

## STREPTOCOCCAL M PROTEIN EXTRACTED BY NONIONIC DETERGENT

### I. Properties of the Antiphagocytic and Type-Specific Molecules\*

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M proteins are surface antigens primarily responsible for the virulence of Group A streptococci by virtue of their unique antiphagocytic characteristic. Lancefield, in 1928 (1), first reported the extraction of M protein from whole Group A streptococci. The method employed, namely heating streptococci to 100°C in a solution of HCl at pH 2 for 15 min, provided the starting material for almost all of the preparations of M protein subsequently reported (see review by Fox [2]). Other methods of extracting M protein from whole streptococci or their isolated cell walls have also been attempted, such as sonic oscillation (3, 4), alkaline extraction (5), pepsin digestion (6), streptococcal phage lysin (7), as well as extraction with guanidine hydrochloride (8). Crude M-protein extracts obtained by these procedures have been subsequently purified from contaminating antigens by applying a variety of protein purification techniques (2). Despite these efforts, however, in all cases the purified M-protein molecule exhibited various unexplained heterogeneities.

In this communication, we describe a method for extracting M protein from isolated streptococcal cell walls by the use of a nonionic detergent. The M protein thus extracted is purified and subjected to a variety of physical, chemical, and immunological tests. Evidence is presented indicating that the antiphagocytic M-protein molecule is composed of proteins that appear to be formed from smaller, type-specific molecules. We also present data supporting the view that the type-specific moiety of M protein differs from the antiphagocytic moiety of the molecule.

### Materials and Methods

*Streptococcal Strains.* Type 6 streptococcal strain D471 was from The Rockefeller University collection. This strain that had never been mouse passaged was an isolate from a human throat. However, several passages through human blood were performed to increase the M-protein yield (9).

*Preparation of Streptococcal Cell Walls.* Strains were grown in dialyzed Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) containing 0.2% yeast extract (Difco) and sterilized by filtration through 0.22  $\mu$ m Millipore membrane. 3 liters of an 18-h growth was inoculated into 60 liters of prewarmed broth and grown with agitation for 4 h at 37°C. At this time, sterile glucose was added to a final concentration of 0.2% and the pH adjusted to 7.2 with 5 N NaOH and maintained at this value for the duration of the growth, using a Radiometer pH meter and Titrator

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Model 11 (Radiometer Corp., Copenhagen, Denmark). Cells were thus kept actively growing for an additional 6 h, after which they were centrifuged in a Sharples continuous flow centrifuge. The packed cell paste was collected and washed in saline, resuspended in distilled water containing 0.02% sodium azide, and disrupted in a Vibrogen Cell Mill (RHO Scientific, Commack, N. Y.) at 14°C. Cell walls containing the M protein were separated from other cell constituents by centrifugation at 8,000 *g* for 1 h and washed in 4 liters of saline containing 0.02% sodium azide. The walls were suspended in 500 ml of phosphate-buffered saline at pH 7.5, pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N. J., no. 5679) was added at a concentration of 30  $\mu\text{g}/\text{ml}$  and incubated for 2 h at 37°C with mixing. The digested cell walls were again washed with 4 liters of saline, 1 liter of distilled water, and resuspended in approximately 300 ml of distilled water containing 0.02% sodium azide and stored at -51°C. A 1.0-ml aliquot was lyophilized to determine the dry weight of the cell walls in the suspension.

*Extracting Solution.* A 1% solution (vol/vol) of the nonionic detergent Emulphogene BC-720 (a fatty alcohol derivative, a gift of GAF Corporation) in 0.005 M sodium acetate buffer at pH 5.5 containing 0.02% sodium azide was used for all detergent extractions.

*Detergent Extraction of Type 6 M Protein.* An aliquot of type 6 cell walls (prepared as described above) was sedimented at 10,000 *g* for 15 min and suspended in 1% detergent to a ratio of 10 mg (dry weight) cell walls/ml 1% detergent. The suspension was magnetically stirred at room temperature for 8–18 h, after which time the walls were sedimented at 10,000 *g* for 20 min and the M-containing supernate saved at 4°C. The cell wall pellet was re-extracted several times under the same conditions and the supernate pooled. Extractions were terminated when no further protein could be extracted; 5–10 extractions were sufficient to remove the extractable M protein from the cell wall preparations.

*Purification of M Protein.* The pooled detergent extract of type 6 cell walls (approximately 800 ml) was filtered through a 1.2  $\mu\text{m}$  Millipore membrane, dialyzed (Spectropore 3, Spectrum Medical Industries, Inc., Los Angeles, Calif.) against a 0.005 M Na acetate buffer pH 5.5, and applied at a rate of 8 ml/h to a 1.5  $\times$  20-cm column of CM Cellulose (Whatman CM52 microgranular) equilibrated with the same buffer. The column was then washed with at least 10 column vol of the equilibrating buffer. The material that did not adhere to the CM cellulose consisted of detergent and nonspecific proteins and peptides. Preliminary experiments using linear gradients of increasing pH and sodium ion concentration indicated that a single symmetrical peak of M protein emerged as the pH approached 6.5 and was complete by pH 7.0 at a final sodium ion concentration of 0.1 M; therefore, the elution of the M protein was accomplished in one step using 0.1 M sodium phosphate buffer pH 7.0 containing 0.02% sodium azide. The eluted M protein was pooled, dialyzed against 0.05 M ammonium bicarbonate pH 8.4, and lyophilized. The dried M protein was suspended in 1 ml 0.1 M ammonium bicarbonate buffer and applied to a 1.5  $\times$  90-cm column of Sephadex G-200. Fractions were tested for their ability to remove type-specific opsonic antibodies from human immune serum (see method below). Those fractions containing 4+ activity were pooled, lyophilized, and rechromatographed on a 1.5  $\times$  90-cm column of Sephadex G-100. Fractions were again tested for their ability to remove type-specific opsonic antibodies from human serum. Active fractions were pooled and utilized for further study.

*Absorption of Opsonic Antibodies.* All purification steps were monitored for the presence of M protein by testing fractions for their ability to remove type-specific opsonic antibodies from human or rabbit immune serum. The possibility exists, however, that nonprecipitating or nonsedimenting complexes may be present in the reaction mixture which might subsequently deplete complement from or interfere with the indirect bactericidal test system (10). Since a false positive reaction (10) could result from such an interference, all M-protein preparations were immobilized on *N*-hydroxysuccinimide-activated Sepharose (11) before use for serum absorption.

The test consisted of mixing 200  $\mu\text{l}$  of protein samples at 300  $\mu\text{g}/\text{ml}$  in 10 mg of activated Sepharose beads in a 1.5-ml microfuge tube (Beckman Instruments Inc., Palo Alto, Calif.). The tubes were mixed end-over-end at 16 rpm for 18 h at 4°C. At this time, 200  $\mu\text{l}$  of 2 M glycine was added and the tubes mixed for an additional 2 h at room temperature. The beads were then sedimented in a Microfuge B centrifuge (Beckman Instruments), washed three times with 0.05 M phosphate-buffered saline (PBS)<sup>1</sup> pH 7.4, and resuspended in 220  $\mu\text{l}$  of immune human or rabbit

<sup>1</sup> Abbreviations used in this paper: BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis.

serum. The tubes were again rotated at 4°C for 18 h, after which time the beads were sedimented and the absorbed serum used in the indirect bactericidal test.

*Indirect Bactericidal Test.* This test was carried out as described by Lancefield (12). Stationary tubes without serum were used to control the growth of cells in the donors' heparinized blood. Rotated tubes without serum were used to control for the active growth of the streptococci in the presence of the phagocytes. Runs were considered valid only when both controls exhibited complete (or laked) hemolysis.

