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Simplified Method for the Purification of Group A Streptococcal M-Proteins: Solution of the Multiple Banding Problem

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A simple and rapid procedure for the isolation in high yield (about a 30% recovery based on the total 30 to 60% ammonium sulfate recovery) of homogeneous purified group A streptococcal M-protein is described. M-proteins extracted from whole cells of group A streptococci by treatment with hot HCl were neutralized, fractionated with ammonium sulfate, dialyzed, lyophilized, and then subjected to treatment with hot 60% trichloroacetic acid. This was shown to produce an M-protein preparation, free of group A carbohydrate activity and extraneous antigens, in yields up to 10-fold higher than previous methods in about one-fifth the time. These M-protein preparations were shown to: (i) have similar amino acid compositions to their respective type-specific proteins purified by diethylaminoethyl and *O*-(carboxymethyl) cellulose chromatography, (ii) react with their respective type-specific antisera in Ouchterlony diffusion, (iii) produce antisera in rabbits capable of promoting streptococcal long-chain formation in vitro, and (iv) give only one major band on polyacrylamide gel disk electrophoresis. The data allow for an explanation of the hitherto described multiple banding M-proteins seen on acrylamide electrophoresis.

The biological importance of group A streptococcal M-proteins as phagocytosis-inhibiting factors has been well documented (9), but work relating this function to their structure has been hampered by the existence of over 60 types and the heterogeneity of the purified preparations (3, 14). Workers in this area have always regarded this heterogeneity as an indication of impurity and possibly responsible for the cross-reactions encountered among some strains.

In recent years, several procedures for the isolation and purification of group A streptococcal M-proteins have evolved (5, 7, 12). These procedures are all laborious, involving ammonium sulfate precipitation, fractionation by ion-exchange cellulose chromatography producing low (about 3 to 6%) products demonstrating multiple protein bands upon gel disk electrophoresis (5, 15). Efforts to obtain single molecular species, as demonstrated by disk electrophoresis, produce still lower yields (4). More importantly, these procedures may have been ignoring M-protein fractions only because they appeared heterogeneous.

Previously it has been shown that in M-protein preparations purified by ammonium sulfate precipitation and diethylaminoethyl (DEAE) and *O*-(carboxymethyl)cellulose (CM) fractionation, M-reactive proteins could be found, not in any one isolated area, but throughout the entire length of their gel electrophoresis (15). This agreed with the work of Fox (3) who referred to type 12 M-protein as a multiple molecular structure and with Lange (12) who theorized that type 12 M-protein belonged in all probability to a family of proteins and did not exist as a single molecular species.

A method that would allow for the rapid, high-yield isolation of these M-antigens in a highly purified form would have great advantages in the area of streptococcal typing as well as helping to elucidate the structure and immunochemistry of these proteins. It was the intent of this investigation to demonstrate a method of purification that would allow for the isolation of highly purified M-proteins to be achieved in a simple, rapid manner without the use of extensive fractionation. The M-protein preparations thus obtained are shown to be similar to their respective type-specific proteins (purified by much more difficult and time

consuming procedures), as seen by comparison of amino acid compositions, immunological activity, and ability to produce antisera capable of streptococcal long-chain formation. Furthermore, a possible explanation for the multiple banding rose from the presented data.

MATERIALS AND METHODS

Source of materials. The following strains of group A streptococci were employed in this experimental procedure: type 1 (T1/195/1P2) was from Abbott Laboratories; types 2, 4, 11, and 13 (1000S, R68/1115 Matt, 100068, and SS-31, respectively) were from W. R. Maxted; type 12 (Hektoen) was from the Hektoen Institute; type 29 (1759) was from E. V. Potter; and types 55 and 56 (2004 and SS-743, respectively) were from S. B. Rabinowitz.

