Rat Testicular Testibumin Is a Protein Responsive to Follicle Stimulating Hormone and Testosterone That Shares Immunodeterminants with Albumin†

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ABSTRACT: During a search for hormonally responsive products in media from Sertoli cell enriched cultures, a major follicle stimulating hormone responsive and testosterone-responsive protein was identified and designated CMB-1. The results of the present study indicate that this protein is related immunologically to rat albumin and rat α-fetoprotein (AFP) and is concentrated in the testis of the adult rat. CMB-1 was therefore termed testibumin. Testibumin was purified from Sertoli cell enriched cultures to apparent homogeneity by sequential high-performance liquid chromatography on anion-exchange, chromatofocusing, gel permeation, and hydroxylapatite columns. The purified protein consists of two concanavalin A (Con A) reactive forms: one which does not interact with Con A and the other which binds to this lectin and is eluted with methyl α-mannoside. Testibumin is a monomer with an apparent molecular weight of 69 000 and a p*I* ranging between 4.5 and 4.85. The heterogeneity of this protein was further demonstrated by crossed-immunoelectrophoresis and two-dimensional gel electrophoresis. A monospecific antiserum and highly purified testibumin were used to develop a specific radioimmunoassay which permitted studies of the hormonal responsiveness of Sertoli cell enriched culture and of the content of testibumin in the reproductive tract fluids in vivo. Even though testibumin was found in serum of both sexes, it was highly concentrated in the testicular and epididymal compartments in adult rats. This protein was compared to rat serum albumin and rat AFP immunologically. With the use of immunoblots, antiserum developed against testibumin showed partial cross-reactivity with albumin and AFP when these latter proteins were denatured and were present in amounts several orders of magnitude greater than testibumin. The extent of this cross-reactivity was then examined by comparing the ability of native and S-carboxymethylated albumin to compete with ^{125}I-testibumin for binding to a monospecific testibumin antiserum. It was shown that the unfolded derivative of albumin showed partial cross-reactivity with testibumin. We conclude (i) that testibumin is immunologically related to albumin and AFP as these latter proteins are related to one another and (ii) that testibumin is possibly the homologue of albumin in the seminiferous tubular compartment.

Sertoli cells determine and maintain the composition of the fluid in the seminiferous tubular compartment in which spermatogenesis and spermiogenesis occur. This is made possible by the fact that these cells create a unique barrier which prevents entry of serum protein into the tubular compartment [for reviews see, Fawcett (1975) and Setchell & Waites (1975)]. Thus, the proteins in tubular fluid are primarily of Sertoli cell origin. Results from a variety of studies indicate that these cells secrete both testis-specific and serum proteins (Wright et al., 1981; Kissinger et al., 1982). These proteins include transferrin (Skinner & Griswold, 1980; Skinner et al., 1984), ceruloplasmin (Wright et al., 1981), androgen binding protein (ABP)[1] (Musto et al., 1977, 1980; Feldman et al., 1981; Kierszenbaum et al., 1980) (a homologue to testosterone–estradiol binding globulin) (TeBG), retinol binding protein (Huggenvik & Griswold, 1981; Carson et al., 1984), inhibin (deJong, 1979), seminiferous growth factor (Feig et al., 1980, 1983), P Mod-S protein (Skinner & Fritz, 1985), cyclic proteins (Wright et al., 1981, 1983), T proteins (Wright et al., 1981), dimeric acid glycoprotein (Sylvester et al., 1984), plasminogen activator (Lacroix et al., 1977, 1981), and SCm proteins (DePhilip & Kierszenbaum, 1982). While looking for hormonally responsive products in media from Sertoli cell enriched cultures by HPLC and SDS PAGE, we observed an abundant albumin-like protein which increased in concentration in response to FSH plus testosterone (Cheng et al., 1986). The present paper reports that this protein shares some immunologic properties with albumin and α-fetoprotein (AFP) and is present in large quantities in testes and epididymides; as a consequence, it has been designated testibumin.

Experimental Procedures

Materials

Biochemicals. The serum-free culture medium (F12/DME) was made in the laboratory by using powdered media obtained from Grand Island Biological Co. (Grand Island, NY) or Flow

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[1] Abbreviations: HPLC, high-performance liquid chromatography; FSH, follicle stimulating hormone; Con A, concanavalin A; BSA, bovine serum albumin; hT, human transferrin; rT, rat transferrin; ABP, androgen binding protein; rABP, rat ABP; TeBG, testosterone–estradiol binding globulin; AFP, α-fetoprotein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; RIA, radioimmunoassay; PBS buffer, 10 mM sodium phosphate and 0.15 M NaCl, pH 7.4 at 22 °C; PBS–BSA buffer, PBS buffer plus 0.5% BSA; NPB buffer, 10 mM sodium phosphate, pH 7.4 at 22 °C; *T*, total acrylamide concentration (grams per 100 mL) (=[acrylamide] + [methylenebis(acrylamide)]; C_{DATD}, amount of cross-linker using *N*,*N*′-diallyltartardiamide (DATD); % *C*, percent cross-linker [=100 × [methylenebis(acrylamide)]/([acrylamide] + [methylenebis(acrylamide)])]; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Bis, *N*,*N*′-methylenebis-(acrylamide); TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; Bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

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Biochemicals (Rockville, MD) and used within 2 weeks. The F12/DME medium consisted of Ham's F-12 nutrient mixture and Dulbecco's modified Eagle's medium (1:1 v/v) containing 1.2 g/L sodium bicarbonate, 15 mM Hepes, and 20 mg/L gentamycin. $Na^{125}I$ (specific activity 15.9–17.1 mCi of $^{125}I/\mu g$ of iodine; code IMS 300) was obtained from Amersham (Arlington Heights, IL). Tris, acrylamide, *N,N'*-diallyltartardiamide (DATD), *N,N'*-methylenebis(acrylamide) (Bis), *N,N,N',N'*-tetramethylethylenediamine (TEMED), ammonium persulfate, agarose (electrophoresis grade), prestained high molecular weight marker proteins, and *Staphylococcus aureus* cells (formalin fixed) used as a source of protein A for immunoprecipitation were from Bethesda Research Laboratories (Gaithersburg, MD). Sodium chloride, Bis-Tris [2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol], Tricine, DL-histidine, phosphoric acid (85% w/v), methyl α-D-mannopyranoside (methyl α-mannoside), urea, iminodiacetic acid, citric acid, and silver nitrate were from Aldrich (Milwaukee, WI). Methanol, hydrogen peroxide (30% v/v), and formaldehyde solution (37% w/v) were from Fisher (Fair Lawn, NJ). Methanol (HPLC grade) was from Burdick & Jackson Laboratories, Inc. (Muskegon, MI). Glycine, 2-mercaptoethanol, Bio-lyte 5/7, sodium dodecyl sulfate (SDS), and high and low molecular weight protein standards were from Bio-Rad (Richmond, CA). Deoxyribonuclease 1 (DNase 1) from bovine pancreas (type III), soybean trypsin inhibitor, testosterone, purified rat serum albumin, Hepes, protein A–peroxidase, 4-chloro-1-naphthol, Con A–Sepharose 4B, bovine insulin, bacitracin, bovine serum albumin (fraction V), NP-40 [Nonidet P-40, octylphenoxypoly(ethoxyethanol)], phenylmethanesulfonyl fluoride (PMSF), and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma (St. Louis, MO). Epidermal growth factor (EGF) was obtained from Collaborative Research (Waltham, MA). Gentamycin was from Schering Pharmaceutical Corp. (Kenilworth, NJ), and collagenase/dispase was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Monospecific polyvalent antiserum against rat serum albumin was obtained from Cooper Biomedical, Inc. (Malvern, PA). Monofilament nylon cloth (Nitex) was obtained from Tetko Inc. (Elmsford, NY). Polybuffer 74 and Pharmalyte 3-10 were from Pharmacia (Uppsala, Sweden). Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) and acetic acid were from Pierce (Rockford, IL). Normal rabbit serum (lyophilized) and purified human transferrin were from Calbiochem (La Jolla, CA). p*I* marker protein kit, Coomassie blue G-250, Coomassie blue R-250, and BSA (RIA grade) were from United States Biochemical Corp. (Cleveland, OH). *Staphylococcus aureus* protease V_8 was from Miles Laboratories, Inc. (Elkhart, IN). Nitrocellulose paper (0.15- and 0.45-μm pore size) was from Schleicher & Schuell, Inc. (Keene, NH). Purified ovine FSH-16 was a gift from the NIH Hormone Distribution Program (NIADDK).

HPLC Columns. A Mono Q (HR 10/10) anion-exchange HPLC preparative column (10 × 100 mm i.d., particle size 10 μm) and a Mono P (HR 5/20) chromatofocusing HPLC column (5 × 200 mm i.d., particle size 10 μm) were obtained from Pharmacia. A Bio-Sil TSK-250 gel permeation HPLC column (7.5 × 300 mm i.d.), a Bio-Sil guard column (7.5 × 75 mm i.d.), a Bio-Gel HPHT hydroxylapatite HPLC column (7.8 × 100 mm i.d. packed with hydroxylapatite derivative), and a Bio-Gel HPHT guard column (4 × 50 mm i.d. packed with spherical polymeric matrix) were from Bio-Rad.

Animals and Tissue. Sprague-Dawley male and female rats were obtained from Charles River Laboratories (Kingston, MA). Animals of different ages were killed by asphyxiation in groups of two to six. Trunk blood was collected in glass tubes and allowed to clot at 4 °C overnight. Sera were separated by centrifugation at 2500*g* for 10 min and stored at −20 °C until used. Testes, epididymides, brain, liver, seminal vesicles, kidneys, and ovaries were minced and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) or glass homogenizer in ice-cold TG buffer (20 mM Tris containing 10% glycerol, pH 7.4 at 22 °C) using a tissue to buffer ratio (w/v) of 1:9 for epididymides, 1:4 for seminal vesicles, and 1:2 for testes and other organs. The homogenates were sedimented at 45000*g* for 1 h at 4 °C and the supernatants used for assay. Amniotic fluid was collected and pooled from fetuses at 17-day gestation. All samples were stored at −20 °C until used.

Methods

Preparation of Primary Sertoli Cell Enriched Cultures. Primary Sertoli cell enriched cultures were prepared from 20-day-old rats. Groups of 20 rats were used for each preparation. Sertoli cells were isolated and cultured by using a modification of the method previously reported by Mather and Sato (1979) as modified in this laboratory and detailed elsewhere (Cheng et al., 1986; Rich et al., 1983; Perez-Infante et al., 1986). Sertoli cell aggregates were seeded in 100-mm plastic culture dishes at a density of approximately 4.5×10^6 cells per dish (9 mL) in the serum-free medium supplemented with insulin (10 μg/mL), hT (1 μg/mL), EGF (2.5 ng/mL), bacitracin (5 μg/mL), testosterone (2×10^{-7} M), and ovine FSH (300 ng/mL). Spent media collected at day 4 and day 8 were stored at −20 °C and then pooled for protein fractionation.

