Evaluation of Methods Used to Purify Acid-Extracted Group A Streptococcal M Protein

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The literature includes descriptions of both acid-soluble and acid-insoluble M protein in the preparation of "hot acid-extracted group A streptococcal M protein." We present evidence for the contamination of crude type 1 acid-insoluble M protein. The purification of preparations of crude and partially purified acid-soluble type 1 and type 12 M protein is described. Our quantitative criteria for purification were recovery of M precipitin activity, improvement in specific activity, and removal of carbohydrate. Exclusion of nucleic acid is also discussed. Greater purification in a single passage was found with a carboxymethylcellulose column (with acidic elution) than with hydroxyapatite, diethylaminoethyl-Sephadex, or carboxymethyl-cellulose (with neutral elution) columns or with ammonium sulfate fractional precipitation. Carboxymethylcellulose with acidic elution was found to be a satisfactory standard laboratory procedure for the preparation of purified acid-extracted (acid-soluble) group A streptococcal M protein.

In 1928, Lancefield described hot acid extraction of type-specific M protein from group A streptococci (17). M protein inhibits the phagocytosis of streptococci, and its presence is closely associated with the virulence of streptococcal strains (30). Attempts to separate the protein from the cell wall by a milder procedure have involved the use of lytic enzymes (1), sonic treatment (4, 27), alcoholic solvents (33), lower temperatures (14), and higher pH (12), but acid-extracted M protein remains the basis for the everyday typing of streptococci (25, 36).

The qualitative estimation of M protein by precipitin reaction in capillary pipettes usually involves the use of a relatively crude M protein preparation and a whole-cell antiserum which has been absorbed to remove nonspecific reactions (29). Extensive absorption often results in a decrease in precipitin activity and produces a serum which is type-specific only within the limits of the maximal concentrations of the cross-reacting antigens tested. Thus, it is apparent that a more satisfactory serum requiring little or no absorption might be obtained by immunization with purified preparations of M protein.

Methods for removing contaminating carbohydrate, nucleic acid, and nonspecific proteins have frequently involved precipitation at reduced pH (14), fractional precipitation with ammonium sulfate solutions (21), and differential elution from ion-exchange columns (2, 6, 10, 16). Results have generally been described in terms of reduced reactivity with unabsorbed heterologous sera, minimal nucleic acid contamination as a function of the ratio of optical density (OD) at 280 nm to OD at 260 nm, and lack of reactivity with antigroup sera. However, there has been no quantitative comparison of the various purification procedures.

Our laboratory needed a standard procedure for purifying acid-extracted M protein which would be simple, effective, and applicable to different types of streptococci. To choose the most effective purification procedure, we compared fractional precipitation with ammonium sulfate solutions and chromatography with diethylaminoethyl-Sephadex (DEAE-Sephadex), carboxymethylcellulose, and hydroxyapatite columns. The comparison was based on quantitative recovery of M protein and removal of contaminating protein, carbohydrate, and nucleic acid from preparations of type 1 and type 12 group A streptococcal M proteins.

MATERIALS AND METHODS

Bacterial strains. Streptococcus pyogenes, type 1, strain 0201/B1 (10/18/67), and type 12, strain 0055 (12/3/68), obtained from W. K. Harrell of the Biological Reagents Section, Center for Disease Control (CDC), Atlanta, Ga., were used in all of our studies. These strains were isolated from infected humans and have been good producers of M protein. Stock cultures

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were passed through human blood twice, grown overnight in Todd-Hewitt broth (THB), and passed through human blood enriched with type-specific antiserum (3). The cultures were then grown overnight in Todd-Hewitt broth enriched with defibrinated rabbit blood (THBB), centrifuged, and resuspended in defibrinated rabbit blood. These suspensions were divided into 0.5- to 1.0-ml portions and stored at −70°C.

**Culture techniques.** A 30-ml amount of THB was inoculated with 0.5 ml of a 4- to 6-hr THBB culture and incubated for 4 to 6 hr at 37°C. Supplemented THB (5.4 liters of THB and 600 ml of supplement containing 90 g of glucose/liter, 80 g of NaHCO₃/liter, 30 g of NaCl/liter, and 15 g of Na₂HPO₄/liter) was inoculated with 60 ml of culture and incubated for 10 to 12 hr at 37°C (final pH, 6.4 to 6.6). Some of the cultures were heat-treated in a water bath at 60°C for 1 hr, allowed to settle overnight at 4°C, and centrifuged at 16,000 × g for 10 to 20 min. Other unheated cultures were centrifuged in a Sorvall continuous-flow system at 38,000 × g. Cells were washed three times with 10 to 20 volumes of 0.9% saline in 0.1 M sodium phosphate buffer, pH 7.0. All manipulations were carried out at 5°C.