Preparation of crude M-protein. Colony isolates of each type were mouse-passed to increased virulence. Cells were grown with continuous aeration (16) in a Biokulture Fermentor (Fermentation Design) in 10 liters of a culture medium composed of Todd-Hewitt broth fortified with 2% yeast extract. The cells were heat killed at 56 C for 30 min and collected by centrifugation. The cells were washed three times with distilled water and M-proteins were then extracted by two different techniques. The first technique employed the standard hot acid (HCl) extraction technique of Lancefield and Perlman (10). For complete M-protein removal the cells were extracted three times at pH 2, at 95 C, with a sample of each extract saved for analysis. The second procedure involved hot trichloroacetic acid instead of HCl. In this second procedure, 10 ml of 60% trichloroacetic acid was added to a test tube containing 6 g (wet weight) of group A streptococcal cells. The tube with its contents was placed in a boiling water bath for 20 min and then the tube was cooled immediately in an ice bath. After 15 min in the ice bath, the cells were sedimented by centrifugation at 4 C for 10 min at 4,000 rpm in an IEC Model HN-S centrifuge. The precipitate was discarded and the supernatant fluid represented the crude M-protein extract.

Purification of the M-proteins. The crude M-protein extracts obtained by hot acid (HCl) extraction were processed in either of two ways. In one procedure, initial purification of the combined HCl-extracted crude M-proteins was effected by ammonium sulfate to obtain the fraction precipitating between 30 and 60% saturation. After dialysis and lyophilization the protein was divided in half and subjected to two different procedures. In one, samples were dissolved in 60% trichloroacetic acid (6 ml acid/100 mg of lyophilized protein) and placed in a boiling water bath for 20 min. The other sample was dissolved in sodium phosphate buffer (pH 7.75, 0.01 M) and chromatographed in a stepwise batch elution process (buffers of increasing molarity and decreasing pH) on DEAE-cellulose (11). Fractions were collected at every interval of 1.0 unit pH decrease until a final pH of 4.0 was achieved; six fractions were collected. Each fraction was examined for group and type activity by immunodiffusion (15). Further purification was accomplished by rechromatographing in a stepwise elution procedure (buffers of increasing pH and constant molarity) on CM-cellulose. Fractions were collected at every interval of 0.5 pH unit increase until a final pH of 7.0 was achieved. Six fractions were collected and again each fraction was examined by immunodiffusion for group and type activity.

In the second procedure, involving M-protein in the combined HCl extracts, the lyophilized extract was subjected to hot trichloroacetic acid treatment. All acid-extracted M-proteins were extracted five times with ethyl ether (three volumes of ether per original volume of acid solution) and the precipitate was then dialyzed for 3 days versus distilled water changed twice daily, prior to lyophilization. Fractions were examined before and after acid treatment for group and type activity by Ouchterlony immunodiffusion.

Chemical determinations. Quantitative total amino acid analyses were performed on a Spinco Model 120 C amino acid analyzer as described by Spackman et al. (13) and as previously reported (12).

N-terminal amino acids were determined by use of fluorodinitrobenzene according to the procedure of Frankel-Conrat and Porter (6). The dinitrophenyl (DNP) reaction was performed on 5 mg of the purified protein preparations (12). Chromatographic determinations of the N-terminal amino acids were performed as previously described (15).

Immunochemical determinations. Immunodiffusion, acrylic gel (disk) electrophoresis, and immunoelectrophoretic studies were performed as previously described (12, 15). Each disk electrophoresis for band comparison was performed on 300 μg of protein. The gels were stained, after fixation for 30 min at 65 C in 12.5% trichloroacetic acid, with a 0.2% solution of the Procion dye, Coomassie brilliant blue (code no. 65-72-1; Colab; Glenwood, Ill.) for 30 min at 65 C. Destaining was performed for 20 min at 65 C with a solution composed of absolute ethanol, glacial acetic acid, and water (25:10:65). After several changes of destaining fluid, the gels were placed in 10% acetic acid at 65 C until destaining was complete.

Specific group A antisera and specific anti-M antisera were supplied by the Center for Diseases Control (CDC; Atlanta, Ga.). Specific anti-M antiserum were also prepared as previously reported (12) employing whole-cell vaccines as well as purified M-protein. The M-protein from each purified streptococcal isolate was also used as a booster immunogen on those animals receiving whole-cell vaccines, after an appropriate rest period for each rabbit. Thus, 6 mg (dry weight) of each M-protein, dissolved in 1.5 ml of saline, was incorporated into complete Freund adjuvant composed of two parts Marcol-52 (Humble Oil), one part Falba, and one part antigen solution in saline, 0.75 mg of *Mycobacterium tuberculosis* var. *hominis* strain H37Ra was added to this final adjuvant-antigen mixture. Each rabbit (NZW 2 kg) received a 1-ml injection (equivalent to one mg of M-protein) intramuscularly (IM) 1 month after the final injection of the whole-cell vaccine. Animals were then bled 1 week later to assay for anti-M activity and high-titered animals were exsanguinated to obtain the type-specific antisera. These sera were absorbed with

whole cells of all the different types available to remove cross-reacting activity and with trypsin-treated and pepsin-treated whole cells. The effects of trypsinization were traced by fluorescent antibody techniques to assess loss of M-protein. The cells that were pepsinized were similarly assayed to assess total loss of extracellular coat proteins.

