Experimental Allergic Aspermatogenic Orchitis

1. ISOLATION OF A SPERMATOZOAL PROTEIN (AP1) WHICH INDUCES ALLERGIC ASPERMATOGENIC ORCHITIS

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A unique highly soluble aspermatogenic protein (AP1) was isolated from guinea pig testes and was shown by immunofluorescence to occupy the outer surface of the sperm acrosome. This protein is a potent inducer of allergic orchitis and aspermatogenesis; as little as 0.2 µg induced orchitis in 60% of guinea pig tested. The AP1 protein, relatively small and neutral, is stable under acid conditions, but at pH 8.6 shows a variety of forms due either to aggregation or polymorphism. The purified AP1 protein appeared homogeneous by polyacrylamide gel electrophoresis at pH 2.7 and in sodium dodecyl sulfate and by immunoelectrophoresis using rabbit antiserum to either the purified protein or the testes extract. It also showed a single band on immunodiffusion over a wide concentration range.

The purification procedure consisted of delipidation with chloroform/methanol (2/1); acid extraction at pH 3.0; precipitation with 85% saturated ammonium sulfate; trichloroacetic acid extraction and gel filtration on Bio-Gel A-1.5; gel filtration on Bio-Gel P-10; chromatography on CM52 cellulose; and preparative gel electrophoresis at pH 2.7. Approximately 20 mg of purified AP1 protein were obtained from 5000 g of wet guinea pig testes.

The AP1 protein induced an autoimmune disease characterized by infiltration of mononuclear cells around and within the seminiferous tubules (orchitis), followed by extensive damage and destruction of the germinal cells (aspermatogenesis). The course of the disease induced by this protein (0.5 to 1 µg) was essentially identical with that seen with whole testicular tissue or other purified fractions.

Experimental allergic aspermatogenic orchitis is a classic autoimmune disease (1) which is induced in male animals by appropriate injections of testicular tissue or spermatozoa as shown over 20 years ago independently by Voisin et al. (2) and Freund et al. (3). Like the widely studied allergic encephalomyelitis (4), this disease is organ specific and in some respects mimics human disorders (5). The pathogenesis of allergic aspermatogenic orchitis is yet unclear, however, in part because the responsible antigen(s) has not been isolated. In addition to the early work of Freund et al. (6), the work of Waksman (7), Tung et al. (8, 9), and Levine and Sowinski (10) has characterized the course of pathologic events in the seminiferous tubules as being primarily cell mediated, whereas the work of Brown et al. (11), Willson et al. (12), and Voisin and Toullet (13) has implicated antibody as well. Since these studies were performed with whole testicular tissue or uncharacterized preparations, the results cannot be regarded as conclusive. The immunologic picture should be further delineated with the isolation of purified aspermatogenic antigens such as occurred with allergic encephalomyelitis where isolation of the A1 protein (14, 15) and encephalitogenic peptides therefrom (16, 17) greatly stimulated immunologic efforts (18, 19).

Only modest purification of the antigen(s) causing allergic aspermatogenic orchitis has been achieved (1, 20). Understandably, the purification of any membrane protein is generally difficult; the raw material is scarce since the disease in guinea pigs is species specific (6), and the aspermatogenic assay is time consuming (4 to 5 weeks). The present investigations were initiated for the purpose of preparing from guinea
pig testes a homogeneous aspermatogenic protein in sufficiently large quantity for chemical study and immunologic investigation. The experimental disease is being used as a model for study of immune mechanisms as part of a more general study of tissue rejection.

Since the first description of the antigenicity of sperm by Landsteiner (21) over 70 years ago, the field of immunoreproduction has required sharper focus on the responsible antigens, particularly those leading to immunopathy. The availability in quantity of homogeneous preparations of chemically defined antigens would provide an opportunity for refining studies on the pathogenesis of allergic aspermatogenic orchitis. Practically, this work could bear upon human fertility problems, whether by controlling fertility or correcting infertility. Chemically, the characterization of spermatozoal antigens could aid our understanding of membrane structure and natural antigens and perhaps provide a fruitful approach to clarification of events involved in sperm-ovum interaction. This report describes for the first time the isolation of a potent aspermatogenic protein (AP1 protein) that is localized in the acrosome of the spermatozoal membrane. This is the first of a series of reports on aspermatogenic antigens and their chemical and immunological properties.

**EXPERIMENTAL PROCEDURES**

**Assay for Orchitis- and Aspermatogenesis-Inducing Activity**

Random-bred male guinea pigs (350 to 500 g) were immunized intradermally with 0.05 ml in each hind footpad of a water-in-oil emulsion made with equal parts of complete Freund’s adjuvant and sperm antigen in 0.14 M NaCl. The adjuvant consisted of 8.5 parts mineral oil (Bayol F), 1.5 parts emulsifying agent (Arlaco AI), and 1 mg/ml of killed Mycobacterium tuberculosis. Generally, six guinea pigs or more were used to assay a preparation at any given dose.

