Acid-Precipitated M Protein Compared with a Column-Eluted M Protein Preparation from Type 12, Group A Streptococci

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An M protein preparation of group A streptococci, precipitated with 0.03 M sodium acetate buffer (pH 4.0) was compared with a column-eluted M protein preparation. Absorption spectra and methyl pentose content were similar in both preparations. Acrylamide gel electrophoresis patterns were different. Gel diffusion demonstrated two lines of fusion in the preparations. More antigens could be demonstrated in both preparations by using immunoelectrophoresis. Neither the pH 4 precipitate nor the column-eluted preparation appeared to be a pure M protein preparation.

The presence of M protein in the cell wall of group A streptococci is an important factor in the virulence of the organism and in the development of type-specific, protective antibodies. Only general characteristics of M protein are known, and attempts to purify it have had limited success (10). Acid precipitation of crude extracts containing M protein was used in many early schemes to purify M protein (7, 9, 11, 16, 17). In these schemes, the M proteins were precipitated between pH 2 and 5. More recently, Fox and Wittner (5) reported a procedure for the purification of M protein involving the elution of a semipurified extract from a carboxymethylcellulose column equilibrated at pH 4. The complexity of their M protein preparation was demonstrated by electrophoresis on acrylamide gel, which revealed 12 to 16 protein bands. Each band was serologically reactive with antisera containing type-specific antibodies. Using the technique of Fox and Wittner, similar crude extracts of M protein precipitated on the column equilibrated at pH 4. Since the electrophoretic patterns of the crude extract layered on their column were not given, the possibility arose that acid precipitation of the crude extract might provide a simple means of preparing M protein similar in purity to that eluted from the column.

This study compares the material eluted from the column to material precipitated under conditions at which the column is equilibrated.

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MATERIALS AND METHODS

Strain. Streptococcus pyogenes, group A strain SS-635 (Lancefield's 12/27/1 M type 12), came from the lyophilized reference collection of the National Streptococcal Disease Center (NSDC). Whole-cell preparations of SS-635 are used to produce the diagnostic type 12 antiserum, distributed by the National Communicable Disease Center (NCDC) for M-precipitin tests.

Media. Lyophilized cultures were inoculated into 5.0-ml quantities of Todd-Hewitt broth (Difco) containing rabbit blood and were incubated at 37 C for 18 hr. Loopfuls of these cultures were incubated for 6 hr in 30 ml of Todd-Hewitt broth and then delivered into 4-liter quantities of glucose-salts-supplemented Todd-Hewitt broth (4).

Antisera. Absorbed type-specific M type 12 (lot 6) and T type 12 (lot 1) were furnished by the Biological Reagents Section of the NCDC (13).

Acrylamide gel electrophoresis. The method of Davis was used (2). Sample gels contained samples that varied from 70 to 140 μg of protein nitrogen in 40 to 80 μliters of phosphate-buffered saline (PBS). Large pore solution (0.2 ml) was used for both sample and spacer gels. Running time was 45 min at 5 ma per gel. Dye migration averaged 33 mm in the running gel.

Agar double diffusion. Glass slides (5.08 by 7.62 cm) were coated with 7.0 ml of 0.6% Agarose (Fisher Scientific Co., Atlanta, Ga.) in 0.05 M sodium barbitol buffer (pH 8.5 (14)). The wells were punched 3 mm in diameter and 4 mm apart. A similar diffusion system was used to study protein electrophoresed in acrylamide gels. Immediately after electrophoresis, acrylamide gels were sliced serially at 3-mm intervals and were placed in 5-mm wells, 6 mm apart. Reactions in
both systems were photographically using indirect lighting after incubation for 30 hr at room temperature.

Absorption spectra. Samples containing 1.0 mg of protein nitrogen per ml of 0.01 M potassium PBS (pH 7.0) were read in a DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif,) using the 0.01-mm slit width and a 1.0-cm light path.

Other methods. Protein concentrations were determined by the method of Lowry et al. (12) with bovine serum albumin as a standard. Methyl pentoses were analyzed by the method of Dische and Shettles (3).

Immunoelectrophoresis. The method of Wilson and Wiley (15) was used with the buffered agar described above. The slide was photographed unstained with indirect lighting.