The effectiveness of each type-specific antiserum prepared against the acid-precipitated M-proteins was determined by examining the capability of the antiserum to promote type-specific streptococcal long-chain formation in vitro. This was done according to the procedure of Stollerman and Ekstedt (14).

Fluorescent goat antirabbit IgG was prepared according to the procedure of Clark and Shepard (1).

RESULTS

Chemical and immunochemical data. Examination of the ultraviolet (UV) absorption spectra of all type-specific proteins precipitated by hot trichloroacetic acid indicated the lack of a significant absorption peak near 260 nm, indicating that this treatment had effectively removed all nucleic acid contamination. This shows that treatment of M-protein extracts with a 60% solution of hot acid alleviates the need to treat the protein with ribonuclease (RNAse). When RNAse was employed in other preparations, UV analysis showed these M-protein fractions also to be free of nucleic acids. Significantly, the RNAse added to the crude protein extract was eluted in the first fraction from the DEAE cellulose and was not detected in any of the CM-cellulose-purified fractions. This is consistent with an isoelectric point (pI) of 7.8 for RNAse.

Recoveries of the M-protein by the hot HCl and trichloroactic acid procedure ranged from a low of 22% for type 12 to a high of 33% for type 29, with an average in the range of 30%. These percentages are based on lyophilized recovery weights compared to the original lyophilized weight of the 30 to 60% ammonium sulfate fraction. Ouchterlony double diffusion analyses on all acid-precipitated M-proteins showed that no group A activity could be demonstrated in any sample. The failure to find group A antigen in any of the ether extracts indicated that the group A polysaccharide was completely eliminated from the protein preparation by the hot 60% acid treatment. Table 1 represents the compositions of three different type-specific M-proteins prepared in three separate ways. The one step that these three procedures have in common is precipitation with 60% hot acid. Analyses on the amino acid analyzer confirmed the absence of cell wall carbohydrates in the

TABLE 1. *Amino acid analyses of three different type-specific M-proteins obtained as described (in milligrams per 100 mg of protein)*

Amino acid	Type 1			Type 12			Type 29		
	Acid Ext.[a]	HCl Ext. acid Ppt.	HCl Ext. NH_4SO_4 Frac. acid Ppt.	Acid Ext.	HCl Ext. acid Ppt.	HCl Ext. NH_4SO_4 Frac. acid Ppt.	Acid Ext.	HCl Ext. acid Ppt.	HCl Ext. NH_4SO_4 Frac. acid Ppt.
Lysine	13.8	10.4	9.5	10.6	11.2	11.3	12.4	12.8	9.3
Histidine	2.5	1.8	1.7	1.0	2.3	3.0	2.0	1.6	0.8
Arginine	7.4	6.2	4.6	5.3	6.1	9.7	5.5	6.4	3.0
Aspartic acid	11.2	12.0	14.4	10.6	12.5	11.5	10.9	12.5	18.2
Threonine	5.1	5.8	5.4	5.6	6.3	5.4	5.0	5.6	5.8
Serine	3.6	4.6	4.5	3.7	4.4	3.4	3.4	5.3	5.2
Glutamic acid	15.2	16.3	19.0	14.7	15.4	16.9	14.6	18.8	20.9
Proline	3.4	5.1	3.0	4.0	3.9	2.6	4.5	3.1	5.0
Cystine ($^1/_2$)	0.4	0.4	0.3	—	—	—	0.6	0.6	0.3
Glycine	4.1	4.3	3.5	4.5	4.8	3.5	4.7	3.8	4.7
Alanine	8.5	8.0	8.8	10.4	5.4	7.7	10.4	7.5	7.5
Valine	5.7	6.8	6.1	7.4	7.1	6.2	7.0	5.5	4.5
Methionine	—[b]	—	—	2.2	0.9	0.7	0.4	—	—
Isoleucine	5.2	5.1	5.0	6.0	5.8	5.4	5.9	4.4	3.9
Leucine	6.7	7.6	8.6	7.2	7.2	7.5	6.8	7.9	8.4
Tyrosine	2.6	2.2	2.1	2.8	2.9	2.0	2.2	1.8	1.5
Phenylalanine	3.2	2.7	3.0	3.5	3.6	3.0	3.4	2.2	1.1
Glucosamine	1.2	0.6	—	0.6	0.5	—	0.6	0.5	—

