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A Sulfated Glycoprotein Synthesized by Sertoli Cells and by Epididymal Cells is a Component of the Sperm Membrane[1]

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ABSTRACT

We report here the purification, partial characterization and immunofluorescent localization of a dimeric acidic glycoprotein (DAG-protein) secreted by cultures of Sertoli cells of rat testes. Partially purified protein was obtained after chromatography over Sepharose 4B under conditions which favored a soluble, nonaggregated form of the protein. Rechromatography over the same column under reducing conditions yielded very pure monomers of 41,000 daltons and 29,000 daltons. Antibodies were prepared against the mixed monomers and used to immunoprecipitate proteins in spent medium from cultures incubated with [^{35}S] methionine, $^{35}SO_4^=$ or tunicamycin. DAG-protein and another protein (Band 4, 70,000 daltons) were coprecipitated by the antiserum and all contained $^{35}SO_4^=$ in their structures. It was shown by Western blotting that the antiserum cross-reacted very weakly with Band 4 protein. The DAG-protein polypeptides secreted in the presence of tunicamycin were assumed to lack N-glycosylation and exhibited apparent molecular weights of 27,000 and 21,000 daltons. Immunoprecipitations of media from organ cultures of testis and epididymis yielded DAG-protein of slightly lower molecular weight than the protein secreted in Sertoli cell cultures.

Indirect immunofluorescence of DAG-protein in paraffin sections of testis and epididymis revealed that the protein was concentrated in the cytoplasm of Sertoli cells, on the stereocilia of epididymal principal cells, in the cytoplasm of epididymal halo cells, and was associated with late spermatids and mature sperm. Sperm were specifically labeled on the acrosome, at the neck, and on the endpiece of the tail.

No enzymatic or structural function has been ascribed to DAG-protein as yet, but the protein must play a pivotal role in spermatogenesis because it is secreted by both the testis and epididymis and becomes an integral component of sperm.

INTRODUCTION

Sertoli cells synthesize and secrete a number of glycoproteins which may serve important roles in the regulation of spermatogenesis. Many of these glycoproteins can be obtained from the medium of primary cultures of Sertoli cells and have been characterized by gel electrophoresis and fluorography (Wilson and Griswold, 1979; Kissinger et al., 1982). Most of the radioactivity (as [^{35}S] methionine) which can be incorporated into the secreted glycoproteins in cell culture is found in only six or seven polypeptides. A small amount of radioactivity is also found in a large number of minor constituents. Several of the major secreted polypeptides have been identified and characterized. Polypeptides previously designated Bands 1 and 3 on fluorograms have been shown to be testicular ceruloplasmin (Skinner and Griswold, 1983a) and transferrin (Skinner and Griswold, 1980), respectively. It has been proposed that these metal transport proteins provide appropriate ions to developing germinal cells which are effectively sequestered from contact with lymph or serum by the blood-testis barrier.

Two other secreted polypeptides, designated Bands 5 (41,000 daltons) and 6 (29,000 daltons), were shown to be disulfide-linked monomers of a larger protein (71,000 daltons) which has an isoelectric point of approximately 4.2 and appeared to be heavily glycosylated (Kissinger

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et al., 1982). This protein was designated as dimeric acidic glycoprotein or DAG-protein, because its function is as yet unknown. This report is concerned with the purification and partial characterization of DAG-protein. In addition, antibodies have been used to localize DAG-protein in sections of testis and epididymis tissue, and on spermatozoa.

MATERIALS AND METHODS

Cell Culture and Protein Collection

Sertoli cells from 20-day-old rats were prepared for and maintained in cell culture as previously described. The serum-free Ham's F-12 medium contained 0.1 mM dibutyryl cyclic AMP (cAMP) and 0.1 mM testosterone throughout the culture period. The spent medium which contained the secreted proteins was collected every 4 days and pooled. Medium and hormones were replaced four times over the 16-day culture period. On the fourth day of culture, a portion of the cultures were incubated for 24 h with medium which contained 1 mCi/ml of [^{35}S] methionine ($\sim$ 400 Ci/mmol, New England Nuclear, Boston, MA). The pooled medium was collected and the secreted proteins were concentrated by ultrafiltration through an Amicon (Danvers, MA) YM-10 filter. In general, each liter of spent medium was reduced to 10 ml of concentrated secreted proteins. This 10-ml fraction was desalted by passing it over a column containing P-6 gel (Bio-Rad, Richmond, CA) equilibrated in 10 mM Tris HCl, pH 7.4. The fraction which contained protein was collected and stored frozen at −80°C. In some experiments, [^{35}S]-Na_2SO_4 ($\sim$ 1 Ci/mmol, New England Nuclear) was used rather than [^{35}S] methionine. The medium in these experiments was prepared without sulfates. In other experiments, 0.1 μg/ml tunicamycin (Sigma, St. Louis, MO) was added with media containing [^{35}S] methionine on the fourth day of culture.

Purification Procedure

The concentrated secreted protein fraction which contained approximately 50 mg of protein and was obtained from more than 10 l of spent medium was precipitated by adding solid $(NH_4)_2SO_4$, and the precipitate made 6 M guanidine hydrochloride (Sigma). The solution was applied to a 5 × 100-cm column containing Sepharose 4B (Pharmacia, Piscataway, NJ) equilibrated with 6 M guanidine hydrochloride, 10 mM Tris-HCl, pH 7.5. The column was eluted with the same buffer and 4-ml fractions were collected. The protein elution from the column was monitored by absorbance at 280 nm and by [^{35}S] methionine radioactivity. The major protein peak (Fractions 335 to 385) was collected and concentrated by ultrafiltration on a YM-10 membrane to 20 ml. This solution was made 1% β-mercaptoethanol and reapplied to the same column which had subsequently been equilibrated in 6 M guanidine hydrochloride, 10 mM Tris, pH 7.5 and 1% β-mercaptoethanol. The protein was then eluted in this same buffer.

Antibody Production

Fractions from the second Sepharose 4B column which contained the heavy (H) and light (L) subunits were dialyzed and lyophilized. This protein (a mixture of H and L subunits) was used for three subcutaneous injections into a rabbit. Approximately 300 μg of the protein in complete Freund's adjuvant was used for the first injection. This was followed 2 wk later by an injection of 150 μg of protein in Freund's incomplete adjuvant. The final injection of 750 μg was made in Freund's incomplete adjuvant after an additional 2 wk and the antibody titer was boosted every 2 m by injection of more antigen.

Polyacrylamide Gel Electrophoresis and Fluorography

One-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done in slab gels containing gradients of 5 to 15% acrylamide. The gel formulation was that of Laemmli (1970) and fluorography was performed as previously described (Skinner and Griswold, 1983b). Two-dimensional electrophoresis was accomplished by the method of O'Farrell (1975) with modifications previously described (Kissinger et al., 1982).

Immunoprecipitations

Immunoprecipitations were accomplished by the method described by Kessler (1981). Up to 1-ml samples of spent culture medium were mixed with 10 μl of a suspension of *Staphylococcus aureus*, 10% w/v (Pansorbin, Calbiochem-Behring, La Jolla, CA) and incubated at room temperature for 30 min. The Pansorbin with nonspecifically bound proteins was removed by centrifugation at 12,000 × *g* for 4 min. The supernatant was then incubated with 20 μl of rabbit anti-DAG-protein serum or normal rabbit serum (as a control) overnight at 4°C. Ten microliters of Pansorbin was added to the mixture and incubated 1 h at room temperature. The mixture was then centrifuged at 3000 × *g* for 4 min and the pellet was washed three times by resuspension and centrifugation in 0.05 M Tris, pH 7.4, 0.15 M sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, and 10 μg/ml phenylmethylsulfonyl fluoride. Finally, the immunoprecipitate was suspended in the appropriate electrophoresis sample buffer and analyzed by one- or two-dimensional gel electrophoresis.

