

Rete Testis Fluid (RTF) Proteins: Purification and Characterization of RTF Albumin¹

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ABSTRACT

A major 68-kDa protein in ram rete testis fluid (RTF) is shown to be chemically and immunologically indistinguishable from albumin in ovine serum. Data obtained with two-dimensional gel electrophoresis of RTF demonstrate the presence of additional proteins with a molecular mass of 68 kDa that do not react with antisera against sheep serum albumin. Biochemical characteristics of albumin preparations isolated by immunoaffinity chromatography from ovine serum and from RTF were compared. Albumin from both sources had the same apparent molecular mass of 68 kDa, the same isoelectric point of approximately 4.2, and neither bound specifically to Concanavalin A. Analysis of tryptic peptide maps, obtained with reverse-phase high-pressure liquid chromatography, indicated no significant differences between digests of the two purified albumin preparations. Results indicate that RTF albumin and serum albumin are the same protein, which implies that RTF albumin may originate from serum. Albumin levels in RTF, collected from different rams and measured by radioimmunoassay, varied between 46 and 164 µg/ml, constituting between 11 and 17% of total RTF protein, while albumin levels in sheep plasma were 40,000 µg/ml. The protein composition of RTF is discussed in relation to the relative amounts of various components contributed by testis cells and the amounts derived from serum.

INTRODUCTION

During mammalian spermatogenesis, spermatozoa are released into the lumen of the seminiferous tubule in the presence of testicular fluids. Sperm and fluids are then transported to the rete testis and hence to the head of the epididymis via efferent ducts. Rete testis fluid (RTF), collected from conscious rams by inserting a catheter through the efferent ducts into the extratesticular rete (Voglmayr et al., 1966), contains components secreted by various classes of testicular cells and by rete testis epithelial

cells (Setchell, 1974; Waites, 1977; Waites and Gladwell, 1982). RTF also appears to contain components that are derived from serum. The ionic, carbohydrate, and amino acid composition of RTF have been well characterized (for reviews, see Setchell, 1970, 1978; Waites and Gladwell, 1982). These components provide the chemical environment in which spermatozoa are maintained prior to their transport to the epididymis.

Protein levels in RTF are about 100 times less than those in plasma (Setchell and Wallace, 1972). Although the presence of many protein bands has been detected by electrophoresis of RTF (Koskimies and Korman, 1973; Wright et al., 1981), only a few proteins have been identified and characterized. Clusterin has recently been shown to comprise approximately 15%

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of the protein in ram RTF (Blaschuk et al., 1983; Blaschuk and Fritz, 1984; Fritz et al., 1984). Among other proteins in RTF, many are reported to have the same electrophoretic mobilities as those in plasma (Koskimies and Kormano, 1973; Wright et al., 1981). Transferrin, produced and secreted by Sertoli cells (Skinner and Griswold, 1980), is present in RTF (Sylvester and Griswold, 1984). An albumin-like band that stains intensely with Coomassie Brilliant Blue has been estimated by microdensitometric analysis to represent 41% of total protein in rat serum and 14% of total protein in rat RTF (Koskimies and Kormano, 1973).

Some of the proteins in ram RTF have been shown to originate primarily from testicular or rete cells. This is thought to be the case for clusterin, since both Sertoli cells and rete cells can synthesize this protein, and clusterin levels in plasma are far lower than those in RTF (Blaschuk et al., 1983; Fritz et al., 1984; Tung and Fritz, 1985). A major secretory protein of rat Sertoli cells and epididymal cells, named "dimeric acidic glycoprotein," has been characterized and associated with the surface of spermatozoa (Sylvester et al., 1984). This protein and clusterin share many of the same chemical properties and may be homologous. Other proteins produced by Sertoli cells that are present in RTF include transferrin and androgen-binding protein.

Aside from these proteins released by testicular cells, additional proteins in RTF could be derived from the passage of proteins from plasma and lymph into the rete. Previously, Everett and Simmons (1958) injected labeled serum albumin, and determined radioautographic localization of labeled material in the testis. Mancini et al. (1965) presented data indicating that labeled serum albumin penetrated the seminiferous tubule. However, Christensen et al. (1985) observed, with careful immunocytochemical techniques, the absence of detectable albumin in rat seminiferous tubule fluid. On the other hand, ^{125}I -labeled albumin, administered systemically has been reported to penetrate the rete testis slowly, as measured by the appearance of labeled material in ram and rat rete testis fluid (Setchell and Wallace, 1972). However, the ^{125}I -labeled moiety measured could have included material other than albumin, since the labeled material counted in RTF was not isolated or shown to be identical with albumin. Unfortunately, these data fail to allow unambiguous interpreta-

tion, but they suggest that albumin can penetrate the rete testis.

As indicated previously, RTF has been reported to contain protein(s) with a molecular mass of 68 kDa (Waites and Gladwell, 1982). In this communication, we present data indicating that the 68 kDa band in RTF contains more than one species of protein. Among the proteins in this band, one has chemical characteristics indistinguishable from those of ovine serum albumin. Results to be reported demonstrate that this RTF albumin comprises between 11 and 17% of total proteins in ram RTF and is most probably derived exclusively from serum.

MATERIALS AND METHODS

Rete Testis Fluid

Different samples of ram rete testis fluid were generously provided by Dr. M. Courot (Nouzilly, France), Dr. B. Setchell (Adelaide, Australia); and Dr. J. K. Voglmayr (Melbourne, FL). Free flow fluid was collected from conscious adult rams by methods previously described (Voglmayr et al., 1966). Fluid was frozen, shipped on dry ice, and kept at -20°C until use. Samples were transferred to buffers containing benzamide and phenylmethylsulfanyl fluoride (PMSF) upon use.

Electrophoresis

Electrophoretic analysis of protein was performed using 5 to 15% polyacrylamide gradient slab gels with the Laemmli sodium dodecyl sulfate (SDS)-buffer system (Laemmli, 1970). All samples were reduced with β -mercaptoethanol and heated at 95°C for 10 min prior to electrophoresis. The procedure of O'Farrell (1975) was used for two-dimensional gel electrophoresis. Gels not blotted to nitrocellulose were stained with Coomassie Brilliant Blue.

Immunoblotting

Transfer of protein to nitrocellulose following electrophoresis was accomplished by laying the SDS gel on a strip of nitrocellulose, both having been soaked in transfer buffer, and applying a constant voltage of 6V overnight. The transfer buffer contained 150 mM glycine, 20 mM tris(hydroxymethyl)amino-methane (Tris) base and 20% methanol (Towbin et al., 1979). Immediately after the transfer, nitro-

cellulose strips were either stained with 0.1% amido black in 45% methanol:10% acetic acid or reacted with antibodies for an immunoblot. To immunoblot the proteins bound to nitrocellulose, the strips were soaked for 15 min each in two changes of Tris-buffered saline (TBS:10 mM Tris, 150 mM NaCl, pH 7.5) followed by a 15-min incubation in TBS plus 10% calf serum. The first antibody was added to fresh TBS plus 10% calf serum to a final dilution of 1:50 and incubated at room temperature for 1 h and then overnight at 4°C. The strip was washed twice for 20 min in TBS, 15 min in TBS with 0.5% triton X-100, and then for 10 min in TBS. The second antibody, labeled with ¹²⁵I using the chloramine T method, was added to 15 ml of TBS plus 10% calf serum and incubated with the strip at room temperature for 4 h; then it was washed, as above, to remove unreacted antibody. The immunoblotted nitrocellulose strip was air-dried and applied to preflashed Kodak X-Omat x-ray film for autoradiography.