*Acid Extract.* Acid-extracted M protein was prepared by the method of Lancefield (1). The neutralized supernate containing M protein was filtered through a 0.22  $\mu$ m Millipore membrane and stored at 4°C in the presence of 0.02% sodium azide.

*Polyacrylamide Gel Electrophoresis.* All purification steps were monitored by electrophoresis in 7-30% gradient slab polyacrylamide gels in the presence of 0.1% SDS (sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis [SDS-PAGE]) by the method of Maizel (13) using a discontinuous buffer system. Electrophoresis was carried out at 50 V for 18 h. Gels were fixed and stained for 2 h at 40°C on a Gyrotory water bath shaker (Model no. G76, New Brunswick Scientific, New Brunswick, N. J.) in a solution of 7% acetic acid, 50% methanol containing 0.2% Coomassie Blue. All gels were destained at 40°C with rotation in a solution of 7% acetic acid 10% methanol containing 10 g each of Dowex 50W-X8 and 1-X8 to remove excess dye. Standards were run concurrently on all gels for molecular weight determinations. 10-50- $\mu$ l samples containing 10-20  $\mu$ g of M protein or standards, 1% 2-mercaptoethanol, 1% SDS, and 30% glycerol were placed in a boiling water bath for 2 min before loading on gels.

Samples containing <sup>125</sup>I-labeled M protein were run in a similar manner, except gels were dried as described by Maizel (13) and exposed to Kodak X-ray film (Blue brand, BB54) to determine the radiolabeled bands by radioautography.

*Protein Determination.* All protein determinations were by the method of Lowry et al. (14) using bovine serum albumin (BSA) as a standard. When determinations were performed in the presence of nonionic detergent, the BSA standard was prepared in the 1% detergent. The slight precipitate that formed after the addition of the phenol reagent was sedimented at 800 g before reading.

*Gel Chromatography.* Gel filtrations were performed on columns (1.5  $\times$  90 cm) equipped for reverse flow and run at a rate of 8 ml/h for Sephadex G-200 and 13 ml/h for Sephadex G-100. Both Sephadex G-100 and G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) were equilibrated with 0.1 M ammonium bicarbonate buffer pH 8.4 containing 0.02% sodium azide. Columns were calibrated by passing through them proteins of known molecular weights, and the distribution coefficient (*K<sub>d</sub>*) of each protein peak calculated as described by Andrews (15). Blue dextran 2,000 was used to determine the void volume and <sup>22</sup>Na for the total volume of the columns. Radioactivity was determined in a Packard model 3022 gamma spectrometer. Column fractions were tested for protein concentration by the method of Lowry et al. (14). Blue dextran was assayed by absorption at 650 nm. All volumes (approximately 1.5 ml/Fx) were determined gravimetrically.

*Antisera.* Type-specific antisera were prepared in rabbits by inoculation of whole heat-killed streptococcal vaccines (16). Unabsorbed antisera used were those that exhibited capillary precipitating activity with homologous M-protein extracts but which contained no reactivity to the group carbohydrate (16). Absorbed type-specific antisera were prepared as described by Rotta et al. (16) in which sera were absorbed with heterologous streptococcal M types to remove all cross-reactions with extracts from those types.

Human type 6 antiserum was from an individual who had high titer opsonic antibodies to type 6 streptococci. This individual had no history of rheumatic fever or nephritis.

*Agar Gel Diffusion.* Double diffusion was performed on standard microscope slides in 1% Noble agar buffered with 0.05 M sodium phosphate pH 7.0 in the presence of 0.02% sodium azide. Reactions were allowed to proceed at room temperature for 18 h. Slides were either photographed directly or washed, dried at 37°C, and stained (17).

*Competitive Inhibition of Radioactive Antigen Binding.* The antiphagocytic M 6 protein in Fraction 48 of the G-200 column was labeled with <sup>125</sup>I by the chloramine T method (18). A dilution of human or rabbit antiserum which could bind 50% of the <sup>125</sup>I-labeled antigen at 200 ng/ml concentration was used in the test. G-200 column fractions 48, 60, 70, 80, and 90 (See Fig. 3) were adjusted to 300  $\mu$ g/ml and serially diluted. 25  $\mu$ l of each dilution and 10  $\mu$ l of antiserum was added

to each microfuge tube (Beckman Instruments) along with 10  $\mu$ l of  $^{125}$ I-labeled antigen containing  $^{22}$ Na as a volume marker (19). The tubes were mixed and placed at 4°C for 18 h. At this time, 45  $\mu$ l of 80% saturated ammonium sulfate (saturated at room temperature) was added to each tube and placed at 4°C for 1 h to precipitate the immune complex. Tubes were centrifuged in a Microfuge (Beckman Instruments) for 5 min and approximately 70% of the supernate removed. The percent antigen bound was calculated as described by Gotschlich (19).

*Analytical Procedures.* Quantitative analysis of amino acids was determined on a Durrum amino acid analyzer Model D500. The samples for analysis were dialyzed in distilled water under nitrogen, dried, and hydrolyzed in 6 N HCl for 18 h at 110°C in evacuated sealed tubes. Methyl pentose was determined by the method of Dische and Shettles (20), total phosphorus by the method of Chen et al. (21), and sialic acid as described by Svennerholm (22).

*Radiolabeling Cell-Bound M Protein.* 50 ml of an overnight culture of streptococci in dialyzed Todd-Hewitt broth was centrifuged and resuspended in 50 ml of fresh dialyzed broth containing 0.1% trypsin (Worthington Biochemical Corp., no. 3707). Cells were allowed to grow for 2 h at 37°C. At this time, cells were chilled in an ice bath, centrifuged, and washed in cold PBS containing 0.05% trypsin, after which two additional washes were performed in fresh, cold PBS without trypsin. The trypsinized cells were then suspended in 50 ml of dialyzed Todd-Hewitt broth containing 20 mg of soy bean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo., type 1S, no. T9003) and incubated at 37°C. At timed intervals, a 10-ml aliquot of the cell suspension was removed, sedimented at 4°C, washed with cold PBS, and transferred to a 1.5-ml Microfuge tube, and recentrifuged in a Microfuge B to pellet the cells. The sedimented cells were resuspended in 100  $\mu$ l of 0.5 M phosphate buffer, pH 7.6, to which was added 10  $\mu$ l of  $^{125}$ I (1 mCi). 10  $\mu$ l of chloramine T (20 mg/ml) was added and the solution mixed rapidly. After 15 s, 50  $\mu$ l of sodium metabisulfite (10 mg/ml) was then added to stop the reaction. 10  $\mu$ l of potassium iodide (20 mg/ml) was then added and the cells washed five times in cold PBS to remove most of the free iodine. The sedimented cells were resuspended in 0.4 ml of 1% Emulphogene extracting solution (see above) and heat-killed at 56°C for 30 min. At this time, the tubes were rotated end-over-end at 8 rpm for 48 h at room temperature to extract the M protein from the cells. The cells were then sedimented in a Microfuge B centrifuge and the supernate passed over a Sephadex G-10 column (0.7  $\times$  30 cm, equilibrated in 0.1 M ammonium bicarbonate pH 8.4) to remove all the remaining free iodine. The radiolabeled material taken from the void volume of the column was diluted to equalize counts between samples and loaded on SDS polyacrylamide slab gels and electrophoresed. Gels were dried and exposed to Kodak X-ray film (BB 54) for radioautography (13).

