument we pose is that fertilization with epididymal sperm by insemination in vitro, by no means simulates the events which take place during natural mating, and consequently experiments of this type do not adequately test the role of the seminal plasma.

Evidence supporting an additional role for seminal plasma in the boar is inconclusive but generally supportive, and comes from studies targeting the seminal vesicles. Removal of the seminal vesicles from the boar reduced the conception rate of inseminated sows (60% conception rate in control versus 41% in seminal vesicle depleted boars) however, this was not a statistically significant difference from controls (Davies et al., 1975). The seminal vesicles of the boar, like those of many other species, are a rich source of fructose, and energy source for the spermatozoon. When poorly motile epididymal spermatozoa are brought into contact with seminal vesicle secretion (and simultaneously viewed under the light microscope), the sperm become intensely motile (Mann, 1954), suggesting that either fructose and/or other substances in the vesicular fluid are responsible for the increased motility. Crossed immunoelectrophoretic experiments in this laboratory show that basic antigens markedly affect precipitin patterns in the presence of solubilized zonae pellucidae (Peterson et al., 1981), suggesting that basic polypeptides are apparently one substance responsible for sperm-zona adhesion which occurs during the course of fertilization. The seminal vesicle secretions, in conjunction with those from the bulbouretheral glands, are responsible for the formation of the gel fraction of boar spermatozoa (Boursnell et al., 1970). When the gel of boar semen is fully formed, it is said to comprise about 50% of the seminal plasma. Gel apparently functions in the sow uterus to prevent backflow of semen.

The seminal plasma of the boar, like the cauda epididymal fluid, contains a factor or factors, which when added to capacitated sperm, acts to decapacitate them, thus extending fertilization time (Hunter and Hall, 1974). The need for such a factor to insure reproduction processes in a wide variety of mammals is debated, with some arguing that the time necessary for capacitation insures that only the most viable and vigorous spermatozoa fertilize the egg.

Identifying polypeptides from accessory glands which attach to sperm PM lays the groundwork for future studies. It is now possible to determine which PM polypeptides become associated with accessory sex gland polypeptides and to determine if accessory sex gland polypeptides have a role in capacitation. If they do, the question of the nature of the interaction between PM polypeptides and accessory gland-contributed PM polypeptides may be elucidated.

NOTE ADDED IN PROOF

A recent report by Nagai et al. (1984; J. Reprod. Fertil. 70:271–275) clearly shows that boar seminal plasma components prevent fertilization in vitro. These components are not readily removed under conditions which capacitate epididymal sperm. The in vitro fertilization system developed by Nagai et al. will allow a determination of which accessory gland fluids and fractions thereof are those which prevent fertilization.

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