Albumin Purification

For isolation of albumin from ram rete testis fluid or sheep serum, the dialyzed supernatant from a 50% saturated ammonium sulfate precipitation was added to an anti-sheep albumin affinity gel suspension and rotated end-over-end overnight at 4°C. Rabbit anti-sheep albumin immunoglobulin G (IgG, Cappel Lab., West Chester, PA) and coupled to cyanogen bromide-activated Sepharose 4B as previously described (Skinner et al., 1984). The gel suspension was poured into a column and washed with 2 column volumes of each of the following buffers: A) 50 mM Tris, 0.5 M NaCl, pH 7.5; B) 50 mM sodium acetate, 0.5 M NaCl, pH 4.0; C) 50 mM glycine, 0.5 M NaCl, pH 2.5. Purified material from both serum and RTF was eluted in the wash at pH 2.5. The pH 2.5 eluent was collected, dialyzed for 48 h at 4°C, lyophilized, and reconstituted in a small volume of 10 mM Tris, pH 7.5.

Chromatofocusing and Concanavalin A Chromatography

Purified albumin was iodinated with ¹²⁵I, using the chloramine T procedure as previously described (Skinner and Griswold, 1982), for chromatofocusing and concanavalin A chromatographic analysis. Chromatofocusing utilized 10 ml of a polybuffer exchanger gel (PBE-94, Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 10 column volumes of 25 mM imidazole, pH 7.0. Before addition of

iodinated albumin, one-half column-volume of the first eluent buffer (polybuffer 74, diluted 1:11, degassed and adjusted to pH 4.0) was allowed to pass through the column. The sample was applied to the column and eluted with 10 column volumes of the pH 4.0 polybuffer, followed by elution with 10-column volumes of polybuffer 74 (diluted 1:11 and degassed) adjusted to pH 3.0. Fractions (5 ml) were collected and the radioactivity in each was measured with a gamma counter, and the pH of each was determined with a pH meter.

Concanavalin A (Sigma Chemical Co., St. Louis, MO) chromatography utilized a 10-ml column equilibrated in 50 mM Tris, 0.5 M NaCl, pH 7.5. The sample was applied to the column and eluted with 5 column volumes of the equilibration buffer before elution with 0.1 M α -methylmannoside, 50 mM Tris, 0.5 M NaCl, pH 7.5. Fractions, 2 ml, were analyzed for radioactivity with a gamma counter.

High-Pressure Liquid Chromatography (HPLC) Peptide Mapping

Peptide mapping of purified RTF and serum albumin utilized reverse-phase HPLC on a Brownlee Aquapore C8 column with a Beckman gradient HPLC apparatus (Skinner et al., 1984). Purified protein, 100 μ g, was reduced with 1% β -mercaptoethanol for 4 h at room temperature and then lyophilized. Reduced protein was reconstituted in 100 μ l of 10 mM Tris, 150 mM NaCl, pH 7.5, and incubated in the presence of 5 μ g of tosylphenylchloroketone-treated trypsin (Sigma Chemical Co.) for 12 h at 37°C. The sample was then applied to the HPLC column equilibrated in 15 mM phosphoric acid, pH 3.0, and the peptides were eluted with a 100-min linear gradient to 30% acetonitrile (Burdick and Jackson) in 15 mM phosphoric acid, pH 3.0. Peptide elution was monitored at both 214 nm and 280 nm.

Albumin Radioimmunoassay

Levels of albumin in RTF were determined by a radioimmunoassay using sheep albumin and rabbit anti-sheep albumin (Cappel Lab.) with a procedure previously described (Skinner and Griswold, 1982). Samples were incubated at 37°C for 1 h with 30,000 cpm iodinated albumin and sheep albumin antibody (1:27,000 final dilution) in buffer containing 2.5 mg/ml gelatin, 50 mM Tris, 0.15 M NaCl, pH 7.5, and 1 mM ethylenediaminetetraacetate (EDTA) in 1.8 ml volume. Goat anti-rabbit immunoglobulin (Sigma

Chemical Co.), 200 μ l, was then added (1:1500 final dilution) and incubated at 37°C for 1 h. One ml of polyethylene glycol buffer (132 mg/ml polyethylene glycol 4000 in 50 mM Tris, pH 7.5) was then added and incubated at room temperature for 15 min. Samples were centrifuged at 2000 \times g for 2 h at 4°C, and the amount of radioiodinated albumin in the pellet was determined. The radioimmunoassay was linear in the range from 10 to 250 ng albumin, and had a 10% coefficient of variation. Levels of albumin in RTF were normalized per mg of total protein determined with a modified Lowrey procedure (Hartree, 1972).

RESULTS

After electrophoretic separation of serum and RTF proteins on SDS gels, the proteins were transferred to nitrocellulose and stained (Fig. 1C, D). As expected,

the major protein in serum was a 68-kDa albumin band. The major band in RTF was also a 68-kDa protein (Fig. 1D). An immunoblot of both serum and RTF demonstrated that the 68-kDa protein in RTF was immunologically similar to serum albumin (Fig. 1A, B). Additional stained bands were observed primarily in the serum sample (Fig. 1B). This could be due to nonspecific binding to denatured proteins from the electrophoretic conditions or nonspecific binding of the second antibody. Under more stringent buffer, incubation, and loading conditions, only a 68-kDa band was detected, as shown in Figure 2. Two-dimensional gel electrophoresis was used to determine if more than one 68-kDa protein was present in RTF. A stain of the proteins revealed a minimum of three 68-kDa bands with overlapping isoelectric points (Bands a, b, c, Fig. 2A). An immunoblot of this two-dimensional gel demonstrated that, predominately, Band b was detected with the