Streptococci for pulse chase experiments were grown in 0.1% trypsin as described above (Radiolabeling Cell-Bound M Protein). After cells had been grown and washed free of trypsin, they were suspended in 50 ml dialyzed Todd-Hewitt broth containing 20 mg soy bean trypsin inhibitor and incubated at 37°C for 5 min to allow for the resynthesis of the M protein to begin. At this time, the cells were chilled in an ice bath and sedimented in the cold and washed twice in cold PBS. The pellet was suspended in 1 ml cold 0.5 M phosphate buffer pH 7.6 and transferred to a 1.5 ml Microfuge tube and recentrifuged to pellet the cells. The sedimented cells were labeled with  $^{125}$ I as described above (Radiolabeling Cell-Bound M Protein) and rapidly washed several times in cold PBS, allowing only 30 s for each centrifugation in the Microfuge B. Preliminary experiments revealed that the streptococci continued to grow normally if they were washed promptly after the short exposure to chloramine T. The labeled cells were suspended in 50 ml of warmed, dialyzed Todd-Hewitt broth containing 20 mg of trypsin inhibitor and incubated at 37°C. At timed intervals, 10-ml aliquots were removed, chilled in an ice bath, centrifuged at 4°C, and the cell pellet suspended in 0.4 ml of 1% Emulphogene BC-720 extracting buffer and heat killed at 56°C for 30 min. Extracting procedure and extracts were handled as described above.

*Sucrose Density Gradient Ultracentrifugation.* Sucrose density gradient ultracentrifugation was performed according to the method of Martin and Ames (23). Linear gradients of 5–20% (wt/vol) sucrose in 0.15 M NaCl and 0.05 M phosphate buffer, pH 7.1 were produced in  $9/16 \times 3.5$  inch cellulose nitrate tubes and the gradients stabilized at 4°C for 18 h. Samples (0.1 ml) were layered onto the 2.55-ml linear gradient and centrifuged in a SW 50.1 rotor at 50,000 rpm for 23 h at 4°C in a Beckman Model L5-65 ultracentrifuge. 0.1 ml of each reference protein as internal standards (BSA, soy bean trypsin inhibitor, ovalbumin, myoglobin) containing  $^{125}$ I-labeled M protein were run in separate tubes. In some tubes, radiolabeled M protein was run alone. Fractions of 10 drops

were collected and assayed for radioactivity. In those experiments in which internal standards were employed, the protein was determined by the method of Lowry et al. (14). The position of the peak of the M protein was not affected by the presence or absence of the internal standards.

### Results

*Extraction and Purification of Streptococcal M Protein.* Electron micrographs of M protein on the surface of Group A streptococci (24) suggested to us that the molecule may be composed of subunits held together by noncovalent interactions (25). Treatment of type 6 cell walls with various chaotropic agents such as SDS, Triton X-100, Igepal CA-630, and Emulphogene BC-720 revealed that protein could be removed in quantity from the cell walls with all these dissociating agents. It was found, however, that the nonionic detergents, Triton X-100, Igepal CA-630 (both alkyl-phenol detergents), and Emulphogene BC-720 (a fatty alcohol detergent) all removed protein from the cell walls which retained reactivity to both absorbed and unabsorbed type-specific antisera, even in the presence of the detergent. In our hands, Emulphogene BC-720 had certain advantages and was used throughout these studies.

As can be seen in Fig. 1 A, before extraction the surface of the cell walls of this type 6 streptococcal strain carried a large amount of the "hair-like" projections typical of M protein (24). After eight successive extractions with Emulphogene BC-720, electron micrographs (Fig. 1 B) reveal that these surface projections had been removed from the streptococcal cell walls, suggesting that the molecules that composed these projections were held together by noncovalent interactions. A similar picture was seen (Fig. 1 C) when cell walls were extracted by the classical acid extraction procedure (1); however, the integrity of the walls seems visibly affected by this method.

Immunodiffusion analysis of the detergent and acid-extracted material against absorbed type 6 antiserum revealed a single line of homology between these two preparations, indicating that both extracts contained immunologically identical antigens.

Since it was found (see below) that several proteins having type-specific precipitation activity were extracted from the streptococcal cell walls by the nonionic detergent, we decided to focus our attention on those molecules that also had antiphagocytic activity. Our working definition of this fraction of M protein is that protein or proteins with the ability to absorb type-specific opsonic antibodies from immune serum *in vitro*. This criterion was utilized as an assay for the presence of the antiphagocytic M-protein molecule during our purification procedures.

Molecular sieve chromatography on Sephadex G-200 of the CM cellulose eluted M protein (see Materials and Methods) revealed three major peaks (Fig. 2). When the proteins in the various fractions were tested for their ability to remove type-specific opsonic antibodies from human and rabbit immune serum (Table I), activity was found to be localized in a narrow area of the second peak (Fig. 2, peak B). Agar diffusion and capillary precipitation analysis using absorbed type 6 rabbit antiserum revealed, however, that type-specific reactive material was found to be present throughout this second peak and continued into the third (Fig. 2). SDS-PAGE (Fig. 2, top, and Fig. 3) indicated that the

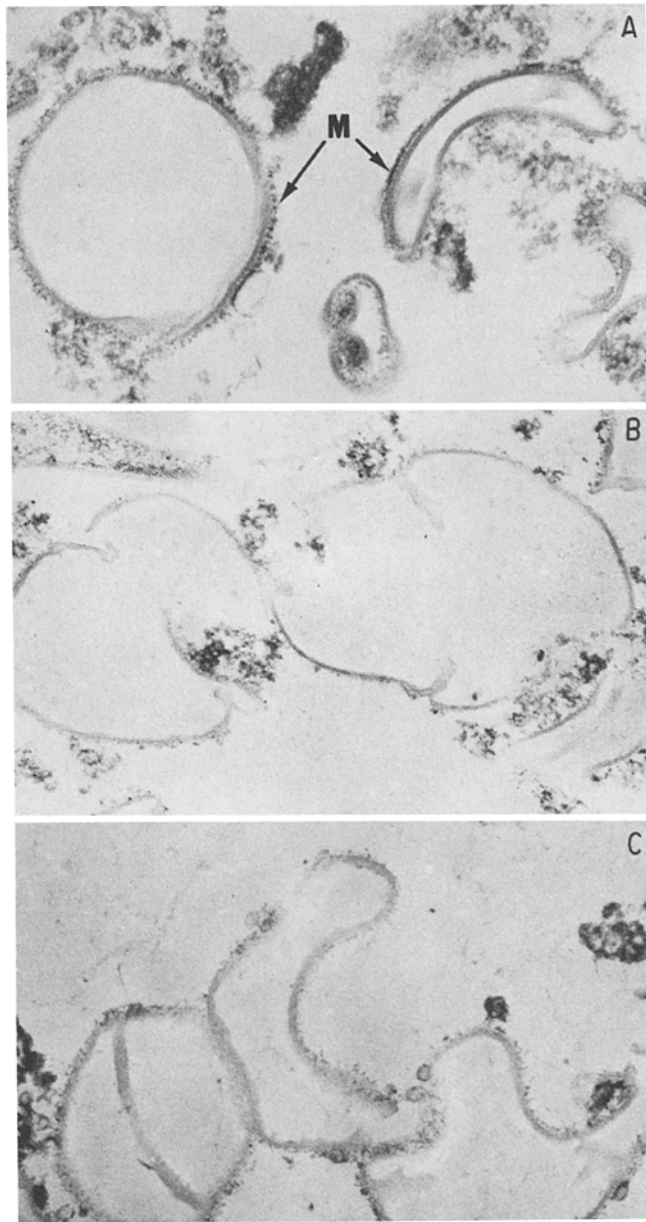


FIG. 1. Electron micrographs of Group A M 6 streptococcal cell walls before and after extraction with nonionic detergent or acid. (A) Before extraction with nonionic detergent, streptococcal cell walls exhibit on their surface the structures typical of M protein (M) (44). After extraction with Emulphogene BC-720 (B), the cell walls are seen to be devoid of these projections. After extraction of the M 6 walls by the acid extraction procedure (C), the M antigen is also removed from the cell walls. Magnification  $\times 70,000$ . (These electron micrographs were kindly taken by Dr. John Swanson, University of Utah.)