**M protein extraction.** Cells were suspended in four volumes of 0.1 or 0.2 M HCl in saline (suspension pH, 1.8 or 2.0, respectively) and heated in a boiling-water bath for 10 min. The suspension was cooled and centrifuged at 16,000 × g for 30 min. The supernatant fluid was decanted, adjusted to pH 7.0 with concentrated NaOH, and centrifuged at 16,000 × g for 1 hr. The supernatant fluid was then taken and the pellet was discarded. The original cells were again extracted, cooled, and centrifuged. The resulting supernatant fluid was neutralized and centrifuged, and the supernatant fluid was again taken. The supernatant fractions from the first and second extractions (18, 21) were pooled and concentrated with Carbowax (polyethylene glycol compound 20-M, Union Carbide Corp., New York, N.Y.). For each ml of original volume, 1 μg of twice-crystallized ribonuclease was added, and the solutions were dialyzed, first for 5 hr at 37°C and then for over 18 hr at 5°C against more than 40 volumes of 0.01 M sodium phosphate buffer (pH 8.0, containing 0.02% sodium azide). The solutions were then centrifuged at 16,000 × g for 1 hr and stored at 4°C.

This crude preparation of acid-extracted M protein was soluble at pH 2 (centrifuged at pH 2 and the supernatant fluid removed); it is referred to as acid-soluble M protein (Ma); 25, 29, 36). When the acid-extraction procedure was altered so that extraction and neutralization were performed prior to centrifugation, the crude product was insoluble at pH 2; it is referred to as acid-insoluble M protein (Mα; 10, 21, 34). The triple-extracted cell residue (used to prepare Ma) was suspended in PBS (0.9% saline in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.02% sodium azide), adjusted to pH 7.0 with concentrated NaOH, incubated overnight at 37°C, and centrifuged at 16,000 × g for 1 hr. The supernatant product is referred to as the acid-soluble supernatant (ASS; Fig. 1).

**Serology.** The following CDC antisera were supplied by W. K. Harrell: M-typing (type 1 lot 6, type 12 lot 7), T-typing (type 1 lot 1, type 12 lot 1), and grouping (group A lot 23).

Type-specific quantitative capillary precipitin tests were performed by a procedure modified from those of Cohen and Pine (7) and Brock (5). Constant-bore glass capillaries (outer diameter 0.78 ± 0.001 mm, inner diameter 0.49 ± 0.01 mm, length 14 cm, from Drummond Scientific Co., Broomall, Pa.) were used. Capillaries were incubated for 1 to 3 hr at 37°C and then for at least 48 hr at 4°C, after which they were centrifuged for 10 min at 1,400 rev/min in an International centrifuge (model CS, head 240). The length of the tightly packed precipitates was read with a Bausch & Lomb measuring magnifier (no. 81-34-38, graduated in 0.1-mm units). A standard curve was constructed with each determination. A combination of linear and semilogarithmic plotting was usually necessary to obtain a straight line over the appropriate concentration ranges, and antigen excess in the type 12 assay often required concentration of the serum and dilution of the antigens. Assays were run in triplicate and the average was taken. Concentrations were read from the standard curves and expressed in units of standard antigen per milliliter.

Agar-gel diffusions were performed in a medium previously described (4). Both macro and micro (32) techniques were used.

Group-specific qualitative precipitin tests were performed with antiserum and antigens as described above. The capillaries were read as positive or negative after 5, 15, and 25 min at room temperature, after 1 hr at 37°C, and after overnight at 4°C.