Guinea pigs were killed routinely at 25 to 30 days. Testes were weighed and, on occasion, along with other tissues such as spleen, lung, kidney, liver, and brain, were fixed in Bouin’s solution embedded in paraffin, sectioned and stained with hematoxylin and eosin, and examined microscopically.

Multiple sections at different levels of both testes from each animal were examined for infiltrating cells (orchitis) and partial or complete destruction of germinal epithelium (aspermatogenesis) within the seminiferous tubules. The epididymides were also checked for infiltrating cells, sloughed off germinal cells, and spermatozoa.

**Chemical Methods**—The polyacrylamide gel electrophoresis was carried out at pH 2.7, 8.6 (22), and also in sodium dodecyl sulfate (23). Briefly, the electrophoresis at pH 2.7 was carried out in 7.5% acrylamide (acrylamide to N,N’-methylenebisacrylamide ratio was 75:1) in 6-mm internal diameter tubes 10 cm in length. Stock solutions were prepared as shown in Table 1.

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>A. 1 N KOH</th>
<th>Glacial acetic acid</th>
<th>Glacial acetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 ml</td>
<td>53.25 ml</td>
<td>48 ml</td>
</tr>
<tr>
<td>B. 1 N KOH</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>2.87 ml</td>
</tr>
<tr>
<td>C. Water to 100</td>
<td>30 g</td>
<td>0.4 g</td>
<td>10.0 g</td>
</tr>
<tr>
<td>D. Water to 100</td>
<td>240.0 mg</td>
<td>4.0 mg</td>
<td>2.5 g</td>
</tr>
<tr>
<td>E. Water to 100</td>
<td>4.0 mg</td>
<td>1.0 ml</td>
<td>3.05 ml</td>
</tr>
<tr>
<td>F. Water to 100</td>
<td>0.05% methyl green</td>
<td>green</td>
<td></td>
</tr>
</tbody>
</table>

**Fractionation Procedure**

**Step 1: Preparation of Testes**—For each preparation the tunica, extraneous muscle, and collagenous material were removed from approximately 5 kg of frozen guinea pig testes after freezing. Usually the epididymides were left intact. All of the following steps were conducted at 4° unless otherwise indicated. All centrifugation was performed for 20 min at 16,000 x g in the Sorvall RC2B centrifuge unless otherwise indicated.

**Step 2: Delipidation**—The testes and epididymides were defatted with methanol prechilled to -20°, homogenized in a Waring Blender for 2 min; 2 volumes of prechilled chloroform were then added and the mixture blended for 2 to 4 min. The chloroform/methanol-treated material was allowed to stand 2 to 4 hours at 4°. The suspension rapidly divided into two layers. The aqueous upper layer contained the material which was later filtered on double filter paper to remove traces of lipid and chloroform and air-dried on the filter paper. The filtrate was discarded.

**Step 3: Acid or Salt Extraction**—At this point two alternatives were employed. (a) The delipidated residue from Step 2 was suspended in 20 to 50 volumes (v/v) of water and adjusted to pH 3.0 by dropwise addition of concentrated HCl while stirring. This suspension was stirred for 2 hours and the pH was readjusted to 3.0 if necessary. The suspension was cooled overnight and the solution was divided into two parts. The extract was either filtered on two sheets of Whatman No. 41 filter paper or centrifuged. The residue was discarded since re-extraction did not yield a significant amount of material. The acid extract was neutralized to pH 6.0 with concentrated ammonium hydroxide which sometimes resulted in the formation of a visible precipitate which was removed by filtration. (b) In this case the delipidated residue was suspended in 20 to 50 volumes (v/v) of 0.5 M NaCl, pH 7.0. The suspension was stirred overnight and either filtered or centrifuged as described above (Step 3a). The filtrate was kept at 4° or frozen.

**Step 4: Ammonium Sulfate Precipitation**—The filtrates from Steps 3a or b were precipitated by the addition of solid ammonium sulfate to 85% saturation. The mixture was stirred overnight and the precipitate was removed by filtration on double thickness Whatman No. 41 paper or centrifugation. The precipitate was dissolved in distilled water and added to a Bio-Gel P-2 column (in water) with a bed volume at least 5 to 10 times the volume of sample applied. Protein and glycoprotein were detected by monitoring absorbance at 280 and 220 nm; salt was detected by measuring conductivity with a Radiometer conductivity meter. Salt-free fractions were combined, lyophilized, and stored at -20°.

**Step 5a: Trichloroacetic Acid Extraction**—To the material from Step 4, 50% trichloroacetic acid (w/v) was added dropwise with stirring until a final concentration of 5% was obtained. The visible precipitate was removed by centrifugation and resuspended in water. The supernatant fluid and resuspended precipitate were extracted with ether.
concentrated, and desalted by Bio-Gel P-2 column chromatography as described above. The salt-free materials were lyophilized.