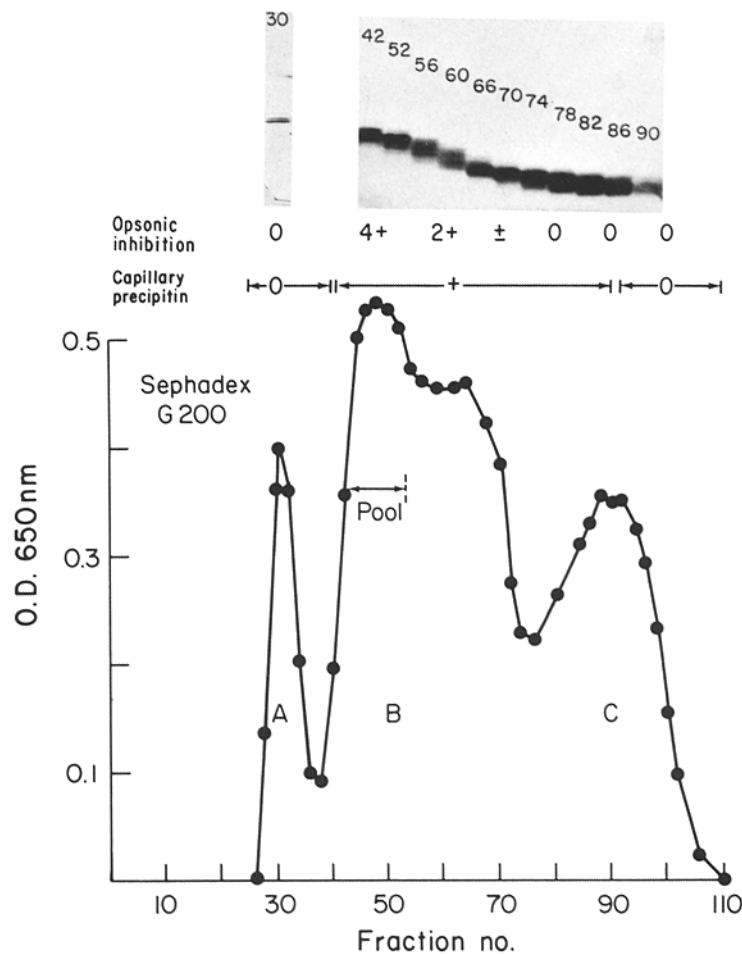


FIG. 2. Sephadex G-200 chromatograph of detergent extracted M 6 protein. Fractions were tested for ability to remove opsonic antibodies from human and rabbit immune serum; 4+ signifies complete removal of opsonic antibodies (see Table I). Fractions were also tested for capillary precipitin activity against absorbed type-specific M 6 antiserum. Top: SDS-PAGGE of representative fractions along the column where the numbers signify corresponding column fraction (see Fig. 3 for detailed enlargement).

various fractions were composed of multiple proteins which varied slightly in molecular weight (2-4,000 daltons between bands) and ranged from 35,000 daltons in Fraction 42 down to 6,000 daltons in Fraction 90. Those fractions having high opsonic inhibitory capacity (Fractions 42-56) were pooled, lyophilized, and rechromatographed on Sephadex G-100. As seen in Fig. 4, a single protein peak was isolated (B') reacting with absorbed type-specific antiserum. However, when the samples were tested for their ability to remove type-specific opsonic antibodies from human serum, the majority of the activity was localized in the leading edge and the peak of the G-100 column fractions. SDS-PAGGE of the various fractions indicated that the larger molecular weight protein bands had the opsonic inhibitory activity (Fig. 4, top). Those fractions with 4+ opsonic

TABLE I  
*Indirect Bactericidal Test: Absorption of M 6 Opsonic Antibodies from Human Serum\* with Sephadex G-200 Fractions†*

Fraction	No. colonies	
	Inoculum	
	200	94
	Bactericidal test	
CM§	Laked	Laked
Sepharose bead control	90	27
30	53	80
48	Laked¶	Laked
58	1,000	600
68	280	100
90	200	100

\* Similar results were obtained with rabbit immune serum.

† For details of procedure, see Materials and Methods section (Absorption of Opsonic Antibodies).

§ CM cellulose eluted M 6 protein before G-200 chromatography.

|| Activated Sepharose beads processed without M antigen bound to the surface.

¶ Opsonic inhibitory activity was scored as follows: 4+ = laked plate; 3+ = ~1,000 colonies; 2+ = 501-900 colonies; 1+ = 100-500 colonies; 0 = <100 colonies (refers to Fig. 2 and 4).

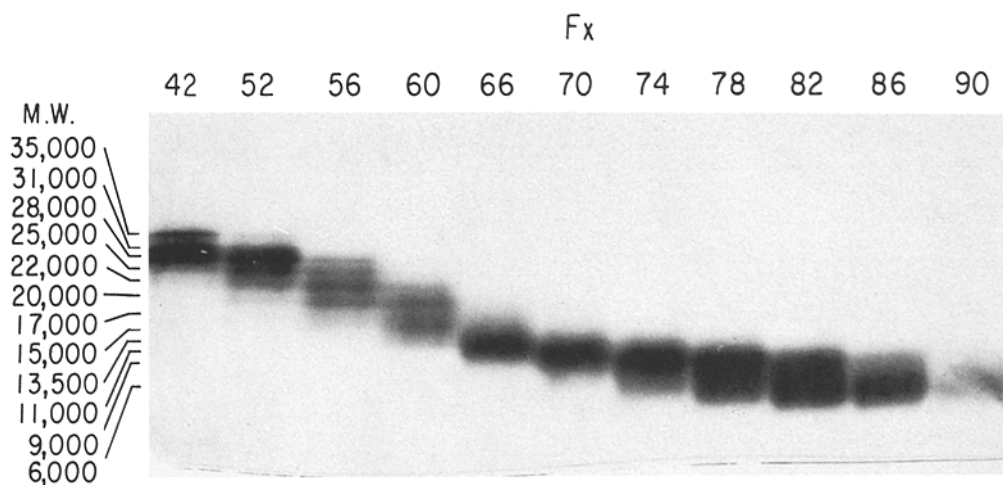


FIG. 3. SDS-PAGE of representative fractions from the Sephadex G-200 chromatogram of M 6 protein (same as Fig. 2, top). On the figure are indicated molecular weight estimates of the protein bands calculated from the ratio of the distance migrated by standard proteins run on the same slab gel and the distance migrated by the unknown bands. (M. W., molecular weight.)

inhibitory activity (Fractions 30-43) were pooled and utilized for further physical, chemical, and immunological studies.

