

The Soluble Proteins of Rat Seminal Vesicle Fluid

SOME PHYSICO-CHEMICAL AND IMMUNOLOGICAL PROPERTIES

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Summary. The native secretion of rat seminal vesicles was found to contain about 290 mg of protein/ml. The ionic strength of the secretion was low (33 millimho. cm^{-2}). Some protein (about 13 per cent) precipitated shortly after collection. The soluble proteins were resolved by electrophoresis into four very basic and two minor acidic fractions. Three of the proteins were eluted from a Sephadex G-200 column at a position indicating a molecular weight much in excess of 150,000 daltons; the others were eluted at a position intermediate between IgG and bovine serum albumin (BSA) markers. Ten bands were resolved in polyacrylamide gel electrophoresis following dissociation of the soluble proteins into subunits. Eight of these had molecular weights ranging between 18,500 and 100,000 Daltons.

Antisera raised in rabbits against seminal vesicle fluid (SVF) formed, in immunoelectrophoresis (IE) six precipitin bands with SVF. They did not react at all with rat serum proteins, nor did antiserum to rat serum proteins react with SVF. A small amount of rat serum proteins was, however, detected by radioimmunoassay in the insoluble fraction.

INTRODUCTION

The seminal fluid of the rat is formed by the admixture of the secretions of the seminal vesicles, the prostate and the bulbo-urethral glands. The coagulating glands, which are applied to the medial surfaces of the seminal vesicles, are prostatic derivatives. Of these glands, the seminal vesicles are the most impressively developed. They contain the largest volume of isolable secretion and presumably contribute a major portion of the seminal plasma of the rat. Together with the secretion of the coagulating gland, vesicular fluid clots, resulting in formation of the vaginal plug. In addition, it appears to enter the uterus where it participates in the gelation of the ejaculum (Joshi, Yaron and Lindner, 1968). This suggests a possible involvement in capacitation.

The proteins of the seminal vesicle fluid (SVF) of the rat have received little attention (Gasser, 1972). In several other species, seminal vesicle proteins have been shown to coat the spermatozoa (Weil and Rodenburg, 1962). Because of its strong antigenicity this coating has been referred to as the spermatozoa-coating antigen. It has recently been suggested

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that removal of the proteins coating the spermatozoa is an essential part of the capacitation of rabbit sperm (Johnson and Hunter, 1971; Oliphant and Brackett, 1972).

This study had two objectives: to characterize the proteins of SVF of the rat and to determine the contribution of blood plasma to the SVF. Electrophoretic and immunological techniques were employed.

MATERIALS AND METHODS

Collection of seminal vesicle fluid (SVF)

Adult, male rats of our colony (Wistar derivation) were killed by cervical dislocation. The efferent ducts of the exposed seminal vesicles were clamped and the coagulating glands removed by blunt dissection. Each seminal vesicle was then incised and the fluid aspirated. Pooled SVF of two to three animals was diluted with nine volumes of sodium cacodylate-HCl buffer, pH 7.1, 0.1 M. The solution was vigorously mixed, then centrifuged at room temperature for 15 minutes at 1000 *g*. The precipitate (SVP) was separated and the supernatant, which will be referred to as stock solution was stored at -20°C.

Physico-chemical studies

The protein concentration in stock solutions of SVF was determined by measuring the absorbancy at 280 nm (in 0.1 M NaOH), assuming an extinction coefficient $E_{280\text{ nm}}^{0.1\text{ per cent}} = 1.4$, or by the Lowry method (Lowry, Rosenbrough, Fan and Randall, 1951). An approximation of the protein concentration in SVP was obtained by measuring the difference between the absorbancies at 280 and 360 nm at great dilution in 0.1 M NaOH, assuming the same extinction coefficient.

Conductivity measurements were performed with a Radiometer CDM-3 conductivity meter (Radiometer, Copenhagen), equipped with CDC 314 and CDC 114 cells. All measurements were performed at 27°. Saturated NaCl served as reference solution.