**Ammonium sulfate fractional precipitation.** The solution was brought to 30% saturation with crystal (NH₄)₂SO₄, allowed to form a precipitate overnight at 4°C, and then centrifuged at 16,000 × g for 1 hr. The supernatant fluid was brought to 60% saturation with

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**Fig. 1. Steps involved in the preparation of acid-soluble M protein (Ma), acid-insoluble M protein (Mα), and acid-soluble supernatant (ASS).**
(NH₄)₂SO₄, allowed to form a precipitate overnight at 4 °C, and centrifuged at 16,000 × g for 1 hr. The precipitates were taken up in a small volume of distilled water, and both supernatant fluids and dissolved precipitates (except for the 60% precipitate) were dialyzed against PBS. The dissolved 60% precipitate was dialyzed against 0.01 M sodium phosphate buffer, pH 8.0, and the procedure was repeated. The fraction which was twice soluble in 30% and insoluble in 60% saturated ammonium sulfate is referred to as partially purified (30 to 60% fraction, second fractionation), and the fraction initially soluble in 60% saturated ammonium sulfate is referred to as high-carbohydrate (> 60%; fraction, first fractionation). The procedure of ammonium sulfate fractional precipitation is referred to as AmSulf.

**Column chromatography.** Resins were equilibrated with the appropriate buffers and the fines were removed (all buffers contained 0.02% sodium azide unless otherwise stated). An amount containing 5 to 35 mg of protein of the solution to be chromatographed was dialyzed against the appropriate buffer and applied to the column as a clear solution or as a slurry of solution and precipitate. Preparations used were (i) crude (ribonuclease-treated), (ii) partially purified, and (iii) high-carbohydrate type 1 and type 12 M protein. Elution was stepwise, with 10 to 30 column volumes each of the initial buffer and succeeding buffers. All chromatography was carried out at room temperature. Fractions of approximately 3 ml were collected at a rate of one drop every 6 to 15 sec, and OD was monitored continuously at 254 nm. Tubes were pooled according to peaks of OD or M protein capillary precipitin activity, or both. Pools were concentrated against Carbowax, dialyzed against PBS, and assayed for quantitative and qualitative M protein activity (capillary precipitin and agar-gel diffusion, respectively), protein (22), hexose (9), approximate nucleic acid content (275 to 260 OD ratio), and qualitative group A capillary precipitin activity. If columns were to be reused, they were eluted with large volumes of concentrated buffers, with 1 M HCl and 1 M NaOH, or with both, and were reequilibrated.

We used four column procedures: DEAE-Sephadex A-50, carboxymethylcellulose (with both acidic and neutral elution), and hydroxyapatite.

(i) For the DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) procedure, we used 0.8 g of resin in a column 1.1 by 9.5 cm. Our procedure was modified from that of Beachey, Alberti, and Stoller- lerman (2). The initial buffer was 0.02 M sodium phosphate, pH 6.8, and successive elutions were with 0.12 M NaCl in buffer, 0.33 M NaCl in buffer, and 0.37 M NaCl in buffer. [Steps were chosen for maximal recovery of type 1 M protein by use of linear and exponential gradients of 0 to 0.4 M NaCl in 0.02 M sodium phosphate buffer, pH 6.8, and 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 6.8. Because of the decreased solubility of the M protein preparations in the Tris buffer, phosphate buffer was used.] 55 ml

(ii) For the carboxymethylcellulose (Whatman CM-52, H. Reeve Angel, Inc., Clifton, N.J.) procedures, we used 1.0 g of resin in a column 1.1 by 6 cm. Our procedure involving acidic elution was modified from the procedure of Vincent, Borman, and Pranitis (31). The initial buffer was 0.02 M sodium acetate, pH 4.5, and successive elutions were with 0.01 M HCl and 0.02 M HCl. (iii) Our procedure involving neutral elution was modified from the procedures of Fox (10) and Fox and Wittner (11). The initial buffer was 0.02 M sodium acetate, pH 4.5, and successive elutions were with 0.02 M sodium acetate or sodium phosphate buffer, pH 5.5, and 0.02 M sodium phosphate buffer, pH 6.8.

(iv) For the hydroxyapatite (Bio-Rad Laboratories, Richmond, Calif.) procedure, we used a column 1.2 by 3.4 to 7.5 cm. Our procedure was taken from that of Johnson and Vosti (16). The initial buffer was 0.01 M sodium phosphate, pH 6.8, and successive elutions were with 0.1 and 0.3 M sodium phosphate buffers, pH 6.8. No more than 90 cm of water pressure was used.