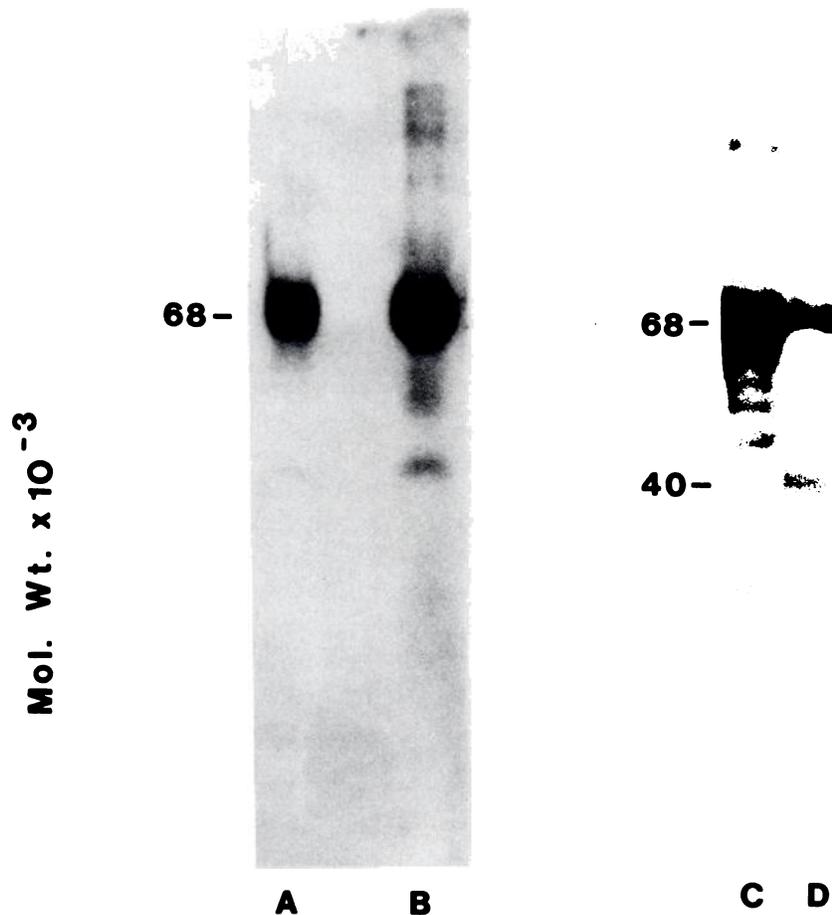


FIG. 1. Albumin immunoblot of rete testis fluid (RTF, Lane A) and sheep serum (B) on 5 to 15% polyacrylamide gradient SDS gels. A rabbit anti-sheep albumin was visualized using an iodinated second antibody and autoradiography. Lanes C (serum) and D (RTF) show transferred proteins stained with amido black.

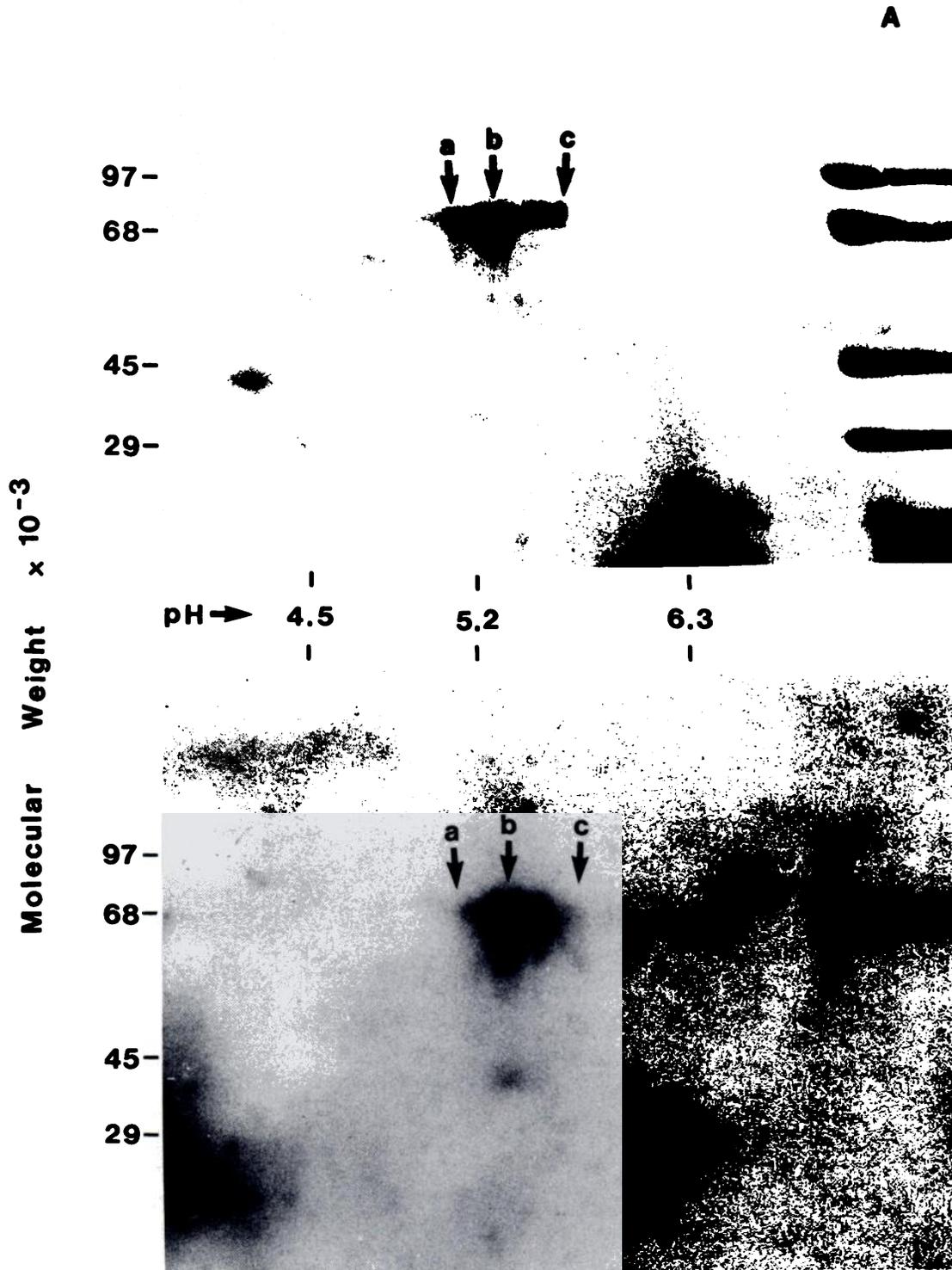


FIG. 2. Two-dimensional gel electrophoresis and albumin immunoblot of rete testis fluid. (A) Proteins transferred to nitrocellulose and stained with amido black. (B) Immunoblot using rabbit anti-sheep albumin and autoradiography. Arrows denote proteins with differential reactivity with amido black and the immunoblot. Protein bands on the right side of figure are molecular weight markers.

anti-albumin while Bands a and c had minimal reactivity (Fig. 2B). These results indicate that RTF contains a 68-kDa protein that is immunologically similar to serum albumin and that additional 68-kDa proteins are also present.

To determine whether the albumin in RTF was a modified form of serum albumin or possibly a different gene product, albumin was isolated from RTF and serum for comparison. Immunoaffinity chromatography was used to isolate both RTF albumin and serum albumin, and SDS-gel electrophoresis was used to assess purity. A Coomassie Brilliant Blue stain of both RTF and serum are shown in Figure 3A, C. RTF was found to have major protein staining bands at 70, 68, 50, 40 and 25 kDa. Immunoaffinity-purified RTF and serum albumin had a homogeneous 68-kDa band after electrophoresis (Fig. 3B, D). This purified albumin was used for subsequent biochemical characterization.