Protein Blotting and Immunodetection

Radioactive secreted proteins were submitted to two-dimensional electrophoresis and then electrophoretically transferred to nitrocellulose sheets as described by Burnette (1981). Nonspecific protein binding sites were blocked by incubating the sheets in 3% bovine serum albumin (BSA) (Fraction V), 150 mM NaCl, 50 mM Tris, pH 7.4, for 2 h at 37°C. The nitrocellulose sheets were then incubated with rabbit antirat DAG-protein 1:2000 in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.25% gelatin and 0.05% Triton X-100 for 4 h at room temperature and then buffer alone for 2 h. The sheets were then incubated in a similar manner sequentially with biotinyl goat antirabbit immunoglobulin and avidin-peroxidase (Vector Lab., Burlingame, CA). The sheets

were developed with HankerYates reagent (Sigma) and autoradiographed.

Organ Culture

Selected portions of testis and epididymis were obtained from anesthetized rats and chopped finely. The tissue was then suspended in Hank's balanced salt solution, shaken, and allowed to settle 5 min. The supernatant was discarded and the process was repeated twice more in an attempt to remove as many spermatozoa as possible. Approximately 25 mg of wet tissue was incubated in 1 ml of Ham's F-12 medium containing 0.2 mCi of [^{35}S]methionine for 5 h at 32°C. The tissue was removed by centrifugation at 12,000 × *g* for 4 min. The supernatant was immunoprecipitated as described above and then analyzed by gel electrophoresis.

Immunofluorescence and Histochemical Methods

Unmated adult male Sprague-Dawley rats were anesthetized and killed by cervical dislocation. A testis, epididymis, and vas deferens were removed and placed in Bouin's fixative. Selected portions of tissues were embedded in paraffin and sectioned at 4 μm. To make smears of spermatozoa, portions of fresh testis, epididymis, or vas deferens were minced briefly and shaken in phosphate-buffered saline (PBS). Rats were also electroejaculated (Lawson et al., 1967) and the sperm plugs obtained were shaken in PBS for 5 min to release sperm. After the tissue pieces or sperm plugs settled out, the supernatants (containing spermatozoa) were collected and centrifuged at 3000 × *g* for 4 min. The pellets were washed twice with PBS and then suspended in a small volume of PBS. Single drops of the cell suspensions were smeared on cleaned microscope slides and allowed to air dry for 30 min. After 5 min fixation in absolute methanol, the slides were dried and used immediately or stored at room temperature for not more than 5 days.

Rehydrated tissue sections and smears were incubated in phosphate buffered saline, 3% calf serum, pH 7.4 (PBS-CS) for 10 min at room temperature prior to staining. The sections were then incubated overnight at 4°C with rabbit anti-DAG-protein or normal rabbit serum, both diluted 1:100 in PBS-CS. After three washes over a 30-min period at room temperature, all sections were treated with biotinylated goat antirabbit immunoglobulin (Vector Labs.) 1:100 in PBS-CS overnight at 4°C. The sections were washed three times as before and then treated with fluorescein-conjugated avidin (Vector Labs.) 1 μg/ml in PBS-CS for 30 min at room temperature. The slides were washed again and then coverslips were mounted with 10% n-propyl-gallate in glycerol (Giloh and Sedat, 1982). Related sections were stained with hematoxylin and eosin for reference.

Immunofluorescence microscopy was performed using a Nikon Model L-Ke microscope equipped for epifluorescence illumination and photomicroscopy. Photomicrographs were recorded with Kodak Tri-X or Kodak High-Speed Ektachrome with exposure indices of 800. All fluorescence exposures were made for 60 sec to assure uniform comparison of antibody-treated and control slides.

RESULTS

Purification of DAG-Protein

Previous work from this laboratory has characterized the secreted glycoproteins from cultured Sertoli cells by gel electrophoresis and fluorography. In Fig. 1, the results of a typical fluorogram from this type of analysis are shown. The bands labeled *Tf* and *Cp* have been identified as testicular transferrin and ceruloplasmin, respectively (Skinner and Griswold, 1980, 1983a). The proteins in bands designated *H* (heavy) and *L* (light) have been shown to be linked by disulfide bonds to form a larger protein which has been designated dimeric acidic glycoprotein or DAG-protein (Kissinger et al., 1982). DAG-protein is the only protein in the mixture which separates into monomers upon reduction with mercaptoethanol. The protein labeled Band 4 has not been well characterized but is structurally related to DAG-protein and will be discussed later.

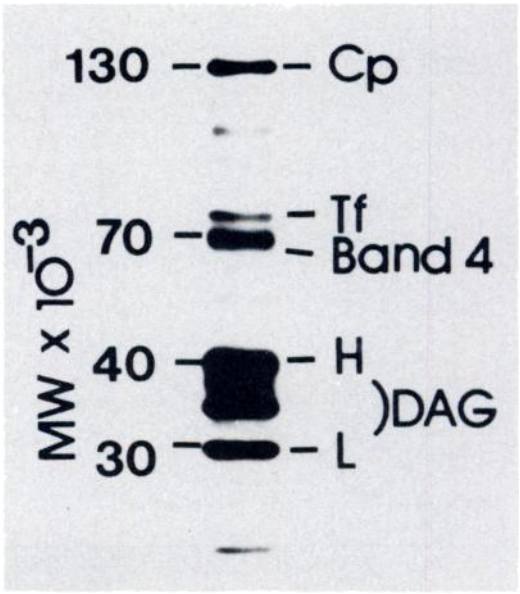


FIG. 1. Fluorograph of polyacrylamide gel electrophoresis of radiolabeled proteins secreted by rat Sertoli cells in culture. Sertoli cell cultures were prepared as described in *Materials and Methods*. Cultures were incubated with medium containing [^{35}S]methionine and the spent medium obtained after 24 h was concentrated, desalted, and loaded onto a slab gel with a polyacrylamide concentration gradient of 5 to 15%. The proteins designated *Cp* (ceruloplasmin), *Tf* (transferrin), and *DAG* (dimeric acidic glycoprotein) have been identified in previous publications.

