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Sertoli cells secrete both testis-specific and serum proteins

(blood–testis barrier/rat testis/immunoprecipitation)

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ABSTRACT **The secretions of the Sertoli cell were examined with two polyvalent antisera—one prepared against proteins in rat serum and the other against testis-specific proteins in rete testis fluid. These antisera detected 12 serum and 9 testis-specific proteins in rete testis fluid. To determine the origin of these proteins, primary cultures enriched in Sertoli cells were incubated with [^{35}S]methionine, and the radiolabeled proteins in the medium were immunoprecipitated. Gel electrophoresis of the two immunoprecipitates resolved eight serum and nine testis-specific proteins. These two sets of proteins were specifically bound by their respective antiserum and were immunologically distinct. Medium from Sertoli cell cultures contained 10 times more of the testis-specific proteins than did cultures enriched for testicular myoid or interstitial cells. The concentration of the serum proteins in Sertoli cell medium was 5 and 10 times greater, respectively, than in myoid or interstitial cell preparations. The proteins from Sertoli cells were next characterized on two-dimensional gels. Seven of the proteins recognized by antiserum against serum proteins had identical molecular weights and isoelectric points as serum proteins. Three of these proteins were ceruloplasmin, transferrin, and glycoprotein 2. In addition to the proteins immunoprecipitated by the two antisera, more than 60 other proteins were detected on two-dimensional gels of the total secretory proteins. We conclude that the Sertoli cell secretes many proteins, some of which are specific to the testis and others of which are similar to serum proteins.**

The fluid within the seminiferous tubules contributes to the physiologically unique environment in which spermatogenesis occurs. Present evidence indicates that the Sertoli cells determine and maintain the composition of this fluid. These cells accomplish this in part by formation of the blood–testis barrier, which segregates the fluid in the adluminal compartment of the tubules from the surrounding blood and lymph (1–3). Sertoli cells also regulate the selective flow of water, ions, steroids, and carbohydrates from the vascular compartment into the lumen (4), but the origin of the proteins in tubular fluid is less clear. In this regard, electrophoretic analysis of the contents of the seminiferous tubules and their extensions, the rete testis, showed that some proteins are unique to the tubules whereas others are similar to serum proteins (5, 6). It was assumed that Sertoli cells could synthesize the testis-specific proteins; however, the origin of the putative serum proteins in tubular fluid was uncertain. As the blood–testis barrier is relatively impermeable to most serum proteins (4), it seemed pertinent to determine whether these proteins were also synthesized by Sertoli cells. In the experiments reported here, we have used two polyvalent antisera, anti-rat serum proteins (anti-SP) and anti-rete testis fluid (RTF) proteins (anti-RTP), in conjunction with two-dimensional gel electrophoresis to show that Sertoli cells secrete serum and tubule-specific proteins.

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MATERIALS AND METHODS

Preparation of Primary Cell Cultures. Three populations of cells (enriched for Sertoli, interstitial, or myoid cells) were prepared from the testes of 11-day-old rats. Decapsulated testes were incubated for 10 min in 0.03% collagenase/dispase (Boehringer Mannheim) and 0.003% soy trypsin inhibitor (Sigma), and the seminiferous tubules were separated from the interstitial cells by unit gravity sedimentation. Interstitial cells were filtered through fine Nytex cloth, washed, and placed in serum-free medium designated TC medium [1:1 (vol/vol) Ham's F-12 nutrient mixture/Dulbecco's modified Eagle's medium (GIBCO) supplemented with 15 mM Hepes, sodium bicarbonate (1.2 g/ml), and gentamycin (10 mg/l)]. To prepare myoid cell-enriched cultures, the tubules were again treated for 30 min with enzymes, and cells in suspension were collected, filtered through fine Nytex, and plated in TC medium with 5% (vol/vol) horse serum (Kansas City Biologicals) and 2.5% (vol/vol) newborn calf serum (Flow Labs). To prepare Sertoli cell-enriched cultures, after the first collagenase treatment, the tubules were minced into small pieces, treated again for 15 min with enzymes, and the cells were filtered through Nytex (no. 160) cloth. Cells in the filtrate were washed and then plated in TC medium. Medium for all cells was changed after days 1 and 3 of culture, and growth factors [insulin (10 μg/ml), transferrin (5 μg/ml), and epidermal growth factor (10 ng/ml)] were added to the Sertoli and interstitial cell preparations. On day 5, the medium was changed to Ham's F-12 nutrient mixture with 1/10th of the normal methionine concentration plus 100 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine (1194 Ci/mmol; New England Nuclear). After 18 hr, medium was collected and concentrated 15-fold at 4°C with a collodion dialysis bag (molecular weight cutoff, 10,000) in a vacuum condensor (Schleicher and Schuell). Dialysis was against phosphate-buffered saline (pH 7.4).