To determine the production of testibumin by Sertoli cell enriched cultures as a function of time in vitro, a total of 14 individual dishes were prepared from 20-day-old rats. One set of two individual dishes was terminated at each time point, and an aliquot of 30 μL per dish was used to assay for testibumin concentration. The dose–response effects of FSH and/or testosterone on testibumin production by Sertoli cells were studied by using 24 individual dishes for each hormonal treatment. One set of four dishes was used for each treatment. The spent media were collected at day 4, and 30 μL from each dish was assayed for testibumin concentration by radioimmunoassay. The different hormonal treatments used were as follows: (1) FSH (0–1000 ng/mL); (2) FSH (0–1000 ng/mL) plus 2×10^{-7} M testosterone; (3) testosterone [(0–2) × 10^{-5} M]; (4) testosterone [(0–2) × 10^{-5} M] plus 300 ng/mL FSH.

Analytical Polyacrylamide Gel Electrophoresis (PAGE). Analytical PAGE in the presence of SDS was performed by the method of Laemmli (1970). The resolving gel consisted of either 7.5% or 10% *T* (total acrylamide concentration) and 2.6% cross-linker using methylenebis(acrylamide) (% C_{Bis}) with a stacking gel of 5% *T* and 15% *N,N'*-diallyltartardiamide (% C_{DATD}). For peptide mapping (Cleveland et al., 1977), the resolving gel consisted of 15% *T* and 2.6% C_{Bis} while the stacking gel was the same as above. Analytical PAGE without SDS was prepared and run essentially as described by Chrambach et al. (1976) using buffer system 2860.0.X and acrylamide concentrations of 6.2% *T* and 3.2% C_{Bis}. Gels were routinely stained with silver nitrate according to the procedures of Wray et al. (1981).

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was performed as previously described (O'Farrell, 1975). Isoelectric focusing was performed in 1.5-mm (i.d.) acrylamide gels containing 9 M urea and 2% (w/v) ampholines (pH 3.5–10) and focused for 5600 V·h. The second dimension was performed on a polyacrylamide slab gel

with a linear gradient of 10–20% *T*.

Purification of Testibumin. (*A*) *Preparation of Primary Culture Medium from Sertoli Cell Enriched Cultures for HPLC.* For each of four different experiments, about 1.2 L of spent medium was used as the starting material. Spent media from day 4 and day 8 cultures were thawed, pooled, concentrated, desalted, and equilibrated against 20 mM Tris, pH 7.4 at 22 °C, in an Amicon high-performance ultrafiltration system (Model TCF-10) equipped with an end point controller (Model EC 22) and a PM-10 membrane (molecular weight cutoff at 10000). This and all subsequent procedures, unless otherwise specified, were performed at 4 °C. The desalted sample was concentrated to about 55 mL and filtered through a 0.45-μm nylon-66 membrane filter in a solvent filtration apparatus from Rainin (Woburn, MA).

(*B*) *Anion-Exchange HPLC of Proteins in Sertoli Cell Enriched Culture Medium.* All buffers used for HPLC were prepared with water purified from a Darco water system (Model 344-D, Darco Water Systems, Inc. Durham, NC) and degassed by vacuum. Fractionation was performed on an anion-exchange preparative column (Mono Q HR 10/10, 10 × 100 mm i.d., particle size 10 μm) from Pharmacia using a chromatographic system from LKB (Bromma, Sweden) equipped with an HPLC controller (Model 2152), two pumps (Model 2150), and a single-wavelength UV detector (Uvicord S11, Model 2238). A Rheodyne sample injector (Model 7125) equipped with either a 2-mL or a 100-μL sample loop was from Rainin. The chromatogram was recorded on a Kipp & Zonen Model BD 41 dual-channel recorder from Rainin. Additional parameters of the chromatograms were obtained and analyzed with an HPLC integrator (Model 3392 A) from Hewlett-Packard (Palo Alto, CA). Connecting tubing was 0.25-mm i.d. stainless steel, except in the low-pressure zone between the solvent systems and the injector where 0.75-mm i.d. tubing was used. This and all HPLC fractionations were performed at ambient temperature (22 °C). Solvent A (20 mM Tris, pH 7.4 at 22 °C) was prepared freshly each day and used as the initial solvent in the gradient separation. The second solvent (solvent B) consisted of 20 mM Tris and 600 mM NaCl, pH 7.4 at 22 °C. The normal operation pressure for the Mono Q preparative column was about 20 bar. The concentrated sample (55 mL) was pumped onto the Mono Q column at a flow rate of 4 mL/min. After the protein profile of the chromatogram returned to the base line, proteins were eluted by using a linear gradient from 0% to 80% solvent B at a flow rate of 4 mL/min for 45 min. Fractions (4 mL each) were collected with an LKB Superrac (Model 2211) fraction collector. The effluent was monitored by the UV absorbance at 280 nm.

(*C*) *Chromatofocusing HPLC.* Fractions containing testibumin obtained from the anion-exchange HPLC fractionation were pooled, concentrated, and desalted against 25 mM Bis-Tris buffer, pH 7.1, adjusted with iminodiacetic acid at 22 °C in an Amicon ultrafiltration cell (Model 8010) using a YM-10 membrane. The desalted sample was concentrated to about 2 mL and filtered through a 0.22-μm filter unit (Millex-GV) from Millipore. The sample was then injected onto the Mono P chromatofocusing HPLC column (5 × 200 mm i.d.) using the 2-mL sample loop. The column was then washed with about 6 mL of 25 mM Bis-Tris buffer at a flow rate of 1 mL/min. Elution of proteins was then performed by using about 60 mL of Polybuffer 74 [Polybuffer 74:H_2O (1:10 v/v), pH 4.0, with iminodiacetic acid at 22 °C]. The normal operation pressure for the Mono P column was about 25 bar. Fractions (1 mL each) were collected, and samples containing testibumin were concentrated to 100 μL with a Centricon-10 microconcentrator (Amicon) and equilibrated against 10 mM sodium phosphate and 0.15 M NaCl, pH 6.8 at 22 °C. The entire concentration step was operated in a Beckman J2-21 refrigerated centrifuge using a JA20.1 rotor head at 4640*g* at 4 °C. The entire procedure routinely took 2–4 h to complete depending on protein concentrations in the starting material. The recovery of proteins from the Centricon-10 microconcentrator was greater than 90%.

(*D*) *Gel Permeation HPLC.* The concentrated sample containing testibumin was injected onto a Bio-Sil TSK-250 gel permeation HPLC column (7.5 × 300 mm i.d.) equipped with a Bio-Sil TSK guard column (7.5 × 75 mm i.d.) using a 100-μL sample loop from Rainin. Proteins were eluted under isocratic condition using 10 mM sodium phosphate and 0.15 M NaCl, pH 6.8 at 22 °C, at a flow rate of 0.5 mL/min. The normal operation pressure ranged between 5 and 10 bar depending on the protein content of the starting materials. Fractions (0.5 mL each) were collected, and proteins were monitored by the UV absorbance at 280 nm.

(*E*) *Hydroxylapatite HPLC.* Samples containing testibumin obtained from the gel permeation HPLC fractionation were concentrated on an Amicon Centricon-10 microconcentrator and equilibrated against 10 mM sodium phosphate and 0.01 mM $CaCl_2$, pH 6.8 at 22 °C, containing 0.05% NaN_3 (solvent A). The concentrated sample (about 1 mL) was degassed for about 10 min in an ice bath under a vacuum and then loaded onto a Bio-Gel HPHT hydroxylapatite system consisting of a guard column (4 × 50 mm i.d. packed with a spherical polymeric matrix) and a Bio-Gel HPHT column (7.8 × 100 mm i.d. packed with a hydroxylapatite derivative). Elution of proteins was performed by using a linear gradient from 0% to 100% solvent B (350 mM sodium phosphate and 0.01 mM $CaCl_2$, pH 6.8 at 22 °C, containing 0.05% NaN_3) at a flow rate of 1 mL/min. The normal operation pressure for the Bio-Gel HPHT column was about 20 bar. Fractions (1 mL each) were collected, and proteins were monitored by the UV absorbance at 280 nm.

Con A–Sepharose Chromatography. Con A–Sepharose 4B was packed in a column (1.5 × 9 cm) and washed extensively with Con A buffer (50 mM Tris, pH 7.4 at 22 °C, containing 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$) as previously described (Cheng et al., 1984). Sertoli cell enriched culture medium was applied to the column under unit gravity. The column was then eluted with Con A buffer at a flow rate of 10 mL/h. The Con A bound glycoproteins were eluted with Con A buffer containing 100 mM methyl α-mannoside. Fractions (1.5 mL each) were collected, and aliquots were assayed in duplicate for testibumin by radioimmunoassay.

Preparation of Testibumin Antiserum. Purified testibumin (60 μg of protein in 1 mL of NPB buffer) was emulsified with an equal volume of Freund's complete adjuvant and injected into a New Zealand White female rabbit in multiple sites intradermally. After 6 weeks, the rabbit received a booster injection of testibumin (50 μg in 1 mL of NPB buffer) emulsified with an equal volume of Freund's incomplete adjuvant and was bled 10 days after the boost. The rabbit was then bled every 7–10 days for a period of 2 months. Antiserum was stored at −20 °C until used. For radioimmunoassay, the antiserum was diluted in BSA–PBS buffer (10 mM sodium phosphate and 0.15 M NaCl, pH 7.4 at 22 °C, containing 0.5% BSA) containing 2% normal rabbit serum.

Crossed-Immunoelectrophoresis. Crossed-immunoelectrophoresis was performed at 12 °C according to the procedures

of Weeke (1975). Glass plates (10 × 10 cm) were covered with 15 mL of 1% agarose in Tris–Tricine–calcium lactate buffer (TTC buffer: 0.32 M Tris, 2.5 mM Tricine, and 1.5 mM calcium lactate, pH 8.6) containing 0.02% sodium azide. About 2 μg of purified testibumin in 10 μL of NPB buffer was added to a 4-mm-diameter well, and electrophoresis was carried out in the first dimension at 100 V for about 120 min. The agarose gel above the testibumin sample was replaced with 10 mL of 1% agarose containing 1% testibumin antiserum in TTC buffer. Electrophoresis was then carried out in the second dimension of 60 V for 24 h. Plates were washed, dried, and stained with Coomassie blue R-250.