A Bio-Gel A-1.5 column (96.5 × 2.6 cm) equilibrated with 0.1 M ammonium acetate, pH 7.0. Selected tubes were assayed for aspermatogenic activity and combined. The tubes were also examined by gel electrophoresis, and combined into two fractions; one was rich in glycoproteins and will be described elsewhere and another contained low molecular weight proteins.

Step 5f: Ultrafiltration (Optional Alternative)—The Amicon hollow fiber dialysis/concentrator DC2 was used to fractionate the ammonium sulfate precipitate from Step 4. The material, dissolved in 0.1 M ammonium acetate, pH 5.0 was first subjected to dialysis or concentration using a HIX50 hollow fiber cartridge (molecular weight limit 30,000). The retentate was washed several times with buffer, lyophilized, and stored. The filtrate or dialysate was then applied to a second hollow fiber cartridge, HDI10 (molecular weight limit 10,000 to 15,000) and concentrated to a small volume. The retentate was washed, lyophilized, and stored as above. The HDI10 filtrate was concentrated and desalted by rechromatography three times.

Step 6: Bio-Gel P-10 Fractionation—Material eluting most slowly from the Bio-Gel A-1.5 fractionation of the trichloroacetic acid-soluble material (from step 5d) was dissolved in 0.1 M ammonium acetate, pH 7.0 and applied to a Bio-Gel P-10 column (95 × 2.5 cm) equilibrated in 0.1 M ammonium acetate, pH 7.0.

Step 7: CM52 Cellulose Chromatography—A Whatman CM52 cation exchange cellulose column (26 × 2.2 cm) was used to further fractionate appropriate aspermatogenic material obtained from Step 6. This fraction was solubilized in 0.002 M ammonium acetate-acetic acid, pH 3.5. After washing thoroughly with 2 to 3 bed volumes of starting buffer, elution was accomplished by applying a linear gradient from 0 to 0.1 M ammonium acetate-acetic acid, pH 3.5; each chamber contained 2.0 liters.

Step 8: Preparative Gel Electrophoresis—As a final step following cellulose CM52 chromatography, aspermatogenic material from Step 7 was dissolved in water and brought to a glycerol concentration of 10%. The fraction was run on a modified Ortec preparative polyacrylamide slab gel electrophoresis unit, the capacity of which was at least 4 fold increased. Separation of up to 75 mg of protein per cell was carried out by vertical descending electrophoresis on acrylamide slabs (7.5%) at pH 2.7 using the same buffer system described for the analytical electrophoretic assay. The two gels were prerun overnight (16 hours) using methyl green as a marker at 135 ma (42 volts). The samples were applied and 125 ma of current was applied for 90 min (80 volts); then the current was increased to 175 ma (80 to 100 volts) and electrophoresis continued. The sample zones were located by cutting very thin slices from the center of the gel slab and staining with 1% Amido schwarz dye in 7% acetic acid.

Gel sections containing the desired component zones were excised and homogenized in a Potter Elvehjem homogenizer in 0.5 M formic acid, pH 2.0, or ammonium acetate, 1 M, pH 8.0. The homogenate was extracted by stirring overnight at 4°, centrifuged at 5000 rpm, and filtered (0.45-µm Millipore filter). The filtrate was evaporated at 30° and then each sample was desalted on a Bio-Gel P-2 column in H₂O and lyophilized. Elution of the samples from the gels by ascending gel slab electrophoresis into a chamber on top of the cell formed by dialysis membrane was successful for larger proteins. Yields of smaller proteins (molecular units 10,000 to 15,000) were very low, and it was assumed that the materials were lost through the dialysis tubing. Use of gelatin tubing did not improve the yields.

Immunological Methods—In all cases, rabbit antisera were obtained following four immunizations by intramuscular route using a total of 6 mg of purified aspermatogenic protein 1 (API). The initial injection, containing 2 mg of protein in Freund's complete adjuvant, was followed 1 week later in Freund's incomplete adjuvant (2 mg). The rabbit was rested 1 month and then reinjected with 1 mg; 3 weeks later a final injection of 1 mg was given. One week after the last injection, the rabbits were bled and the serum tested by immuno electrophoresis. When the acid extract of the testes was used for immunization, the same procedure was followed except that 18 mg were used. The acid extract was first neutralized to pH 6.0, dialyzed, and lyophilized prior to injection.

Double diffusion was carried out according to the method of Ouchterlony (31). For each plate of 5-cm diameter, 7 ml of 1% agarose in 0.15 M sodium chloride was used. Patterns were made with a gel punch (Grafar Corp., Auto-Gel T/M); the antigen and antibody wells were 4-mm in diameter and placed 6 mm apart. After the addition of appropriate antigen and antibody the plates were incubated in the cold (4°).

For immunofluorescent studies, the indirect staining method of Coons (32) was used. Whole guinea pig sperm were collected from turgescent epididymides and washed three times by centrifugation in saline at 750 × g, and suspended at 1 to 2 × 10⁸ sperm/ml of saline. The sperm were placed on a microscope slide and a thin smear was air dried. Appropriate dilutions (1:10 to 1:900) of rabbit anti-API protein and fluorescein labeled goat antirabbit immunoglobulin (Cappel Labs., Downingtown, Pa.) were added after the standard washing procedures. Controls consisted of preimmunization serum and buffer.