To obtain a nontesticular cell preparation, spleens of saline-perfused rats were minced in phosphate-buffered saline, and cells that remained in suspension were collected. These cells were pelleted by centrifugation (190 × *g*), resuspended in Ham's F-12 medium, and immediately plated with growth factors and [^{35}S]methionine.

Antisera. The polyvalent antiserum, anti-SP, was prepared by using serum from castrated male rats (>2 wk) that had been depleted of 70% of its albumin by passage through a column of Cibacron blue-Sepharose (7). This was mixed with Freund's complete adjuvant and 8.4 mg of immunogen injected intradermally into each rabbit as described (8). The animals were boosted 3 wk after the first injection with 4.2 mg of immunogen in Freund's incomplete adjuvant. Animals were bled thereafter

Abbreviations: RTF, rete testis fluid; anti-RTP, polyvalent antiserum against testis-specific proteins in RTF; anti-SP, polyvalent antiserum against serum proteins.

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at weekly intervals, and antisera with high titers, as determined by crossed immunoelectrophoresis, were pooled. The IgG fraction was prepared from a portion of this antiserum by protein A affinity chromatography (9) and was used as an immunoadsorbent for preparation of the RTF antigen.

Polyvalent antiserum, anti-RTP, was generated against testis-specific proteins in RTF collected by micropuncture of the rete testis of mature Sprague–Dawley rats (10). Serum proteins in RTF were removed by immunoadsorption with the IgG fraction of anti-SP, centrifugation, and fractionation of the supernatant on protein A-Sepharose. This procedure removed all serum proteins from RTF that could be detected by crossed-immunoelectrophoresis against anti-SP. Two rabbits were injected with the immunogen (≈450 μg/injection), boosted five times at 3-wk intervals, and bled weekly after the second injection. In order to remove any antibodies that crossreacted with the rat serum, anti-RTP was fractionated on a column of dealbuminated rat serum coupled to Sepharose 4B.

A hyperimmune antiserum also was prepared by immunization of a rabbit with Freund's complete and incomplete adjuvants. The IgG fractions of the three antisera were isolated by precipitation with 33% $(NH_4)_2SO_4$ for use in the immunoassays.

Immunoassays. Radioinert proteins were detected by crossed immunoelectrophoresis (11). Antigens were separated in the first dimension by electrophoresis at 10 V/cm in 1% agarose that contained a 1:4 dilution of electrophoresis buffer (324 mM Tris/96 mM Tricine/1.4 mM calcium lactate/0.08% NaN_3, pH 8.6). Electrophoresis in the second dimension was conducted at right angles to the first at 2 V/cm in 1% agarose/3–5% antiserum. After 16 hr, the gels were washed in phosphate-buffered saline and dried, and immunoprecipitates were stained with 0.05% Coomassie blue R-250.