Zone electrophoresis of SVF was performed in 2 per cent w/v agarose (Agarose 37A, L'Industrie Biologique Française, SA, Gennevilliers, France) gels on microscope slides (Scheidegger, 1955). Sodium barbiturate-barbituric acid buffer, pH 8.2, 0.05 M, was used throughout. Electrophoresis was carried out at room temperature, at a constant current of 3.1 mA/cm (initial voltage: 32 V/cm). Proteins were stained with methanolic solutions of amido black or nigrosin. Polyacrylamide gel electrophoresis of protein subunits was performed as described by Maizel (1966). 0.1 ml of diluted stock solution containing 0.2 mg of soluble SVF protein was incubated for 12 hours at room temperature in a solution containing sodium dodecyl sulphate (0.5 per cent), urea (0.5 M) and 2-mercaptoethanol (2-ME) (0.1 per cent). Before application to the gel, an equal volume of dilute bromophenol blue in glycerol was added to the sample. Markers of known molecular weight used for calibration were BSA (mol. wt = 67,000), pepsin (mol. wt = 35,000), trypsin (mol. wt = 23,800) and myoglobin (mol. wt = 17,500), all from Mann Research Laboratories, New York, U.S.A.). A current of 5 mA/gel was applied until the bromophenol blue marker reached the bottom of the gel. The proteins in the gel were stained with Coomassie brilliant blue; the mobilities of the marker proteins were used to determine the molecular weights of the subunits of SVF (Fig. 5).

Sephadex gel filtration. A column (1.5 cm × 58 cm) of Sephadex G-200 beads equilibrated in phosphate-buffered saline (PBS) (0.15 M sodium chloride; 0.01 M sodium phosphate buffer, pH 7.5) was used. The column was packed and operated at 4°. The sample con-

tained 40 mg of soluble SVF protein and two markers: ^{125}I -labelled rabbit IgG ($0.5\ \mu\text{g}$, 5×10^4 cpm) and ^{125}I -labelled BSA ($0.1\ \mu\text{g}$, 8×10^4) cpm, all in the 1.5 ml of cacodylate buffer. The column was eluted with PBS. Flow rate was 12.5 ml/hour; 0.5 ml fractions were collected. The effluent was optically monitored at 280 nm. Radioactivity in each of the effluent tubes was determined in an Elron well-type gamma counter (ELRON Electronics, Haifa, Israel). The eluted protein fractions were separately pooled, concentrated by pervaporation, dialysed against cacodylate buffer and each fraction then subjected to zone electrophoresis in agarose gel.

Immunological studies

Production of antisera. Antisera to rat SVP and to normal rat serum (pool of four adult, male rats) were produced in female, randomly bred rabbits of local origin. One millilitre of serum or 10 mg of SVF proteins in 0.5 ml PBS, emulsified in an equal volume of Freund's complete adjuvant (FCA) (Difco Laboratory, Detroit, Minnesota), were injected into the two hind foot-pads and into the skin of the flanks of the animals; 3 weeks later an equal amount of antigen, emulsified in Freund's incomplete adjuvant (FIA) (Difco), was reinjected at multiple intradermal sites. The animals were exsanguinated about 3 weeks following the second injection. Serum was stored at -20° .

Purification of the IgG fraction of rabbit antiserum against rat serum proteins (RaRSP) and of normal rabbit serum (NRS): the globulin fraction of RaRSP and of NRS were precipitated, at 0° , by the addition of neutralized $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 40 per cent saturation. The precipitates were washed 3 times in cold, 40 per cent saturated ammonium sulphate, dissolved in 1 ml of 0.01 M Na phosphate buffer, pH 7.5 and dialysed exhaustively against this buffer. The sample was then chromatographed on DEAE-cellulose (exchange capacity 0.92 mEq/g; Cellex-D, Biorad Laboratory, Richmond, California); using 0.22 ml of packed resin/mg of protein, equilibrated in the same buffer. Only the protein which did not bind to the column under these conditions was used for radiolabelling.

Absorption of the antisera. Antisera to SVF were absorbed with normal rat serum, insolubilized with glutaraldehyde (Avrameas and Ternynk, 1969). Each antiserum sample (about 5 ml) was incubated with 1 ml of packed insoluble normal rat serum for 1 hour at 37° with occasional mixing, the insoluble material was then removed by centrifugation. This process was repeated three times for each serum sample.