Initially, the purification of DAG-protein involved the collection of large amounts of spent medium from cultured Sertoli cells and the concentration of the glycoproteins in that medium by ultrafiltration. The mixture of glycoproteins in the concentrated medium tended to aggregate. This aggregation could not be reduced or prevented in high or low salt buffers, or in 8 M urea. However, 6 M guanidine hydrochloride reversed the aggregation and allowed the chromatographic separation of the proteins. Therefore, the first purification step of the concentrated proteins involved chromatography over Sepharose 4B equilibrated in 6 M guanidine hydrochloride and 10 mM Tris pH 7.5. The elution profile of the proteins was monitored by absorbance at 280 nm and by the radioactivity due to the incorporation of [^{35}S] methionine (Fig. 2). The composition of the eluted fractions was also analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The results showed that the major absorbance peak which corresponds with Fractions 335 to 385 contained 3 major radioactive polypeptides which were transferrin and the 2 monomers of the dimeric acidic glycoprotein (see Lane 1, Fig. 4). Band 4 protein was very labile on storage and thus did not appear in analysis of these columns. These fractions (335 to 385) were pooled, concentrated by ultrafiltration, reduced with β-mercaptoethanol and rechromatographed over the same Sepha-

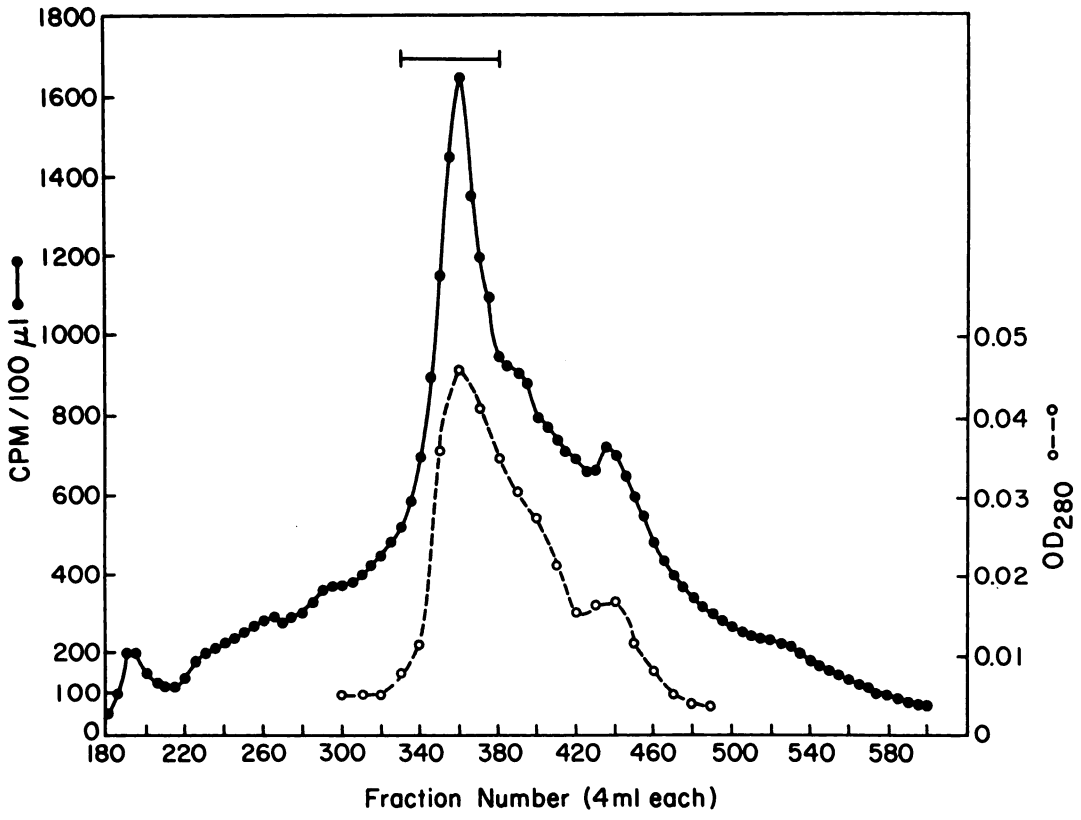


FIG. 2. Sepharose 4B column chromatography of radiolabeled proteins secreted by Sertoli cells in culture. Approximately 10 l of spent medium from cultured Sertoli cells was desalted and concentrated by ultrafiltration to 210 ml. This sample contained 34.5 mg of total protein and 3.4 X 10^6 cpm of [^{35}S] methionine incorporated into the protein. Solid $(NH_4)_2SO_4$ was added to the sample until saturation occurred and the protein precipitate was centrifuged at 10,000 X *g* for 15 min and resuspended in 9.5 ml of 6 M guanidine hydrochloride, 10 mM Tris, pH 7.5 (Buffer A). This sample was applied to a column (2.5 cm X 40 cm) of Sepharose 4B equilibrated in Buffer A. Fractions (4 ml) off the column were analyzed for radioactivity by monitoring a 100-µl sample in a liquid scintillation counter. The absorbance profile for the eluted protein was monitored by an ISCO UV monitor at 280 mn. Fractions 335 to 385 were pooled for further chromatography.

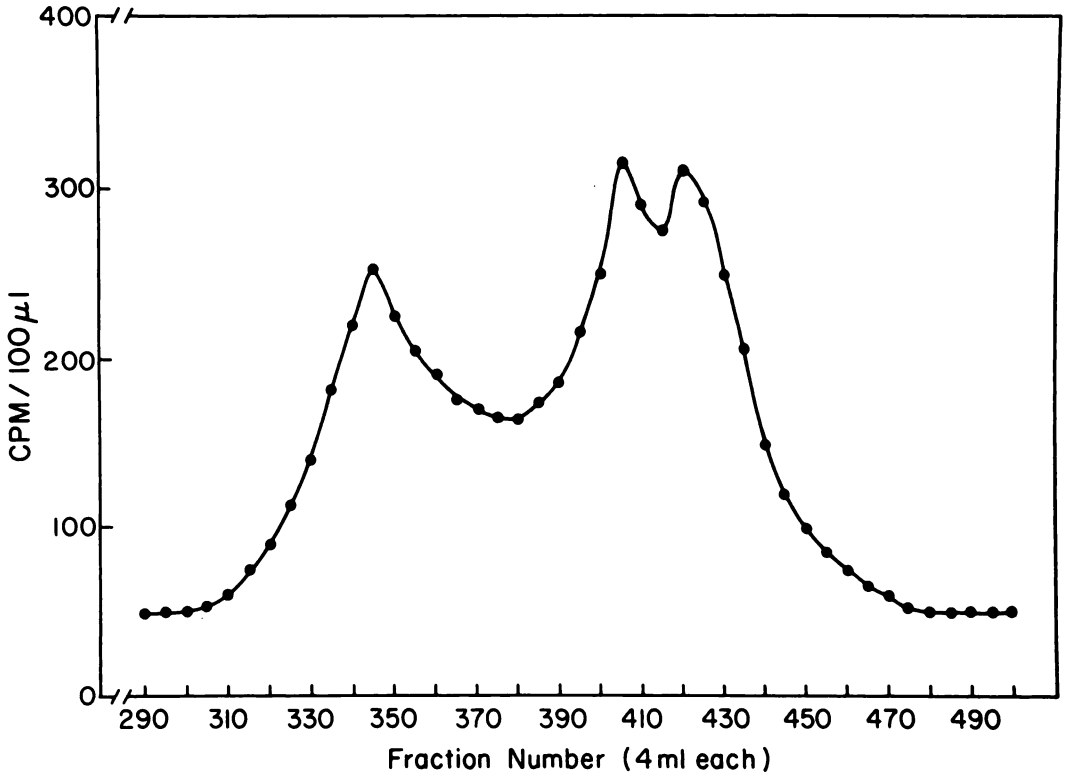


FIG. 3. Sepharose 4B column chromatography of partially purified DAG-protein under reducing conditions. Fractions 335 to 385 from the column profile shown in Fig. 2 were concentrated to 10 ml by ultrafiltration over a YM-10 membrane. The sample was made 1% mercaptoethanol and chromatographed over the same column described in Fig. 2 except that the column was equilibrated in 6 M guanidine hydrochloride, 10 mM Tris, pH 7.5, and 0.15 M β-mercaptoethanol. All other conditions were identical to those described. The protein elution was monitored by determining the radioactivity in 100 μl of the 4-ml fraction.