The Leitz orthoplan (Ploem incident light system) fluorescent microscope equipped with the Orthomat II camera was used in this study.

Immunoelectrophoresis was performed with a Gelman apparatus utilizing Scheidegger's modification (33) of the technique of Grabar and Williams (34, 35). A gel containing 1% agarose (General Biochemicals, Chagrin Falls, Ohio) prepared in 0.025 M barbital acetate buffer (ionic strength 0.05) at pH 8.6 was used. Approximately, a 3-mm thick layer of gel was placed on microscopic slides (7.5 × 2.5 cm). The well in which the antigen was placed was 2 mm in diameter. The electrode compartments contained 0.05 N barbital acetate buffer at pH 8.6. Selection of the antigen was ensured by adding 7.5 ml of a 1 mg/ml suspension of sperm to a 2-mm wide area on the gel disc and allowing electrophoresis, proceeded in the cold; slides were stored in a moist chamber and checked periodically to observe patterns.

RESULTS

Preparation—After each step in the fractionation of the aspermatogenic protein (API protein), the various fractions were assayed for orchitogenic and aspermatogenic activities as shown in Table I. Initially, the guinea pig testicular tissue was shown to induce orchitis and aspermatogenesis at 50-mg doses and no special effort was made to titrate the crude tissue. Although various investigators (6, 36) have used from 0.3 to 250 mg of whole testicular tissue in order to induce orchitis and aspermatogenesis, generally 10 to 20 mg are used (7, 8, 11). Freund et al. (6) found that 5.6 to 11 mg of testes tissue induced severe tubular damage, while 0.7 mg induced minimal damage. Apparently, therefore, 0.7 mg of testes tissue can be considered a borderline dose. Although most fractions were tested at several dosages, the results shown in Table I represent only the lowest fully effective dose for the various fractions except for the ammonium sulfate precipitate.

The first step in the preparation of the API protein, the lipid extraction with chloroform/methanol, greatly facilitated the extraction of aspermatogenic protein material. Other techniques, therefore, of initial treatment were not explored except for autoclaving, which led to a partially active supernatant fluid (Table I). Presumably, therefore, the responsible antigen(s) was not seriously damaged by the lipid extraction. The next procedure generally employed was acid extraction at pH 3.0, a pH which should avoid extraction of basic proteins (37) from the head piece. Very little difference was noted in the protein profile on gel electrophoresis at pH 2.7 or 8.6 when extraction was carried out at pH 3.0 or with salt. The ammonium sulfate step, which concentrated the active antigen, was used routinely because of the high aspermatogenic activity of the precipitate (Table I).

At this point, precipitation with trichloroacetic acid was used because it removed many of the nonorchitogenic and nonaspermatogenic protein contaminants as shown in Fig. 1 where comparison is made with the acid extract of the testes.

* The protein isolated as described in this report will henceforth be referred to as the API protein. It is highly aspermatogenic, non-glycosylated and localized in the spermatozoal acrosome.

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4 Gradient of 0.4 M, with respect to ammonium ions without regard for acetate ions, is usually consistent at pH 3.5.
and sperm proteins. In pH 2.7 gels, the AP1 protein appears just above the fastest moving band; clearly, the trichloroacetic acid supernatant fluid shows a great concentration in this protein as compared to the acid extract where it is very faint even with 200 μg applied. The AP1 band is also barely noticeable in the sperm profile. Clearly, the trichloroacetic acid precipitate contains most of the contaminating proteins. The aspermatogenic activity of this precipitate is correspondingly low, having no activity at 5 μg. Gel electrophoresis at pH 8.6 of the trichloroacetic acid supernatant fluid shows essentially the same picture as at pH 2.7; most contaminating proteins were removed by treatment with trichloroacetic acid. The trichloroacetic acid treatment was very effective in further purifying the AP1 protein and gave a supernatant fluid with high aspermatogenic activity. However, some AP1 co-precipitates along with other proteins in the trichloroacetic acid-insoluble material. Glycoproteins, which do not stain with the Amido schwarz procedure and thus do not appear in Fig. 1, are also concentrated in the trichloroacetic acid supernatant fluid. It was possible, however, to separate the glycoproteins from the nonglycosylated aspermatogenic AP1 protein on a Bio-Gel A-1.5 column; the elution profile is shown in Fig. 2. Most of the glycoprotein elutes by tube 68 in the first two peaks as shown by the carbohydrate analyses and gel electrophoresis (Fig. 3a). The first peak contains several large glycoproteins, which have a low absorbance at 280 nm and which do not stain with Amido schwarz reagent as shown by Fig. 3b. Absorbance at 215 nm was not done because of absorption due to ammonium acetate. The next peak, tubes 50 to 70, also contains large glycoproteins but few other proteins. The main peak (tube 73) contains the slowest moving protein band (Gel 6, Fig. 3b) and virtually no glycoprotein. While the glycoprotein material, Fig. 2 (tubes 52 to 68), was highly aspermatogenic, so also was the material in tubes 78 to 90, designated Fraction II, which contains primarily two major protein bands (Gels 8 and 9, Fig. 3b). Thus, the Bio-Gel A-1.5 fractionation separates the glycoproteins, which are larger molecules, from the smaller nonglycosylated proteins, one of which is the aspermatogenic AP1 protein.