M protein purification. The method of Fox and Wittner (5) was followed closely. After incubation at 37 C for 18 hr, the type 12 culture was processed live. The cells were harvested in a KSB (Ivan Sorvall, Inc., Norwalk, Conn.) continuous-flow system at 4 C at 30,000 X g, with a flow rate of 120 ml per min. The cells were washed three times in 1.3 liters of distilled water at 10,000 X g and weighed wet.

Cell walls were prepared by the method of Bleiweis et al. (1) in a Braun MSK homogenizer (Bronwill Scientific, Rochester, N.Y.). The type 12 cells required 3 min at 4,000 oscillations per min for disruption, as demonstrated by Gram stain of the homogenate. The disruption was followed by three washes in PBS (pH 7.0). Resuspending was effected by a hand-operated plunger without addition of antifoam agents. Cell walls were separated from intracellular material by differential centrifugation (5). A 20% suspension of cell walls in PBS was treated with ribonuclease (10 µg per ml of suspension). The digestion mixture was maintained without preservative at 37 C for 4 hr and then washed twice in PBS. A 25% suspension of cell walls was acid-extracted and centrifuged twice; the neutralized supernatant fluids were pooled (15).

A 33 to 60% ammonium sulfate fractionation of the pooled supernatant fluids was done at 4 C. Ammonium sulfate, 17.0 g per 100 ml of supernatant fluid, was dissolved in the supernatant fluids, and the solution was centrifuged for 30 min at 17,000 X g. The resulting supernatant fluid was adjusted to pH 8.0 with 1 N NaOH and brought to 60% saturation adding 13.9 g of ammonium sulfate per 100 ml of supernatant fluid. This solution was mechanically stirred for 1.5 hr at 4 C and then was centrifuged at 17,000 X g for 30 min at 4 C. The 33 to 60% pellet was solubilized in 10.0 ml of distilled water and dialyzed against PBS for 18 hr at 4 C.

After dialysis, a portion of the 33 to 60% ammonium sulfate fraction was frozen, and the remainder was divided into two equal parts. One part (33.3 mg) was dialyzed against 0.03 M sodium acetate buffer (pH 4.0). A precipitate appeared in the dialysis bag after 15 min. After 6 hr in dialysis, the material precipitated at pH 4.0 was centrifuged for 30 min at 27,000 X g and suspended in 4.0 ml of PBS. Both the pH 4 precipitate and the pH 4 supernatant fluid were dialyzed in PBS for 18 hr at 4 C and were saved for analysis.

The remaining 33.3 mg of the 33 to 60% ammonium sulfate fraction was layered on a carboxymethyl cellulose column (1.5 x 24 cm) (Cellex-CM, exchange capacity 0.68 meq/g; BioRad Laboratories, New York, N.Y.) equilibrated at 4 C with 0.03 M sodium acetate buffer (pH 4.0). The column size was adjusted, in relation to the amount of protein added, to correspond with that used by Fox (4). Fractions of 5.0 ml were collected with an LKB 7000A UltraTec fraction collector (LKB-Produkter, Stockholm, Sweden). Protein peaks were monitored with the LKB Uvicord II photometer at 280 nm with a 3-mm optical path. The column flow rate was regulated between 0.5 and 1.0 ml per min by a peristaltic pump. The pH of material collected in each tube was measured.

The 33 to 60% ammonium sulfate fraction was handled according to the revised technique of Fox et al. (6). Elution was begun at tube 1 with 0.1 M sodium acetate buffer (pH 5.5). When the effluent reached pH 5.5, a three-chambered gradient elution was initiated by using 50 ml of 0.1 M potassium phosphate buffer at pH 6.0, 6.5, and 7.0. The gradient was continued until the effluent reached pH 7.0. After fractionation, each peak was lyophilized and reconstituted in a small volume of PBS and was dialyzed against PBS for 18 hr at 4 C. The fractions were stored at -20 C.

RESULTS

Cell wall preparation and extraction. Whole cells (186 g, wet weight) were recovered initially from 60 liters of supplemented Todd-Hewitt broth. After the final wash, 61.6 g (wet cell weight) remained for preparation of cell walls. The 33 to 60% ammonium sulfate precipitate obtained from the acid extract of the cell walls contained 86.0 mg of protein nitrogen.