Radiolabeled proteins were immunoprecipitated by the technique of Kessler (12) as modified by Ivarie and Jones (13). The concentration of total radiolabeled proteins was first established by trichloroacetic acid precipitation (14). Proteins (500,000 cpm) were then mixed 2:1 (vol/vol) with 150 mM NaCl/50 mM NaH_2PO_4/10 mM methionine/1% deoxycholate/1% Triton X-100, pH 7.5, and 10:1 (vol/vol) with hyperimmune serum (Hyp). This mixture was incubated for 4 hr at 4°C, treated 1:0.67 (vol/vol) with 10% (wt/vol) *Staphylococcus aureus* fixed in formalin (SAC, Pansorbin, Calbiochem), and incubated for 30 min at 4°C. The bacteria were then pelleted by centrifugation (800 × *g* for 15 min), and anti-SP, anti-RTP, or hyperimmune serum were added (1:10, vol/vol) to the supernatants. These mixtures were incubated overnight at 4°C, and then the SAC preparation was added as before. After centrifugation and three washes with 0.5 ml of 0.025 M KH_2PO_4/100 mM NaCl/1 mM EDTA/0.25% Nonidet P-40, pH 7.5, the antibody–antigen complexes were extracted into the appropriate sample buffer for electrophoresis, and an aliquot of each extract was taken to measure total radioactivity by liquid scintillation spectrometry.

Polyacrylamide Gel Electrophoresis. One-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis was conducted as described (15). The proteins in sample buffer [6.25 mM Tris·HCl/2.5% (wt/vol) dithiothreitol/3% (wt/vol) $NaDodSO_4$/10% (wt/vol) glycerol, pH 6.8] were heated for 5 min at 95°C. A polyacrylamide gel of 5% T (total monomers), 15% C (cross-linker in the total monomers) as *N,N'*-diallyltartardiamide was used to stack the proteins before they were resolved on a gradient polyacrylamide gel (8.2%–20.5% T with 2.59% C as *N,N'*–methylenebisacrylamide).

Two-dimensional gel electrophoresis was performed as described (16, 17). Proteins in sample buffer (0.05 M cyclohexylaminoethanesulfonic acid/2% $NaDodSO_4$/1% dithiothreitol/10% glycerol, pH 9.5) were mixed with 4 μg of isoelectric focusing standards, heated at 95°C for 5 min, and then focused for 7500 V·hr in 2.5-mm acrylamide gels that contained 9 M urea and 2% (wt/vol) ampholines (LKB; pH 3.5–10). The second dimension of electrophoresis was on an 8.2–20.5% T polyacrylamide gel.

Radiolabeled and radioinert molecular weight standards were purchased from Bethesda Research Laboratories (Rockville, MD), and radioinert isoelectric focusing standards and acetylated cytochrome *c*, were from United States Biochemical (Cleveland, Ohio). Radioinert proteins were detected by staining with Coomassie blue R-250 (18); radiolabeled proteins were detected by fluorography (19). Protein content of the various samples was determined as described (20), with bovine serum albumin as a standard.

RESULTS

Demonstration of Serum and Testis-Specific Proteins in RTF. Crossed-immunoelectrophoresis of rat serum (0.078 μl; 5.8 μg of protein) and RTF (0.92 μl; 5.8 μg of protein) against anti-SP (Fig. 1 *A* and *B*) indicated that this antiserum, which detects 14 distinct serum proteins, also identifies 12 proteins in RTF. The concentration of these proteins in RTF was only 1/10th (or less) of that in serum. In addition, the relative amount of each protein in RTF was different than in serum. When RTF and serum (15.8 μg of total protein) were compared by crossed immunoelectrophoresis against anti-RTP, 11 proteins were distinguished in RTF and none in serum. However, when the size of the serum sample was increased 46-fold, two proteins of low concentration were detected (data not shown). The demonstration of both testis-specific and serum proteins in RTF led us to examine the kinds of proteins synthesized by Sertoli cells.

Immunoprecipitation of Radiolabeled Proteins from Cultured Cells. Primary cultures enriched in Sertoli cells secreted eight proteins that were immunoprecipitated by anti-SP and nine proteins by anti-RTP (Fig. 2). The specificities of and differences between these antisera were demonstrated by (*i*) no precipitation of radiolabeled proteins by the hyperimmune antiserum, (*ii*) competition by rat serum and RTF but not rabbit serum with the radiolabeled proteins for binding to their respective antiserum (Fig. 2), and (*iii*) no significant competition by rat serum for the binding of the radiolabeled proteins to anti-RTP (Fig. 2). The following lines of evidence suggested that the radiolabeled proteins were actually secreted and not present in the media due to cell lysis. First, most of the immunoprecipitable proteins were undetectable in cell homogenates. Second, accumulation of [^{35}S]methionine into cellular protein began immediately after the addition of the radiolabeled amino acid and was linear for 18 hr, whereas accumulation of radiolabeled proteins in the media was negligible for the first 4–6 hr and then was substantial and linear for the following 12–14 hr.