An alternative to the trichloroacetic acid and Bio-Gel A-1.5 steps, which gave material similar to Fraction II (Fig. 4a, Gel 1) of the Bio-Gel A-1.5 column, was the use of ultrafiltration followed by column chromatography on Bio-Gel P-10. In Fig. 4b, the protein profile of the eluate from a Bio-Gel P-10 column is shown; polyacrylamide gel patterns of appropriate fractions and tubes are shown in Fig. 4a. The major aspermatogenic activity was localized in tubes 57 to 59, presumably corresponding to the major protein band seen on Gel 6 of Fig. 4a. Tubes 49 to 62 containing this band, were combined as shown by the cross-hatched area. It must be noted, however, that tubes 25 to 48 all showed considerable activity, presumably due to some other factor. According to the Bio-Gel P-10 fractionation spectrum, four major protein bands were observed on gel electrophoresis. It appears that the band just behind the fastest moving protein represents the major aspermatogenic material. The material of tube 66 (Gel 10), representing the

### TABLE I

<table>
<thead>
<tr>
<th>Material</th>
<th>Dose (μg)</th>
<th>Orchitic and aspermatogenic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole testes</td>
<td>50,000</td>
<td>6/6</td>
</tr>
<tr>
<td>Autoclaved supernatant</td>
<td>2,000</td>
<td>6/6</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate</td>
<td>50</td>
<td>6/6</td>
</tr>
<tr>
<td>Trichloroacetic acid supernatant</td>
<td>2</td>
<td>6/6</td>
</tr>
<tr>
<td>Trichloroacetic acid supernatant, a of sheep, rabbit, dog, bull, or hamster</td>
<td>1,500</td>
<td>0/24</td>
</tr>
<tr>
<td>Trichloroacetic acid pellet</td>
<td>20</td>
<td>0/6</td>
</tr>
<tr>
<td>Bio-Gel A-1.5, Fraction II</td>
<td>50</td>
<td>6/6</td>
</tr>
<tr>
<td>HIDP10 ultrafiltrate</td>
<td>20</td>
<td>6/6</td>
</tr>
<tr>
<td>Bio-Gel P-10 (tubes 48 to 62)</td>
<td>20</td>
<td>6/6</td>
</tr>
<tr>
<td>Cellulose CM (tubes 610 to 780)</td>
<td>1</td>
<td>6/6</td>
</tr>
</tbody>
</table>

a Dosage to animals was based on lyophilized weight.

b Recorded as number of guinea pigs showing lesions over total animals tested. In every case, both destruction of germinal cells (aspermatogenesis) and inflammatory lesions (orchitis) were observed.

c Whole testes were autoclaved, centrifuged, and the supernatant fluid was lyophilized.

d Doses from 1 to 500 μg were tested; all results were negative. With the exception of this entry all other materials were derived from guinea pig testes.

![Fig. 1. The polyacrylamide gel pattern at pH 2.7 is shown from left to right for sperm (400 μg), acid extract (200 μg), and trichloroacetic acid supernatant fluid (200 μg). The sperm was obtained from guinea pig epididymides, washed three times in 0.15 M NaCl, defatted with chloroform/methanol (2/1), and extracted at pH 3 as done with the testicular tissue. The AP1 protein migrates just above the fastest band as shown by the arrow. Gels were run from anode (top) to cathode (bottom).](http://www.jbc.org/fig1.png)
FIG. 2. Chromatography of the trichloroacetic acid supernatant fluid from Step 5a on a Bio-Gel A-1.5 column (96.5 x 2.6 cm) in 0.1 M ammonium acetate, pH 7.0, monitored for protein (O), hexose (x), and hexosamines (Δ). The absorbance was measured at 280 nm; carbohydrate refers to either hexosamine or hexose. Of 355 mg applied, 310 mg were recovered. Fraction I (tubes 52 to 68) and Fraction II (tubes 78 to 80) were derived based on aspermatogenic assay and gel electrophoresis. Fractions of 10 ml per tube were collected.

fastest band, was not aspermatogenic at 20 μg and was therefore ignored.

When the material of tubes 49 to 62 was applied to a CM52 cellulose column, two main peaks were obtained (Table II). When examined by gel electrophoresis (Gel II, Fig. 4a) a highly purified protein band was evident which migrated identically with the major protein band of the most active material from the Bio-Gel P-10 column (Gel 6). This result offers strong evidence for the presence of a major aspermatogenic protein, referred to as the API protein. The CM52 cellulose step was essential because it removed an inactive contaminant (tubes 120 to 160, Fig. 5), which nonetheless migrated on gels at the same position as the API protein. The peak preceding the API contained material which comprised the fast moving band on gel electrophoresis, Fig. 4a. Thus the CM52 cellulose step resolved the two main protein components from the Bio-Gel P-10 column.