Radioiodination of Testibumin. The radioiodination of purifed testibumin was performed by using Iodogen as previously described (Fraker & Speck, 1978). The labeling reaction was stopped by loading the sample onto a Sephadex G-50 column (0.8 × 22 cm), which had been washed with 1 mL of 2% BSA in NPB buffer and reequilibrated with 20 mL of the NPB buffer. The elution was carried out with the same buffer. The fractions containg ^{125}I-testibumin were pooled and stored at −20 °C until used.

Standard Testibumin. A laboratory standard was established and used in each assay to estimate the concentrations of testibumin in unknown samples. A pool medium (EP125-130) derived from Sertoli cell enriched cultures grown for 8 days in the presence of both FSH (300 ng/mL) and testosterone (2×10^{-7} M) was compared to a highly purified testibumin preparation (CYCPR3A-IV), using a dye binding assay (Macart & Gerbaut, 1982), and was found to contain 3.8 ng of purified testibumin per 1 μL equiv of EP125-130.

Radioimmunoassay of Testibumin. Each assay tube contained 200 μL of BSA–PBS buffer, 100 μL of standard or sample diluted in BSA–PBS buffer, 100 μL of antiserum at a working dilution of 1:300, and 100 μL of radiolabeled testibumin (about 10 000 cpm) purified by DEAE chromatography immediately before use to give a final volume of 500 μL. The procedures to purify iodinated testibumin by DEAE chromatography were essentially the same as those previously described for human TeBG (Cheng et al., 1983) except that a gradient of 0–0.5 M NaCl in NPB was used to elute the bound ^{125}I-testibumin which was eluted at about 0.1 M NaCl. The assay mixture was vortexed briefly and then incubated at 4 °C for 36 h. Thereafter, the bound and free iodinated testibumins were separated by adding 30 μL of formalin-fixed *Staphylococcus aureus* cells which had been washed vigorously 3 times with protein A buffer (50 mM Tris, 150 mM NaCl, 5 mM Na_2EDTA, and 0.5% NP-40, pH 7.4 at 22 °C, containing 2 mg/mL BSA), and the tubes were incubated for 1 h at 4 °C. Thereafter, the tubes were centrifuged at 4 °C for 20 min at 2000*g*, the supernatants were aspirated carefully under suction, and radioactivity was measured in the pellets. Each standard or sample was run in duplicate or triplicate and analyzed as previously described (Cheng et al., 1983). ^{125}I was quantified by γ scintillation spectrometry (Packard, Model 5110) at 70% counting efficiency. The RIA intraassay coefficient of variation was 8%; the interassay coefficient of variation was 12%. The minimal detectable dose was less than 0.4 μL equiv/assay tube, and 50% displacement was at 5 μL equiv. To estimate the concentrations of testibumin in Con A column fractions, the nonspecific interference by components of chromatography buffer was determined in two additional sets of tubes containing 0.3 mL of Con A buffer and Con A buffer plus 100 mM methyl α-mannoside. These reagents did not produce any detectable interference to the immunoassay. The cross-reactivity of testibumin in testicular cytosol, epididymal cytosol, and serum in the testibumin RIA was determined by simultaneous fitting of displacement curves using the computer program ALLFIT as previously described (DeLean et al., 1978).

Analysis of Testibumin, Rat Serum Albumin, and Rat AFP by Electrophoretic Transfer and Immunostaining. Highly purified testibumin, rat serum albumin, and rat AFP and their proteolytic digests were fractionated on SDS–polyacrylamide slab gels and transferred to nitrocellulose paper using an electrophoretic transfer technique as previously described (Burnette, 1981). After the gel was equilibrated in the transfer buffer [25 mM Tris and 192 mM glycine, pH 8.3 at 22 °C, containing 20% methanol (v/v)] for about 10 min, the proteins were transferred onto nitrocellulose paper at 60 V (approximately 0.3 A) for at least 18 h at 15 °C using a Transphor cell (Model TE42) from Hoeffer Scientific and a Model 250/2.5 power supply from Bio-Rad. All subsequent procedures were carried out at room temperature, unless otherwise specified, with agitation on a reciprocating shaker (Eberbach Corp., Ann Arbor, MI) at 70 cycles/min. For immunostaining, the nitrocellulose paper was washed twice for 10 min each with Tris–PBS buffer (10 mM Tris, 10 mM sodium phosphate, and 0.15 M NaCl, pH 7.4 at 22 °C) and then incubated with 0.5% Tween 20 in Tris–PBS buffer for 1 h. The paper was then incubated overnight with 1% rat testibumin antiserum, rat serum albumin antiserum, or rat AFP antiserum in Tris–PBS buffer containing 0.1% BSA and 0.05% NaN_3, washed twice (10 min each) in Tris–PBS buffer, and incubated with protein A–peroxidase (1 μg/mL) in Tris–PBS buffer containing 0.1% BSA. After a 5-min wash in Tris–PBS buffer containing 0.05% NP-40 (v/v) and a 10-min wash in Tris–PBS buffer to remove unbound protein A–peroxidase, the proteins that bound antibodies against testibumin, albumin, or AFP were visualized by using 4-chloro-1-naphthol (Hawkes, 1982).

Peptide Mapping of Testibumin, Rat Serum Albumin, and Rat AFP. To generate peptide maps for testibumin, rat serum albumin, and rat AFP, highly purified proteins were resolved on a 7.5% SDS–polyacrylamide gel and stained with Coomassie blue. The proteins were cut from the gel and resuspended in protease buffer [0.125 M Tris, pH 6.8 at 22 °C, containing 1 mM EDTA and 0.1% SDS (w/v)]. Peptide maps were generated by using protease V_8 on a second SDS–polyacrylamide slab gel containing 15% *T* in the resolving gel using an enzyme to substrate ratio of 1:10 (Cleveland et al., 1977). The peptide fragmentation patterns were visualized either by silver nitrate or by electrophoretic transfer to nitrocellulose paper and subsequent immunologic staining with either anti-testibumin, anti-rat serum albumin, or anti-AFP antiserum.

RESULTS

Purification of Rat Testibumin. Each 1.2-L sample of spent medium was first fractionated by anion-exchange HPLC on a preparative Mono Q column. Such a preparation had a total protein content of about 27 mg of which 1.5 mg was subsequently shown to be testibumin by radioimmunoassay (Table I). A total of 15 major protein peaks were eluted from the ion-exchange column (Figure 1A). Using the radioimmunoassay and SDS–PAGE, it was shown that testibumin was eluted in fractions between 22 and 37 (Figure 1B) under peaks 7 through 13 (Figure 1A). This step produced a cumulative 2.5-fold purification over the starting material (Table I). The fractions containing testibumin were then concentrated and fractionated onto a Mono P chromatofocusing HPLC column. Twelve protein peaks were routinely observed (Figure 2A).

Table I: Summary of the Purification Scheme for Testibumin from Rat Primary Sertoli Cell Enriched Culture Medium

step	[testibumin][a] (nmol)	[protein][b] (mg)	sp act.[c] (nmol/mg)	x-fold purification: from previous step	x-fold purification: cumulative	recovery (%): from previous step	recovery (%): cumulative
pooled and concn culture medium[d]	21.7	27.4	0.7919		1		100
Mono Q anion-exchange HPLC	17.0	8.43	2.0	2.53	2.53	78	78
Mono P chromatofocusing HPLC	12.4	2.54	4.9	2.45	6.2	73	57
Bio-Sil TSK-250 gel permeation HPLC	9.8	0.816	12.0	2.45	15	79	45
hydroxylapatite HPLC	5.3	0.373	14.2	1.20	18	54	24

[a] The testibumin concentration was measured by radioimmunoassay using an apparent molecular weight of 69 000. [b] The protein concentration was determined by the dye binding assay with bovine serum albumin as a standard (Macart & Gerbaut, 1982). [c] Specific activity was expressed as nanomoles of testibumin per milligram of protein. [d] Sertoli cell enriched culture medium (1.2 L) concentrated to 55 mL with a PM-10 membrane.