Immuno-electrophoretic analyses and double diffusion studies were performed by the micromethod of Scheidegger (1955). Preparation of the gels and electrical conditions during the runs were as described for zone electrophoresis.

Radioimmunoassay. The IgG fractions of RaRSP and of NRS (10 mg of each) were labelled with $\text{Na-}^{125}\text{I}$ in dilute NaOH (specific activity > 14 Ci/mgI, The Radiochemical Centre, Amersham, U.K.) by the chloramine-T method (Hunter and Greenwood, 1962). Unreacted ^{125}I was removed from the reaction mixture by dialysis. Immediately before use, the labelled proteins were centrifuged at 17,000 rev/min for 10 minutes to eliminate aggregates.

Freshly prepared SVP from 2.2 ml of SVF was suspended in 5 ml of cacodylate buffer. For the binding assay, 0.5 ml of this suspension plus the labelled IgG fractions of either RaRSP or NRS (0.77 mg of each, 820,000 cpm and 900,000 cpm respectively) and buffer to make up 1 ml, were incubated at room temperature for 1 hour. The precipitates were collected by centrifugation (10 minutes at 4000 rev/min), washed twice in PBS and counted

in 1 ml of PBS in a Packard Auto Gamma spectrometer. In the experiments in which both unlabelled (5 mg) and labelled protein (0.77 mg) were added to SVP, incubation after addition of the unlabelled protein was 1 hour, followed by an additional incubation of 1 hour after addition of the labelled compound. Non-specific binding of iodinated protein to the plastic tubes accounted for 4000 cpm in tubes which had contained only labelled protein; when labelled protein was mixed with unlabelled protein (e.g. ^{125}I -labelled NRS + rat serum) non-specific binding dropped to 1200 cpm. Because of the large amount of insoluble material in each vial, it was unnecessary to use cold carrier protein to bring down the precipitates. Normal rat serum (25 μl) precipitated, under similar conditions (and equally without cold carrier), 38,000 cpm and 1240 cpm of ^{125}I -labelled IgG of RaRSP and ^{125}I -labelled IgG of NRS respectively.

RESULTS

In the process of obtaining SVF the coagulating gland was removed by dissection prior to incision of the seminal vesicle, thus minimizing the danger of contaminating SVF with coagulation-promoting material. Nevertheless, some of the SVF protein tended to drop

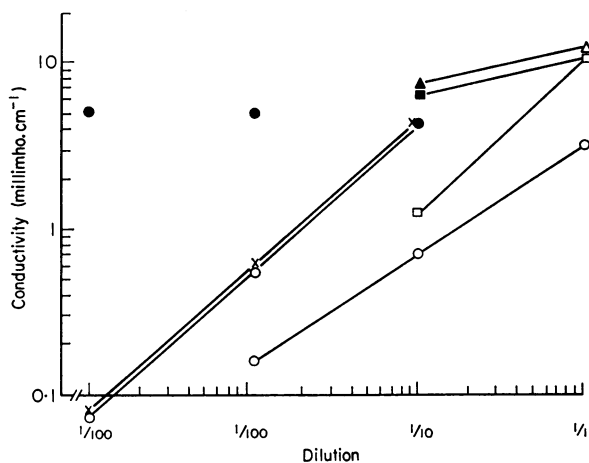


FIG. 1. Conductivity of seminal vesicle secretion. The solid symbols represent dilutions in 0.1 M Na cacodylate buffer, pH 7.1, conductivity 4.5 millimho/cm. The open symbols represent dilutions in deionized water, conductivity 0.5×10^{-3} millimho/cm. (○) Seminal vesicle secretion. (A dilution of 1/1 corresponds to the native secretion; a dilution of 1/10 to the concentration of SVF.) (Δ) Rat serum. (□) Human serum. (×) Na cacodylate buffer. Dilutions were made with water. (A dilution of 1/10 corresponds to 0.1 m.)

out of solution spontaneously. This process is different from blood coagulation; it was not inhibited by citrate or heparin added to the diluent. Different buffers, with pH between 3.5 and 9.5 and molarities ranging from 0.01 to 0.2, were compared as diluents of seminal vesicle secretion. When the collected fluid was diluted with nine volumes of Na cacodylate-HCl buffer, pH 7.1, 0.1 M, less precipitation of the proteins was observed, as judged visually, than in any of the others tested. Six different samples of stock solution contained between 23 and 27 mg of protein/ml, independently of the method used for protein determination, thus indicating a concentration of about 250 mg/ml of soluble protein in

the native secretion. The cacodylate-insoluble fraction (SVP) from each millilitre of native secretion contained 40 mg of protein.