**Definition of purification factors.** The purification factors, PF and PF' (see below), were developed to compare purification by the different techniques. They represent a combination of the parameters by which we compared the effectiveness of our procedures: increase in specific activity, retention of M capillary precipitin activity, and removal of carbohydrate. The product of the factor of increase in specific activity (to be referred to as specific activity increase) and the fraction of M recovered divided by the percentage of carbohydrate retained will be referred to as the purification factor (PF):

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PF = \frac{\text{specific activity increase}}{\text{percentage of carbohydrate retained}} \times \text{(fraction M recovered)}
\]

Since the partially purified products contained very little carbohydrate (see Results), further removal by column chromatography could not be accurately determined; PF is then replaced by PF':

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PF' = \frac{\text{specific activity increase}}{\text{(fraction M recovered)}}
\]

PF, which weights differences in carbohydrate removal more heavily than changes in specific activity or M retention, applies to procedures starting with crude or high-carbohydrate M protein. PF', which does not contain a factor for carbohydrate removal, applies to further purification of partially purified preparations.

**Miscellaneous.** Absorption spectra were determined with a Gilford 2000 recording spectrophotometer. Bactericidal and indirect bactericidal inhibition tests were performed according to the method of Stoller- lerman and Ekstedt (28). T antigen was prepared according to the procedure of Lancefield and Dole (20). All variations are expressed as ± range/n, where n is equal to the number of determinations.

**RESULTS**

Acid-soluble and acid-insoluble M protein. Although acid-insoluble M protein (M₈) is almost completely insoluble at pH 2 (2, 23), preliminary experiments showed that type 1 M₈ became soluble at this pH after AmSulf purification.
in gel diffusion (Fig. 2 and 3). Apparent identity was also found between T antigen and the ASS fraction (Fig. 3 and 4). However, the sensitive Wadsworth microimmunodiffusion procedure revealed: (i) complete identity between ASS and some of the outer band antigens, none of which were present in our T preparation; (ii) complete identity between the remainder of the outer band antigens and some of the antigens in our T preparation; and (iii) the presence in our T preparation of some antigens which were not present in the outer band (Fig. 5). On rare occasions, concentrated preparations of Ms gave a second band on diffusion against MI lot 6 antiserum, but this band was not identical to the bands discussed above.

The two type 1 M1 bands were still present after purification by AmSulf and DEAE-Sephadex column chromatography. Preliminary experiments indicated that, for a given weight of cells, the total M precipitin activity of M1 was approximately equal to the sum of that of Ms plus that of ASS (from the Ms residue). This suggested that the precipitin activity of the combined inner and outer bands equaled the precipitin activity of the Ms band plus the ASS band.

Preparations of type 12 M1 showed an extremely weak outer band with anti-M serum, and reacted to a very small extent with anti-T serum.

Since we felt that the outer M1 bands represented contamination of the M protein, Ms preparations were used in all of our comparative purification experiments.

Crude Ms. Ribonuclease-treated type 1 and type 12 preparations ranged from 3.1 to 4.2 mg of protein per ml and 0.9 to 1.4 mg of carbohydrate

Our early work with the type 1 strain involved only M1 preparations. These reacted strongly with type-specific sera in capillary precipitin and bactericidal tests, and repeatedly produced two bands on agar-gel diffusion (Fig. 2). Ms preparations were also active in capillary precipitin and bactericidal tests, but showed only one band on agar-gel diffusion (Fig. 2). One of the M1 bands (referred to as the "inner band") appeared to be identical to Ms, and the other M1 band (referred to as the "outer band") appeared to be identical to T antigen

**Fig. 2.** Gel diffusion pattern of type 1 anti-M (aM) and anti-T (aT) sera with type 1 Ms, M1, and T antigens.

**Fig. 3.** Gel diffusion pattern of type 1 anti-M (aM) and anti-T (aT) sera with type 1 T, M1, and ASS antigens.

**Fig. 4.** Gel diffusion pattern of type 1 anti-T serum (aT) with type 1 M1, Ms, T, and ASS antigens.
per ml, and had specific activities between 240 and 330 units of M/mg of protein (each crude preparation defined separately as 1,000 units of M/ml). Substantial amounts of nucleic acid remained despite treatment with ribonuclease, as shown by 275 to 260 OD ratios between 0.65 and 0.97. Each preparation reacted strongly with its M-typing serum in capillary precipitin and gel diffusion tests, and selected pools of the preparations were positive in indirect bactericidal tests.