Both RTF albumin and serum albumin were

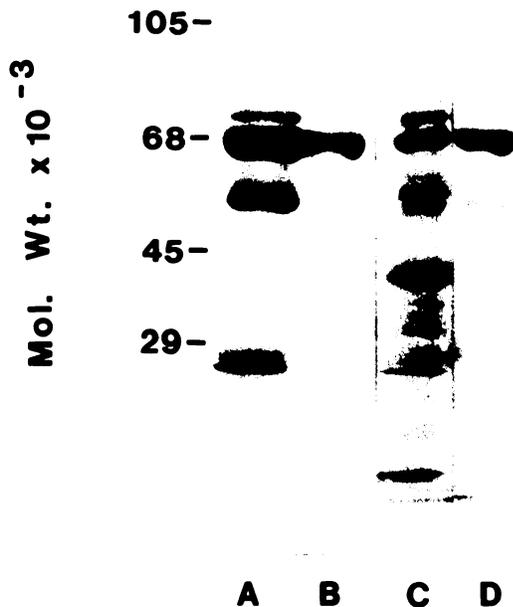


FIG. 3. Electrophoretic profile of serum and rete testis fluid (RTF) proteins. Coomassie Brilliant Blue stained 5 to 15% polyacrylamide gradient-SDS gel of serum (Lane A) and RTF (Lane C). Immunoaffinity purified albumin from serum (Lane B) and RTF (Lane D) was eluted from a rabbit anti-sheep albumin affinity column at pH 2.5.

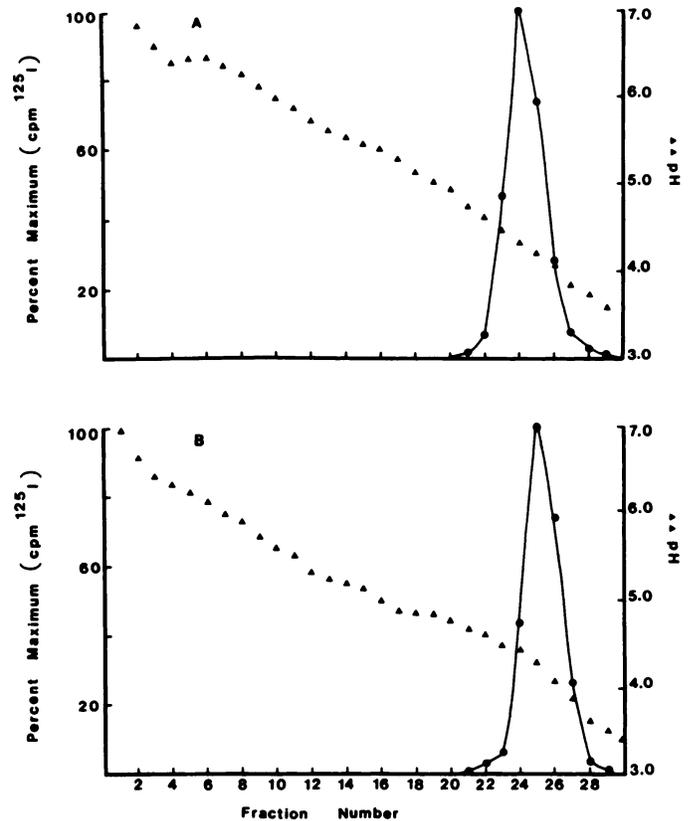


FIG. 4. Chromatofocusing of immunoaffinity purified iodinated albumin from sheep serum (A) and rete testis fluid (B). Fractions collected were assessed for radioactivity (\bullet), expressed as percent of maximum $\text{cpm } ^{125}\text{I}$, and for pH (\blacktriangle).

iodinated to assist in data analysis of isoelectric point determination and concanavalin A chromatography. Chromatofocusing of the iodinated albumin demonstrated that both RTF and serum albumin have the same isoelectric point of approximately 4.2 (Fig. 4). Concanavalin A-affinity chromatography was utilized to assess the possible glycoprotein nature of RTF albumin. Both the RTF and serum albumin eluted in the void volume of the concanavalin A column with no detectable specific binding (data not shown). As a positive control, iodinated serum transferrin was found to bind specifically to the concanavalin A column and was eluted with α -methylmannoside (data not shown). These results indicate that RTF albumin and serum albumin have the same isoelectric point and no apparent specific binding to concanavalin A.

A more rigorous comparison of serum albumin and RTF albumin was made using a tryptic peptide map analyzed by reverse-phase HPLC. Absorption spectra at 214 nm revealed approximately 65 different

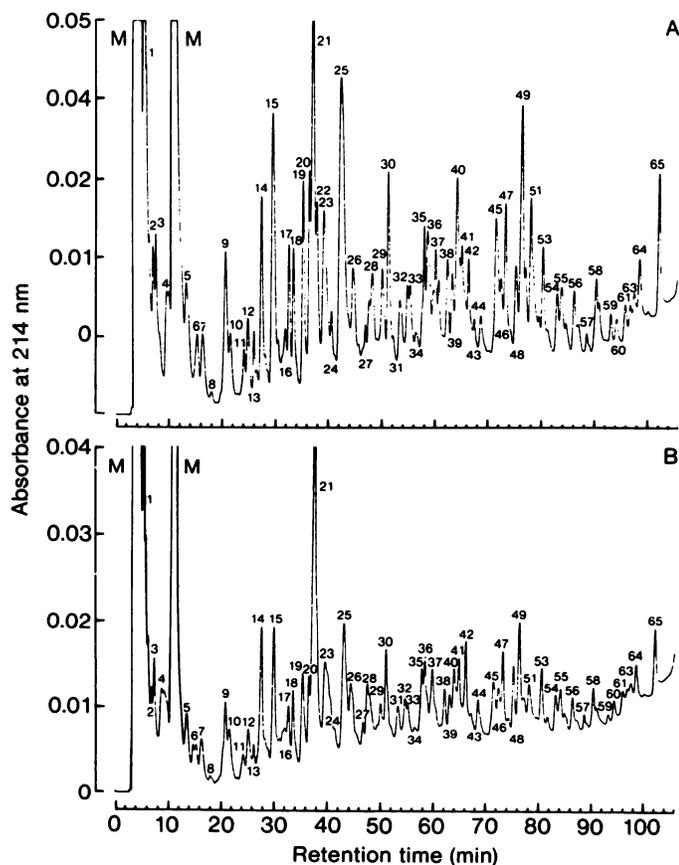


FIG. 5. High-pressure liquid chromatography (HPLC)-tryptic peptide map of serum albumin (A) and rete testis fluid (RTF) albumin (B). Peptides were eluted from a C8 reverse-phase column with a 100-min linear gradient to 30% acetonitrile in the presence of 15 nM phosphoric acid. Peptide elution was monitored at 214 nm and retention time expressed in min. Peaks designated M were due to β -mercaptoethanol reduction of the proteins prior to digestion with 5% trypsin (w/w). The profiles presented are selected profiles of similar profiles from three separate experiments. Numbered peaks were present in all profiles obtained and were similar between the serum albumin and RTF albumin.

tryptic peptides that were similar for both RTF and serum albumin (Fig. 5). The profiles presented are representative of a minimum of three separate experiments in which the magnitudes of individual peaks varied slightly but the same peaks were consistently present with similar retention times. Analysis at 280 nm also revealed the same peaks, numbered 1 through 8, for both RTF albumin and serum albumin (Fig. 6). No significant difference between RTF albumin and serum albumin was detected using this peptide-mapping analysis. Therefore, factors such as contaminating proteins and digestion conditions do not appear as major variables.