Table II summarizes the results of the extraction and purification of the antiphagocytic M 6 protein from 8 g of isolated streptococcal cell walls. As can be



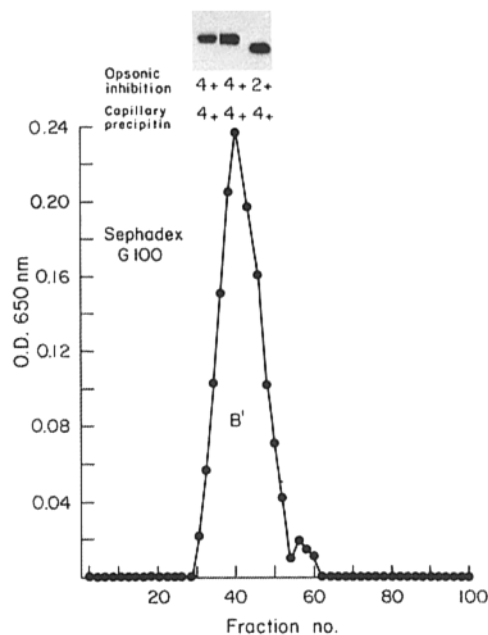


FIG. 4. Sephadex G-100 chromatogram of pooled antiphagocytic M 6 protein fractions of G-200 column (Fig. 2). Fractions were tested for ability to remove opsonic antibodies from human and rabbit serum, and 4+ signifies complete removal of these antibodies. Fractions were also tested for capillary precipitin activity against absorbed type-specific M 6 antiserum. Top is an SDS-PAGGE of representative fractions along the column: Fraction 35, left; Fraction 40, center; Fraction 48, right.

TABLE II  
*Purification of Antiphagocytic Streptococcal M Protein*

Step	Procedure	Volume	Protein		Yield	
					Protein	Wall*
		<i>ml</i>	<i>mg/ml</i>	<i>total mg</i>	<i>%</i>	
1.	1% detergent extract‡	800§	0.48	384	100	4.8
2.	CM cellulose chromatography	27	1.6	43.2	11.3	0.5
3.	Sephadex G-200 (Fractions 42-56)	29.7	0.4	11.9	3	0.2
4.	Sephadex G-100 (Fractions 30-43)	33.0	0.22	7.3	1.9	0.1

\* Based on the dry weight of the cell walls.

‡ From 8.0 g dry weight of M 6 cell walls.

§ Eight consecutive extracts of 100 ml each.

seen, this represents almost 2% of the extractable protein from the cell wall and is 0.1% of the total weight of the walls. The protein yield appears unusually low; this is due, however, to the large amount of Folin-reactive material not retained by the CM cellulose. This material was found to be composed of small peptides when analyzed on SDS-PAGGE.

*Chemical Analyses.* Purified M-protein preparations contained no detectable levels of methyl pentose, phosphorus, or sialic acid. Also, no amino sugars were found in these preparations during the amino acid analysis.

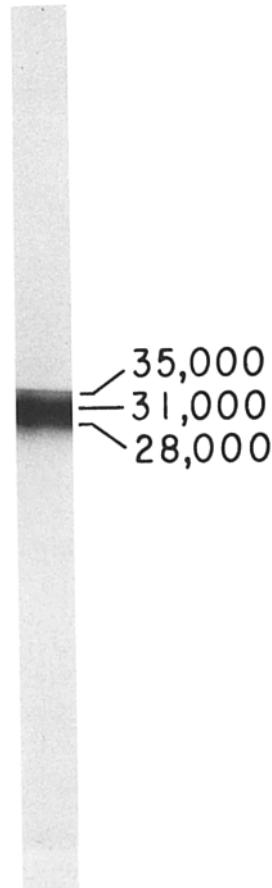


FIG. 5. SDS-PAGE of the antiphagocytic molecules of M 6 proteins. Fractions of the Sephadex G-100 column (Fig. 4) having 4+ absorption of opsonic M 6 antibodies were pooled and run on SDS-PAGE. This high resolution gel system indicates that the antiphagocytic M 6 antigen is composed of three protein molecules of 28,000, 31,000, and 35,000 daltons.

*Physical Properties of Purified Antiphagocytic M Protein.* Calibration of the Sephadex G-100 column with globular proteins of known molecular weight (BSA, ovalbumin, cytochrome *c*) indicated that the antiphagocytic M protein isolated from Peak B' had a mol wt of 88,000 daltons (26). Upon analysis by SDS-PAGE, we found, however, that this peak contained 3 closely spaced protein bands having mol wt of 28,000, 31,000, and 35,000 daltons (Fig. 5). The sedimentation coefficient of these molecules was estimated by sucrose gradient centrifugation (25). Results from six separate runs indicated that these antiphagocytic M antigens had an *S* value of  $1.68 \pm 0.07$  SD, suggesting a smaller molecule than that observed on the G-100 column.

Utilizing the *K<sub>d</sub>* values obtained for the reference proteins (BSA, ovalbumin, cytochrome *c*) by Sephadex G-100 chromatography, along with the known

Stokes radii for these proteins (27), the Stokes radius of the M protein was calculated to be 4.70 Å (27, 28). With this figure, using a partial specific volume ( $\bar{v}$ ) of 0.726<sup>2</sup> calculated from the results of the amino acid analysis (30) and the sedimentation value ( $S_{20,w}$ ) of 1.68, the average mol wt ( $M$ ) of the antiphagocytic M protein was calculated<sup>3</sup> to be 33,000 daltons (27). This agrees with the average mol wt of 31,300 daltons obtained by SDS-PAGE for these three molecules. Therefore, the mol wt obtained by direct analysis from the G-100 column (88,000 daltons) was misleading. This is reflected by the calculated<sup>4</sup> frictional ratio ( $f/f_0$ ) of 2.2 for this material (27), indicating that the molecules were actually asymmetrical and not globular.

Comparison of the SDS-PAGE patterns of the detergent-extracted M 6 and M 14 proteins chromatographed on the same G-200 column revealed an identical pattern of multiple bands distributed across the column. In addition, the anti-phagocytic as well as the type-specific activity of the type 14 proteins were distributed within the same fractions as the type 6 proteins (Fig. 2), indicating that the molecular size distribution of the proteins between these two M types was the same even though the antigenic configuration differed.

We were still concerned, however, that the multiple bands may have been an artifact of our methods. The possibility existed that these bands were actually due to different amounts of detergent molecules on the protein which caused the protein to appear as multisized bands on the SDS gel. To answer this question, radiolabeled nonionic detergent (Triton X-100) was prepared (31). The iodinated detergent was added to BSA and the mixture allowed to remain at room temperature for 2 h. SDS-PAGE of the <sup>125</sup>I-Triton-BSA mixture revealed that the radiolabeled band was in the exact position as the stained BSA band (31). These results demonstrate that even under the dissociating conditions of SDS, nonionic detergent remains bound to the protein molecule yet does not alter the position of the protein on the SDS gel, nor does it cause any multiple banding pattern of the BSA molecule. (Similar results were also obtained with ovalbumin and cytochrome *c*.) Therefore, based on these data along with the correlation of size between the M protein on the G-200 column and the SDS gel (i.e., the larger molecules on the G-200 column were the larger molecules on the SDS gel), we were quite confident that the multiple protein bands which were extracted were not due to an artifact of the bound detergent.

*Radiolabeling Cell-Bound M Protein.* Despite our gentle treatment of the M protein during its extraction and purification, the fact still remained that the antiphagocytic and type-specific molecules spanned a broad molecular weight range. Inasmuch as bound detergent molecules had been eliminated as a possible cause for these multiple bands, two possible mechanisms remained which

<sup>2</sup> This does not take into account the effect, if any, of bound detergent (29).

$$^3 M = (6\pi\mu aNS_{20,w})/(1-\bar{v}\rho), \quad [1]$$

where  $M$  = molecular weight;  $a$  = Stokes radius ( $4.7 \times 10^{-7}$ );  $S_{20,w}$  = sedimentation coefficient ( $1.68 \times 10^{-13}$ );  $\bar{v}$  = partial specific volume (0.726);  $\mu$  = viscosity of the medium ( $1.002 \times 10^{-2}$ );  $\rho$  = density of the medium (0.9988);  $N$  = Avogadro's number ( $6.02 \times 10^{23}$ ).

$$^4 \text{Frictional ratio } (f/f_0) = a/(3\bar{v}M/4\pi N)^{1/3}, \quad [2]$$

where  $M$  = 33,000 daltons from the results of Eq 1.