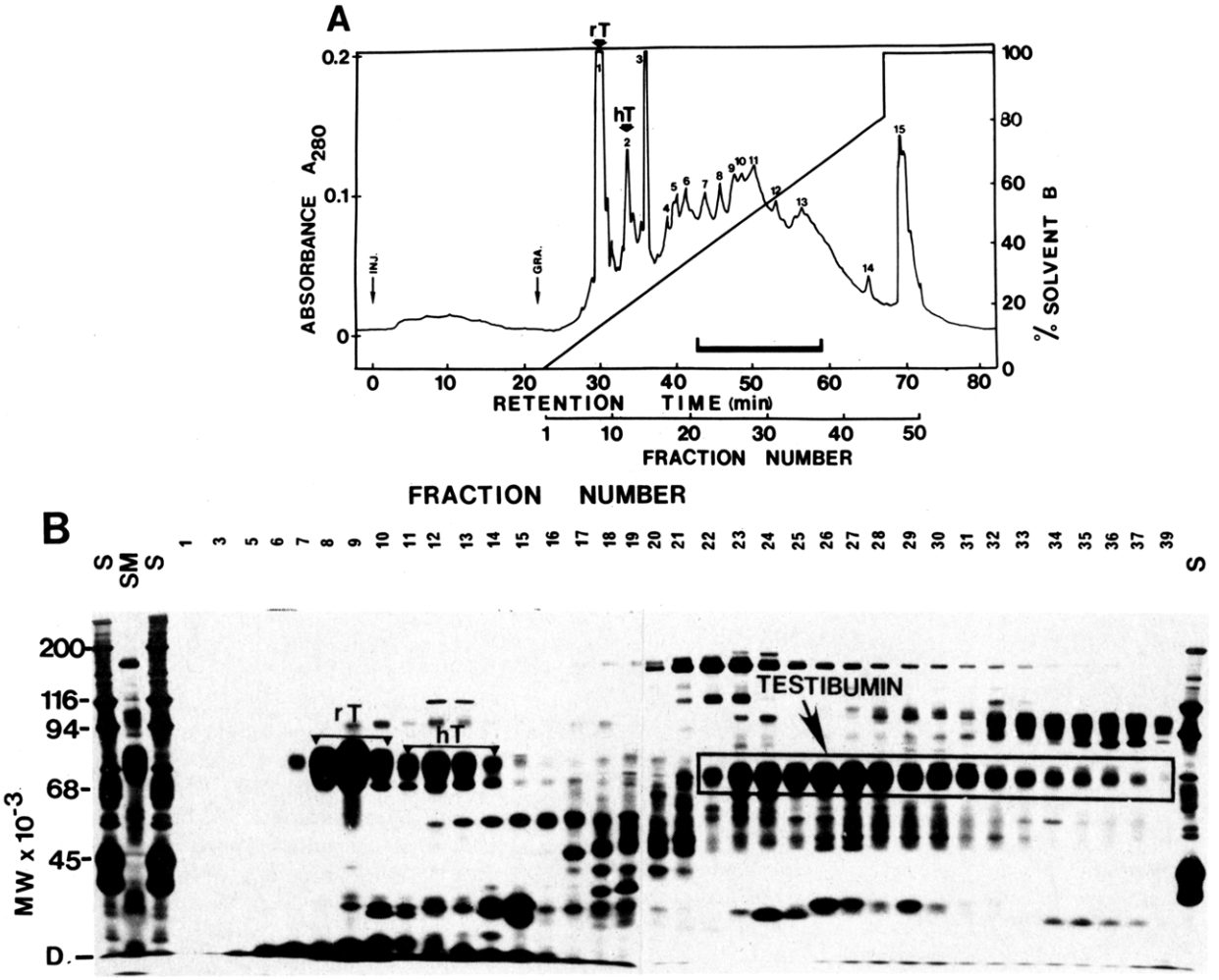

FIGURE 1: Fractionation of proteins contained in Sertoli cell enriched culture medium by anion-exchange HPLC (A) and SDS–PAGE (B). (A) Anion-exchange HPLC of proteins in Sertoli cell enriched culture medium. Culture medium (1.2 L) was concentrated and equilibrated with solvent A (20 mM Tris, pH 7.4 at 22 °C) in an Amicon ultrafiltration cell using a PM-10 membrane as described under Experimental Procedures. The concentrated sample (about 55 mL) was injected onto a preparative Mono Q column at a flow rate of 4 mL/min. The protein profile was allowed to return to the base line, and a gradient of 0–80% solvent B (20 mM Tris and 600 mM NaCl, pH 7.4 at 22 °C) was applied to the column over 45 min at a flow rate of 4 mL/min. Fractions (4 mL) were collected. The effluent was monitored by the UV absorbance at 280 nm. A total of 15 major protein peaks were resolved. INJ. indicates where the sample was loaded; GRA. indicates where the gradient started. (B) Silver-stained SDS-containing polyacrylamide gel (7.5% *T*) of proteins from fractions obtained in (A). An aliquot (10 μL) from each fraction from the anion-exchange HPLC fractionation was resolved by SDS–PAGE, and proteins were visualized by silver nitrate. The S lanes are molecular weight markers consisting of 0.3 μg each of myosin (M_r 200 000), β-galactosidase (M_r 116 500), phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 68 000), and ovalbumin (M_r 45 000). The SM lane is unfractionated Sertoli cell enriched culture medium (1.5 μg of total protein). The numbers across the top of the gel correspond to the fraction numbers shown in Figure 1A. Human transferrin (hT) and rat transferrin (rT) were identified by immunoassays as previously described (Cheng et al., 1986). Testibumin eluted between fractions 22 and 37. These fractions were pooled and concentrated for further purification. D, dye front.

Testibumin was eluted in fractions between 41 and 45 along with three major contaminating proteins (Figure 2B). The p*I* of native testibumin was estimated to be about 4.2 (Figure 2B). Fractions containing testibumin were then concentrated and fractionated on a gel permeation HPLC column. Although 10 protein peaks were eluted (Figure 3A), testibumin was present in the major protein peak in fractions 20–23 (Figure 3B). These fractions also contained at least two to three minor proteins as detected by silver stain (Figure 3B). Fractions containing testibumin were then concentrated and

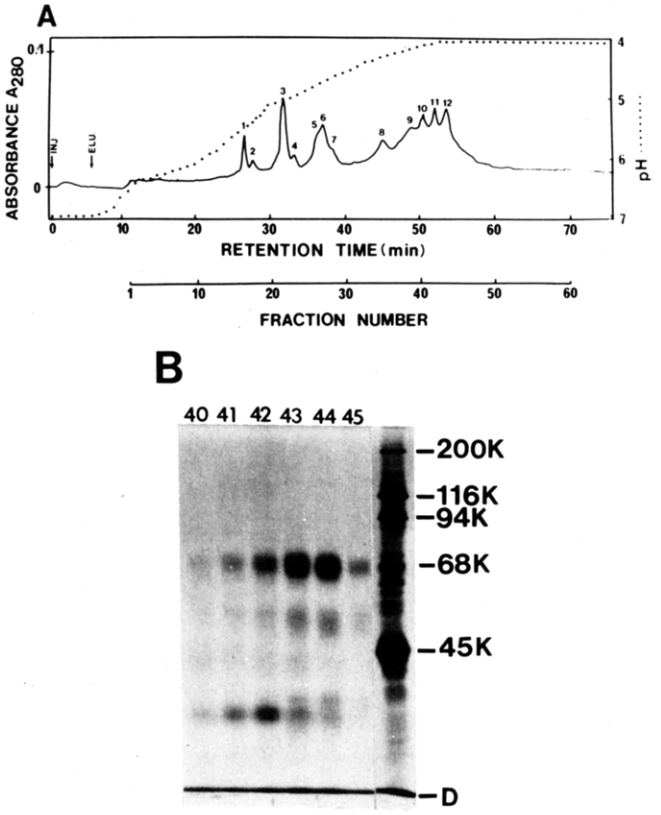

FIGURE 2: Purification of testibumin by chromatofocusing on a Mono P column. (A) Partially purified testibumin obtained from anion-exchange HPLC was equilibrated against 25 mM Bis-Tris buffer, pH 7.1 at 22 °C, concentrated, and applied to a Mono P column. Proteins bound to the column were then eluted with Polybuffer 74 as described under Experimental Procedures. The eluate was monitored by the UV absorbance at 280 nm at a flow rate of 1 mL/min; 1-mL fractions were collected. A total of 12 major protein peaks were detected. (B) Silver-stained SDS–polyacrylamide gel (7.5% *T*) of proteins from fractions 40–45 under peaks 10–12 shown in Figure 2A. Testibumin was present in fractions 41–45. Molecular weight markers were the same as those shown in Figure 1B. D, dye front.

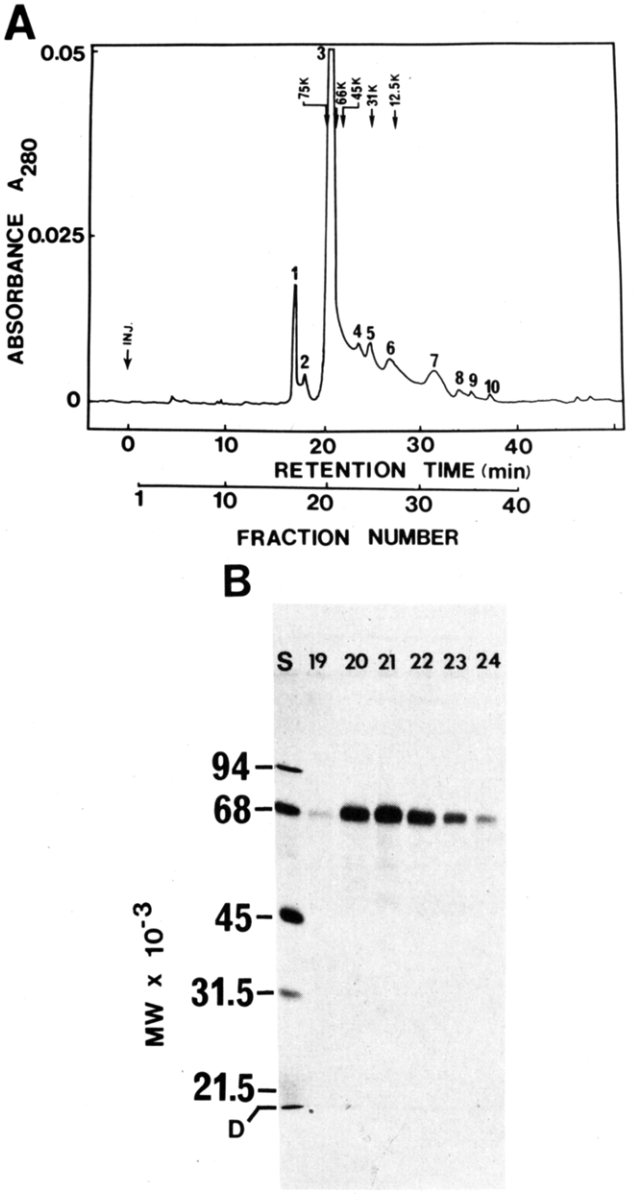

FIGURE 3: Purification of testibumin by gel permeation HPLC on a Bio-Sil TSK-250 column. (A) The fractions from the chromatofocusing HPLC (fractions 41–45) were pooled, equilibrated against 10 mM sodium phosphate and 0.15 M NaCl, pH 6.8 at 22 °C, and concentrated to 100 μL by using a Centricon-10 microconcentrator. The sample was applied to a Bio-Sil TSK-250 column and eluted with the same sodium phosphate buffer. The eluate was monitored by the UV absorbance at 280 nm at a flow rate of 0.5 mL/min. Fractions (0.5 mL) were collected. Ten protein peaks were identified; testibumin was in peak 3. (B) Silver-stained SDS–polyacrylamide gel (10% *T*) of proteins from fractions 19–24 shown in Figure 3A. Molecular weight markers used were the low molecular weight markers from Bio-Rad consisting of 0.1 μg each of phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 500), and soybean trypsin inhibitor (M_r 21 500). D, dye front.

equilibrated against 10 mM sodium phosphate, applied to a hydroxylapatite HPLC column, and eluted at about 165 mM sodium phosphate (Figure 4A). The specific activity of this preparation suggested that it contained only testibumin (Table I). Analysis of portions of the peak (fractions 15 and 17) from the hydroxylapatite column by SDS–PAGE showed only one protein band (Figure 4B, lanes 2 and 3). The purification scheme of rat testibumin from primary Sertoli cell enriched culture medium is summarized in Table I. Even though the specific activity was increased only 18-fold, four HPLC columns were required due to the marked heterogeneity of this protein.