rose column equilibrated in 6 M guanidine hydrochloride, 10 mM Tris, pH 7.5, and 1% β-mercaptoethanol. The elution profile of the radioactive proteins after chromatography under these reducing conditions is shown in Fig. 3. Analysis of this profile by SDS-polyacrylamide gel electrophoresis and fluorography showed that the first peak (Fractions 335 to 365) contained transferrin and other minor contaminants of a similar molecular weight. Fractions 395 to 430 contained the reduced subunits of the dimeric acidic glycoprotein (Fig. 4). Previous analysis of the Sertoli cell-secreted proteins showed that only one major glycoprotein (DAG-protein) in the entire mixture contained disulfide-linked monomers (Kissinger et al., 1982). As a result, the Fractions 395 to 435 were not contaminated by any other proteins as determined by SDS-polyacrylamide gel electrophoresis. Some of the fractions (385 to 405) contained only the heavy subunit (H), some (430-435) contained only the light subunit (L), while the intermediate fractions contained mixtures of both subunits. The fluorogram is shown in Fig. 4, but the staining of total protein with Coomassie blue gave identical results.

Immunoprecipitations of DAG-Protein

Fractions which contained mixtures of both subunits were used to produce an antiserum in rabbits to the DAG-protein. The antiserum produced to denatured reduced subunits reacted strongly in double diffusion gel analysis with concentrated mixtures of Sertoli cell-secreted proteins producing only one precipitin line.

The antiserum was used to immunoprecipitate radioactive DAG-protein from mixtures of Sertoli cell-secreted proteins. The secreted proteins were obtained from a variety of

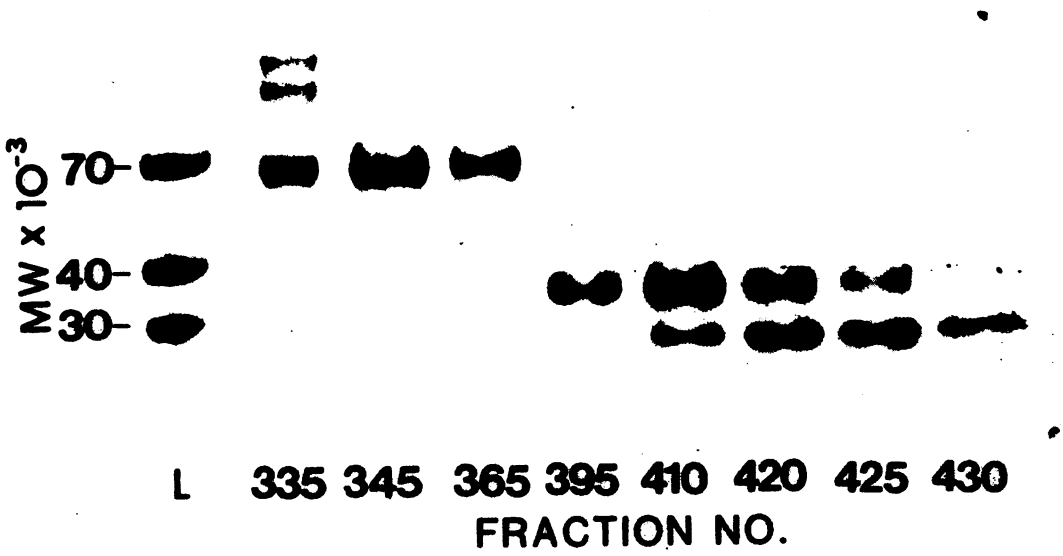


FIG. 4. Polyacrylamide gel electrophoresis and fluorography of selected fractions from the column profile shown in Fig. 3. Aliquots (100 μl) of fractions (4ml) were dialyzed for 48 h against H_2O and analyzed by gel electrophoresis. The fluorogram of the gel is shown. One lane (*L*) represents the pooled fractions from the column in Fig. 2.

culture conditions and had incorporated several different isotope precursors. The immunoprecipitates were analyzed by gel electrophoresis and fluorography. First, if the secreted proteins were labeled with [^{35}S] methionine, the antiserum immunoprecipitated the DAG-protein and coprecipitated a small amount of Band 4 protein (Fig. 5). The amount of Band 4 protein that coprecipitated appeared to be only a fraction of that present in the original sample. When the cultured Sertoli cells were labeled with [^{35}S] methionine in the presence of tunicamycin and analyzed by polyacrylamide gel electrophoresis, the molecular weights of the secreted proteins were generally reduced. If the antiserum was used to immunoprecipitate DAG-protein and Band 4 protein, the molecular weight of each was also decreased (Fig. 5). The monomers which normally migrate on these gels with apparent molecular weights of 41,000 and 29,000 were reduced to molecular weights of 27,000 and 21,000. Band 4 protein appeared to decrease from a molecular weight of 71,000 to 51,000. The radioactivity shown running at the dye front in Fig. 5, Lane *C* may have resulted from some proteolysis of the nonglycosylated proteins.

In order to determine if the antiserum to DAG-protein was cross-reacting with or was coprecipitating the Band 4 protein, an additional analysis was done. Sertoli cell-secreted proteins labeled with [^{35}S] methionine were subjected to two-dimensional gel electrophoresis. The proteins were then electroblotted onto nitrocellulose sheets and after appropriate blocking procedures, were incubated with anti-DAG-protein immunoglobulins. The nitrocellulose was washed and the anti-DAG-protein immunoglobulins were detected with biotinyl goat antirabbit immunoglobulins and avidin-peroxidase enzyme. The results of this analysis (Fig. 6) showed that the antiserum reacts strongly with DAG-protein subunits and only very weakly with the Band 4 protein. Note that the DAG-protein subunits displayed a pronounced charge heterogeneity when analyzed by two-dimensional gels. This pattern of multiple spots characteristic for DAG-protein has been previously reported (Kissinger et al., 1982).

When the Sertoli cells were cultured in the presence of $^{35}SO_4^=$, the radioactivity was incorporated into some of the secreted proteins. If the macromolecules from this medium were concentrated and analyzed by gel electrophoresis and fluorography, it was apparent that significant amounts of $^{35}SO_4^=$ are incorporated into 3 bands of molecular weights of 70,000,