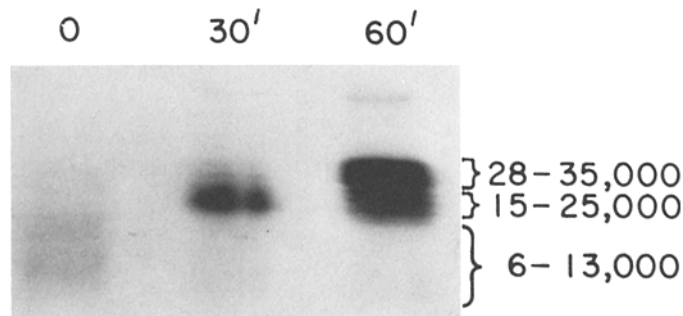


FIG. 6. Autoradiograph of SDS-PAGE of cell-bound M 6 protein radioiodinated on growing streptococci at timed intervals. Cells labeled immediately after trypsinization (0 time) exhibited only the small mol wt proteins on their surface (6-13,000 daltons). After 30 min of resynthesis, the 15-25,000 dalton proteins predominate; however, after 60 min, the 28-35,000 dalton proteins appeared.

could produce such an array of molecules: (a) The breakdown of a single large protein into smaller units during our methods of cell wall preparation, extraction, and purification or (b) the covalent polymerization of smaller subunits into larger, more complex molecules. To distinguish between these possibilities, we proceeded to radiolabel cell-bound M protein during its synthesis on the cell wall of living streptococci.

Actively growing streptococci were trypsinized to remove existing M protein and radiolabeled by the chloramine T method (18) at timed intervals during the resynthesis of the M antigen. The cells were then extracted with Emulphogene BC-720 and the extracts examined by SDS-PAGE. As can be seen from the radioautograph in Fig. 6 when the cells were labeled immediately after trypsinization, only a small amount of the lower mol wt (6-13,000 dalton) proteins were present on the wall. After 30 min of resynthesis, the 15-25,000 dalton proteins seem to predominate; however, 60 min after trypsinization, the 28-35,000 dalton proteins appeared. These results suggest two possibilities: (a) We were looking at incompletely synthesized as well as completed M antigens or (b) at M molecules after completion with subsequent cross-linking on the cell wall generating these multiple forms.

To determine which mechanism was actually at work, we decided to follow the development of these molecules, particularly the larger antiphagocytic moieties, by pulse labeling the M protein on the surface of living streptococci 5 min after trypsinization. The cells were then allowed to continue growth and the label followed during the remainder of the resynthesis of the M antigens. At timed intervals after labeling, cells were removed, heat killed, and the M protein extracted with detergent and analyzed by SDS-PAGE and radioautography. Fig. 7 illustrates that, at the time of labeling, after 5 min of resynthesis, only the smaller mol wt molecules (15-28,000 daltons) were present on the cell. However, after 45 and 75 min of resynthesis, the label had moved into the larger mol wt molecules (31,000 and 35,000 daltons), strongly suggesting that the smaller molecular weight molecules were used in the formation of the larger proteins. In support of these results, the following chemical and immunological tests were performed.

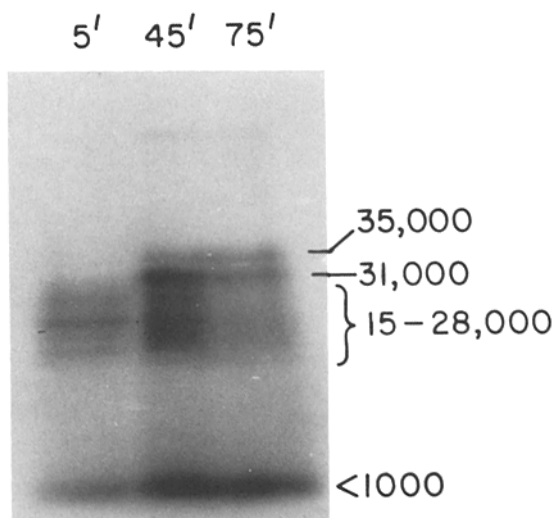


FIG. 7. Autoradiograph of SDS-PAGE of cell-bound M 6 protein pulse labeled with  $^{125}\text{I}$  on growing streptococci, and the label chased at timed intervals. At the time of labeling (5 min after resynthesis of the M protein), only the small mol wt 15-28,000 dalton proteins were present on the cell wall. After 45 min, the label was found in the 31,000 and 35,000 dalton proteins, which persisted at 75 min. Small mol wt peptides (<1,000 daltons) were found to be present at all time periods.

*Amino Acid Analysis.* The amino acid composition of the antiphagocytic molecules, listed in Table III, indicates that these molecules were rich in glutamic acid, lysine, threonine, and aspartic acid. They were found to be low in the aromatic amino acids and contained no cysteine. This composition was compared to that of the type-specific molecules (15-22,000 daltons) found in Fraction 60, the 11-13,000 dalton proteins in Fraction 70, as well as the 6-11,000 dalton molecules found in Fraction 82 of the G-200 column (see Fig. 3). The results of this comparison revealed that the 11-22,000 dalton M-protein molecules in both Fractions 60 and 70 had an almost identical amino acid composition to that of the 28-35,000 dalton antiphagocytic molecules, further supporting the idea that the smaller molecules were used to construct the larger antiphagocytic proteins. When the amino acid composition of the proteins in Fraction 82 were compared to the other three protein fractions, a lack of homology was observed with several of the amino acids. This may be explained by the fact that, even though the proteins in this fraction exhibited type-specific activity, they could be contaminated by some nonspecific peptides or other small proteins.

*Type Specificity and Its Relation to the Antiphagocytic Molecule.* The G-200 chromatogram (Fig. 2) revealed that type-specific and antiphagocytic activity depend upon the size of the M-protein molecules involved in the reaction. M protein of any molecular size was able to react with type-specific antiserum, whereas the antiphagocytic activity was limited only to the M protein of the higher molecular weight. As illustrated in Fig. 8, if type-specific rabbit antiserum was absorbed with either the large molecular weight antiphagocytic mole-

TABLE III  
Amino Acid Composition of M 6 Protein

Amino acids	Purified anti-phagocytic molecules*	G-200 column fractions†		
		60	70	82
	<i>nm/100 nM</i>	<i>nm/100 nM</i>		
Glu	20.40	20.85	20.67	21.54
Lys	17.59	16.72	17.14	14.20
Thr	15.13	14.69	14.86	11.82
Asp	14.51	15.57	14.46	12.53
Leu	8.69	9.31	9.12	8.13
Ala	6.80	6.83	6.58	8.03
Ilu	3.85	3.00	3.68	3.02
Gly	3.52	3.88	3.52	6.94
Ser	3.42	2.68	3.46	6.32
Val	2.73	2.62	2.49	4.02
Arg	1.96	2.04	1.98	1.60
Tyr	0.51	0.71	0.87	0.63
Met	0.51	0.47	0.51	0.40
Phe	0.37	0.55	0.59	0.49
His	0	0	0	0.25
Pro	0	0	0	0

\* Sephadex G-100 pool (Fractions 30-43, see Fig. 4).

† Type-specific proteins (See Fig. 2 and 3).