Ammonium sulfate fractional precipitation (AmSulf). Our initial studies (24) involved M₈ preparations from types 1, 8, 9, 12, and 22, with protein concentrations ranging from 1.2 to 1.3 mg/ml. A single precipitation between 30 and 60% saturation removed 98.5 to 99.1% of carbohydrate (which largely remained in the 60% saturated solution). Although at least 90% of the recovered precipitin activity was contained in the 30 to 60% fraction, specific activity increased little: an average of 54% of the M activity and 52% of the protein was recovered in this fraction. Considerable amounts of nucleic acid were removed, since the 275 to 260 OD ratios rose to a mean of 1.20.

With the more concentrated preparations of this study (see above), AmSulf removed 98.9 to 99.1% of the carbohydrate after a single precipitation, and 99.9% after reprecipitation. Two precipitations were required for the preparations to become nonreactive with grouping serum by capillary precipitin test. After the first precipitation, 29 to 35% of the M activity was recovered in the 30 to 60% fraction, but 35 to 57% was recovered along with the bulk of the carbohydrate in the >60% fraction. When the ammonium sulfate was raised to 60% saturation, there was a considerable amount of fine white material in addition to the expected heavy white precipitate; the fine material layered on the supernatant fluid and was mainly collected in the >60% fraction. The fine precipitate was easily dissolved when the >60% fraction was dialyzed against PBS. When the 30 to 60% fraction was put through the procedure again, the fine white precipitate was not observed, and 74 to 81% of the M activity was recovered in the 30 to 60% fraction. This represented an overall recovery of 23 to 26% of the M activity after two precipitations. Specific activity increased an average of 280% with the first precipitation but rose only slightly after reprecipitation. Nucleic acid removal was less effective than in our earlier experiments (see above); the mean 275 to 260 OD ratios of the 30 to 60% fractions rose only to 1.01 after the first and 1.14 after the second precipitation.

Column chromatography. Ion-exchange columns were used to purify both crude preparations of M protein and M protein which had been through the AmSulf procedure. Since the AmSulf procedure divided the M protein into two major fractions, high-carbohydrate and partially purified (see above and Materials and Methods), both were used. From each elution, the pooled eluate fraction containing the largest amount of total M precipitin activity was selected. The fractions were then compared with respect to recovery of M precipitin activity, increase in specific activity, and removal of carbohydrate. All of the selected fractions reacted strongly with type-specific serum in capillary precipitin and gel diffusion tests, and each of the most highly purified fractions (from chromatography of a partially purified preparation) was positive in the indirect bactericidal test.

Since the contaminating protein and carbohydrate usually came off the columns in the first and second elution steps, effective purification of the M protein depended in part on its coming off the column in a later elution step. Although type 1 and type 12 M protein behaved in a similar manner on hydroxylapatite (elution with 0.1 M buffer) and on carboxymethylcellulose with acidic elution (0.02 M HCl), this was not the case on DEAE-Sephadex or on carboxymethylcellulose with neutral elution. With DEAE-Sephadex, type 1 M protein was mainly eluted with buffer plus 0.33 M NaCl and type 12 M protein with buffer plus 0.12 M NaCl; with carboxymethylcellulose with neutral elution (CM-neutral), type 1 M protein was mainly eluted with pH 5.5 buffer and type 12 M
protein with pH 6.8 buffer. This difference in elution patterns made it impossible to retain reliably the majority of the M activity by simply collecting tubes containing a peak of OD; instead, it became necessary to measure the M activity in each tube before the tubes could be pooled. Moreover, the earlier elution of type 1 M protein on CM-neutral and type 12 M protein on DEAE-Sephadex tended to reduce purification.

The elution of crude type 1 M protein on DEAE-Sephadex produced an increase in specific activity greater than on any other column (or with any other M protein preparation; Fig. 6 and 7). We chose elution steps for maximal recovery of type 1 M protein on DEAE-Sephadex after analysis of the effect of linear and exponential gradients of increasing pH and ionic strength. These elution steps proved to be much less satisfactory for type 12 M protein. We did not repeat the gradient analysis; it might have revealed more optimal conditions for elution of type 1 and type 12 M protein from the other columns.

Carboxymethylcellulose with acidic elution (CM-acid) was the most effective column in removing carbohydrate; however, even the least effective column, DEAE-Sephadex, usually removed 98 to 99% (Fig. 6 and 7). All columns removed nucleic acid from preparations of crude M protein, since the 275 to 260 OD ratios rose to a mean of 1.20. But only CM-neutral was effective in removing additional nucleic acid from partially purified preparations, with the 275 to 260 OD ratios rising to a mean of 1.32.