The tissue specificity of the proteins recognized by anti-RTP was next examined by incubating [^{35}S]methionine with spleen cells or with testicular cell preparations enriched for either myoid or interstitial elements. Substantial amounts of radioactive proteins were recovered from the media of all cell preparations. When these proteins were immunoprecipitated with anti-RTP, it was found that myoid and interstitial cells secreted (per cell) only 10% of the amount of tubule-specific proteins secreted by cultures enriched for Sertoli cells. Spleen cells did not secrete these proteins. In contrast, when media from these cultures were incubated with anti-SP, the relative amounts of immunoprecipitable proteins were: Sertoli, 1.0; myoid, 0.2; interstitial, 0.1; spleen, 0.02.

Characterization of the Proteins Secreted by Sertoli Cell-Enriched Cultures. An immunoprecipitate of the radiolabeled proteins was prepared with anti-SP and fractionated by two-dimensional gel electrophoresis (Fig. 3A); this was replicated

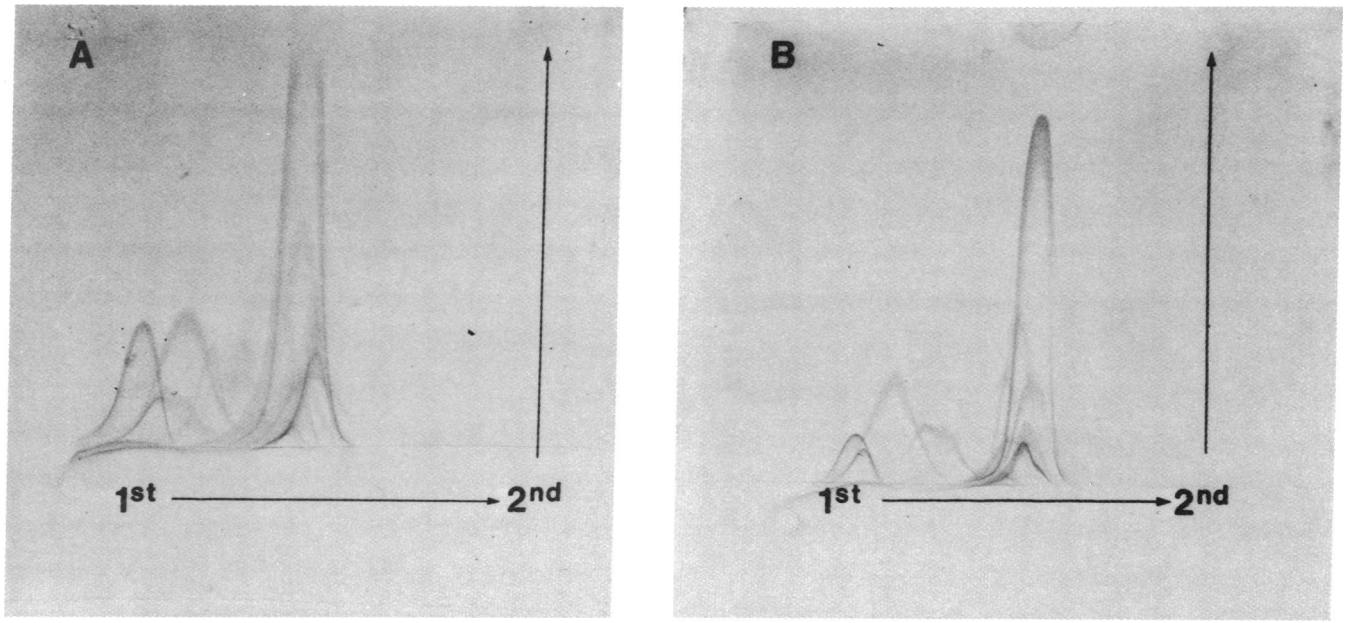

FIG. 1. Crossed immunoelectrophoresis of 5.8 μg (0.078 μl) of rat serum proteins (*A*) or 5.8 μg (0.92 μl) of RTF proteins (*B*). Antigens were separated in the first dimension by electrophoresis in agarose and then forced into a gel that contained antiserum against rat serum proteins. Immunoprecipitates were detected by staining with Coomassie blue R-250; 14 separate proteins were resolved in rat serum and 12 in RTF.