Examination of the CM52 cellulose material (tubes 610 to 780) by immunoelectrophoresis, using rabbit antiserum to the ammonium sulfate precipitated material, revealed one major and two minor bands. In order to obtain homogeneous API protein, and remove traces of other components, the final step consisted of preparative gel electrophoresis. The eluted API protein was lyophilized; aliquots were taken as required for testing.

Yield—It was not possible to determine the amount of API protein in the testes by the purification procedure because some of the steps are inefficient and because of the presence of other aspermatogenic factors. From 1000 g of frozen testes, approximately 15 g of ammonium sulfate precipitate were obtained. Ultimately, this precipitate gave approximately 70 mg of Fraction II (Fig. 2) from the Bio-Gel A-1.5 column when the trichloroacetic acid procedure (Step 5a) was utilized. Generally, from 1000 g of testes, 80 to 120 mg were obtained from the Amicon H10P10 ultrafiltrate depending on the number of washings. Of the 400 mg of Fraction II applied to the Bio-Gel P-10 column (the yield from a 6000-g testes preparation), 165 mg of material rich in API protein were recovered (tubes 49 to 62, Fig. 4b). On CM52 cellulose chromatography, 30 mg of API protein were recovered (tubes 610 to 780, Fig. 5) and estimated to be 95% pure. Had more attention been given to yield rather than purity, possibly 50 mg of API protein could have been obtained. Thus we estimate that from 1000 g of original material approximately 6 to 10 mg of API protein (95% pure) could be obtained by this procedure. The yield likely reflects a lower value than actually exists in testicular tissue. The amount of API protein obtained will depend on the number of sperm present since this figure may vary with age and status of the animals.

Immunological Studies—Immunoelectrophoresis was carried out as shown in Fig. 6a. Only one line of precipitation is observed when antisera to either the purified API protein or the crude ammonium sulfate precipitate are reacted with the API antigen. In addition, antisera to the API protein shows only a single line of precipitation when reacted with the trichloroacetic acid supernatant fluid and the crude ammonium sulfate precipitate. These results suggest that the API
protein could be highly homogeneous. Any immunogenic contaminant to which antibody had been elicited would have been detected by this procedure. It is apparent from Fig. 6b that a minimum of eight antigens are present in the crude ammonium sulfate precipitate. During the purification procedure, it was found that these potential contaminating antigens were removed. For example, only four lines were observed with Fraction II from the Bio-Gel A-1.5 column, and three lines were observed from the active material from CM52 cellulose column when reacted against antircrud ammonium sulfate precipitate.

Immunodiffusion of the AP1 protein was studied using antiserum to the ammonium sulfate precipitate. A single immunoprecipitant band was formed over wide concentrations of the AP1 protein (0.63 to 10 mg/ml). In addition, antisera to the purified AP1 protein gave the same results when tested against varying dilutions of the antigen. These data further support the results obtained in immunoelectrophoresis and indicate a high degree of immunologic purity.

Localization of the AP1 protein in the spermatozoa was determined with the indirect immunofluorescence technique (Fig. 7). In order to avoid nonspecific reactions, dilutions higher than 1:20 of both the normal and immune sera were used; dilutions of antiserum ranged from 1:20 to 1:400. At dilutions as low as 1:200, acrosomal fluorescence was observed in every case. Controls, consisting of normal rabbit serum and buffer, were consistently negative. These results demonstrate that the AP1 protein is positioned in the crescent-shaped structure atop the sperm head. It is not likely that the sperm acrosome was appreciably disrupted by drying. Thus the AP1 protein probably occupies an external position in the acrosomal membrane.

Homogeneity—The immunodiffusion and immunoelectrophoresis procedures possibly suggest a high degree of purity of the AP1 protein. In each case only a single immunoprecipitin band was found. Further evidence that the AP1 protein is homogeneous and lacking contamination was shown by polyacrylamide gel electrophoresis (Fig. 8). Only a single band was found by electrophoresis in sodium dodecyl sulfate or at pH 2.7. Even when applied in high concentration, no contaminating proteins were evident. Over the range of 20 to 400 μg, only one band was observed. At pH 8.6, however, several bands were observed. It is possible that these bands are a result of aggregation, but since the protein is quite close to its isoelectric point at pH 8.6, it is possible that the bands may represent various polymorphic species, a phenomenon known to occur with many proteins (38). Deamidation is thought to account for this finding in some cases (39) since different protein species differing by a single charge would migrate significantly differently near their isoelectric point.