The levels of albumin in RTF were determined with an albumin radioimmunoassay. RTF from three

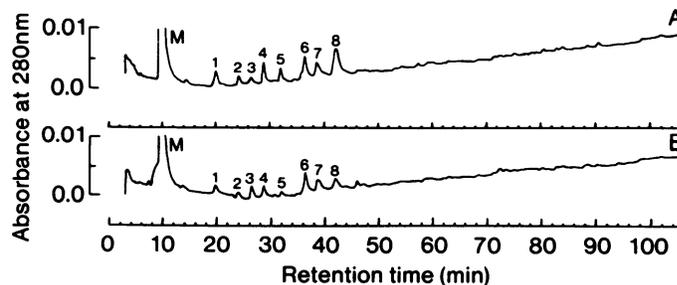


FIG. 6. High-pressure liquid chromatography (HPLC)-tryptic peptide map of serum albumin (A) and rete testis fluid albumin (B). The same conditions were used as outlined in the legend for Figure 5, except the peptide elution was monitored at 280 nm.

different sources was examined and designated RTF A, RTF B and RTF C. The concentration of albumin in sheep serum was determined to be approximately 40 mg per ml, which is similar to that previously reported (Peters, 1975). Levels of albumin in RTF from different samples ranged from 46 to 164 μ g/ml, constituting 11 to 17% of total RTF protein (Table 1). These results confirm the presence of albumin in RTF and demonstrate that levels of RTF albumin ranged between 0.12 and 0.41% of values in serum. In the establishment and validation of the albumin radioimmunoassay, it was demonstrated that RTF and ovine albumin have parallel displacement curves in the assay (Fig. 7). This information indicates that the antigen detected in the RTF by the radioimmunoassay is immunologically similar to ovine albumin.

To determine the possible presence of additional serum proteins in RTF, the presence of IgG was investigated. Immunoblots of serum and RTF proteins with rabbit anti-sheep IgG revealed that the same proteins were detected in both serum and RTF (data not shown). For unknown reasons, exact amounts could not be determined reproducibly by radioimmunoassays with antibodies available against sheep

TABLE 1. Levels of albumin and protein in rete testis fluid (RTF).

Sample	μ g Albumin/ml ^a	μ g Protein/ml ^b	Albumin/protein (%)
RTF A	46 \pm 4	265	17
RTF B	164 \pm 6	1470	11
RTF C	73 \pm 4	611	12

^aValues for albumin were determined with a radioimmunoassay and are expressed as μ g albumin/ml RTF and represent the mean \pm SEM for n=5.

^bValues for protein are expressed as μ g protein/ml RTF.

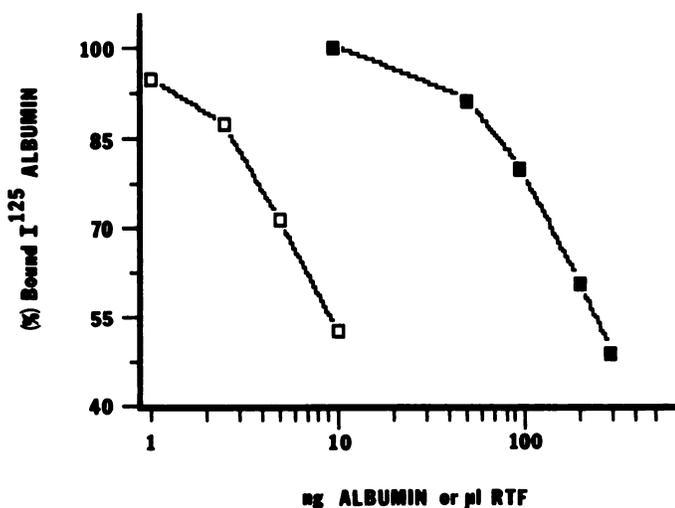


FIG. 7. Albumin radioimmunoassay displacement curves for ovine albumin (■) or rete testis fluid (RTF, □). The relative percentage bound 125 I-albumin was determined with increasing concentrations of ovine albumin, expressed as ng albumin, or RTF, expressed as μ l RTF. Values are the mean of a triplicate determination from a representative experiment that was repeated a minimum of three times with different RTF preparations. In all experiments, RTF and ovine albumin had parallel displacement curves.

IgG. However, a minimum of 25 μ g/ml IgG was detected in RTF. Further studies will be required to determine the biochemical characteristics and exact quantities of immunoglobulin present in RTF.

DISCUSSION

The biochemical characteristics of the albumin-like molecule from ram RTF have been compared with those of sheep serum albumin. In both cases, the proteins have been isolated by immunoaffinity chromatography, using antibodies against sheep serum albumin linked to sepharose. Purified albumin from serum and RTF each had the same molecular mass (68 kDa) and isoelectric point (4.2). Chromatofocusing provides a more accurate measure of the physiological isoelectric point than two-dimensional gel electrophoresis. High concentrations of urea (9M) present during the isoelectric focusing is likely to alter the apparent pI. We therefore place greater reliability on the pI value of 4.2 determined by chromatofocusing than on the apparent pI value of 5.3 determined by two-dimensional electrophoresis. The influence of iodination on the pI of the albumins remains to be determined. With either procedure, however, the pI value for serum albumin was indistinguishable from that of RTF albumin.

The 68-kDa albumin-like molecule was not the only protein in RTF with this molecular mass. Analysis by two-dimensional gel electrophoresis revealed the presence of three species of protein in RTF that had an apparent molecular mass of 68 kDa. However, only one was reactive with antibodies against sheep serum albumin. Two other species of proteins, having the same molecular mass, are also present in RTF, and are immunologically distinct from serum albumin. Therefore, estimates of albumin levels by microdensitometric analysis of 68-kDa bands after electrophoresis of RTF would be inaccurate.

Neither serum albumin nor RTF albumin specifically bound to concanavalin A, indicating the absence of available mannosyl linkages and suggesting that neither albumin is a glycoprotein. However, the presence of non-mannosyl residues remains to be determined. Analysis of tryptic peptide maps of serum albumin and of RTF albumin, isolated by reverse-phase HPLC, revealed no significant differences between the two proteins in apparent peptide amino acid sequences. No modified peptides were detected in either protein. Combined results demonstrate that RTF albumin is indistinguishable from serum albumin by the immunological and chemical criteria employed. It therefore appears plausible that albumin in RTF originates from albumin in serum.