The conductivity of the native SVF was 3.3 millimho/cm, contrasting with that of 12.8 millimho/cm of normal rat serum and 11 millimho/cm of normal rabbit and normal human serum (Fig. 1). Stock solution and dilutions thereof in deionized water displayed slightly lower conductivities than the corresponding dilutions of cacodylate buffer alone. The intrinsic conductivity of SVF is, therefore, low.

THE PROTEIN COMPONENTS OF SVF

These were separated by zone electrophoresis in agar gels. At the pH employed, 8.2, almost all of the proteins were located on the cathodic side; no anodic fractions were detected. Four cathodic fractions were distinguished by comparing the electrophoretic

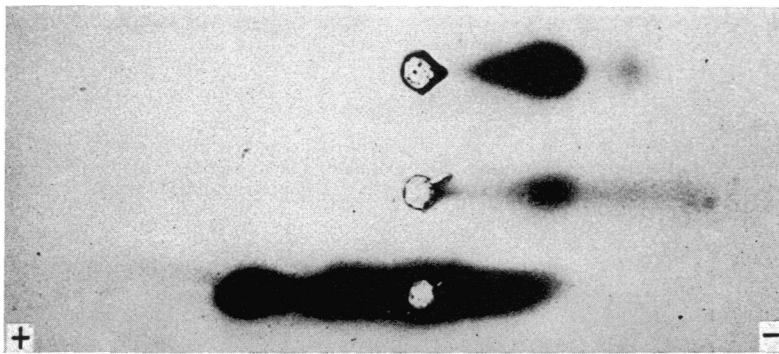


FIG. 2. Agar gel electrophoresis of SVF. The upper well contains a stock solution of SVF. The middle well contains a stock solution of SVF, diluted 1/5. The lower well contains normal rat serum. The cathode is to the right.

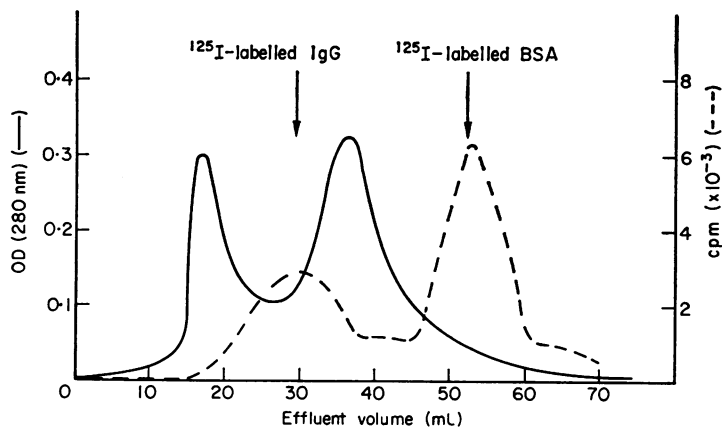


FIG. 3. Elution pattern of SVF from Sephadex G-200. To 40 mg of SVF in 1.5 ml cacodylate buffer two markers were added: ¹²⁵I-labelled rabbit IgG (0.5 μ g, 5×10^4 cpm); and ¹²⁵I-labelled BSA (0.1 μ g, 8×10^4 cpm). The column was equilibrated and eluted with PBS. 0.5 ml fractions were collected.

separations at different protein concentrations. Curiously, the most basic of these four was seen clearly only in the more dilute SVF (Fig. 2).