[a] Ext., Extraction of whole heat-killed cells. Ppt., Precipitation of lyophilized protein by hot 60% Acid. Frac., Fraction of the crude protein extract by obtaining the fraction that precipitates between 30 and 60% ammonium sulfate saturation.

[b] Dash indicates recovery less than 0.05 mg/100 mg of protein.

proteins obtained by subjecting the 30 to 60% ammonium sulfate fraction to precipitation with trichloroacetic acid. Small amounts (0.5 to 1.2%) of glucosamine were found in the proteins prepared by acid extraction alone of the whole cells and by hot acid precipitation of the proteins extracted by HCl alone. The amino acid analyses of the type-specific proteins showed them to be quite similar in composition to their respective type-specific proteins which were purified by extensive cellulose chromatography. In each case, here as well as with types 2, 4, 11, 13, 55, and 56, the prevalent amino acids were glutamic acid, aspartic acid, lysine, leucine, and alanine, in decreasing order of concentration. These five amino acids generally comprised approximately 60% of the total amino acid content.

The results of the DEAE stepwise batch elution performed on the 30 to 60% ammonium-sulfate-saturated fractions showed (upon Ouchterlony analysis versus group- and type-specific antisera) that the group A activity was found in the first two fractions, whereas the M-protein activity could be found in all six fractions (Table 2). When these first two fractions were rechromatographed by a stepwise CM batch elution, Ouchterlony analysis versus group- and type-specific antisera indicated that the group activity and type activity were effectively separated, with the group A activity being found in fraction I, whereas the major M-protein peaks were found in fractions IV, V, and VI (Table 3). When the M-positive, A-negative fractions obtained from the prior DEAE and CM purifications were pooled and rechromatographed in a stepwise CM batch procedure, Ouchterlony analysis indicated that M-protein activity could be present in any of the final six fractions (Table 4). However, the major protein peaks

TABLE 2. *Batch DEAE Cellulose purification of 415 mg of type 12 M-protein*

Fraction	Starting pH	Buffer molarity	Eluted pH	Recovery (mg)[a]	Group A activity	M activity
I	7.75	0.01	7.52	132	2+	2+
II	7.00	0.02	7.32	18	1+	1+
III	6.00	0.03	6.50	9	–	1+
IV	5.00	0.05	5.80	10	–	2+
V	4.00	0.05	5.20	10	–	2+
VI	4.00	0.50	4.30	27	–	2+

[a] Total, 206; percent recovery, 50%.

TABLE 3. *Batch CM cellulose purification of fractions I and II from DEAE cellulose (150 mg of protein)*

Fraction	Starting pH	Buffer Molarity	Eluted pH	Recovery (mg)[a]	Group A activity	M activity
I	4.00	0.1	3.80	27	+	–
II	4.50	0.1	4.30	2	+	–
III	5.00	0.1	4.85	5	–	1+
IV	5.50	0.1	5.30	8	–	2+
V	6.00	0.1	6.00	26	–	3+
VI	7.00	0.1	6.80	8	–	2+

[a] Total is 75 and percent recovery is 28% based on the 415 mg employed initially (Table 2).

TABLE 4. *Batch CM cellulose purification of combined fractions III through VI from DEAE cellulose and fractions III and VI CM cellulose (100 mg total)*

Fraction	Starting pH	Buffer molarity	Eluted pH	Recovery (mg)[a]	Group A activity	M activity
I	4.00	0.1	3.90	7	–	1+
II	4.50	0.1	4.40	3	–	1+
III	5.00	0.1	4.90	2	–	1+
IV	5.50	0.1	5.25	4	–	2+
V	6.00	0.1	6.10	18	–	3+
VI	7.00	0.1	6.90	4.5	–	2+

[a] Total is 39 and percent recovery is 9.47 based on the 415 mg employed initially (Table 2); for fraction V alone the recovery is 4%.

were found to be eluted from the CM at a pH between 5.5 and 6.5. This corresponded to the pH range reported by Fox (3) and Lange et al. (12) working with CM-cellulose-purified proteins.