four times with identical results. Seven of the radiolabeled proteins had molecular weights and isoelectric points identical with those of proteins from a serum sample that was fractionated on a similar gel (Fig. 3*B*). Three of these serum proteins have been designated by Anderson and Anderson (21) as ceruloplasmin (protein S2), transferrin (protein S5), and glycoprotein 2 (protein S4).

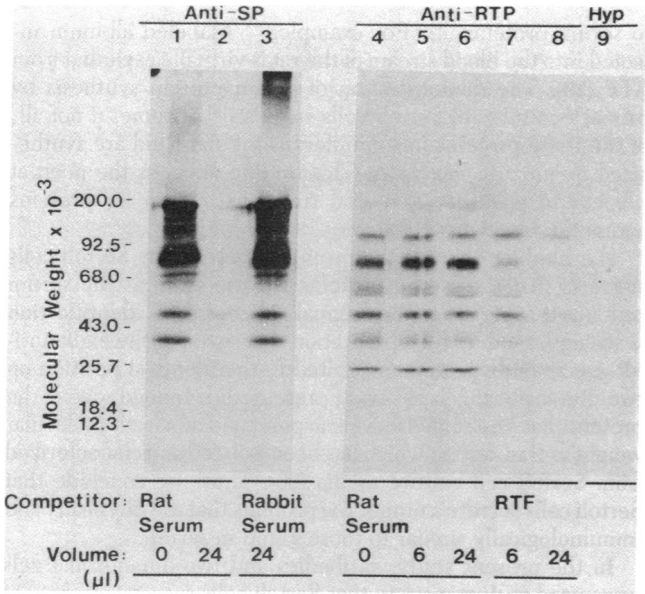

FIG. 2. Specific immunoprecipitation of [^{35}S]methionine-labeled proteins secreted by Sertoli cell-enriched cultures. Immune complexes were isolated, the proteins were fractionated on 8–20% gradient acrylamide gels, and the radiolabeled proteins were detected by fluorography. Eight proteins were immunoprecipitated by anti-SP (lane 1), nine by anti-RTP (lane 4), and none by the control, hyperimmune (Hyp) antiserum (lane 9). The addition of 6 or 24 μl of rat serum to anti-SP reduced the immunoprecipitable labeled proteins by 95% (lane 2). When 24 μl of serum were incubated with the anti-RTP, the reduction was only 15% (lane 6). Addition of 24 μl of RTF to anti-RTP reduced the radiolabeled protein that was bound by 85% (lane 8). The addition of rabbit serum had no influence on immunoprecipitation by either antiserum (lane 3).

Of the proteins immunoprecipitated by anti-RTP (Fig. 4), none have the isoelectric point and molecular weight of androgen binding protein (22). All of these proteins except one (T8) have different molecular weights from those immunoprecipitated by anti-SP. It seems unlikely that T8 was the same protein as the comparable protein immunoprecipitated by anti-SP, S8, because rat serum proteins competed with S8 for binding to anti-SP, whereas they had no influence on the binding of T8 to anti-RTP. Thus, T8 and S8 are probably different proteins with similar physical properties.

When the total culture medium from Sertoli cells was fractionated on two-dimensional gels, >70 proteins were resolved (Fig. 5*A*). Based on the molecular weights and isoelectric points of the proteins recognized by the two antisera, we have classified the total secreted proteins as being recognized by anti-SP, anti-RTP, or not recognized by either antisera (Fig. 5*B*).