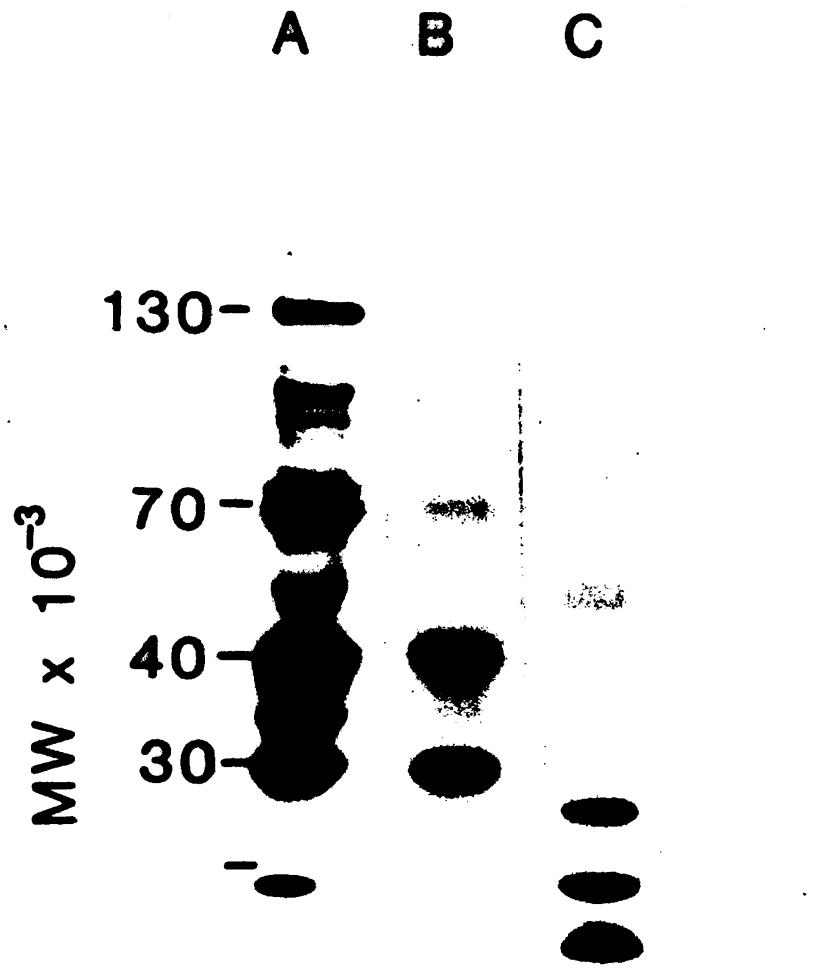


FIG. 5. Fluorograph of polyacrylamide gel electrophoresis of immunoprecipitated DAG-protein. Sertoli cell cultures were treated with [^{35}S] methionine or [^{35}S] methionine and tunicamycin. The spent media from these cultures was immunoprecipitated and the precipitates were analyzed by electrophoresis as described in *Materials and Methods*. The lanes are: *A*) [^{35}S] methionine-labeled, Sertoli cell-secreted proteins obtained from cultured Sertoli cells; *B*) immunoprecipitation of *A* using anti-DAG-protein; *C*) immunoprecipitation of DAG-protein from medium from Sertoli cells cultured in the presence of tunicamycin.

41,000 and 29,000 daltons (Fig. 7). These three bands represented Band 4 protein and DAG-protein since they could be immunoprecipitated by anti-DAG-protein antiserum (data not shown). The radioactivity incorporated into the heterogeneous high molecular weight bands at the top of the gel represent proteoglycans and glycosaminoglycans (M.K.S., unpublished observations).

Immunofluorescence Localization of DAG-Protein

To determine the cellular location of DAG-protein in vivo, indirect immunofluorescence studies were carried out. The method selected involved the use of a biotinylated second antibody and fluorescein-conjugated avidin to achieve a high ratio of fluorescence to immunoreactive site.

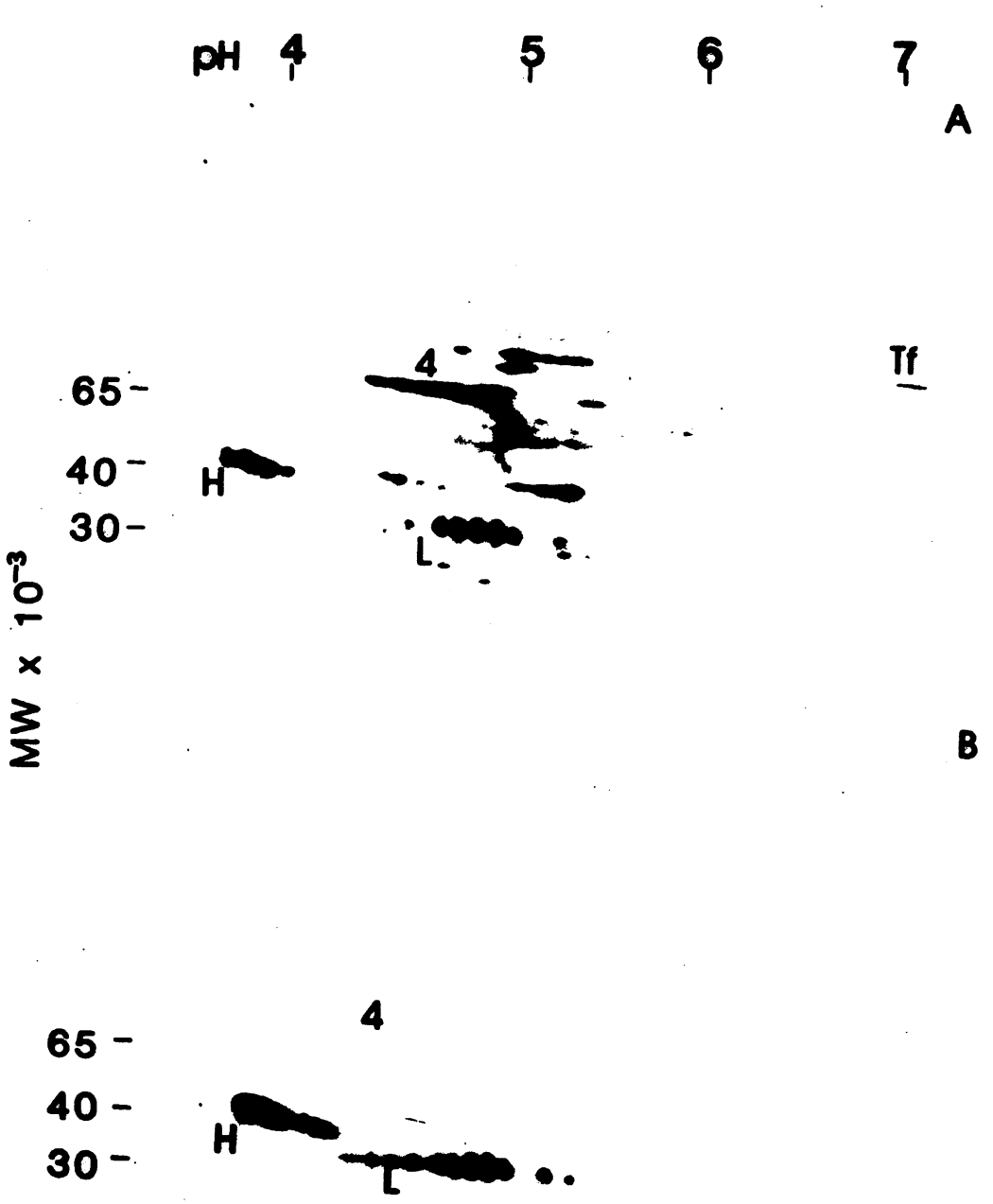


FIG. 6. Two-dimensional electrophoresis and immunoblot analysis of Sertoli cell-secreted proteins and anti-DAG-protein antiserum. The secreted proteins were labeled with [^{35}S]methionine and analyzed by two-dimensional gel electrophoresis. The proteins were then blotted onto nitrocellulose and the blotted proteins were detected by autoradiography (*A*) and by incubation with anti-DAG protein antibody which was detected by an avidin-biotin immunoperoxidase system as described (*B*). *A*) The autoradiograph of the nitrocellulose paper shown in *B*.