Gel filtration of stock solution on Sephadex G-200 resolved the SVF proteins into two

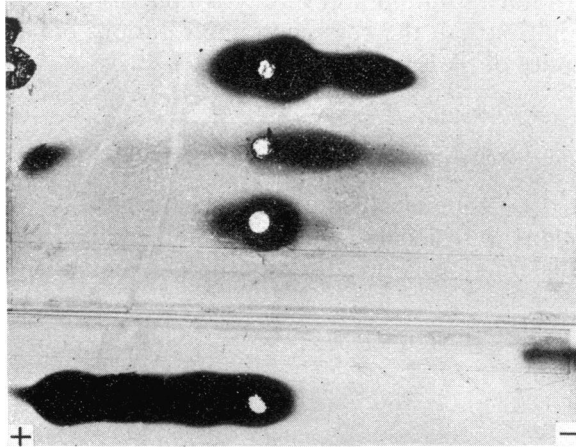


FIG. 4. Agar gel electrophoresis of the two fractions of SVF separated by chromatography on Sephadex G-200. The upper well contains a stock solution of SVF. The second well contains fraction I of SVF. The third well contains fraction II of SVF. The fourth well contains normal rat serum. The cathode is to the right.

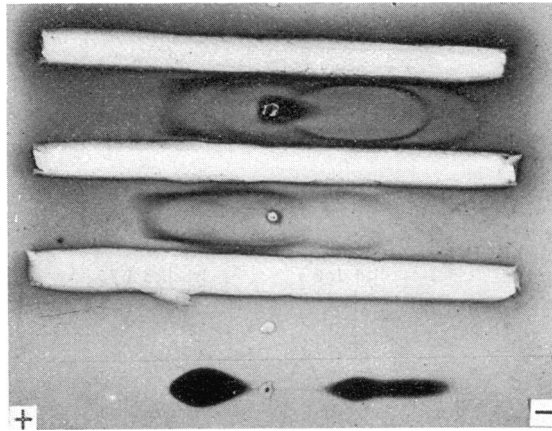


FIG. 5. Immunoelectrophoretic analysis of SVF. The upper well contains normal rat serum. The second well contains a stock solution of SVF. The third well contains a stock solution of SVF, diluted 1/5. The fourth well contains normal rat serum, diluted 1/5. The fifth well contains normal rat serum, electrophoresed simultaneously, and immediately fixed and stained. All the troughs contain rabbit anti-rat SVF serum. The cathode is to the right.

peaks, one emerging before the IgG marker, the other at an intermediate position between IgG and BSA (Fig. 3). The heavier proteins (the first peak to emerge from the Sephadex column) resolved into two very cathodic fractions and an anodic one with a mobility similar to serum albumin (Fig. 4). The proteins were of high molecular weight as judged

by their very limited diffusion in the agar. The proteins of the second peak resolved poorly in agar gel electrophoresis. There were apparently two cathodic fractions (Fig. 4). Thus, after Sephadex gel filtration an anodic-migrating protein fraction appeared, among the higher molecular weight proteins, which was not seen in the unfractionated stock solution.

In IE of SVF proteins, using rabbit anti-SVF antiserum, five clear precipitin bands were regularly observed. Three were cathodic, while two appeared on the anodic side (Fig. 5). Their localization coincided with that of the bands revealed by simple zone electrophoresis