Each purified M-protein showed strong precipitin activity with its homologous antiserum prepared with either whole-cell vaccine or purified protein. Additionally, no cross-reactions could be detected with the other type antiserums. There were, however, considerable cross-reactions when the unadsorbed serums were allowed to react with a 30 to 60% ammonium sulfate fraction of a different type. These cross-reactions were removed after absorptions with different type cells, although a one-way faint line of cross-reaction between types 1 and 29 appeared that could not be removed by repeated whole-cell absorptions. Pepsinization was seen to cause a marked decrease in the amount of coat protein on the intact cell, as traced by the indirect fluorescent antibody technique. These pepsin-treated cells proved effective in adsorbing out any group A activity from the type-specific sera. Similarly, trypsin-treated whole cells, used to adsorb any group activity from homologous serums, did not remove the type-specific reactivity of the antisera.

Each acid-purified M-protein preparation showed strong precipitin activity against its homologous serum and no cross-reactions could be detected with the other type antisera. These acid-purified M-proteins also reacted strongly with their respective type-specific antisera obtained either from the CDC or the prepared homologous typing sera.

Table 5 represents the amino acid analyses and Fig. 1 represents the disk electrophoresis of the six fractions from the final CM purification performed on the type 12 protein. Although the data seemed to indicate a very strong similarity among the six fractions, it should be noted that the total basic amino acid composition increases from fraction I to fraction VI, whereas the corresponding total acidic amino acid composition decreases. This also affected the staining patterns of the disk electrophoresis as seen in Fig. 1 since Procion dyes are known to bind covalently and most actively to free amino groups (2).

These same fractions on immunoelectrophoresis and Ouchterlony analysis yielded critical results. Most important, regardless of the number of bands in the disk electrophoresis, only one line was obtained on Ouchterlony (Fig. 2). Fractions II and III gave the weakest lines on Ouchterlony and on immunoelectrophoresis. Fractions I, IV, V, and VI showed that M-reactive antigens could exist along the entire length of their disk electrophoretic gels, indicating the relatedness of all the bands. This was demonstrated in another fashion as seen in Fig. 3

TABLE 5. *Amino acid analyses on type 12 protein preparations obtained as described in Table 3 (in mg/100 mg of protein)*

Amino acid	Fraction					
	I	II	III	IV	V	VI
Lysine	9.1	10.3	10.1	12.6	14.2	13.2
Histidine	1.4	1.5	1.9	2.2	1.9	2.4
Arginine	3.7	3.3	3.8	4.6	6.3	6.3
Aspartic acid	12.6	13.8	13.6	13.0	11.3	11.0
Threonine	6.1	5.5	6.3	4.5	4.2	5.4
Serine	4.5	4.4	4.4	3.9	3.9	3.7
Glutamic acid	21.1	20.1	18.3	17.5	19.6	16.0
Proline	3.6	2.2	2.3	2.9	1.5	2.5
Cystine (½)						
Glycine	3.5	3.7	4.0	2.9	2.9	4.3
Alanine	8.7	10.1	7.6	6.8	8.3	7.6
Valine	4.5	4.4	4.9	4.9	4.8	6.6
Methionine	1.9	2.4	2.2	0.9	0.9	0.8
Isoleucine	4.4	4.3	5.6	4.9	4.5	5.6
Leucine	8.2	7.2	7.5	9.5	9.9	7.5
Tyrosine	1.8	1.9	2.3	2.6	2.0	1.5
Phenylalanine	2.7	3.5	3.6	3.1	2.8	3.7