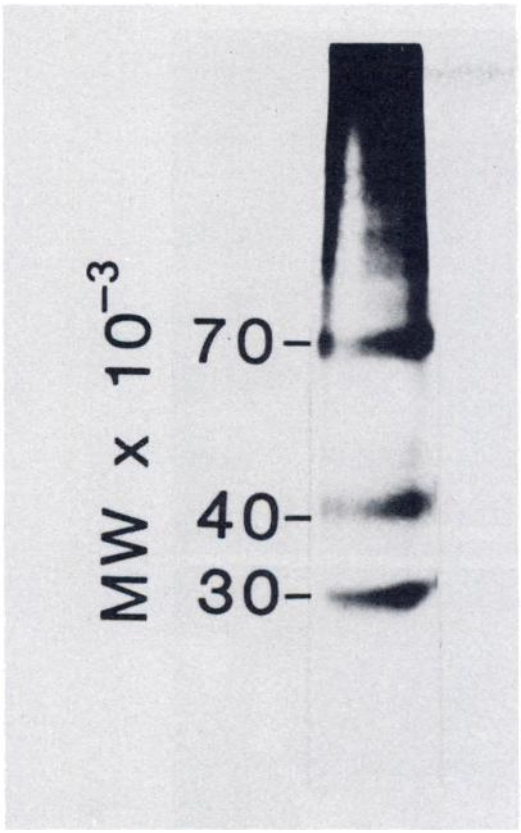


FIG. 7. Incorporation of $^{35}SO_4^=$ into Sertoli cell-secreted proteins. Cultured Sertoli cells were incubated with [^{35}S]-Na_2SO_4 and the secreted proteins were collected, concentrated and desalted. The radioactive material was analyzed by gel electrophoresis and fluorography as described.

The most intense staining in testis thin sections was observed in three locations of the seminiferous tubules: in the Sertoli cell cytoplasm, on the heads and tails of late spermatids, and in the residual bodies (see Fig. 8a and b). Spermatogonia, spermatocytes and early spermatids were not stained significantly, nor were peritubular cells.

The staining in the epididymis was quite complex. The epididymis can be divided morphologically into four areas: the initial segment, the caput, the corpus and the cauda. In the initial segment, the cytoplasm of apical cells (found only in this segment) and principal cells were lightly stained (Fig. 9a). In the caput, the cell membranes and stereocilia of the principal cells were brightly fluorescent. The staining in the corpus was similar to that in the caput but less intense (Fig. 9b). In the cauda, the halo cells stained brightly and the principal cells were lightly stained along the stereocilia (see Fig. 9c). Clear cells in the cauda were devoid of staining as could be seen by the breaks in fluorescence along the luminal surface of the tubule. The tissue between the tubule walls did not stain significantly. The heads and tails of luminal spermatozoa were stained throughout the organ with varying intensity (Figs. 9a-c).

The epithelium of the vas deferens was stained only along the luminal border (not shown). The sperm in the vas deferens remained stained and may have contributed to the staining observed along the luminal border.

Smears of spermatozoa from the testis, epididymis, vas deferens and also from ejaculates were stained to reveal fine detail. Immunoreactive DAG-protein was concentrated in the acrosomal region of the head, at the neck, and on the endpiece of the tail (Figs. 10a and b). The principle piece of the tail was stained lightly but significantly more than in control slides. By continuously adjusting the focus and using high-powered optics, the staining of the spermatozoa was found to be consistent with a membrane-associated antigen. (This analysis does not lend itself to photographic documentation.)

Control slides of tissue sections or smears treated with nonimmune rabbit serum were stained uniformly and very lightly such that photomicrographs were dark with barely discernible images. Further, sections treated with an antibody to an unrelated protein exhibited a unique staining pattern, suggesting that the antisera in this study was specific for DAG-protein (Sylvester and Griswold, 1984).

Epididymal Secretion

The epididymis is known to absorb fluid, protein and fragments of spermatozoa (Mason and Shaver, 1952). To determine if the staining of DAG-protein in the epididymis was due to digested sperm or due to synthetic products of the epididymis, organ culture studies were initiated. Figure 11 shows a fluorograph of one-dimensional SDS-gel electrophoresis of anti-DAG-protein immunoprecipitates of [^{35}S] methionine-labeled proteins from spent organ culture media. It can be seen that the testis and caput epididymidis secrete relatively more DAG-protein than the cauda epidi-

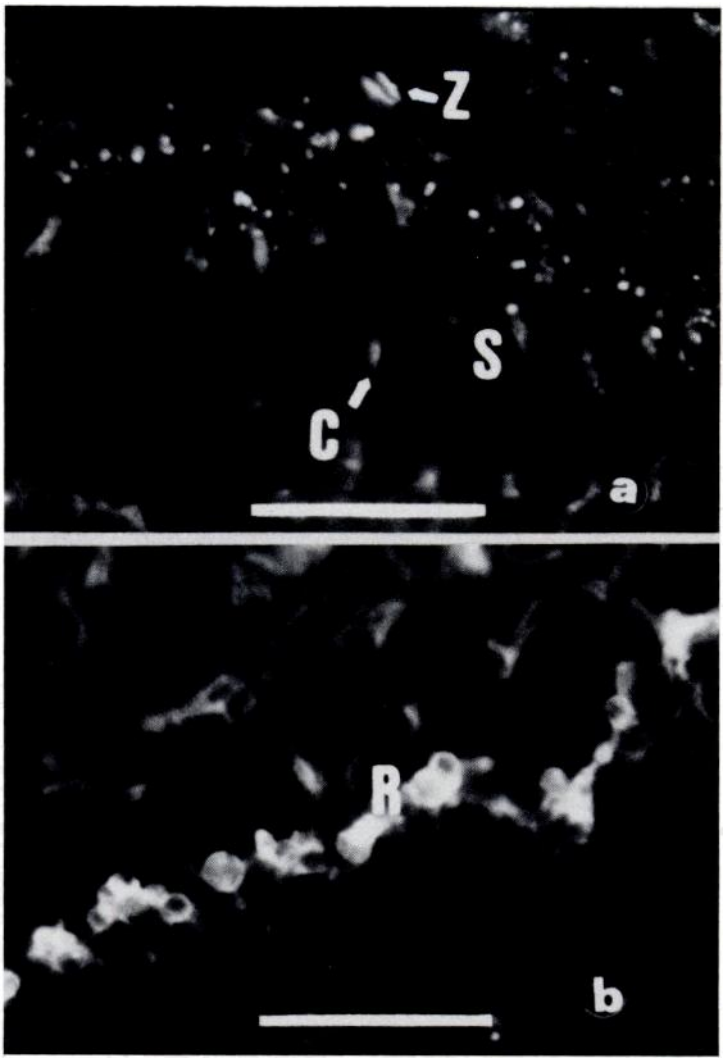


FIG. 8. Indirect immunofluorescence of DAG-protein in rat testis. A testis from a 60-day-old rat was fixed with Bouin's solution and embedded in paraffin. Rehydrated sections (4 μm) were treated with antibody to DAG-protein, biotinylated second antibody, and fluorescein-conjugated avidin as described in *Materials and Methods*. *a*) Sertoli cell cytoplasms (*C*) and heads and tails of spermatozoa (*Z*) are heavily stained. *b*) In tubule cross section where they occurred, residual bodies (*R*) stained brightly. Bars=25 μm.

dymidis. No detectable DAG-protein was secreted by the vas deferens. Also, it is apparent that DAG-protein monomers and Band 4 secreted in organ cultures of adult tissues are of slightly lower molecular weight (27,000, 35,000, and 60,000 daltons, respectively) than those secreted in Sertoli cell cultures from immature testes. Note that the first lane of this gel is substantially overexposed to the film and some minor contaminants or proteolytic degradation products in the immunoprecipitate are visible.