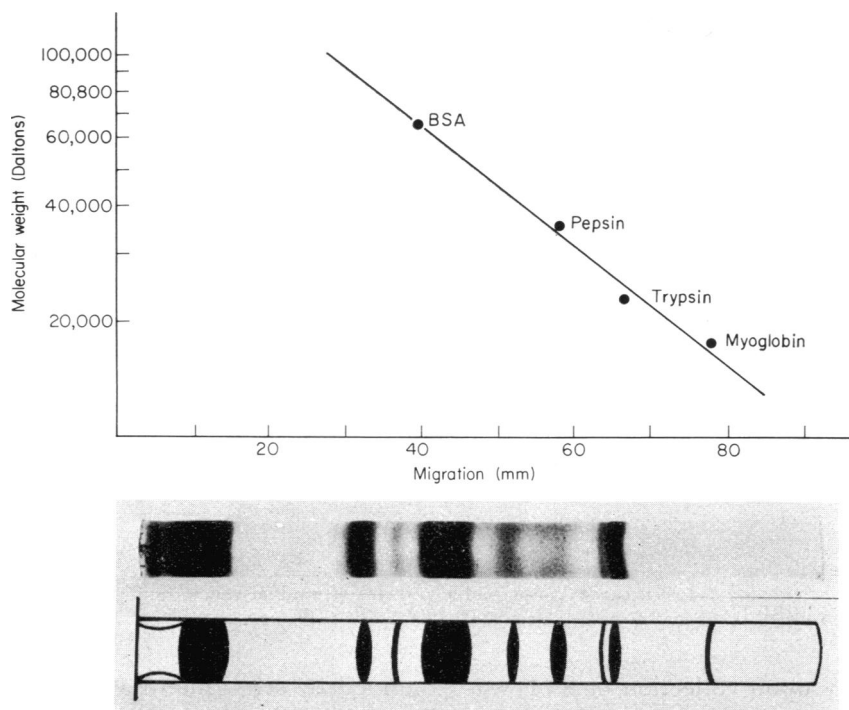


FIG. 6. Electrophoretic separation of the subunits of SVF proteins and of four marker proteins on sodium dodecyl sulphate polyacrylamide gel.

of the first Sephadex fraction. Proteins contained in the second Sephadex peak were not positively identified.

The subunits of SVF proteins, produced by dodecyl sulphate + urea + 2-ME dissociation were separated according to molecular weights in polyacrylamide gel electrophoresis. At least eight bands ranging in molecular weight between 18,500 and 98,000 Daltons were found (Fig. 6). In addition, one or two bands close to the origin were prominent. Because of their high molecular weight, these were assumed to represent the aggregates and/or incompletely dissociated molecules commonly seen in this technique.

SERUM PROTEINS IN SVF

Two different rabbit antisera to normal rat serum did not react with any dilution of

stock solution tested in double diffusion or in IE. Neither did two anti-SVF sera react with rat serum under similar conditions. This indicates that: (a) there are no serum proteins in the cacodylate-soluble fraction of SVF; (b) the soluble proteins of rat SVF do not share any antigenic determinants with rat serum proteins. To further confirm this latter conclusion, antiserum to SVF, was absorbed with rat serum, insolubilized with aqueous glutaraldehyde (Avrameas and Ternynk, 1969). This absorption of antiserum produced no difference in the pattern of precipitin bands elicited in gel diffusion or in IE.

The failure to detect serum proteins in the stock solution may indicate their removal from the SVF by coprecipitation with or by absorption to the precipitate (SVP) which forms after the dilution of SVF with cacodylate buffer. Therefore, the SVP which formed

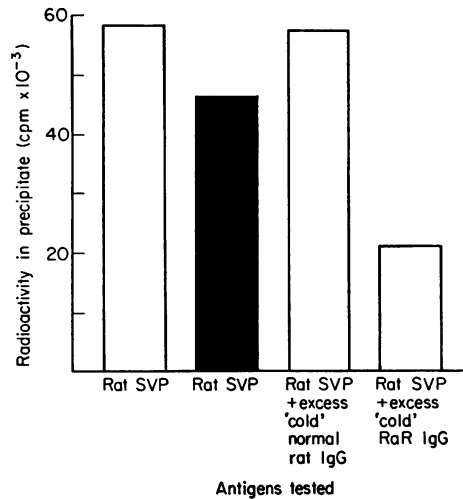


FIG. 7. Interaction between cacodylate-insoluble proteins of seminal vesicle fluid (SVP) with ¹²⁵I-labelled RaRSP IgG (□) and with ¹²⁵I-labelled IgG of NRS (■).

immediately upon collection of SVF was examined by semi-quantitative radioimmunoassay for the presence of serum proteins. Design of this test was complicated by the finding that SVP binds radioiodinated IgG from normal rabbit serum and IgG from rabbit antiserum to rat serum proteins to almost identical extent (Fig. 7). In an attempt to eliminate this nonspecific binding of IgG by SVP, an excess of unlabelled IgG from normal rabbit serum was preincubated with SVP. The binding of labelled antibody to rat serum was not significantly decreased. The specificity of this reaction with labelled antibody was confirmed by the highly significant decrease in binding obtained when SVP was preincubated with unlabelled antibody to rat serum proteins. These results are consistent with the presence of small amounts of rat serum proteins or of proteins sharing common antigenic determinants with serum proteins in the insoluble fraction of seminal vesicle proteins.