DISCUSSION

Prior to this report, only a few secretory proteins from Sertoli cells had been identified: androgen binding protein (23, 24), plasminogen activator (25), transferrin (26), and a mitogen (27). This communication indicates that Sertoli cells in primary culture are able to synthesize a large number of proteins, some of which are recognized by antiserum against the proteins in rat serum and others by antiserum against testis-specific proteins in RTF. We detected 12 serum and 9 testis-specific proteins in RTF, as compared with 8 serum and 9 testis-specific proteins in culture media from Sertoli cells. The number of proteins in these two classes are in remarkably good agreement with those in reports on the composition of RTF collected by micropuncture: 9 proteins with electrophoretic mobilities similar to serum proteins and 11 unique proteins (5). It is probable, however, that the numbers of proteins in RTF and seminiferous tubule fluid are greater than these data suggest, because >70 proteins were detected by two-dimensional gels of total culture media from Sertoli cells.

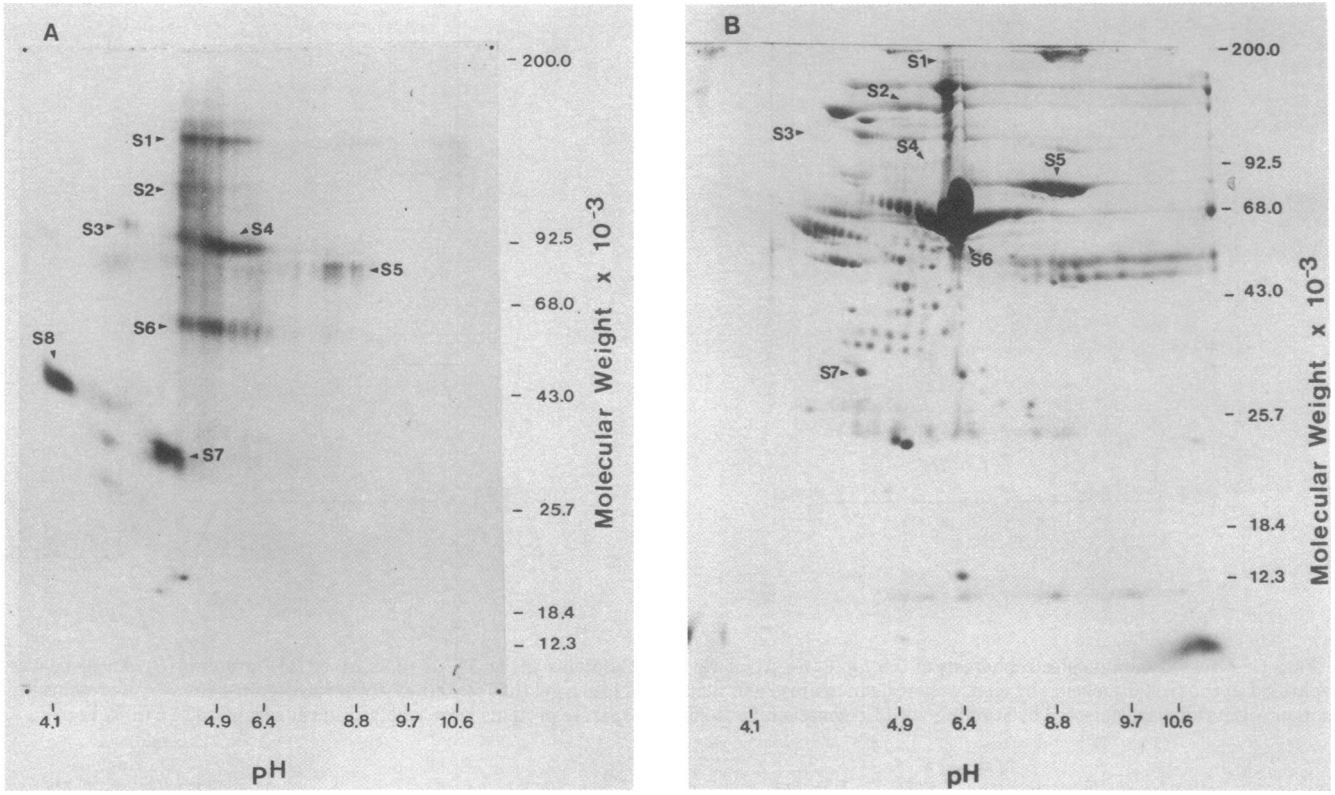

FIG. 3. Two-dimensional gels of serum proteins. (*A*) Two-dimensional gel electrophoresis of ^{35}S-labeled proteins immunoprecipitated by anti-SP from Sertoli cell culture medium. Molecular weight and pH gradients were determined by the use of internal standards. Proteins S1–S7 had identical pIs and molecular weights as those found in serum. (*B*) Two-dimensional gel electrophoresis of proteins in rat serum. Protein S2 is ceruloplasmin, S5 is transferrin, and S4 is glycoprotein 2 (21).

The presence of serum proteins in tubular fluid has been attributed in the past to the transport of these proteins across the blood–testis barrier (28). However, the seminiferous and rete testis epithelia have proven to be relatively impermeable to serum proteins (4). For example, ^{125}I-labeled albumin injected into the blood stream of the rat is virtually excluded from RTF (29). The demonstration of serum protein synthesis by primary cultures of Sertoli cells suggests that some, if not all, of the these proteins in seminiferous tubular fluid are synthesized *de novo* in the testis. This finding obviates the need at present to postulate a special transport system for proteins across the blood–testis barrier.