Analysis of immunoprecipitates from caput epididymidis organ culture by two-dimensional gel electrophoresis shows the monomers of DAG-protein to be of more basic isoelectric point than the Sertoli cell culture counterparts (data not shown). The basis for the variability in forms of the protein is currently under investigation.

DISCUSSION

Although many proteins involved in male reproduction have recently been identified, the

roles which these proteins play in the reproductive processes are unknown or often speculative. We have purified and partially characterized a protein secreted in relatively large amounts by cultured Sertoli cells and localized it in the male reproductive tract in an attempt to gain insight into its biological functions.

From the data presented, we conclude that DAG-protein is a sulfated, heavily glycosylated protein composed of heterodimers linked by disulfide bonds. The evidence that DAG-protein is glycosylated is as follows: [^{3}H] fucose is incorporated into the DAG-protein in culture (Wilson and Griswold, 1979), the charge heterogeneity of the protein is greatly altered by neuraminidase (Kissinger et al., 1982), and tunicamycin results in the secretion of subunits of significantly reduced molecular weights. Sulfation probably occurs on a component of the oligosaccharide chains. The sulfation of specific glycoproteins such as luteinizing hormone has been well documented (Anumula and Bahl, 1983) and other sulfated glycoproteins have been reported in oviductal epithelium and oviductal fluid (Basa and Oliphant, 1981; Hanscom and Oliphant, 1976).

Antiserum made to the purified monomers of DAG-protein weakly recognized an additional protein termed Band 4. Our interpretation is that the DAG-protein and Band 4 are associated in a complex and thus Band 4 can be coprecipitated with antiserum to DAG-protein. Since the antiserum does not react strongly with Band 4 (the immunodetection on Western blots is barely discernible), it is unlikely that the binding of antibody to Band 4 contributes to the results obtained from our immunofluorescence studies. In these studies the antiserum bound to antigens in fixed, embedded, dehydrated tissue and only the strongest antibody-antigen interactions should have survived the procedure. Nevertheless, the conclusions from these studies must be tempered by the possibility that Band 4 protein is localized differently than DAG-protein and contributes to the observed immunofluorescence. Band 4 protein and DAG-protein are structurally related (both are acidic, sulfated glycoproteins of approximately 70,000 mol. wt.) and may be functionally related. The clarification of these relationships must await further analysis.

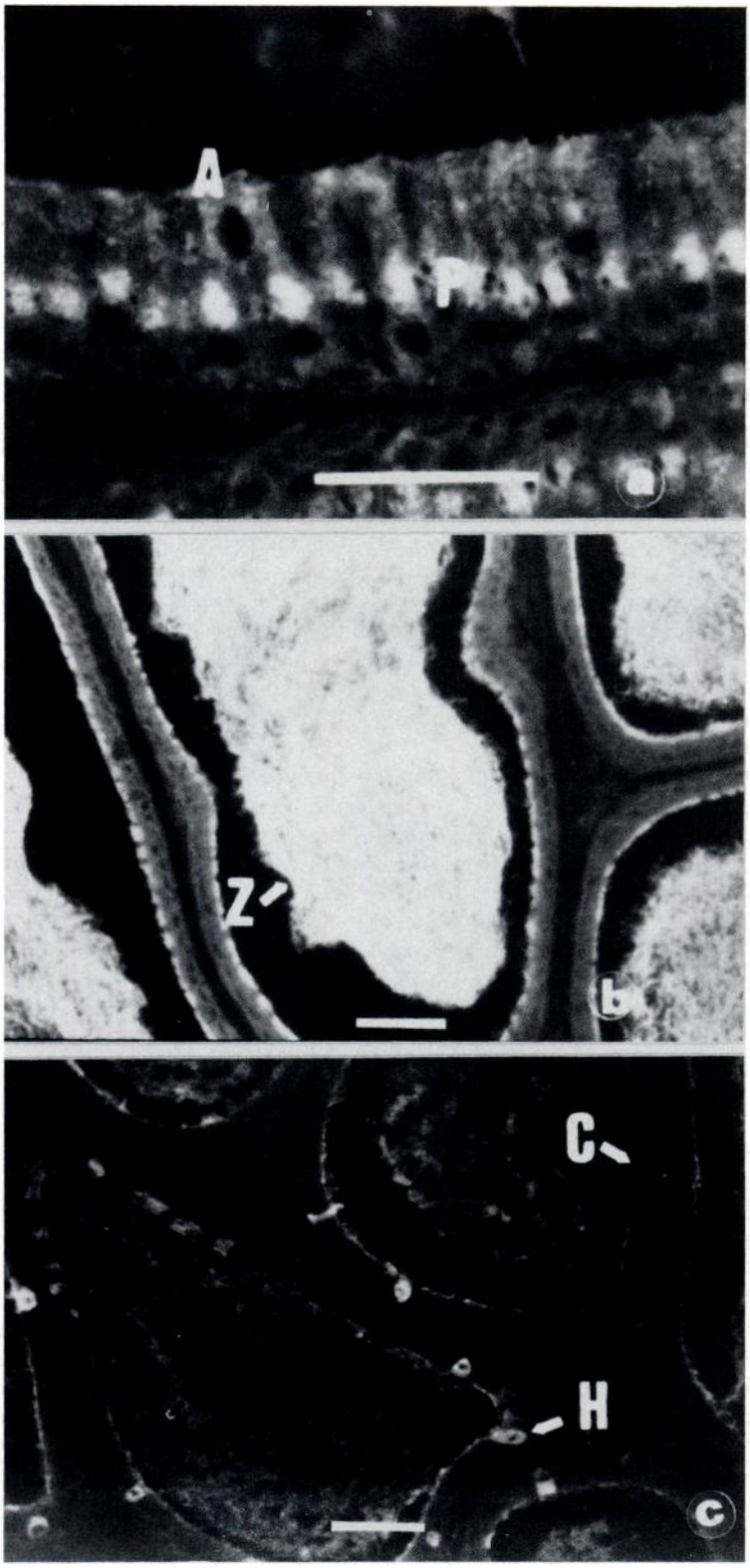
Immunofluorescence studies revealed that the DAG-protein was localized within the Sertoli cell cytoplasm, became a lasting component of the sperm, and also appeared in the epithelium of the epididymis and vas deferens. This is the first demonstration of a major protein product secreted by the Sertoli cell which becomes an integral part of the mature sperm. Organ culture of epididymal tubules confirmed that the protein was also a major secretion product of the caput epididymidis and not present solely as a result of the resorbtive property of the organ.

Brooks (1983a) has described a protein secreted by epididymal organ cultures which is of slightly lower molecular weight than the protein we have observed in Sertoli cell cultures. This protein (Protein F), upon gel electrophoresis under reducing conditions, separates into two bands similarly to DAG-protein. The antiserum which we prepared against DAG-protein derived from Sertoli cell cultures, immunoprecipitates a protein from epididymal organ cultures which behaves similarly to the proteins described by Brooks when analyzed by two-dimensional gel electrophoresis. Brooks suggests that the protein is secreted chiefly by the caput region of the epididymis. The results from our immunoprecipitates of organ cultures concur with this observation. Further, the immunofluorescence staining we observed in the epididymis was brightest in the caput. Brooks also suggests that the secretion of Protein F in the initial segment of the epididymis is under hormonal control. We have not tested this observation.