Disease Induction—The AP1 protein showed extremely potent orchitogenic and aspermatogenic activity (Table II). Generally the loss of testicular weight roughly correlated with loss of germinal cells but not with the presence of inflammatory cells. Even at 0.2 μg, approximately 60% of the guinea pigs showed the typical inflammatory lesions in the testes characterized by inter- and intratubular cellular infiltration generally
beginning around Day 10 to 13 after sensitization. The cells are primarily mononuclear with only very few polymorphonuclear cells evident and in this respect appear virtually identical with that seen in brain or sciatic nerve in response to the A1 protein (allergic encephalomyelitis) or peripheral nerve myelin (allergic neuritis), respectively (5). A typical lesion, found at day 22 to 25, shown in Fig. 9a shows a more advanced pathology where cellular invasion of the tubules has occurred as well as considerable loss of germinal epithelium. At 30 days, Fig. 9b, it is clear that the tubules are devoid of most of the germinal cells in contrast to a normal seminiferous tubule (Fig. 9c). The diseased tubules are left as empty vesicles except for the Sertoli cells. These cells, which border the tubular membrane, appear resistant to the degradative immunopathologic events. Likewise, the Leydig cells appear to be normal. A detailed study of the course and mechanism of pathologic events induced by the AP1 protein will be published elsewhere. These results show that the AP1 protein is at least as active on a weight basis in producing inflammatory lesions as in the A1 protein of myelin in eliciting allergic encephalomyelitis (5). It should be noted that the AP1 protein, on a weight basis, is far more active in induction of aspermatogenic orchitis material heretofore isolated (1).

Histologic examination for lesions in other organs such as liver, kidney, brain, spleen, and lung was also made, but all of

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<tr>
<th>Dosage*</th>
<th>Orchitic and aspermatogenic activity*</th>
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<tbody>
<tr>
<td>µg</td>
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<tr>
<td>0.1</td>
<td>0/6</td>
</tr>
<tr>
<td>0.2</td>
<td>4/6</td>
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<tr>
<td>0.5</td>
<td>(orchitis only)*</td>
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<tr>
<td>1.0</td>
<td>6/6</td>
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<td>16/18</td>
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<tr>
<td>10.0</td>
<td>24/24</td>
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*Dosage was measured as lyophilized material dissolved in saline and mixed 1:1 with complete Freund’s adjuvant and injected as previously described to give the final quantities shown above.

*Measured as the ratio of guinea pigs showing orchitis and aspermatogenesis over number of animals tested at 30 days following immunization.

* Only cellular infiltration (mononuclear cells) was observed in this case.

Fig. 6 (top), a, immunoelectrophoresis (cathode to right) of the purified AP1 protein using rabbit antiserum to the crude ammonium sulfate precipitate (upper trough), and antiserum to the purified AP1 protein (lower trough). Concentration of AP1 is 5 mg/ml. b, immunoelectrophoretic (cathode to right) pattern of the crude ammonium sulfate precipitate reacted against rabbit antiammonium sulfate precipitate. Concentration of crude ammonium sulfate precipitate is 10 mg/ml.

Fig. 7 (bottom). The interaction of antiserum (1:200 dilution) to the purified AP1 protein with guinea pig spermatozoa using the indirect immunofluorescent technique. The fluorescein-labeled goat anti-rabbit immunoglobulin was diluted 1:20. Only acrosomal fluorescence was observed.

![Image](http://www.jbc.org/)

TABLE II

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the results were negative. This result agrees with early conclusions of Freund and co-workers (3) and Toulet et al. (40) that their testicular extracts induced inflammatory lesions only in the testes.

**DISCUSSION**

This report describes for the first time a definitive purification of an aspermatogenic protein, referred to as the API protein. This protein appears homogeneous by gel electrophoresis in two systems as well as by immunoelectrophoresis and immunodiffusion. Antibody to crude testicular extract, as shown by immunoelectrophoresis, shows multiple bands against the crude extract, and only a single band when reacted against the API protein. Therefore, we conclude that this preparation contains a single protein species and thus is appropriate for immunologic and chemical studies.
Histologic studies show destruction of germ cell and spermatid zones exclusively 25 to 30 days after a single injection of purified AP1 protein. Therefore, it is highly probable that the responsible antigen is a germ cell-specific protein and not a general testicular protein. Gel electrophoresis of the sperm proteins indicated a faintly staining band migrating identically with the AP1 protein. Immunofluorescent data, utilizing antisera to the purified AP1 protein, provides direct proof of its acrosomal localization. A protein similar to the AP1 protein also exists in the spermatozoa of other animal species examined (dog, rabbit, hamster, bovine, and sheep) as shown by both immunoelectrophoresis and polyacrylamide gel electrophoresis. But these heterologous preparations do not elicit aspermatogenic activity in the guinea pig. Thus the AP1 protein is species specific as well as organ specific in regard to disease induction.