DISCUSSION

The most striking features of rat SVF can be summarized as follows.

(1) The concentration of protein is surprisingly high. That of the cacodylate-soluble proteins was approximately 250 mg/ml. Since the precipitate (SVP) represented an

additional 40 mg of protein/ml, the native secretion *in situ* must contain at least 290 mg of protein/ml.

(2) SVF contains no free serum proteins. Using extremely sensitive methods, traces of serum proteins were detected bound to SVP.

(3) Only six distinct soluble protein fractions were detected. Two of these are major components and two others are also present in important concentrations. These four major proteins are basic.

(4) At least, some of the proteins of SVF are strongly immunogenic in the rabbit. Three of the cathodic and both of the anodic proteins separated in agarose electrophoresis reacted strongly with antiserum.

The unusually high concentration of protein in SVF and the large volume of stored secretion in the seminal vesicles imply that SVF is the major source of seminal plasma proteins in the rat.

It would be of interest to pinpoint the structural features which enable the gland (a) to maintain such strikingly high protein concentrations and (b) exclude serum proteins from the lumen of the gland. We are not aware of any other body fluid which contains no serum albumin.

In this study we found that the *insoluble* proteins did, in fact, contain traces of serum protein antigens; the possibility of these arising from contamination by blood during isolation of the glandular contents cannot be excluded.

The major SVF protein has a mobility similar to that of the most basic IgG. This basic protein, unless shielded by high concentration of ions would exercise an electrostatic attraction towards acidic proteins. In fact, the ionic strength of SVF is only one quarter of that of serum, as measured by their conductivities, suggesting that such an electrostatic attraction may indeed be operative.

The number of rat vesicular fluid proteins is small, at most six. Four are major components; the two others are more acidic proteins which were detected only in immunoelectrophoresis or after separation of SVF on Sephadex G-200. On the other hand, the most basic protein of SVF was detectable only after dilution (see Fig. 2). This suggests that dilution may play an important role in the association or dissociation of protein-protein complexes within SVF. However, the possibility that the minor fractions represent degradation products formed upon storage can not be ruled out. Thus, in fact, in native rat vesicular secretion, the number of proteins may even be less than six. The number of SVF proteins has also been reported to be low in other species of rodents such as the guinea-pig (Orsini and Shulman, 1971), and the mouse (Plat and Wolfe, 1969).

Only eight bands representing dissociated subunits were resolved by polyacrylamide gel electrophoresis. Two others were localized close to the origin where molecular weights can only be roughly estimated; they may represent aggregated material and/or incompletely dissociated protein ranging between 150,000 and 200,000 Daltons. The first clearly resolved subunit has a molecular weight of about 100,000 Daltons. On the other hand, two of the proteins of SVF eluted from Sephadex G-200 at a position indicating a molecular weight much greater than 150,000 Daltons. Obviously, these two heavy proteins must have dissociated into several, possibly similar, subunits. The formation of the quaternary structures of the SVF proteins is thus achieved by the association of a limited number of subunits.

Antisera, prepared in rabbits against rat SVF, produced six precipitin arcs in IE of rat SVF. This, however, can be explained by assuming either the presence of six immunogenic

proteins in SVF, or by the formation of antibodies to a smaller number of subunits which are common to some of the six proteins separated by agarose electrophoresis. That the number of immunogens may be less than the number of proteins has been reported in the guinea-pig, where only one of six proteins elicited antibodies in rabbits (Orsini and Shulman, 1971).

Certain proteins of the seminal vesicle have been found to bind firmly to the membrane of ejaculated spermatozoa. Due to the high immunogenicity of this coating protein it has been termed the spermatozoon:coating antigen (Weil and Rodenburg, 1962). It can be speculated that this binding results from the basic charge of these proteins. Another function of the basic SVF proteins is probably to complex with some or all of the acidic serum proteins present in the female genital tract. As a result, the composition of the microenvironment in the female, in which sperm cells are capacitated is determined in some measure by these proteins.

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