Column elution. The column was loaded with 33.3 mg of protein nitrogen. The first peak (I) appeared in tubes 5 and 6 coincident with a sharp drop in pH to 3.5 (Fig. 1). A second peak (II) appeared in tubes 10 to 12 at pH 3.6, and a third peak (II) appeared in tubes 16 to 19 between pH 4.4 and 5.4. After initiation of the gradient, a

![Fig. 1. Elution pattern of type 12 M protein from a carboxymethyl cellulose column (1.5 x 24 cm). The percentage transmittance at 280 nm is recorded by the solid line, pH by the broken line.](image)
**TABLE 1. Recovery and analysis of type 12 M protein**

| Determination                | Protein nitrogen | Methylpentose<sup>a</sup> mg | Ultraviolet absorption<sup>b</sup> |
|------------------------------|------------------|-------------------------------|----------------------------------|
| Starting material            |                  |                               |                                  |
| Ammonium sulfate fraction (33 to 60%) | 33.3             | 25/7,750                      | 1.31                             |
| Material recovered           |                  |                               |                                  |
| Precipitate (pH 4)           | 5.6              | 2.5/1,600                     | 1.06                             |
| Fraction IIIB                | 3.64             | 9/700                         | 1.25                             |

<sup>a</sup> Expressed as the amount of methylpentose found (micrograms) compared to the amount of protein (micrograms).

<sup>b</sup> Ratio of absorption at 280 nm to that at 260 nm.

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**FIG. 2. Gel diffusion pattern of absorbed antisera with starting material and products.** Antigen wells contain (A) 33 to 60% ammonium sulfate fraction, (T) T'-12-absorbed antisera, (M) M'-12-absorbed antisera, (3) fraction IIIB, and (4) pH 4 precipitate.

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broad double-humped peak (IIIA, IIIB) appeared at pH 5.6 at tube 34 and slowly tailed off to tube 47 at pH 6.6. The final peak (IIIB) in tubes 38 to 43 occurred at pH 5.9. Of the 33.3 mg applied to the column, only 12.6 mg of protein nitrogen was recovered in the various fractions eluted from the column up to pH 7.0.

**Quantitative.** Table 1 shows the protein recovery and the methylpentose contamination of the materials examined. Fraction IIIB and the material precipitated at pH 4 represent only small amounts of the protein present in the starting 33 to 60% ammonium sulfate fraction. Contamination of these products with methylpentose was minimal. The ultraviolet absorption spectra revealed a peak response at 276 nm for the 33 to 60% fraction, 272 nm for the pH 4 precipitate, and 276 nm for fraction IIIB. Their maximum to minimum absorption ratios were correspondingly 1.56, 1.35, and 1.42. Minimal nucleic acid contamination was demonstrated by ultraviolet absorption ratios at 280 and 260 nm.

**Gel diffusion.** Figure 2 shows a pattern of fusion formed by two bands between absorbed type-12 antiserum and both the starting material and the derived products. This demonstrated the anti-

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**FIG. 3. Gel diffusion pattern of absorbed antisera with type 12 protein fractions.** Central wells (M) contain absorbed type 12 antisera. Peripheral wells contain (A) 33 to 60% ammonium sulfate fraction, (1) T'-12-absorbed antisera, (2) fraction II, (3A) fraction IIIA, (3B) fraction IIIB, (4) pH 4 precipitate, and (4S) pH 4 supernatant fluid.

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**FIG. 4. Acrylamide gel electrophoresis patterns of type 12 M proteins.** From left to right: 33 to 60% ammonium sulfate fraction, pH 4 supernatant fluid, pH 4 precipitate, and fraction IIIB.
genic similarity of these three materials, as tested with absorbed type-specific antiserum. No reaction appeared between the absorbed T-12 antiserum and the protein preparations. Figure 3 presents the relationship between the absorbed M type-12 antiserum and material from steps in the purification procedure. Antigens eluted from the column precipitated with this antiserum and gave reactions of identity with the antigens present in the pH 4 supernatant fluid. A faint line, not connected with other bands, appeared between fraction I and the antiserum.

**Acrylamide gel electrophoresis.** Figure 4 illustrates the acrylamide gel electrophoresis patterns of the starting material, the pH 4 precipitate and the two products. The 33 to 60% fraction contained 11 bands with a dense grouping of the second, third, and fourth bands, and also a dense band 0.58 of the way from the origin to the terminal dye marker. The gel patterns of the pH 4 supernatant fluid were similar to that of the ammonium sulfate fraction. In contrast, the material precipitated at pH 4 contained primarily the 0.58 band with less prominent slow bands but more prominent fast bands in the lower one-third of the gel. Fraction IIIB was characterized by a heavy grouping of three bands similar to the second, third, and fourth bands of the 33 to 60% fraction, but only faint bands in the 0.58 area and beyond.