Physicochemical Characterization of Testibumin. (*A*) *Apparent Molecular Weight of Testibumin.* The apparent molecular weight of highly purified testibumin was determined by gel permeation HPLC using a Bio-Sil TSK-250 column (7.5 × 300 mm i.d.) as described in Figure 3A. Purified testibumin and molecular weight standards were applied individually as well as in a mixture to the column. The variation in retention time for each of these standards and testibumin over a period of 10 months was less than 5%. Linear relationships between retention times and logarithms of molecular weights for standard proteins and testibumin were noted. The apparent molecular weight of native testibumin was estimated to be 70 000. The apparent molecular weight of native testibumin was also determined by the method of Lambin and Fine (1979) using PAGE with a 5–30% *T* linear gradient and estimated to be 68 000 (data not shown) which is consistent with the result obtained by gel permeation HPLC. The apparent molecular weight was also estimated under reducing conditions using SDS-containing polyacrylamide gels (7.5% *T*) by plotting the log of molecular weights of standard proteins against the relative mobilities. This analysis showed that the apparent molecular weight of testibumin was about 69 000. These results suggest that testibumin is a protein consisting of one polypeptide chain.

(*B*) *Heterogeneity of Testibumin on Con A–Sepharose Chromatography.* When Sertoli cell enriched culture medium

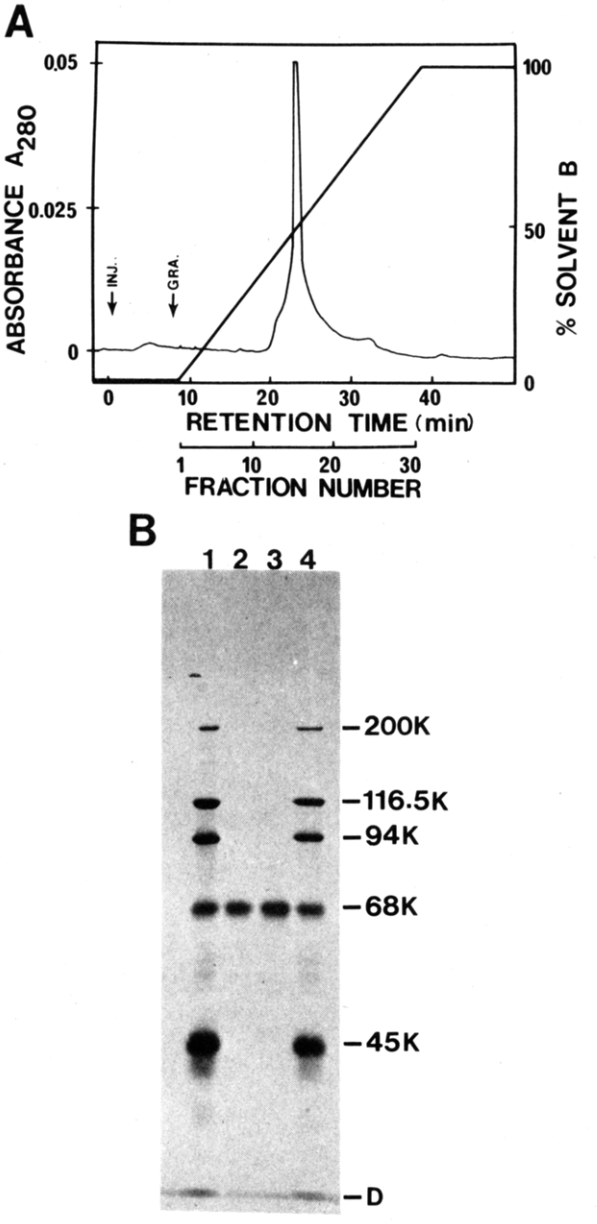

FIGURE 4: Purification of testibumin by hydroxylapatite HPLC. (A) Highly purified testibumin obtained from the gel permeation HPLC (fractions 19–24) was pooled, concentrated, and equilibrated against solvent A (10 mM sodium phosphate and 0.01 mM $CaCl_2$, pH 6.8 at 22 °C, containing 0.05% NaN_3) using a YM-10 membrane in an Amicon concentrator. A 2-mL sample was applied to a Bio-Gel HPHT column, and proteins were eluted by using a gradient of 0–100% solvent B (350 mM sodium phosphate and 0.01 mM $CaCl_2$, pH 6.8 at 22 °C, containing 0.05% NaN_3) at a flow rate of 1 mL/min. The eluate was monitored by the UV absorbance at 280 nm, and 1-mL fractions were collected. INJ. indicates where sample was loaded onto the column, and GRA. indicates where the gradient elution started. (B) Silver-stained SDS–polyacrylamide gel (7.5% *T*) of highly purified testibumin. Lanes 1 and 4 are molecular weight markers consisting of 0.2 μg each of myosin (M_r 200 000), β-galactosidase (M_r 116 500), phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 68 000), and ovalbumin (M_r 45 000). Lanes 2 and 3 represent the ascending (fraction 15) and descending (fraction 17) portions of the protein peak shown in Figure 4A consisting of about 0.2 μg of protein. D, dye front.

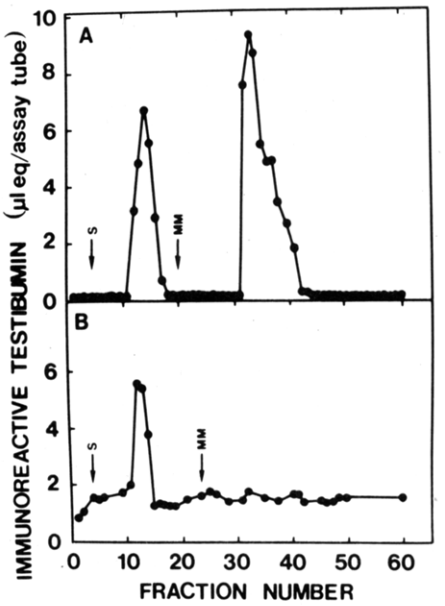

FIGURE 5: Con A–Sepharose affinity chromatography of medium from Sertoli cell enriched culture. Crude Sertoli cell enriched culture medium (EP147/148; 0.12 mg of protein) which contained about 1880 μL equiv of immunoreactive testibumin material was chromatographed on a column of Con A–Sepharose (1.5 × 9 cm), and fractions (1.5 mL) were collected at a flow rate of 10 mL/h. (A) An aliquot (0.1 mL) from each fraction was withdrawn, and the concentration of testibumin in each sample was estimated by radioimmunoassay. (B) To estimate that the first peak observed in Figure 6A was not due to overloading of the column, portions (1 mL each) of fractions 12–17 from Figure 6A were pooled and concentrated. This sample, containing 230 μL equiv of immunoreactive testibumin, was then rechromatographed on a separate Con A–Sepharose column (1.5 × 9 cm), and fractions (1.5 mL) were collected. An aliquot (0.2 mL) of each fraction was withdrawn and assayed for testibumin concentration. The amount of immunoreactive testibumin recovered under the peak was about 129 μL equiv. S indicates where sample was applied; MM indicates where elution of glycoproteins began using Con A buffer containing 100 mM methyl α-mannoside. The recovery of immunoreactive testibumin in (A) and (B) was 60% and 55%, respectively.

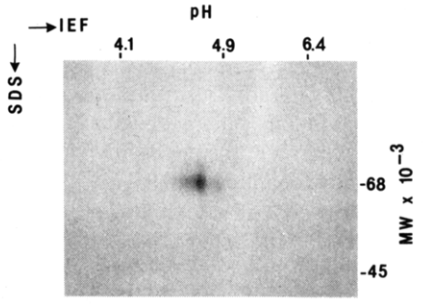

FIGURE 6: Two-dimensional electrophoresis of purified testibumin isolated from Sertoli cell enriched culture medium. About 5 μg of protein of purified testibumin was electrophoresed in an isoelectric focusing gel of pH 3.5–10. The second dimension was performed on a 10–20% linear gradient polyacrylamide gel. The gel was stained with Coomassie blue.

was fractionated on Con A columns, the immunoreactive testibumin was eluted in two peaks (Figure 5A). The first peak was eluted in the void volume, while the remainder was retained by the lectin and eluted with methyl α-mannoside. The immunoreactive activity in peak 1 was not due to overloading of the column, since when a fraction of this peak was concentrated and rechromatographed on a separate Con A column, it also did not bind to the column (Figure 5B). These analyses suggest that testibumin is a glycoprotein consisting of Con A reactive and Con A nonreactive forms.

(*C*) *Heterogeneity of Testibumin on Two-Dimensional Gel Electrophoresis.* Purified testibumin resolved by two-dimensional gel electrophoresis migrated as a broad heterogeneous band consisting of several isoelectric variants with p*I*'s ranging between 4.60 and 4.85 (Figure 6).

(*D*) *Heterogeneity of Testibumin on Crossed-Immunoelectrophoresis.* The antiserum raised against highly purified testibumin produced an immunoprecipitin arc when examined

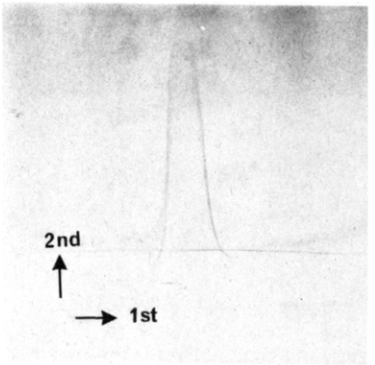

FIGURE 7: Crossed-immunoelectrophoresis of purified rat testibumin. In the first dimension (1st, horizontal), 2 μg of purified testibumin was electrophoresed in a 1% agarose gel. The gel containing the protein was removed and applied to another glass plate, and electrophoresis was then performed in a second dimension (2nd, vertical), using a 1% agarose gel containing 1% rabbit anti-testibumin antiserum.

against purified testibumin by crossed-immunoelectrophoresis (Figure 7). The presence of a single large immunoprecipitin arc suggested that most of the epitopes on this heterogeneous protein are common. The spur which fused with the ascending portion of the immunoprecipitin arc suggested that a portion of the testibumin molecules might have distinctive epitopes.

Radioimmunoassay of Testibumin. (*A*) *Radioiodination of Testibumin.* Purified testibumin was iodinated, separated from free iodine on a Sephadex G-50 column, and stored at −20 °C until used. Prior to its use for radioimmunoassay, the iodinated protein was loaded on a DEAE column and eluted with a gradient of 0–0.5 M NaCl in NPB buffer; immunoreactive ^{125}I-testibumin was eluted at about 0.1 M NaCl.

(*B*) *Purity of Testibumin Tracer.* The purity of the ^{125}I-testibumin preparations for use in immunoassays was assessed by SDS–PAGE and crossed-immunoelectrophoresis. When iodinated testibumin obtained from DEAE chromatography (about 80 000 cpm) was resolved by SDS–PAGE and visualized by autoradiography, only a single band was observed which corresponded to the silver-stained protein band on the SDS-polyacrylamide gel. In addition, with crossed-immunoelectrophoresis, the antiserum raised against testibumin produced one immunoprecipitin arc as visualized by autoradiography.