It is possible that Sertoli cells or rete cells might be a source of RTF albumin. However, we have not been able to detect the presence of serum like albumin molecules in the medium during culture of Sertoli cells or rete testis cells (unpublished observations). Immunoprecipitates of rat Sertoli cell-radiolabeled secreted proteins with an antisera to rat serum albumin gave negative results (unpublished observation). These results imply that neither of these cell populations synthesize the serum-like albumin in RTF. The presence of an albumin-like protein that may be functionally similar but chemically and immunologically distinct from serum albumin remains a possibility to be investigated. A preliminary report by Cheng and Bardin (1986) has implied that such a protein may be present in Sertoli cell-conditioned medium. These authors isolated a 68-kDa protein they called testibumin and produced an antisera to the protein. Denatured carboxymethylated serum albumin cross-reacted with the testibumin antisera. However, the native proteins, serum albumin or testibumin, appeared not to be cross-reactive with

either the testibumin antisera or serum albumin antisera. Therefore, testibumin and albumin appear to be chemically and immunologically distinct, but upon denaturation, they show some immunological similarities. Radiolabel amino acid incorporation into testibumin by Sertoli cells was not demonstrated, and the functional similarities between testibumin and albumin are not presently known. The results of Cheng and Bardin (1986) support our unpublished observations and confirm that Sertoli cells do not appear to produce albumin nor are they the source of the serum-like albumin in RTF. The possibility that an albumin-like protein, distinct from serum albumin, may be produced by Sertoli cells has some interesting physiological implications. Due to the lack of serum albumin beyond the blood-testis barrier, such an albumin-like protein may be needed within the seminiferous tubule. Because the serum albumin concentration in the RTF is relatively high, the need for an additional albumin-like protein would not appear to be required unless some unique function was present that could not be performed by serum albumin. Therefore, it would not appear necessary that the reproductive tract, excluding possibly the seminiferous tubules, would require a locally produced albumin-like protein to carry out the functions of serum albumin.

Levels of albumin in RTF obtained from different rams ranged between 46 and 164 $\mu\text{g/ml}$, and constitute 11 to 17% of total proteins in these RTF samples (Table 1). Available information is insufficient to provide an explanation for the relatively large range of these values. In addition to biological variations among animals, many factors could have influenced the albumin levels, including the possible contamination by non-rete fluids during one or more of the collections. In spite of these variations, it is nevertheless evident that the albumin concentrations in all samples of RTF tested are far lower than albumin levels in serum (approximately 40,000 $\mu\text{g/ml}$). Since the albumin concentration in serum is 250 times greater than the highest albumin concentration measured in RTF, it follows that the barrier to the passage of albumin in the rete testis appears relatively efficient. However, the rete testis barrier is not absolute by this criterion. In contrast, the immunocytochemical observations of Christensen et al. (1985) would suggest that the seminiferous tubule barrier is absolute, since no albumin could be detected in the albuminal compartment. Albumin was able to

pass readily through the boundary layer of the seminiferous tubules, but the tight junctional complexes between Sertoli cells excluded further penetration of albumin (Christensen et al., 1985).

The protein concentrations in rat seminiferous tubular fluid (about 5 to 10 mg/ml) are approximately five to ten times higher than those in RTF (Hinton and Keefer, 1983). However, levels of albumin-like protein are reported to be much lower in seminiferous tubular fluid than in RTF (Koskimies and Kormano, 1973). Assuming that "band 27," made visible for microdensitometry by staining proteins separated by step-gradient acrylamide gel electrophoresis, represents only albumin, Koskimies and Kormano (1973) concluded that albumin comprised 14% of total stained protein in RTF and 3% of total stained protein in seminiferous tubule fluid. These calculations provide semi-quantitative estimates at best, since "band 27" consisted of a weakly stained doublet in seminiferous tubule fluid and was often visible as a doublet in RTF. In addition, data presented in this paper demonstrate that more than one protein with a molecular mass of 68 kDa is present in RTF (Fig. 2). The protein concentrations in rat RTF reported by Koskimies and Kormano (1973) was 5.7 mg/ml , a value indicating probable contamination by lymph or interstitial fluid (Hinton and Keefer, 1983). The concentration of albumin in rat RTF was estimated to be about 800 $\mu\text{g/ml}$ (14% of 5.7 mg/ml) from the data of Koskimies and Kormano (1973). In contrast, our data indicate an albumin concentrations in ram RTF of $\sim 100 \mu\text{g/ml}$ (Table 1).

These authors indicated an "albumin" to "globulin" ratio of 0.3, but "globulin" was relatively loosely defined as high molecular weight proteins (Koskimies and Kormano, 1973). We used a radioimmunoassay against sheep IgG to estimate IgG levels in RTF, but were unsuccessful in obtaining consistent analytical results for reasons yet to be determined. However, from available data, IgG levels were never any greater than those of albumin concentrations in RTF (unpublished observations). The 50 kDa and 25 kDa bands stained by Coomassie Brilliant Blue after SDS-gel electrophoresis of RTF (Fig. 3) are thought to represent the heavy and light chains, respectively, of immunoglobulins. These bands, which are prominently stained, have the same migration as the IgG bands in serum. Immunoblot analysis of IgG was performed and, as has been found previously, the electrophoretic transfer of the light chain of IgG (25

kDa) to nitrocellulose is inefficient with the conditions used. However, the 50-kDa heavy chain of IgG was detected in both RTF and serum (data not shown). Both electrophoretic and immunoblot analysis suggest that IgG is present in RTF and support previous observations that immunoglobulins are present in RTF (Johnson, 1972).

The composition of RTF, and its regulation, have been reviewed (Setchell, 1970, 1974, 1978, 1982; Waites and Gladwell, 1982). The current information suggests that about 90% of RTF is derived from seminiferous tubule cell secretions and only 10% from rete testis secretions. Some proteins secreted by seminiferous tubule cells must be reabsorbed in the rete to account for the five- to ten-fold lower protein concentration in RTF than in seminiferous tubular fluid (Hinton and Keefer, 1983). Alternatively, there could be bulk transport from rete testis epithelial cells to the rete of fluid having a low protein concentration. A high pinocytotic bulk transport capacity is suggested by the large number of vesicles in rete epithelial cells (Nykanen and Kormano, 1978). If this occurred, it could account for the fall in protein concentrations observed as seminiferous tubule fluid enters the rete, but it would be inconsistent with observations supporting the interpretation that 90% of rete testis fluid originates from seminiferous tubule secretion. Resolution of this paradox will be aided by experiments to determine the possible presence of specific transport sites for resorption or secretion of proteins by rete testis epithelial cells.