Figure 5 demonstrates lines of precipitation from a serially sliced acrylamide gel reacted with absorbed type-12 antiserum. Although the slide illustrates the sliced gel patterns of the 33 to 60% fraction, the precipitation patterns were the same for the pH 4 precipitate and fraction IIIB. The
appearance of the double precipitin line from wells 6, 7, and 8 coincided with the position of the 0.58 band. No precipitin lines were detected from the protein bands in the lower one-fourth of the acrylamide gel.

**Immunoelectrophoresis.** The precipitin lines formed by the reaction of the fraction IIIB and the pH 4 precipitate with absorbed type-12 antiserum are shown in Fig. 6. Fraction IIIB developed a pattern in the gel of at least two inner and two outer arcs of precipitation, each joined by a reaction of identity. However, in the pH 4 precipitate the inner band nearest the origin did not appear.

**DISCUSSION**

Since protein was precipitated on the top of the carboxymethylcellulose column at pH 4, it was of interest to see whether that precipitate was similar to the protein fraction IIIB eluted from the column at pH 6 by the technique of Fox et al. (5, 6). We hoped that this acid precipitate would contain M protein in sufficient purity to eliminate the column procedure. Therefore, fraction IIIB and the material precipitated at pH 4 were examined for similarities. Absorbed antiserum was chosen for antigenic analysis to characterize only the M components. Antigenic similarities were found among the pH 4 precipitate, fraction IIIB, and the 33 to 60% fraction by the gel diffusion and sliced gel techniques. However, using immunoelectrophoresis, there appeared to be at least one antigenic difference between fraction IIIB and the pH 4 precipitate. This difference may have contributed to the alterations in patterns, as shown by the acrylamide gel electrophoresis. The basic pattern of the 33 to 60% parent compound was reflected in both the pH 4 precipitate and fraction IIIB, but relative densities of the major bands differed in intensity.

The purified type-12 protein preparation described by Fox et al. (5, 6) closely resembled our fraction IIIB prepared by his method. Both were eluted from the carboxymethylcellulose column at pH 6. The acrylamide gel patterns were similar (5). Both had identical absorption ratios at 280 and 260 nm. Fraction IIIB had a slightly higher methylpentose content than the Fox preparation. The two lines of precipitation found in the diffusion studies differed from the preparation of Fox and Wittner (5), who found only one. By using the sliced-gel technique, the two lines of reactivity could be localized to a dense band in the gel pattern 0.58 of the way to the end of the gel. The 0.58 band in fraction IIIB gave strong serological reactions despite staining weakly in the acrylamide gel. Also, proximal unstained portions of the gels produced serological reactions. This phenomenon is probably due to the relative insensitivity of the Amido Swartz dye and the sensitivity of the double-diffusion technique in demonstrating protein antigens. The failure of Fox and Wittner to find more than one band in their preparations could be due to differences in techniques, strain and antiserum selection, and protein concentration.

The claims of M protein purity rest on showing a material which reacts with type-specific antisera and promotes the production of a protective antibody. Fox and Wittner suggest that M protein is a family of closely related molecules (5), whereas Johnson and Vosti (8) suggest that it may represent a repeating unit. Alternatively, the multiple serologically reactive bands seen on the acrylamide gel could represent serologically reactive type-specific non-M proteins which contaminate the preparations. Each band on the acrylamide gel pattern may not actually be a "virulence" protein or protein which assists the streptococcus in resisting phagocytosis. Antibodies to non-M proteins might be expected to be present in our antiserum according to its method of preparation, since it was prepared with a whole cell vaccine and absorption of the cross-reacting antibodies with other M-types. Immunization with purified preparations could also result in serological reactivity to a non-M type-specific protein which may not have the biological characteristics of M protein. In our preparations, the distal bands in the disc electrophoresis were not serologically reactive. This result agrees with the findings of Johnson and Vosti (8) and could mean contamination with non-M protein antigens or the presence of nonreactive M protein degradation products. Since both the pH 4 precipitate and the column-eluted fraction have serologically nonreactive terminal bands, this would indicate that neither should be considered a pure M protein preparation.

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