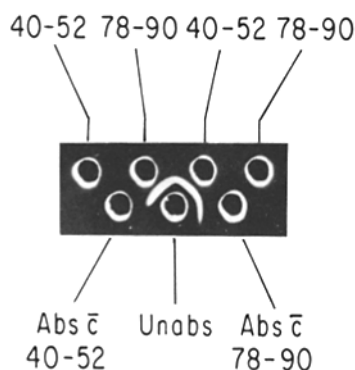


FIG. 8. Double diffusion in agar of pooled M 6 fractions from Sephadex G-200 column. Type-specific M 6 antiserum (unabsorbed) was absorbed with either the large molecular weight antiphagocytic proteins in Fractions 40-52 or the small molecular weight type-specific proteins in Fraction 78-90 (see Fig. 2 and 3). Before absorption, a line of homology is seen between both large and small molecules. After absorption with either molecule, reactivity is lost to both large and small molecular species. Reaction between unabsorbed serum and serum absorbed with Fraction 78-90 is due to antigen excess in the absorbed serum. The 40-52 absorbed serum contained no excess antigen.

cules (Fractions 40-52<sup>5</sup>) or the smaller type-specific proteins (Fractions 78-90<sup>5</sup>), type-specific precipitation activity was lost to both the large and small molecules, suggesting that the antigenic sites present on smaller type-specific mole-

<sup>5</sup> These pooled fractions refer to the G-200 chromatogram in Fig. 2.

TABLE IV  
*Removal of Type-Specific Precipitation Reaction with Type-Specific M 6 Protein\*  
 Without Altering the Opsonic Power of the Rabbit Serum*

Serum	Precipitation reaction with M 6 antigens‡		Indirect bactericidal test	
	Antiphago- cytic mole- cules	Type-spe- cific mole- cules	No. colonies	
			Inoculum	
			54	19
			Bactericidal test	
Normal rabbit serum	Neg	Neg	Laked	Laked
Rabbit opsonic serum	4+	4+	3	1
First absorption§	Trace	Neg	6	6
Second absorption	Neg	Neg	1	2

\* Sephadex G-200 Fraction 78-90 (type-specific molecules).

‡ From pooled fractions of G-200 chromatogram: antiphagocytic (Fractions 40-52); type specific (Fractions 78-90).

§ 1 ml of type 6 rabbit opsonic serum was absorbed at 4°C for 18 h with 120 µg of type-specific M protein bound to Sepharose.

|| 0.5-ml aliquot of serum after first absorption was reabsorbed with 60 µg protein under the same conditions as the first absorption.

cules were also present on the larger antiphagocytic proteins. However, even after multiple extractions of type-specific opsonic antiserum were performed with the small molecular weight type-specific molecules (Fractions 78-90) bound to activated Sepharose in order to remove all traces of type-specific precipitation activity, the opsonic power of the serum was not altered (Table IV). These results indicate that the opsonic antibody is, in all likelihood, nonprecipitating, possibly explaining why a spur was not observed in the immunodiffusion experiment illustrated in Fig. 8. These results also suggest that there are additional antigenic sites on the large antiphagocytic molecules which are not present on the smaller type-specific ones.

Further support for this idea comes from a competitive inhibition experiment in which Fraction 48 of the G-200 column (the antiphagocytic molecules) was radiolabeled and its binding to human opsonic antiserum inhibited with the smaller, type-specific proteins found in the subsequent fractions of the G-200 column, i.e., Fractions 60, 70, 80, and 90 (See Fig. 2 and 3). In Fig. 9, it can be seen that as little as 0.9 µg of unlabeled Fraction 48 (the homologous antigen) inhibited 100% of the binding. Serial dilutions of Fractions 60, 70, 80, and 90 were also able to inhibit the binding of radiolabeled Fraction 48, but to a lesser degree. Even Fraction 90, which has the smallest molecular weight, was able to inhibit 50% of the binding of Fraction 48.

Therefore, by two independent immunological methods, a similar conclusion can be drawn: that is, the smaller molecular weight molecules contain antigenic sites similar to the larger antiphagocytic proteins since they can both absorb or inhibit antibody reactivity to this larger species. However, these smaller molecules do not contain the antigenic determinants necessary to remove the opsonic

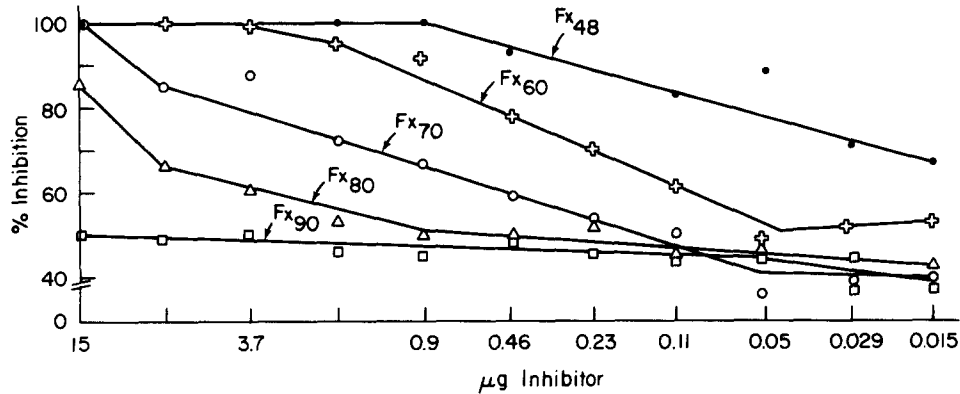


FIG. 9. The inhibition of the binding of  $^{125}\text{I}$ -labeled antiphagocytic M 6 protein (G-200 Fraction 48) to human immune serum by various fractions (60, 70, 80, and 90) of M 6 protein from the Sephadex G-200 chromatogram (see Fig. 2 and 3). Decreasing protein concentrations of each fraction, including the homologous fraction (Fraction 48), were used as inhibitors in the system.

antibodies present in the large molecule. These data, along with our physical and chemical studies, suggest that the smaller molecular weight type-specific proteins may be incompletely assembled molecules of the larger antiphagocytic proteins.

### Discussion

Our original observation that the large complex "hair-like" structure on the surface of M-positive Group A streptococcal cell walls could be removed with nonionic detergents indicated that these molecules were bound to the wall by noncovalent interactions (25). Since the extraction of protein molecules by nonionic detergents has been shown to be one of the gentlest methods of solubilization, usually not disturbing the immunological or biological activities of these proteins (32-34), it was likely that the M-protein molecules isolated by the method presented here were as close to their native configuration as possible.

Even though it has been observed by several investigators that acid extraction yielded multiple molecular forms of the M antigen which still had type-specific and/or antiphagocytic determinants (35-37), a coherent picture as to the structure of M protein has yet to emerge. One reason for this obscurity probably lies in the fact that M protein is a heterogeneous group of molecules made more heterogeneous by the acid-extraction procedure. In this regard, we have observed that acid extraction causes a good portion of the detergent-extracted M-protein molecules to be hydrolyzed into small fragments, a phenomenon also observed by Fox and Wittner (5) as well as Myoda et al. (37) in the acid treatment of alkaline-extracted M protein. This type of fragmentation is, in all likelihood, caused by random cleavage at acid-sensitive aspartic acid residues (38).

We feel that it is important to stress the significance of the SDS gel system used throughout these studies. In our earlier experiments, we employed the



Weber and Osborn continuous SDS gel system (39) to analyze our M-protein preparations. However, it was not until we shifted to a discontinuous buffer system (13) in slab gels that we realized that what we believed was a single broad band in the Weber and Osborn system (39) was actually composed of closely spaced proteins. Therefore, the resolution of the discontinuous slab gel system in conjunction with the ability to compare protein samples side by side on the same gel facilitated these studies.