(*C*) *Dilution of Antiserum.* To determine the amount of antiserum to be used for RIA, different dilutions were incubated for 24 h at 4 °C with a constant amount of tracer (about 10 000 cpm in 100 μL). Fifty microliters of precipitating antibody was added, and the tubes were further incubated for 24 h at 4 °C. A working dilution of 1:300 was selected, which gave 25% binding of radioactivity at a final dilution of 1:1200.

(*D*) *Specificity of the RIA.* Serial dilutions of medium (EP-125-130) derived from Sertoli cell enriched cultures grown for 8 days in the presence of both FSH and testosterone were compared with dilutions of testicular cytosol, epididymal cytosol, and serum from adult male rats for their ability to compete with ^{125}I-testibumin for antibody binding sites. In all four samples tested, parallel displacement curves were obtained (Figure 8). A pool of rete testis fluid from mature rams showed no cross-reactivity with testibumin (Figure 8).

Immunologic and Structural Comparison of Testibumin, Serum Albumin, and AFP. (*A*) *Immunoblots.* With immunoblots, 0.1 μg of each protein was optimally visualized on nitrocellulose paper by its respective antiserum (1%) but not by the others (Figure 9A). However, when the concentrations of albumin and AFP were 50 times that of testibumin (5 μg vs. 0.1 μg), antiserum developed against testibumin partially

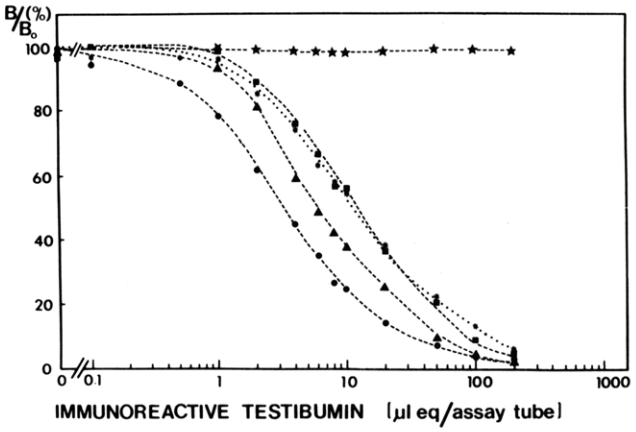

FIGURE 8: Standard curve for testibumin radioimmunoassay using Sertoli cell enriched culture medium (EP125–130) (▲) and displacement curves using male serum (■), testicular cytosol (●), and epididymal cytosol (*) from mature rats and rete testis fluid pooled from mature rams (★). The abscissa is the log dose of competitor. The response is expressed as B/B_0, where B and B_0 are the counts bound in the presence (B) and absence (B_0) of unlabeled competitor.

cross-reacted with albumin (Figure 9B, lane 3 vs. lanes 1 and 2) and with AFP but to a lesser extent (Figure 9B, lane 4 vs. lanes 1 and 2). Since the albumin contained two contaminants as shown by SDS–PAGE, we sought to determine whether the immunostaining was specific by reacting increasing amounts of the albumin preparation (5–20 μg) with testibumin antiserum. The albumin was stained with increasing intensity while the two contaminants did not stain (Figure 9C).

(*B*) *Radioimmunoassays.* To further investigate the cross-reactivity between albumin and testibumin, these proteins were used to generate displacement curves in the testibumin immunoassay (Figure 10A,B). The native rat albumin did not show any cross-reactivity with testibumin; however, reduced and S-carboxymethylated rat albumin showed partial cross-reactivity (Figure 10A). Both native and reduced BSA did not react with testibumin antiserum (Figure 10B). These data along with the immunoblots indicated that only denatured albumin was recognized with testibumin antiserum.

(*C*) *Peptide Maps.* Highly purified testibumin, rat albumin, and rat AFP were separated by SDS–PAGE and stained with Coomassie blue. The protein bands were cut from the gel and transferred to another slab gel for proteolysis using protease V_8. The peptide fragmentation patterns were identified by using silver nitrate staining and immunologic staining with the different antiserum following electrophoretic transfer to nitrocellulose paper. All three proteins showed distinctive fragments, and all had some that were similar (Figure 11A). Testibumin had a unique peptide above the enzyme lane (Figure 11A, lane 2) which was absent in the maps of AFP (Figure 11A, lane 1) or rat albumin (Figure 11A, lane 3). When peptide maps of these proteins were visualized by their respective antiserum, even more distinctive features were evident (Figure 11B). The testibumin antiserum recognized primarily the peptide fragment at 31K (Figure 11B, lane 1) while albumin and AFP antiserum recognized four and three smaller peptides, respectively (Figure 11B, lanes 5 and 9). These results indicate that the homologies between these proteins are not sufficient to produce similar peptide maps.

Physiology of Testibumin. (*A*) *Testibumin Production by Sertoli Cell Enriched Cultures as a Function of Hormone Supplement.* To determine the optimal conditions for testibumin secretion in primary culture, the effects of adding various concentrations of FSH and testosterone were examined by using cultures prepared from 20-day-old rats. FSH alone

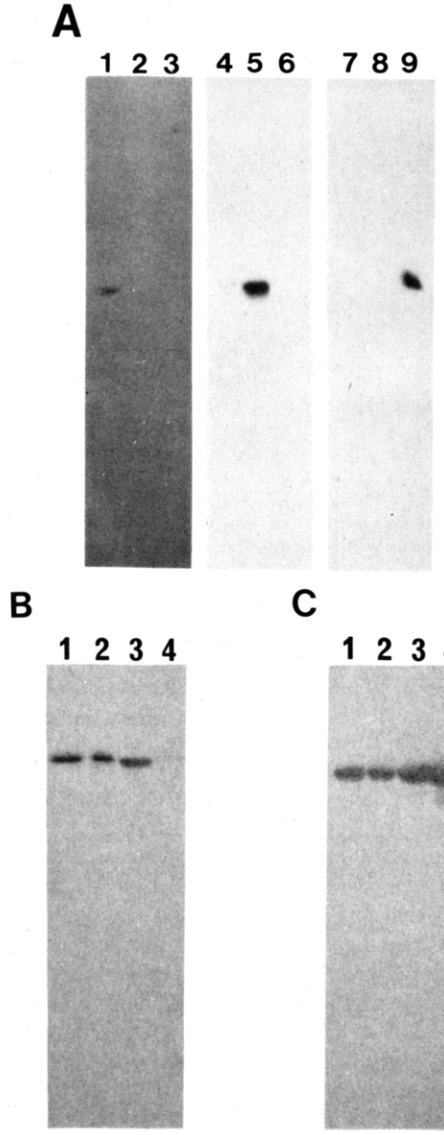

FIGURE 9: Analysis of testibumin, rat serum albumin, and rat AFP by immunoblots. (A) Highly purified rat testibumin (0.1 μg in lanes 1, 4, and 7), rat albumin (0.1 μg in lanes 2, 5, and 8), and rat AFP (0.1 μg in lanes 3, 6, and 9) were resolved on an SDS–polyacrylamide slab gel (7.5% *T*) and transferred electrophoretically to nitrocellulose paper. The nitrocellulose paper was immunologically stained by using either anti-testibumin antiserum (lanes 1–3), anti-albumin antiserum (lanes 4–6), or anti-AFP antiserum (lanes 7–9). In this study, each protein is stained only with its respective antiserum. (B) Highly purified rat testibumin (0.1 μg from two different preparations, lanes 1 and 2), rat albumin (5 μg, lane 3), and rat AFP (5 μg, lane 4) were resolved on an SDS–polyacrylamide slab gel (10% *T*), transferred electrophoretically to nitrocellulose paper, and stained with anti-testibumin antiserum. In this study, serum albumin stains as intensively as testibumin, and AFP is faintly stained. (C) Increasing concentrations of albumin (5, 10, 15, and 20 μg of protein in lanes 1, 2, 3, and 4, respectively) were stained with anti-testibumin antiserum.

produced maximal secretion at 300 ng/mL. The addition of 2×10^{-7} M testosterone produced an added stimulation of testibumin secretion (Figure 12A). Testosterone alone was not as effective as FSH. However, FSH (300 ng/mL) plus testosterone produced a 2.5-fold stimulation on the testibumin production by Sertoli cell enriched cultures (Figure 12B). We next determined the cumulative production of testibumin by Sertoli cell enriched cultures as a function of time in vitro; in the presence of FSH and testosterone, the production of testibumin peaked at day 7 (Figure 13). These observations confirm that testibumin accumulation in culture is regulated by testosterone and FSH.

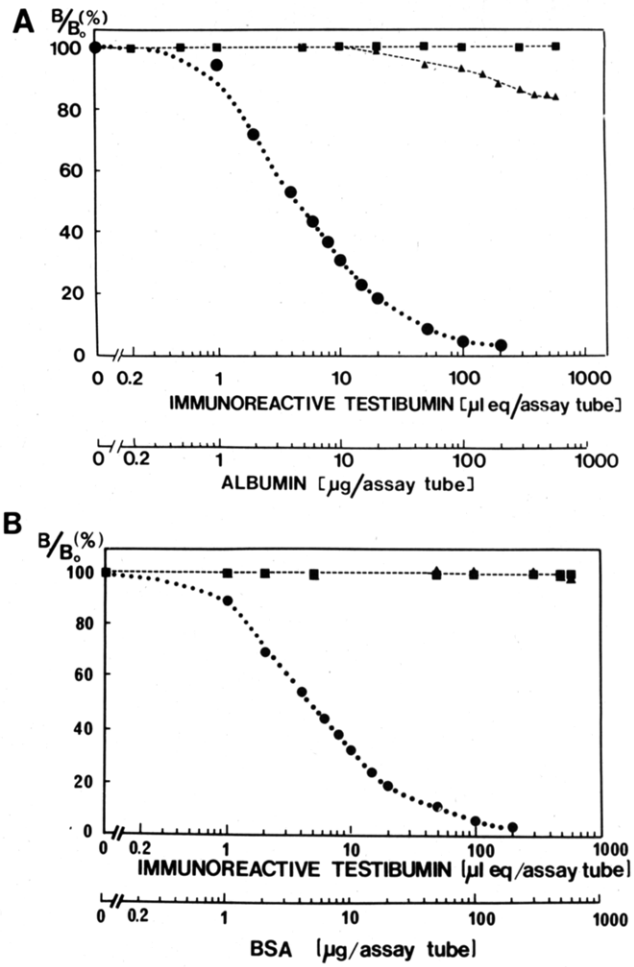

FIGURE 10: Immunologic analysis of rat testibumin, rat albumin, rat AFP, bovine serum albumin, and their reduced and alkylated derivatives by radioimmunoassay. (A) Displacement of ^{125}I-testibumin by a pool of Sertoli cell enriched culture medium (EP125–130) (●), native rat albumin (■), and reduced and S-carboxymethylated rat albumin (▲). (B) Displacement of ^{125}I-testibumin by Sertoli cell enriched culture medium (EP125–130) (●), native bovine serum albumin (■), and reduced and S-carboxymethylated bovine serum albumin (▲). The abscissa is the log dose of competitor in microliters. Values are expressed as B/B_0 where B and B_0 are the counts bound in the presence (B) and absence (B_0) of unlabeled competitor.