In a recently published study, Brooks (1983b) has shown that radiolabeled Protein F from epididymis organ culture will bind to spermatozoa harvested from the testis and also to washed erythrocytes. The immunofluorescence localization of DAG-protein on the sperm membrane lends tenability to Brooks' observation.

All of the above observations lead us to believe that DAG-protein and Brooks' Protein F are similar, if not identical. The changes in molecular weight and isoelectric point observed may be a result of incomplete protein modification in Sertoli cell cultures or extra processing as a result of cell-cell interactions in cellular complex organ cultures. Proteinases and glycosidases may be secreted into organ culture media by cells external to the epididymal lumen. These enzymes may thus have access to the secreted protein.

The observation that DAG-protein is secreted by Sertoli cells of the testis and also by the epithelium of the epididymis suggests that the


A
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a
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FIG. 9. Indirect immunofluorescence of DAG-protein in the rat epididymis. *a*) In the initial segment staining was light in the perinuclear golgi region of principal cells (*P*). The apical cells (*A*) were lightly and uniformly stained in their cytoplasms. *b*) The staining in the caput and corpus epididymidis was mainly along the stereocilia of principal cells. The staining was heaviest in the caput and the luminal spermatozoa (*Z*) were very brightly stained. The view shown is of the upper corpus. *c*) The stereocilia of principal cells in the cauda epididymidis continued to be stained but with less intensity. The cytoplasm of halo cells (*H*) were brightly stained but clear cells (*C*) were devoid of staining. Bars=25 μm.

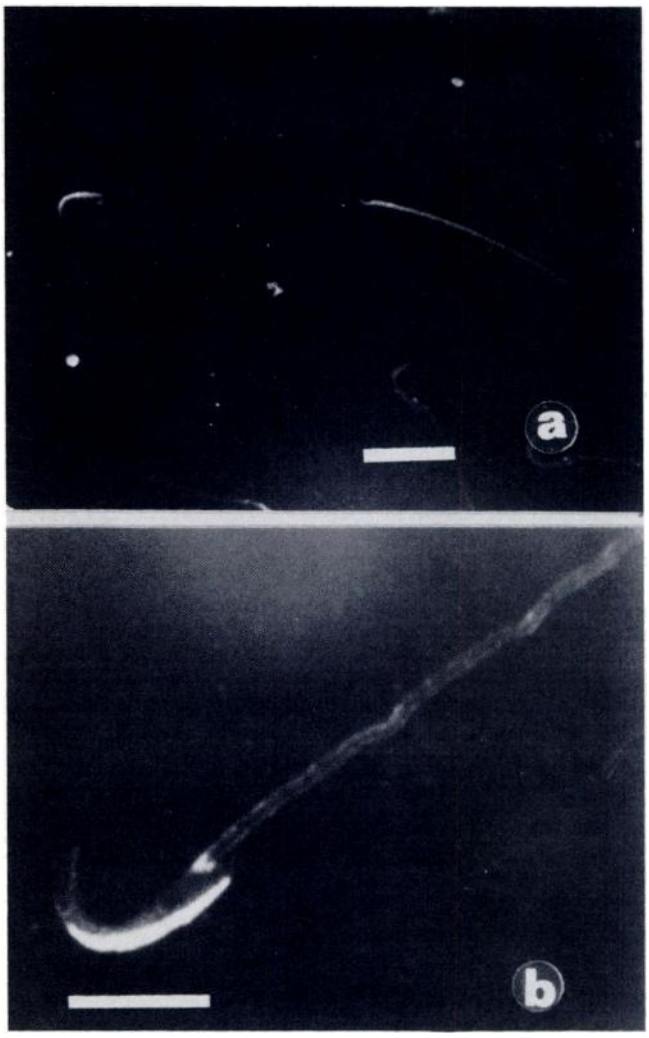

FIG. 10. Indirect immunofluorescence of DAG-protein on rat spermatozoa. Spermatozoa isolated from testis, epididymis, vas deferens and ejaculates were washed, smeared on slides and fixed as described in the *Materials and Methods*. The smears were then treated with rabbit antirat DAG-protein, biotinylated second antibody and fluorescein-conjugated avidin as described. *a*) Spermatozoa were brightly stained on the acrosome, at the neck, and on the endpiece of the tail. The principle piece of the tail was also stained lightly, significantly more than in control smears. The sperm shown were from the cauda epididymidis. *b*) Oil immersion view of intense staining on acrosome and neck of spermatozoa from the caput epididymidis. Bar in *a*=25 μm and bar in *b*=10 μm.

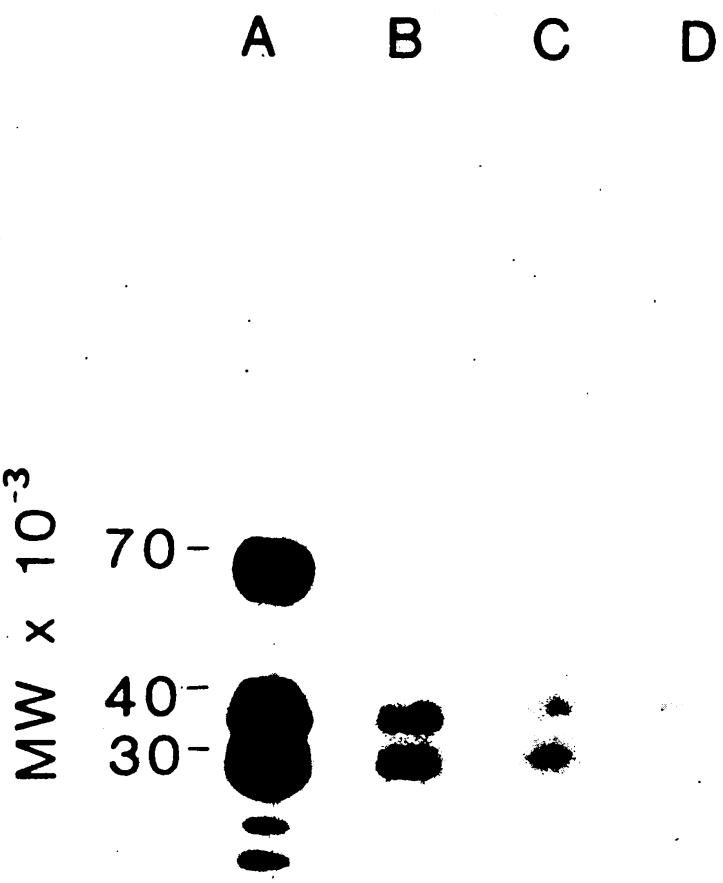

FIG. 11. DAG-protein immunoprecipitated from organ cultures of rat testis, epididymis, and vas deferens. Organ cultures were prepared as described in *Materials and Methods* and incubated with [^{35}S] methionine. Immunoprecipitates of medium were analyzed by polyacrylamide gel electrophoresis and fluorography as described. Organ culture media from testis (*A*), caput epididymidis (*B*), corpus epididymidis (*C*) and cauda epididymidis (*D*) was analyzed. Similar amounts of tissue (approx. 25 mg wet weight) were treated identically.

protein plays an integral role in the process of sperm maturation. The presence of the highly acidic protein on sperm may contribute to the highly negative charge observed on sperm (Yanagimachi et al., 1972) or may aid in the process of fertilization in the female tract. The presence of the membrane protein may also determine whether a sperm is absorbed by the epididymis or allowed to proceed through the tract unharmed.

More detailed studies on the structure and function of the DAG-protein are currently underway.

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