Increases in specific activity with column purification of crude M protein were expected to be greater than those of the partially purified or high-carbohydrate preparations (Fig. 6 and 7), but we were surprised to find that the overall increases in specific activity were comparable. The mean increase in specific activity for crude M protein purified by only one column elution (averaged over all four columns) was approximately equal to the total mean increase after column elution of the partially purified AmSulf preparations: for type 1, crude (5.5 ± 1.6) ∼ AmSulf (3.4) + column purification (2.2 ± 0.2); for type 12, crude (4.5 ± 0.8) ∼ AmSulf (2.6) + column purification (2.3 ± 0.6; Fig. 6 and 7). Further column purification of
high-carbohydrate type 12 M protein also provided a comparable mean increase in specific activity, and in addition was approximately additive [crude (4.5 ± 0.8) ≈ AmSulf (2.5) + column purification (2.5 ± 0.3)]. But chromatographic purification of high-carbohydrate type 1 M protein gave a lower mean specific activity increase, and was not additive (Fig. 6 and 7). This difference was due largely to the hydroxylapatite column, which removed less carbohydrate and contaminating protein than in other experiments. (None of the resins removed as high a percentage of carbohydrate from the type 1 high-carbohydrate preparation as they did from other preparations.)

Using our criteria for purification, we found that CM-acid was the most effective column with either crude high-carbohydrate or partially purified preparations of type 1 or type 12 M protein (Fig. 8 and 9). With the crude preparations, CM-acid was the only column which provided a product of higher PF than that from the AmSulf procedure: 24.5 ± 3.2 and 16.3 ± 4.2 for CM-acid with type 1 and type 12 M protein, respectively, and 7.8 and 6.7 for the partially purified AmSulf preparations of type 1 and type 12, respectively (Fig. 8 and 9). Carbohydrate was removed almost as effectively from both types of M protein in one column elution with the CM-acid procedure as it was with two 30 to 60% ammonium sulfate precipitations. Thus, we obtained 99.8% carbohydrate removal and negative group precipitins in 9 of 10 runs with CM-acid, as compared with 99.9% carbohydrate removal and totally negative group precipitins with ammonium sulfate. CM-acid was as effective as any other column in removing nucleic acid from crude preparations of M protein, but less effective than CM-neutral with partially purified preparations (see above).

**DISCUSSION**

The reliable measurement of the M protein content of our crude and purified preparations was made possible by a technique for type-specific quantitative capillary precipitin tests. A standard
Preparations used to curve pressed and treatments, purification factors defined only precipitate concentrations expected separation of lower antigen concentrations. This was subsequently removed in the process of purification. This would have produced an error suggesting reduced purification, since the loss of precipitin activity would decrease the calculated protein content.

Both M1 (1, 2, 10, 21, 27, 33, 34) and M8 (17, 25, 29, 36) have been described in the preparation of "hot acid-extracted Group A streptococcal M protein." Nevertheless, their chemical properties differ: crude M8 is soluble and M1 is insoluble at pH 2. Some investigators have described the precipitation of crude M1 preparations at pH 2 as an early step in the purification of their M protein preparations (1, 2, 21), but our preliminary experiments indicated that purified M1 was soluble at pH 2. Likewise, Wahl and Drach (33) described the elution of M1 from carboxymethylcellulose columns at pH 2 and 1.7, which again implies that purified M1 is soluble at pH 2. Moreover, Lancefield and Perlman (21) noted that neutralization prior to centrifugation of the crude pH 2 extract may bring into solution cellular components other than M protein. It seems likely that insolubility at pH 2 is not an inherent property of M1 but instead curve made it possible to compare the amount of precipitate obtained with an unknown preparation to that obtained with a standard preparation. Any precipitin curve is truly linear only over a small range of lower antigen concentrations, and a semilogarithmic plot can be expected to approximate linearity only over a relatively small range of higher antigen concentrations (15). The wide range of antigen concentrations we encountered could not be adequately measured with a single graph, but required a combination of linear and semilogarithmic plotting. Antigen excess was rarely a problem with our type 1 antigens (7), but our type 12 antigens often required dilution prior to measurement with our weaker type 12 antiserum.