Our objective in this work was to isolate and purify a sperm protein capable of specifically inducing allergic orchitis and aspermatogenicity. The paucity of material and suspected low yield necessitated using testes rather than sperm as a starting material. The purification was demanding because the assay is long (20 to 30 days), requires histologic evaluation, and while only semiquantitative, is reproducible with experience. The difficulties are compounded because more than one aspermatogenic resides in testicular tissue, and in addition some inactive proteins also present in the testes show properties similar to the AP1 protein. The fractionation on CM52 cellulose appears essential since it removes a contaminant which migrates identically with the AP1 protein on electrophoresis. Fortunately, the AP1 protein, like the AI protein of myelin, resists denaturation through the delipidation and acid extraction and thus retains a conformation, essential for disease induction.

Immunologically, the AP1 protein is far more active in inducing orchitis and aspermatogenicity than any material reported. At 0.2 μg, mild lesions were observed (interstitial infiltration of mononuclear cells and some shedding of mature germinal cells), at 0.5 to 1 μg, severe lesions (desquamation of all germinal cells occurs in addition to extensive cellular infiltration) were seen. On this basis, the AP1 protein is at least 10-fold more active than the “CPM” fraction of Freund et al. (6) which showed no activity at 2 μg and good activity at 8 μg in only 50% of the guinea pigs. The preparation of Brown et al. (11) which chemically is probably similar to the CPM material, reportedly produced significant lesions at 5 μg in guinea pigs.

Voisin and Toulet (13) provided a significant advancement by preparing from spermatozoa three aspermatogenic fractions (S, P and T) with different chemical and immunological properties. Unlike the AP1 protein which contains no carbohydrate, the fractions of both Freund and Brown contain glycoproteins and it is likely that they may have very similar characteristics to Voisin’s fraction S. Both fractions T and P are insoluble in trichloroacetic acid and thus are distinguished from the AP1 protein. However, it should be noted that in all of the preparations to date little evidence for chemical homogeneity has been presented. Based on our experience with trichloroacetic acid soluble supernatant materials, we suspect considerable contaminating protein and possibly DNA (11). In addition, these other preparations (6, 11, 13) probably contain only traces of the AP1 protein since extensive dialysis was used.

In our studies, high purity correlates with high aspermatogenic activity. If this were not the case, a trace of a potent aspermatogenic factor along with inactive material could be misleading. The history of the search for the allergic encephalitogenic antigen emphasizes this point (4). Claims of other aspermatogenic materials have also been made, particularly for the enzyme sorbitol dehydrogenase (41). Since sorbitol dehydrogenase induces a very mild lesion after 3 months, it is highly unlikely that a comparison can be made with the AP1 protein (a potent inducer of orchitis and aspermatogenesis).

Perhaps the best effort to date to purify a testicular antigen was made by Katsh et al. (42). Material was obtained by a series of steps including pepsin digestion, precipitation with antibody followed by dissociation, and final isolation by gel filtration. This material apparently showed one major band on gel electrophoresis. Although the antigen induced good antibody titters as shown by passive hemagglutination, it appeared relatively inactive in the aspermatogenic assay since 500 μg were required to produce aspermatogenesis based on loss of testicular weight. We would interpret these results to indicate that while the material is a good antigen, in that it has the ability to induce antibody production, it is inactive as an aspermatogenic factor.

It appears that the purification of the AP1 protein from testicular tissue represents an enrichment of approximately 1000-fold or more by comparison with Freund’s data which was based on the activity of crude testicular tissue (6). Therefore, it probably represents a small portion of the spermatozoal proteins but must be a significant component of the spermatozoal acrosome and germinal cells. Whether it is a structural component or endowed with a special biological activity must await further research. It does not appear to have proteolytic activity and thus is probably not the sperm trypsin-like enzyme (43) that appears important in early penetration of the ovum. Other proteins which have also been purified from spermatozoa, include the small basic proteins (37, 44), and the enzyme lactic dehydrogenase—X (45), a unique form exclusive to sperm. It is unlikely that any of these proteins are aspermatogenic because of their internal location within the spermatozoa.

The striking feature of the AP1 protein is its strong aspermatogenic activity. The lesion it elicits at 22 to 30 days is not unlike that induced with whole tissue or the preparations from other laboratories referred to above. The orchitis picture generally varied from slight to intense infiltration of mononuclear cells which were inter- and intratubular. Sometimes mononuclear cell infiltration was massive within the epididymus as well. Depending on the dose, we observed widespread impairment of spermatogenesis and often severe damage destroying all germinal cells, even spermatogonia. A detailed report on the course of immunopathologic events elicited by the AP1 protein will be described elsewhere. Thus the AP1 fits the category of an autoantigen since the homologous protein induces an organ- and species-specific (of those tested) autoimmune disease in the guinea pig.

Chemically, the AP1 protein is distinguished from most aspermatogenic antigens because it is not a glycoprotein; it is soluble and stable to acid conditions. Fortunately, it can be dissociated from the spermatozoal membrane under acidic or neutral conditions and still retain the conformation necessary to produce the autoimmune disease. At pH 8.6, however, it shows a series of bands on gel electrophoresis. Detailed characterization of the AP1 protein will be published elsewhere.2

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