While several of the proteins in RTF appear to be derived from serum, some are unique to RTF (Koskimies and Kormano, 1973; Wright et al., 1981; Olsen and Hinton, 1985). The latter could be derived from cells in the seminiferous tubule, with Sertoli cells being regarded as particularly good candidates, and/or from rete testis epithelial cells. Among the unique proteins detected in the electrophoresis profile of RTF (Fig. 3), several have been identified and characterized. The prominent 40-kDa band is a cell-aggregation molecule named clusterin, a dimeric protein of 80 kDa that comprises about 15% of ram RTF (Blaschuk et al., 1983; Fritz et al., 1983, 1984). It is synthesized and secreted by Sertoli cells (Blaschuk and Fritz, 1984) and by rete testis cells (Tung and Fritz, 1985). Serum clusterin levels are low (about 1 $\mu\text{g/ml}$) (Fritz et al., 1984), and therefore unlikely to contribute to the high levels in RTF (about 100 $\mu\text{g/ml}$).

Androgen-binding protein (ABP) is present in RTF (Danzo et al., 1977; Turner et al., 1984) and is synthesized by Sertoli cells (Fritz et al., 1976). Its concentration in RTF is too low to permit detection by Coomassie Brilliant Blue staining after gel electrophoresis (Fig. 3). A lactalbumin-like protein has been identified in RTF (Hamilton, 1981), and it is secreted by Sertoli cells under defined conditions (Skinner and Fritz, 1986). This was demonstrated by showing that an antibody against the epididymal lactalbumin-like protein (Klinefelter and Hamilton, 1984) immunoprecipitated a radiolabeled 20-kDa protein secreted by stimulated Sertoli cells in culture. The concentration of this 20-kDa protein in ram RTF was also too low to be detected by Coomassie Brilliant Blue staining (Fig. 3).

A 70-kDa protein band is prominent in electrophoretic profiles of serum and RTF (Fig. 3). In serum, this band has been identified as transferrin (Aisen and Litowsky, 1980). Testicular transferrin, synthesized and secreted by Sertoli cells (Skinner and Griswold, 1980), appears to be the same gene product as serum transferrin. However, the glycosylation of the two proteins is different (Skinner et al., 1984). Although it appears plausible that the 70-kDa band in RTF is transferrin derived primarily from Sertoli cell secretions, it is not yet possible to determine directly the relative amounts of transferrin derived from serum. The concentration of transferrin in RTF, seminiferous tubule fluid, and serum have been estimated to be 47, 141, and 3700 $\mu\text{g/ml}$, respectively (Sylvester and Griswold, 1984). If the ratio of RTF transferrin to serum transferrin were the same as the ratio of RTF albumin to serum albumin (1:400), then the level of transferrin RTF derived from serum would be approximately 9 $\mu\text{g/ml}$, or about 20% of the observed concentration of transferrin in RTF. From these considerations, it is postulated that transferrin derived from Sertoli cell secretions comprises the bulk of RTF transferrin. This interpretation is consistent with the suggestions of Holmes et al. (1982) that most of the transferrin in human ejaculates is derived from the testis rather than from serum. It is also consistent with the observations that ^{125}I -labeled transferrin, injected intravenously, did not appear in seminiferous tubule fluid, but that levels in RTF were approximately 10% of those in blood (Sylvester and Griswold, 1984).

As with transferrin, ceruloplasmin is also present in serum and is produced by Sertoli cells in culture

(Skinner and Griswold, 1983). Its presence as a 130-kDa band was not detected in the SDS-gel electrophoresis profile stained by Coomassie Brilliant Blue (Fig. 3), indicating that its concentration was less than 10 $\mu\text{g/ml}$.

Among the bands stained by Coomassie Brilliant Blue after SDS-gel electrophoresis of RTF (Fig. 3), we tentatively identify the 70-kDa protein as transferrin, and postulate that 80 to 90% is derived from the testis and 10 to 20% is from serum; a significant part of the 68-kDa band is albumin, derived exclusively from serum; the 50-kDa and 25-kDa bands appear to be subunits of immunoglobulins, derived exclusively from serum; and the 40-kDa protein is clusterin, produced by Sertoli cells and by rete testis epithelial cells, and most probably derived exclusively from these sources as indicated above. Ceruloplasmin, ABP, and a lactalbumin-like protein are also present in RTF, but at levels too low to be detected by stain with Coomassie Brilliant Blue. RTF is therefore composed of a mixture of proteins, some derived exclusively from the testis (ABP and clusterin), some presumably derived exclusively from serum (albumin and IgG), and some derived from both sources (transferrin and ceruloplasmin). From available data, we estimate that albumin comprises about 15% of RTF protein (Table 1), that clusterin comprises another 15% (Fritz et al., 1984), and that transferrin comprises about 10% (Sylvester and Griswold, 1984). If we assume that IgG comprises approximately 10% of RTF protein, these four proteins together (clusterin, albumin, transferrin, and IgG) would account for about half of the major proteins present in RTF. Low levels of ceruloplasmin, ABP, and lactalbumin-like protein could account perhaps for 1 or 2%. This leaves nearly half the proteins in RTF to be identified and characterized. Possible functions of these RTF proteins in the maintenance and maturation of spermatozoa remain to be elucidated.