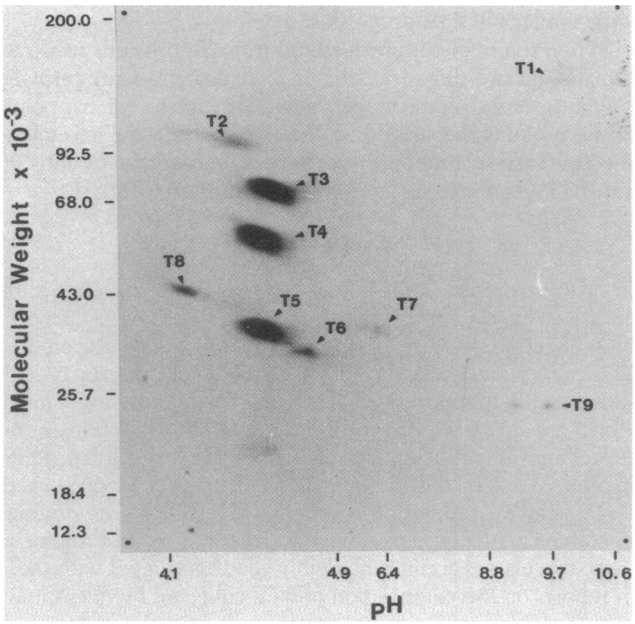

FIG. 4. Two-dimensional gel electrophoresis of the nine ^{35}S-labeled proteins immunoprecipitated by anti-RTP from Sertoli cell culture medium. One protein, T8, had a molecular weight and pI similar to those of a protein immunoprecipitated by anti-SP (Protein S8, Fig. 3*A*).

The binding to anti-SP of proteins secreted by Sertoli cells suggests that the bound proteins are the same as the serum proteins that served as the original antigens. This identification is strengthened by the observation that seven of the eight anti-SP precipitable proteins migrated to the identical position on two-dimensional gels as serum proteins did. Indeed, one of the proteins had the same isoelectric point and apparent molecular weight as transferrin, which has been isolated and characterized from Sertoli cell culture media (26). Thus, we conclude that Sertoli cells secrete a number of proteins that are physically and immunologically similar to those found in serum.

In the present study, antibodies and two-dimensional gels were used to demonstrate that Sertoli cells secrete a variety of unique proteins. In our attempt to determine whether other cells could synthesize the testis-specific proteins in RTF, we detected them in cultures enriched for interstitial or myoid cells, though at 10% of the amount found in media from Sertoli cells. However, these two preparations were contaminated by Sertoli cells, and it is possible that these cells accounted for much, if not all, of the testis-specific proteins found in these cultures. The potential for the synthesis of these proteins by myoid or interstitial cells cannot be finally resolved until clonal lines of testicular cells are developed for the rat, as they have been for the mouse (30).