Our analysis involved the basic assumption that all precipitin activity was due to the reaction of the M protein antigens with their specific antibodies. Because of this, a small error may have been introduced by the use of whole-cell sera which were absorbed to the limit of nonreactivity with unconcentrated heterologous antigens: these sera can still precipitate more concentrated heterologous antigens (L. S. Phillips and A. J. Berry, unpublished data). Thus, our concentrated preparations of crude M protein may have had some nonspecific precipitin activity which was subsequently removed in the process of purification.

**Fig. 8. Relative effectiveness of column purification of type 1 acid-soluble M protein.** PF and PF' refer to purification factors defined in Materials and Methods. Preparations used were (a) crude, (b) partially purified, and (c) high-carbohydrate M protein. Variation expressed as ± range/n.

![Graph showing relative effectiveness of column purification](image)

**Fig. 9. Relative effectiveness of column purification of type 12 acid-soluble M protein.** PF and PF' refer to purification factors defined in Materials and Methods. Preparations used were (a) crude, (b) partially purified, and (c) high-carbohydrate M protein. Variation expressed as ± range/n.

![Graph showing relative effectiveness of column purification](image)
represents coprecipitation with contaminating cellular components (39).

The two bands seen on gel diffusion of type 1 M₁ against type-specific whole-cell serum may well be a manifestation of contamination with cellular components. The inner band of M₁, continuous with the single band of M₈, appeared to represent a common M antigen. But the outer band of M₁ was not represented in M₈ and contained sub-bands which were continuous with ASS (obtained from the pH 7 PBS incubation of the cellular residual from the centrifugation of M₈ at pH 2). This suggests that the outer M₁ antigens and the ASS antigens may represent a common cellular constituent (attached to the cells at pH 2 but released at pH 7) that contributes to the outer M₁ antigens if centrifugation takes place after neutralization and to the ASS antigens if centrifugation takes place prior to neutralization.

It is striking that only the outer M₁ antigens are insoluble at pH 2 in the presence of cells, but both inner and outer M₁ antigens are insoluble in the absence of cells. As discussed above, coprecipitation with a contaminant may explain the insolubility of crude M₁ preparations at pH 2 when purified preparations of type 1 M₁ (still containing both inner and outer bands) are soluble at pH 2. It seems reasonable that the outer M₁ antigens are more closely associated with a contaminant than the inner M₁ antigens, since the outer antigens are precipitated at pH 2 in both the presence and absence of cells. The differential solubility of the outer and inner M₁ antigens in the presence of cells would be explained if the contaminant were limited in binding sites and tended to bind more tightly to cells than to the inner M₁ antigens.

We have not studied our preparations of ASS antigen further, and can only speculate on its identity at the present time. Streptococcal typing is most often complicated by R and T antigens, and an R antigen would probably be more likely in this case. Despite the reaction with anti-T serum, T antigens are usually destroyed by hot acid extraction (20, 26); however, Hambly (13) has reported a trypsin-resistant protein which survives hot acid extraction. The R antigens vary in their resistance to trypsin and, like the T antigens, are unrelated to virulence, but usually survive hot acid extraction (19, 35). A variety of other antigens have been described, including polyglycerophosphate (23), E₄, F, and E antigens (38), but the R and T antigens have been reported more frequently.

The most common contaminants of preparations of M protein are nucleic acid and carbohydrate. Since highly purified preparations of M protein have an absorption maximum at 276 nm (16, 21) and the highest reported 275 to 260 OD ratios were consistently in the range 1.34 to 1.37 (1, 16, 21), we accepted these ratios as representing maximal nucleic acid removal. Maximal removal was first effected by ribonuclease digestion and several reprecipitations with ammonium sulfate (21). It was later reported (1) with ribonuclease digestion and careful washing of broken cell walls followed by a single ammonium sulfate precipitation. Recently, Johnson and Vosti (16) reported comparable results with two precipitations with ammonium sulfate and a single hydroxyapatite column elution; they avoided ribonuclease digestion entirely. Since we found column elution to be effective in removing considerable amounts of nucleic acid and since others have produced satisfactory preparations without the use of columns (above), our difficulty in achieving maximal nucleic acid removal probably involved ammonium sulfate precipitation. This procedure was effective with dilute preparations, but removed nucleic acid poorly from concentrated preparations of crude M protein.