Table II: Distribution of Testibumin in Tissues of Male and Female Sprague-Dawley Rats

sex	age (days)	organ (*n*)[a]	testibumin concn: μL equiv/g of tissue	testibumin concn: μL equiv/organ or pair of organs
male	10	testes (5)	1330 ● 140[b]	60 ● 10
		epididymides (5)	1640 ± 74	31 ± 2
		brain (1)	1690	1609
		liver (2)	1440 ± 450	1130 ● 353
	90	testes (2)	8220 ± 830	24410 ± 3769
		epididymides (2)	8030 ● 330	7710 ± 130
		brain (1)	1270	2270
		liver (2)	1750 ● 150	22710 ● 1960
		seminal vesicles (3)	30 ● 10	29 ± 16
		kidneys (1)	2240	6180
female	90	uterus (1)	1840	520

[a] Denotes the number of rats used; all tissues were obtained from the same animal. [b] Values are expressed as mean ± SD. All assays were done in duplicate.

(*B*) *Distribution of Testibumin in Tissues and Body Fluids of Male and Female Sprague-Dawley Rats of Different Ages.* The distribution of testibumin in organs and body fluids of Sprague-Dawley rats is shown in Tables II and III. In 10-

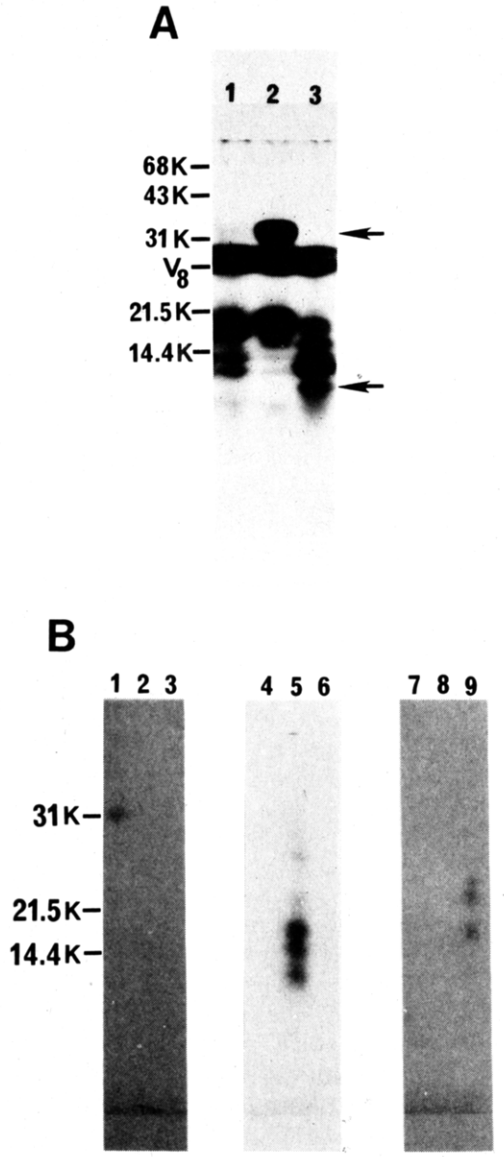

FIGURE 11: Analysis of rat testibumin, rat albumin, and rat AFP by peptide mapping using *Staphylococcus aureus* protease V8. Highly purified testibumin, albumin, and rat AFP (1 μg of protein each) were resolved on a 7.5% *T* SDS–polyacrylamide slab gel; the protein bands were visualized by Coomassie blue, sliced from the gel. They were then electrophoresed in the presence of protease V8 (0.1 μg of protein) on a second slab gel with 15% *T* in the resolving gel. At the end of electrophoresis, peptide fragments were either visualized with silver nitrate (A) or immunoblotting (B). (A) Peptide fragments of AFP, testibumin, and albumin were visualized with silver nitrate in lanes 1, 2, and 3, respectively. (B) Peptide fragments were electrophoretically transferred onto nitrocellulose paper and then visualized by immunologic staining using either anti-testibumin (lanes 1–3), anti-albumin (lanes 4–6), or anti-AFP antiserum (lanes 7–9). In lanes 1, 4, and 7 are fragments of testibumin; lanes 2, 5, and 8, albumin; and lanes 3, 6, and 9, AFP.

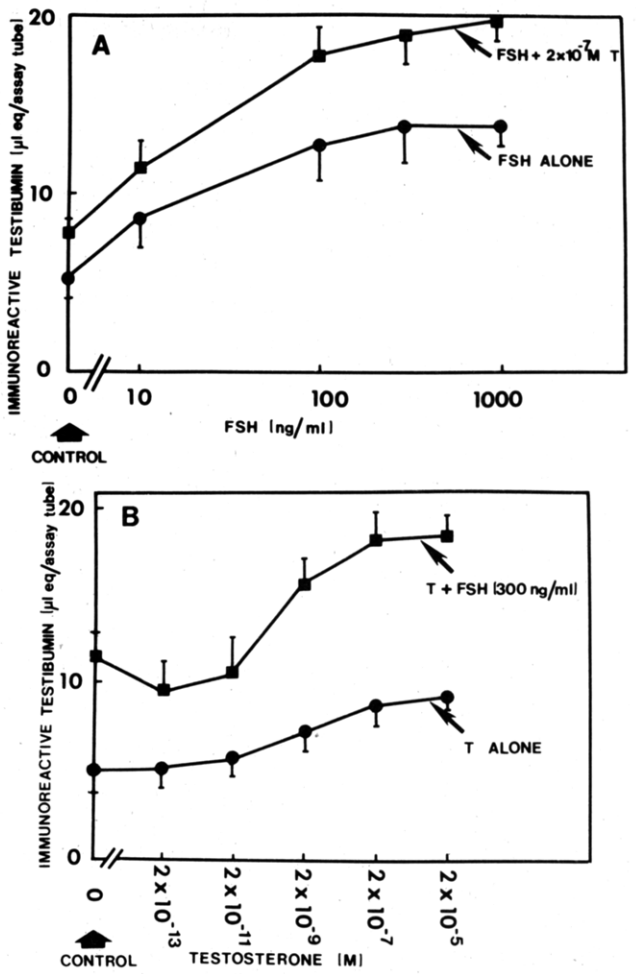

FIGURE 12: Production of testibumin by Sertoli cell enriched cultures from 20-day-old rats as a function of FSH (A) and testosterone (B). Sertoli cells were cultured for 4 days as described under Experimental Procedures in the presence of different doses of FSH and testosterone. An aliquot (30 μL) from each sample was assayed for testibumin concentration by radioimmunoassay. Values given are the cumulative mean ● SD from quadruplicate cultures.

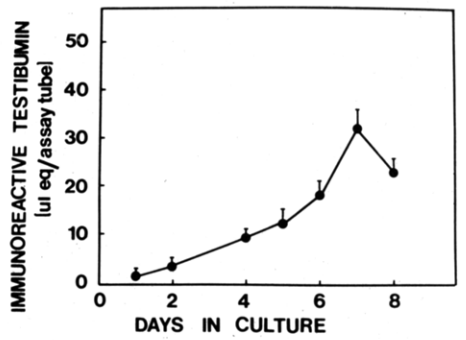

FIGURE 13: Production of testibumin by Sertoli cell enriched cultures from 20-day-old rats as a function of age in vitro. Sertoli cells were cultured as described under Experimental Procedures for different days in the presence of 300 ng/mL FSH and 2×10^{-7} M testosterone. An aliquot of medium from each culture was obtained for testibumin radioimmunoassay on the days indicated. Values given are the cumulative mean ± SD from triplicate cultures.

day-old male rats, the concentration of testibumin in all organs tested was similar to that in serum. By contrast, in 90-day-old male rats, testibumin was concentrated in testis and epididymis and was about 6-fold of that of other organs (Table II). The fact that testibumin was present in serum of both sexes suggests that it is also synthesized in sites other than the male reproductive tract (Table III). Also, the specific activity of testibumin when expressed in microliter equivalents per milligram of protein was much higher in the testicular compartment than in the vascular compartment (Table III). The specific activities of this protein in the seminiferous tubule and rete testis fluids were 10- and 200-fold higher than in the vascular compartment, respectively (Table III), suggesting that testibumin is secreted into the tubular lumen. The fact that the specific activity in the interstitial fluid was higher than that in serum suggests that this protein may also be released from the base of Sertoli cells or from the rete testis.

We next compared the concentration of testibumin in the male reproductive organs and fluids with those of rABP (Table

Table III: Distribution of Testibumin in Body Fluids Obtained from Male and Female Sprague-Dawley Rats at 60 Days of Age

body fluid	protein concn (mg/mL)	testibumin concn (μL equiv/mL)	sp act. (μL equiv/mg of protein)
male rat serum (n = 4)[a]	52 ± 13	1510 ± 140[b]	29
female rat serum (n = 3)	56 ± 14	1310 ± 300	23
male rat thoracic lymph (n = 3)	35 ± 8	950 ± 57	27
interstitial fluid (n = 3)	40 ± 6	1930 ± 220	48
seminiferous tubule fluid (n = 4)	29 ± 8	11540 ± 3040	398
rete testis fluid (n = 3)	0.6 ± 0.4	3710 ± 240	6183
amniotic fluid[c] (n = 2)	3 ± 1	180 ± 19	60

[a] Denotes the number of rats used. [b] All values are expressed as mean ± SD. [c] Amniotic fluid was collected from mother rats at 17-day gestation.