REFERENCES

- Acott TS, Hoskins DD, 1978. Bovine sperm forward motility protein. *J Biol Chem* 253:6744-50
- Aisen P, Litowsky I, 1980. Iron transport and storage proteins. *Annu Rev Biochem* 49:357-93
- Blaschuk O, Burdzy K, Fritz IB, 1983. Purification and characterization of a cell-aggregation factor (clusterin), the major glycoprotein in ram rete testis fluid. *J Biol Chem* 258:7714-20
- Blaschuk OW, Fritz IB, 1984. Isoelectric forms of clusterin isolated from ram rete testis fluid and from the secretions of primary cultures of ram and rat Sertoli cell enriched preparations. *Can J Biochem Cell Biol* 62:456-61
- Brooks DE, 1983. Effects of androgens on protein synthesis and secretion in various regions of the epididymis, as analyzed by two-dimensional gel electrophoresis. *Mol Cell Endocr* 29:255-70
- Cheng CY, Bardin CW, 1986. Rat testibumin is a protein responsive to follicle stimulating hormone and testosterone that shares immunodeterminants with albumin. *Biochemistry* 25:5276-88.
- Christenson AK, Komorowski TE, Wilson B, Ma S, Stevens RW, 1985. The distribution of serum albumin in the rat testis, studied by electron microscope immunocytochemistry on ultra thin frozen sections. *Endocrinology* 116:1983-96
- Danzo BJ, Cooper TG, Orgebin-Crist M, 1977. ABP in fluids collected from the rete testis and cauda epididymis of sexually mature and immature rabbits and observations on morphological changes in the epididymis following ligation of the ductuli efferentes. *Biol Reprod* 17:64-77
- Dym M, Fawcett DW, 1970. Observations on the blood-testis barrier of the rat and on the physiological compartmentation of the seminiferous epithelium. *Biol Reprod* 3:308-28
- Everett NB, Simmons BA, 1958. Measurement and radioautographic localization of albumin in rat tissues after intravenous administration. *Circ Res* 6:307-13
- Fritz IB, Blaschuk OW, Burdzy K, 1984. Properties of clusterin, a glycoprotein which elicits cell aggregation, and immunochemical determination of levels in ovine tissues. In: Sairam MR, Atkinson DE (ed.), *Gonadal Proteins and Peptides and their Biological Significance*. Singapore: World Scientific. pp. 311-25
- Fritz IB, Burdzy K, Setchell B, Blaschuk OW, 1983. Ram rete testis fluid contains a protein (Clusterin) which influences cell-cell interactions in vitro. *Biol Reprod* 28:1173-88
- Fritz IB, Rommerts FFG, Louis BG, Dorrington JH, 1976. Regulation by FSH and dibutyryl cyclic AMP of the formation of ABP in Sertoli cell-enriched cultures. *Exp Cell Res* 123:127-35
- Hamilton DW, 1981. Evidence for α -lactalbumin-like activity in rat male reproductive fluids. *Biol Reprod* 25:385-92
- Hartree EF, 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal Biochem* 48:422-27
- Hinton BT, Keefer DA, 1983. Evidence for protein absorption from the lumen of the seminiferous tubule and rete of the rat testis. *Cell Tissue Res* 230:367-75
- Holmes SD, Lipshultz LI, Smith RG, 1982. Transferrin and gonadal dysfunction in man. *Fertil Steril* 38:600-04
- Howards SS, Jessee SJ, Johnson AL, 1976. Micropuncture studies of the blood-seminiferous tubule barrier. *Biol Reprod* 14:264-69
- Johnson MH, 1972. The distribution of immunoglobulin and spermatozoal antigens in the genital tract of the male guinea pig. *Fertil Steril* 23:383-92
- Jones R, Brown CR, VonGlos KI, Parker MG, 1980. Hormonal regulation of protein synthesis in the rat epididymis. *Biochem J* 188:667-76
- Klinefelter GR, Hamilton DW, 1984. Organ culture of rat epididymal tubules in a perfusion chamber. *J Androl* 5:243-58
- Koskimies AF, Kormano M, 1973. The proteins in fluids from the seminiferous tubules and rete-testis of the rat. *J Reprod Fertil* 34:433-44
- Koskimies AL, Kormano M, 1975. Proteins in fluids from different segments of the rat epididymis. *J Reprod Fertil* 43:345-48
- Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-85
- Mancini RE, Vilar O, Alvarez B, Seiguer AC, 1965. Extravascular and intratubular diffusion of labeled serum proteins in the rat testis. *J Histochem Cytochem* 13:376-85
- Nykanen M, Kormano M, 1978. Early effects of efferent duct ligation on the rat rete testis. *Int J Androl* 1:205-34
- O'Farrell PH, 1975. High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250:4007-21
- Olsen GE, Hinton BT, 1985. Regional differences in luminal fluid polypeptides on the rat testis and epididymis revealed by two-dimensional gel electrophoresis. *J Androl* 6:20-34
- Peters T, 1975. Serum albumin. In: Putnam FW (ed.), *The Plasma Proteins*, Vol. 1. New York: Academic Press, pp. 133-81
- Roosen-Runge EC, 1961. The rete testis in the albino rat, its structure, development and morphological significance. *Acta Anat* 45:1-30
- Setchell BP, 1970. Testicular blood supply, lymphatic drainage and

- secretion of fluid. In: Johnson AD, Gones WR, Van Demark NC (eds.), *The Testis*, Vol. 1. New York: Academic Press, pp. 101–239
- Setchell BP, 1974. Secretions of the testis and epididymis. *J Reprod Fertil* 37:165–77
- Setchell BP, 1978. *The Mammalian Testis*. London: Elek, pp. 233–74
- Setchell BP, 1982. Spermatogenesis and spermatozoa. In: Austin CR, Short RV (eds.), *Reproduction in Mammals*, Vol. 1: Germ Cells and Fertilization, 2nd Edition. Cambridge: Cambridge University Press, pp. 63–101
- Setchell BP, Wallace ALC, 1972. The penetration of iodine-labeled follicle-stimulating hormone and albumin into the seminiferous tubules of the sheep and rats. *J Endocrinol* 54:67–77
- Skinner MK, Cosand WL, Griswold MD, 1984. Purification and characterization of testicular transferrin secreted by rat Sertoli cells. *Biochem J* 218:313–20
- Skinner MK, Fritz IB, 1986. Identification of a non-mitogenic paracrine factor involved in mesenchymal-epithelial cell interactions between testicular peritubular cells and Sertoli cells. *Mol Cell Endocr* 44:85–97
- Skinner MK, Griswold MD, 1980. Sertoli cells synthesize and secrete transferrin-like protein. *J Biol Chem* 255:9523–25
- Skinner MK, Griswold MD, 1982. Secretion of testicular transferrin by cultured Sertoli cells is regulated by hormones and retinoids. *Biol Reprod* 27:211–21
- Skinner MK, Griswold MD, 1983. Sertoli cells synthesize and secrete a ceruloplasmin-like protein. *Biol Reprod* 28:1225–29
- Sylvester SR, Griswold MD, 1984. Localization of transferrin and transferrin receptor in rat testis. *Biol Reprod* 31:195–203
- Sylvester SR, Skinner MK, Griswold MD, 1984. A sulfated glycoprotein synthesized by Sertoli cells and by epididymal cells is a component of the sperm membrane. *Biol Reprod* 31:1087–1101
- Towbin H, Staehelin T, Gordon J, 1979. Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose sheets: procedures and some applications. *Proc Natl Acad Sci USA* 76:4350–54
- Tung KSK, Alexander NJ, 1977. Autoimmune reactions in the testis. In: Johnson AD, Gones WR (eds.), *The Testis*, Vol. 4. New York: Academic Press, pp. 491–516
- Tung PS, Fritz JB, 1985. Immunolocalization of clusterin in the ram testis, rete testis, and excurrent ducts. *Biol Reprod* 33:177–86
- Turner TT, Jones CE, Howards SS, Weing LL, Zegeye B, Gunsalus GL, 1984. On the androgen microenvironment of maturing spermatozoa. *Endocrinology* 115:1925–32
- Voglmayr JK, Waites GMH, Setchell BP, 1966. Studies on spermatozoa and fluid collected directly from the testis of the ram. *Nature* 210:861–63
- Waites GMH, 1977. Fluid secretion. In: Johnson AD, Gones WR (eds.), *The Testis*, Vol. 4. New York: Academic Press, pp. 91–123
- Waites GMH, Gladwell RT, 1982. Physiological significance of fluid secretions in the testis and blood-testis barrier. *Physiol Rev* 62:624–71
- Wright WW, Musto NA, Mather JP, Bardin CW, 1981. Sertoli cells secrete both testis specific and serum proteins. *Proc Natl Acad Sci USA* 78:7565–69