Dixon and Webb (8) have stated that approximately 75% of a relatively pure protein will be precipitated within a 6% increase in saturation of the ammonium sulfate solution. This is easily encompassed by the 30 to 60% fraction commonly used for M protein preparation, although contaminating antigens may also precipitate in this wide range. Thus, Zimmerman, Mathews, and Wilson (38) reported cross-reactions with the 60 and 70% fractions, but not with the 30, 40, and 50% fractions, for a particular type 12 hemagglutinating antigen. Our use of increased initial concentrations of M preparations should have resulted in more complete precipitation of the M protein within the 30 to 60% range (8), but it may also have produced precipitation of some of the contaminating nucleic acid and carbohydrate, which are usually soluble in 60% saturated ammonium sulfate (1, 40).

With our concentrated preparations of crude M protein, the mean 275 to 260 OD ratio rose only to 1.01 after one and to 1.14 after two ammonium sulfate fractionations, almost half of the precipitin activity was lost into the >60% fraction, and a fine white precipitate contaminated the supernatant fraction of the 60% saturated solution. But with our more dilute M protein preparations, the average 275 to 260 OD ratio rose to 1.20 after a single ammonium sulfate fractionation, 90% of the recovered precipitin activity was contained in the 30 to 60% fraction, and the supernatant fluid of the 60% saturated ammonium sulfate solution was clear. Unfortunately, the fine precipitate (in the 60% supernatant) was collected and dissolved with the rest of the >60%
fraction and was not separately analyzed. However, it seems likely that the fine precipitate represented a combination of nucleic acid and M protein (39) that contaminated the 30 to 60% fraction with nucleic acid and carried M protein over into the >60% fraction.

Despite the contamination with nucleic acid, fractional precipitation with ammonium sulfate removed carbohydrate equally well from dilute and concentrated preparations of M protein: a single fractionation removed approximately 99% of the carbohydrate, and a second fractionation removed 99.9% of the carbohydrate and produced nonreactivity with antigen group serum. As other investigators have found (1, 18, 34), fractional precipitation with ammonium sulfate produced relatively little increase in specific activity.

However, use of column chromatography generally resulted in a more substantial specific activity increase. CM-acid, overall the most effective of the columns, was several times more effective than ammonium sulfate fractional precipitation in increasing specific activity. Although Vincent, Borman, and Pranitis (31) have reported that with CM-acid most of the M activity was eluted with 0.01 M HCl, we confirmed instead the reports of Cayeux and Wahl (6) and Wahl and Drach (33) that most of the M activity was eluted with 0.02 M HCl. This difference in elution can probably be explained by differences in the initial composition of the crude preparations, but there is no doubt about the overall applicability of the procedures described by Cayeux and Wahl (6) and Vincent, Borman, and Pranitis (31).

For a variety of reasons, we found the other columns less satisfactory than CM-acid. Hydroxyapatite was less effective in removing carbohydrate and contaminating protein from preparations of crude M protein, but was almost as good as CM-acid with partially purified preparations. [Johnson and Vosti (16) have reported the combination of ammonium sulfate fractional precipitation and hydroxyapatite column elution in the preparation of highly purified M protein.] DEAE-Sephadex and CM-neutral were frequently less effective than CM-acid in removing contaminating protein and carbohydrate, and neither allowed similar elution of both type 1 and type 12 M protein. Since we designed a routine laboratory procedure which would be applicable to different types of streptococci, DEAE-Sephadex and CM-neutral were unacceptable.

Part of the difficulty in preparing pure M protein lies in the limitations of our tests for nonspecificity and heterogeneity (24). Highly purified preparations of M protein have frequently been heterogeneous on polyacrylamide gel electrophoresis (11, 16), and our column eluates would probably have given the same results had this been pursued. Preparations which appear to be pure may still produce cross-reacting sera when used as immunogens (2; Phillips and Berry, unpublished data), and the final decision regarding purity will probably have to be made by the rabbit (21, 37).

Using our criteria for purification (see above), we found CM-acid to be more effective in a single passage than ammonium sulfate fractional precipitation or any other column procedure tested. It is rapid, simple, and appears easy to adapt for use with larger amounts of crude starting material. M activity is eluted in a narrow peak requiring little concentration. We feel that the CM-acid procedure is a satisfactory routine laboratory method for the preparation of purified acid-extracted (acid-soluble) group A streptococcal M protein.

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