The SDS-PAGE data indicated that detergent-extracted M 6 protein is composed of a group of protein molecules ranging from 35,000 down to 6,000 daltons, with only 2-4,000 daltons between each molecule. When these proteins were tested for their type-specific activity by precipitin reaction with absorbed M 6 antiserum, they all gave a positive reaction. However, the ability to remove opsonic antibodies from human or rabbit immune serum was limited to those molecules having a mol wt of 28-35,000 daltons (Fig. 2 and 3). Therefore, the antiphagocytic molecules, which also had type-specific activity, could be separated from those protein molecules which solely had type specificity.

The physical properties of the purified antiphagocytic molecules were determined by various parameters. Gel filtration data on Sephadex G-100 indicated that this M 6 protein had a mol wt of 88,000 daltons, which could lead one to believe that these 28-35,000 dalton molecules were aggregating (25). However, it was shown by Siegel and Monty (27) that the elution behavior of macromolecules on molecular sieves correlates with the Stokes radius of the molecule and not necessarily with its molecular weight.

Using experimental determinations for the Stokes radius and sedimentation coefficient as well as a partial specific volume calculated from the amino acid analysis (30), the molecular weight of the antiphagocytic M protein was calculated to be 33,000 daltons (Eq 1). Using these values in Eq 2, the frictional ratio of the antiphagocytic molecules was calculated to be 2.2, pointing to an asymmetrical protein configuration, which may offer an explanation for the discrepancy between the molecular weight determined directly on Sephadex and that found by SDS-PAGE.

In spite of the fact that these M antigens do contain a certain number of bound nonionic detergent molecules,<sup>6</sup> the 33,000 dalton figure determined by the parameters in Eq 1 was similar to the 31,300 dalton average mol wt estimated by SDS-PAGE. These data suggested that the bound nonionic detergent molecules do not play a major role in altering the physical configuration of the M antigens (31, 34). Therefore, we can assume that the individual proteins separated on the Sephadex columns were not aggregated as previously believed (25), but exist as discrete protein molecules.

We attempted to determine if these multiple proteins were due to the cleavage of a large molecule or were actually partially synthesized or incompletely assembled forms of the larger antiphagocytic proteins. We discovered, through surface iodination of newly synthesized M protein, that these antigens were found in multiple forms on the streptococcal cell wall. By labeling at intervals

<sup>6</sup> Experiments utilizing radiolabeled Triton X-100 (31) to extract M protein from streptococcal cell walls indicated that the M antigens isolated on SDS-PAGE contained bound radiolabeled detergent (unpublished data).

during the synthesis of these antigens, it was observed that the 15–25,000 dalton type-specific proteins were synthesized first, followed by the 28–35,000 dalton antiphagocytic molecules (Fig. 6). Therefore, these multiple proteins were not due to fragmentation during our extraction or purification methods.

Such a heterogeneous pattern could result if nascent M protein was prematurely released from the ribosomes at variable stages of synthesis or if a cross-linking mechanism existed which was operative after transport of a basic unit of the M antigen to the surface of the cell wall. Since the same molecules were also found on isolated cell walls, the latter explanation seems most probable. To distinguish between these possibilities, however, pulse chase experiments were carried out. We learned through these experiments that the label incorporated into the 15–28,000 dalton molecules soon appeared in the 31,000 and 35,000 dalton proteins (Fig. 7). These unexpected results were interpreted to mean that the smaller molecular weight, type-specific proteins were synthesized first and then cross-linked to other similar molecules in the assembly of the larger, antiphagocytic proteins. The results of the amino acid analysis support this view. Both the type-specific molecules in Fractions 60 and 70, as well as the larger antiphagocytic proteins, were nearly identical in composition and had minimal mol wt of 17,000 daltons. Since the amino acid analysis also reveals that these M-protein molecules were rich in lysine and glutamic acid but lacked cysteine residues, cross-linking may be accomplished by an  $\epsilon$ -( $\gamma$ -glutamyl) lysine linkage by means of a transglutaminase. This enzyme is widely distributed in animal tissues (40) and has been suggested to exist in bacterial cells (41). However, other types of cross-linkages also exist in nature which do not involve cysteine, but require lysine and/or glutamic acid residues (42, 43). Therefore, work is presently in progress to determine the exact nature of this cross-link. In addition, since the complete M-protein structure on the cell wall is considerably larger than the individual protein molecules isolated (24), the quaternary structure of these subunits on the streptococcal cell wall awaits further study.

Additional evidence that the small molecular weight type-specific molecules are a part of the larger antiphagocytic antigens is presented in the results of the double diffusion experiments with absorbed antiserum (Fig. 8). When type-specific opsonic antiserum was absorbed with either the small molecular weight type-specific or larger antiphagocytic antigens, type-specific precipitation activity was lost to both the large and small molecules. However, even after complete removal of type-specific precipitating antibodies from the serum with the type-specific molecules, the opsonic power of the serum was not altered (Table IV). These data led to the conclusion that certain antigenic sites present on the larger antiphagocytic molecules (which have the ability to remove opsonic antibodies) are not present on the smaller type-specific moieties. The results also indicated that the opsonic antibodies will bind to but will not form a precipitate with the antiphagocytic molecules. Similar results were presented by Cunningham and Beachey (44) using alkaline-extracted M protein. By isoelectric focusing, they isolated a protein molecule which was able to absorb opsonic antibodies from type-specific serum, but, unlike our antiphagocytic molecule, it did not have the ability to form a type-specific precipitation reaction.

The competitive inhibition studies further strengthened our contention that the smaller molecular weight type-specific molecules were a part of the larger antiphagocytic proteins. The data demonstrated that the type-specific proteins found in the various fractions of the G-200 column were able to inhibit binding of the radiolabeled antiphagocytic molecules to human opsonic serum (Fig. 9). Even the 6–13,000 dalton proteins found in Fraction 90 were able to inhibit 50% of the binding of the larger antigen. This "basic unit" concept has been alluded to in the work of Fox and Wittner (5) in which a comparison was made between alkaline-extracted M protein and acid-treated fractions. Despite the fact that the mechanisms for the alkaline extraction of M protein is unknown, their chemical and immunological data suggest that the large alkaline-extracted molecule may be composed of a smaller basic unit. A similar mechanism was also proposed by Johnson and Vosti (36) in which they suggest that the composition of two fragments of M protein isolated by acid extraction may be explained by a unit structure. Along these same lines, the results of our competitive inhibition experiments indicate that the size of this unit may be as small as 6,000 daltons.

The various experiments present here also support the view (8, 45, 46) that a specific site or sites on the antiphagocytic M molecule are responsible for binding type-specific opsonic antibodies, and that these sites may not be the same as those responsible for binding type-specific precipitating antibodies. Inasmuch as our data indicate that the antigenic sites able to remove opsonic antibodies exist on the larger antiphagocytic molecule and these molecules are formed from cross-links between the smaller type-specific proteins, it may be logical to assume that this covalent interaction may be involved in the formation of the antiphagocytic sites.

### Summary

Group A streptococcal M protein was extracted with nonionic detergent and subjected to a number of physical, chemical, and immunological tests. M protein thus extracted was composed of multiple protein bands, ranging from 35,000 down to 6,000 daltons, all having type-specific precipitating activity. The antiphagocytic proteins, however, were limited to three molecular species having mol wt of 28,000, 31,000, and 35,000 daltons, and could be separated from those proteins that had only type specificity. Physical studies indicated that these proteins existed as individual asymmetrical molecules which were not aggregated.

By radiolabeling M protein on living streptococci, it was determined that these protein bands were found on the streptococcal cell wall in this multiple form. Also, by pulse chase experiments supported by chemical and immunological data, evidence was obtained strongly suggesting that the smaller, type-specific molecules are used to assemble the larger, antiphagocytic proteins.

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