No protein that is synthesized by Sertoli cells has been shown

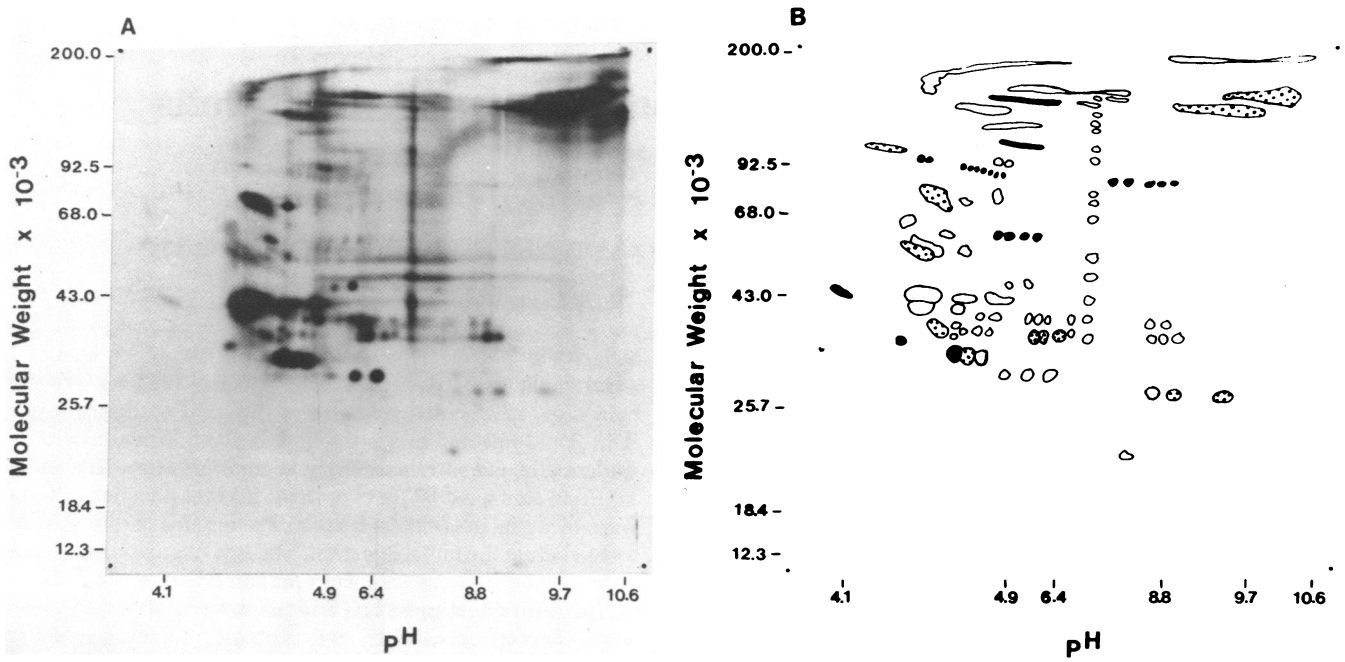

FIG. 5. (*A*) Two-dimensional gel electrophoresis of the total proteins secreted by Sertoli cells in culture. (*B*) Map of the two-dimensional separation of the proteins secreted by Sertoli cells in culture. Black ovals indicate proteins recognized by anti-SP, dotted ovals indicate proteins recognized by anti-RTP, and open ovals indicate some of the proteins recognized by neither antiserum.

to have a particular function in the seminiferous tubules. Nevertheless, the intimate contact of the Sertoli cells with developing germ cells (31) and the exclusion of the germ cells (leptotene and later stages) from the general physiological environment by the blood–testis barrier (2) suggest that some of the proteins secreted by the Sertoli cell may support the growth and differentiation of germ cells. All cells *in vitro* appear to require some serum proteins for survival (32); the most universally required are transferrin and insulin (33). Transferrin from Sertoli cells probably supports germ cells in the same way that serum transferrin supports many somatic cells—by assuring adequate iron transport across the plasma membrane. Recent evidence of insulin synthesis in the testis (34) raises the possibility that Sertoli cells also supply this hormone to the developing spermatogenic cells.

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