Table IV: Concentrations of Testibumin and rABP in Sertoli Cell Enriched Culture Medium, Tissue Extract, and Reproductive Tract Fluids of Sprague-Dawley Rats

sample/tissue extract	[testibumin][a]	[rABP][b]	testibumin:rABP ratio
Sertoli cell enriched culture medium (μg/mL) (EP125–130)	3.8	0.14	27.1
testis (90 day) (μg/g)	31.2	2.36	13.2
epididymides (90 day) (μg/g)	30.5	18.62	1.6
rete testis fluid (60 day) (μg/mL)	14.1	4.76	3.0
interstitial fluid (60 day) (μg/mL)	7.3	0.72	10.1
seminiferous tubule fluid (60 day) (μg/mL)	43.8	3.45	12.7
male serum (60 day) (μg/mL)	5.7	0.20	28.5
amniotic fluid (17-day gestation) (μg/mL)	0.7	0.17	4.1

[a] The testibumin concentration was determined by RIA, as described under Experimental Procedures. One microliter of EP125–130 is equivalent to 3.8 ng of purified testibumin. [b] rABP concentration was determined by RIA as described elsewhere (Cheng et al., 1984). One microliter of a standard epididymal cytosol preparation (81-9-2) for calibration of the standard curve which compared to the NIH rABP standard WR-R1 was found equal to 9.8 ± 0.9 fmol of binding sites (mean ± SEM). Assuming the molecular weight of rABP to be 85 000 (Musto et al., 1980), 1 μL equiv was therefore equivalent to 0.833 ng of purified rABP.

IV). It was of interest to note that the concentration of testibumin in the testis was similar to that in the epididymis; by contrast, the concentration of rABP in testis was only one-seventh of that in the epididymis (Table IV). The ratio of testibumin to rABP is similar in interstitial and seminiferous tubule fluids (10.1 vs. 12.7). As these two proteins pass from the interstitial fluid into serum, the ratio increases from 10.1 to 28.5. By contrast, when they pass from the tubular fluid to the rete testis and then to the epididymis, the ratio progressively decreases (from 12.7 to 3.0 to 1.6). These observations suggest that the transport and/or metabolism of these proteins is strikingly different.

Discussion

We recently developed a technique for identifying proteins of interest in serum-free culture medium using HPLC and SDS–PAGE (Cheng et al., 1986). The approach is similar to that of two-dimensional gel electrophoresis, except that separation of proteins by charge (i.e., the first dimension) was performed on a preparative Mono Q column. This anion exchanger has a large loading capacity so that samples concentrated from as much as 1.2 L of serum-free culture medium can be fractionated in an hour. Proteins in the medium are well resolved and eluted in a small volume to permit their further purification. The fractionation of a small aliquot of each fraction from the HPLC column by SDS–PAGE separated the proteins according to sizes (i.e., the second dimension) and permitted visualization by a highly sensitive silver staining procedure, thus providing clues for purification strategies. We now report the use of such an approach to purify a glycoprotein, which is responsive to FSH and testosterone, from the Sertoli cell enriched culture medium. It should now be possible to purify all the major proteins in such a culture medium.

Sertoli cells secrete serum proteins such as transferrin (Skinner & Griswold, 1980; Skinner et al., 1984), retinol binding protein (Huggenvik & Griswold, 1981; Carson et al., 1984), and ceruloplasmin (Wright et al., 1981; Skinner & Griswold, 1983) into the luminal compartment of the testis; we therefore wondered whether Sertoli cells would also secrete an analogue of serum albumin. In the mammalian fetus, AFP is the dominant plasma protein which is produced in the embryonic yolk sac. Its synthesis is continued in the liver parenchymal cells until after birth when production of AFP decreases dramatically and albumin becomes the predominant protein in the serum [for reviews, see Ruoslahti & Engvall (1978), Ruoslahti & Seppala (1979), and Crandall (1981)]. Both AFP and albumin consist of a single polypeptide chain except that the former is a glycoprotein containing 4% carbohydrate and its molecular size is slightly larger than albumin (M_r 70 000 vs. 68 000, respectively) (Crandall, 1981). The inverse relationship in the expression of AFP and albumin and the physicochemical similarities between these two proteins suggest that AFP may be the fetal analogue of serum albumin. During our survey of Sertoli cell secretory proteins, we encountered two proteins which have sizes similar to rat albumin. These proteins were termed CMB-1 and CMB-9 (Cheng et al., 1986). Since CMB-1 was more abundant and responded to hormones, it was isolated and compared to albumin and AFP structurally and immunologically. Even though this is a major protein in the culture medium, it was difficult to purify because of its heterogeneity. For example, this protein eluted over a wide range of salt concentrations from the anion-exchange HPLC; by contrast, most other proteins in Sertoli cell enriched medium were eluted in two to six fractions as noted in Figure 1B. In addition, several other proteins eluting in the fractions with testibumin from the anion-exchange column had similar pI's and sizes. Therefore, three additional HPLC steps were required to isolate this protein.

Using an immunologic approach, it was shown that testibumin is related to albumin and AFP in the same way as AFP and albumin relate to one another. That is, antibodies against testibumin do not cross-react with native albumin or AFP but recognized their denatured derivatives. Similarly, anti-AFP and anti-albumin antibodies do not cross-react with native albumin and AFP, respectively but do show partial cross-reactivity once these proteins are reduced and alkylated (Ruoslahti & Engvall, 1976). While peptide maps generated for these proteins suggested differences in their primary structure, it is of interest to note that they also share common features in their fragmentation patterns. These data suggest that this protein is related to the AFP/albumin family of proteins.

Studies of the distribution of testibumin in different organs and body fluids suggest that this protein is synthesized in both

sexes. The testis, however, is a major site of synthesis in male rats as evidenced by the fact that it is produced by testicular cells in culture and that the highest specific activity of testibumin is found in rete testis fluid. Moreover, its specific activity in the tubular compartment is several orders of magnitude higher than that of the vascular compartment. Since it is a major protein of the testis and in view of its similarity with albumin and AFP, the name testibumin seems justified.

The use of electron microscopy and immunocytochemistry to study the distribution of rat serum albumin within the testicular compartment showed that this protein localized extracellularly in the interstitial space (Christensen et al., 1985). At the base of the seminiferous epithelium, albumin extended between Sertoli cells but was not localized within these cells and did not traverse the Sertoli–Sertoli junctional complexes (Christensen et al., 1985). These observations also suggested that testibumin is immunologically distinct from albumin. The use of two-dimensional gel electrophoresis to study the proteins in the luminal fluids collected from the seminiferous tubule, rete testis, and epididymides (Olson & Hinton, 1985) has identified a 68K protein which comigrates with serum albumin. The p*I*'s of this protein complex ranged between 6.0 and 6.5 and are thus strikingly different from testibumin (p*I*'s 4.5–4.85). This luminal protein might be CMB-9 (Cheng et al., 1986) which has an apparent molecular weight similar to albumin and testibumin. It is also possible that this 68K protein complex may be a molecular variant of testibumin due to in vitro oligosaccharide processing within the luminal compartment which alters its p*I*'s.

In conclusion, testibumin is a FSH- and testosterone-responsive glycoprotein in medium from the rat primary Sertoli cell enriched cultures prepared from 20-day-old rats. This protein was purified to apparent homogeneity, a specific radioimmunoassay established, and its hormonal responsiveness determined. This protein is immunologically related to albumin and AFP. A better understanding of testibumin and its relation to albumin and AFP must await detailed sequence analysis of this Sertoli cell protein.

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^{1}H NMR Study of Dynamics and Thermodynamics of Heme Rotational Disorder in Native and Reconstituted Hemoglobin A†

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ABSTRACT: The reaction of heme and apoprotein has been studied in detail by ^{1}H NMR spectroscopy in order to elucidate the conditions for reconstitution of hemoglobin (Hb) to yield the native protein. The initially formed holoprotein exists as a mixture of isomers with individual subunits possessing the two heme orientations differing by a 180° rotation about the α,γ-meso axis [La Mar, G. N., Yamamoto, Y., Jue, T., Smith, K. M., & Pandey, R. K. (1985) *Biochemistry 24*, 3826–3831]. We characterize in detail herein the rates and mechanism of heme reorientation and show that the rates differ dramatically for met-aquo and met-azido derivatives and are highly pH dependent in both subunits in a fashion that allows selective equilibration in either subunit. Nonequilibrium mixtures of such isomers can be kinetically trapped in the met-azido form and stored in this metastable form for many months. With kinetically controlled heme orientationally disordered Hb, unambiguous assignment of ^{1}H NMR resonances to individual subunits has been made for the met-azido derivative, which demonstrates ~2% and 10% equilibrium heme disorder in the α- and β-subunits, respectively. Comparison of the ^{1}H NMR spectra of various heme rotationally disordered Hb derivatives indicates that this disorder is observable in all forms studied, but is most easily recognized as heme disorder and most conveniently monitored in the met-azido complex. Structural consequences of heme disorder appear to manifest themselves much more strongly in peripheral than axial interactions at the heme. Preliminary studies reveal that both the rate of autoxidation of oxy-Hb and the azide affinity of met-aquo-Hb depend on the orientation of the heme.

The reaction between apohemoglobin and heme does not yield the pure native holoprotein within a few milliseconds, as originally thought (Gibson & Antonini, 1960; Rose & Olson, 1983), but affords initially a ~1:1 mixture of the holoprotein with the heme rotationally disordered with respect to the α,γ-meso axis (Figure 1) (La Mar et al., 1985). The equilibration of this metastable heme orientation to yield the structure essentially as defined by single crystal X-ray diffraction (Perutz, 1970; Fermi, 1975; Baldwin & Chothia, 1979) takes several hours to many days. Most importantly, the intermediate does not completely disappear with time, and the identification of ^{1}H NMR spectral characteristics of this intermediate in preparations of native Hb[1] A indicate that a significant degree of equilibrium heme orientation disorder exists within at least one of the subunits (La Mar et al., 1985).

The heme orientation disorder was identified in the ^{1}H NMR spectrum of freshly reconstituted Hb A in both the met-aquo and carbonyl form using the met-azido complex as the spectroscopic probe (La Mar et al., 1985). Thus the hyperfine-shifted portion of the ^{1}H NMR spectrum of freshly reconstituted metHbN_3 exhibited twice the number of peaks as observed in the native protein (Davis et al., 1969; Neya & Morishima, 1981), and assignments of the individual heme resonances using isotope labeling revealed the characteristic interchange of environments, 5-CH_3 ↔ 8-CH_3 and 1-CH_3 ↔ 4-vinyl (La Mar et al., 1978, 1980a, 1983; La Mar, 1979; Lecomte et al., 1985), predicted for the two orientations differing by a 180° rotation about the α,γ-meso axis (Figure 1). While the spectral assignment of peaks to the two heme orientations was direct and unambiguous, it was possible to

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[1] Abbreviations: Hb, hemoglobin; metHb, ferric hemoglobin; Mb, myoglobin; metMb, ferric myoglobin; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; IHP, inositol hexaphosphate; ppm, parts per million.

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