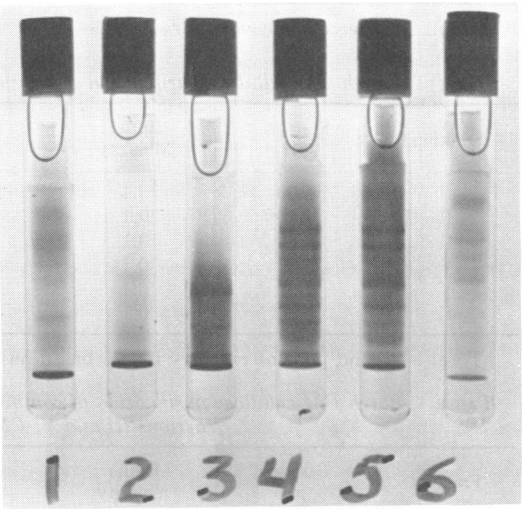

FIG. 1. *Disk electrophoretic studies performed on six M-12 protein preparations purified by DEAE and CM stepwise elution batch procedures. Each disk electrophoresis employed 300 μg of protein and was run at a pH of 8.3. The proteins were stained with the Procion dye Coomassie blue. The heavily concentrated protein areas seem to reflect an increase in the basic amino acid content (lysine and arginine).*

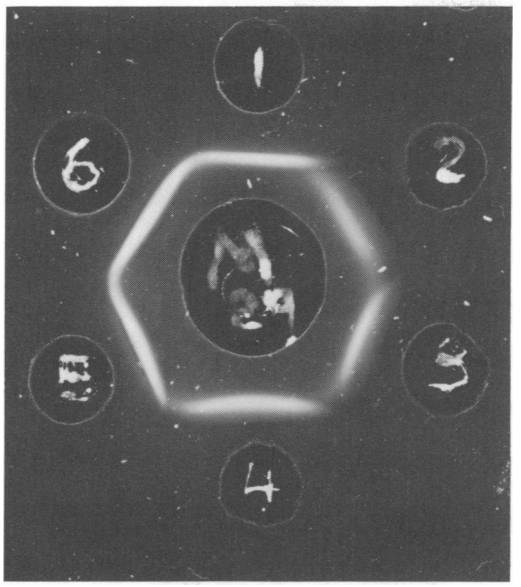

FIG. 2. *Ouchterlony analysis was performed on the six fractions from the final CM cellulose purification. Fractions II and III usually contained the lowest titer of M-proteins, whereas fractions V and VI were the highest. Regardless of the heterogenity of these fractions on acrylamide electrophoresis, only one precipitin line of identity was seen on the Ouchterlony diffusion plates.*

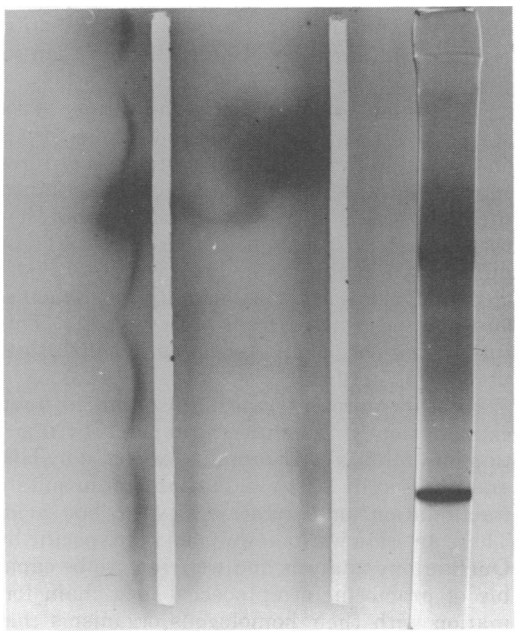

FIG. 3. *Gel disk electrophoretic separations were run in triplicate on M 12 proteins purified by DEAE and CM. The conditions were as in Fig. 1. One gel was sliced into 10 sections and aligned next to a trough containing the respective type-specific antisera. Another unstained gel was laid next to another trough containing the same antisera. The unstained gel was removed after 24 h and the respective stained gel was placed into position for photography. Both gels in this photograph were stained with Buffalo black.*

where fraction V of the type 12 M-protein was subjected to disk electrophoresis and fractions were isolated by transverse sectioning of the gels (50 mm in length) into 10 pieces. Each slice was aligned next to a trough and an unstained whole gel was also layered on the agar slide next to a trough containing the type-specific antisera. With type-specific antiserum, a single line of precipitation occurred, indicating that each of the bands was serologically identical. Gel disk electrophoretic studies and immunoelectrophoreses indicated that the purification of each type-specific protein followed a similar pattern, with the major M-protein peak eluting from the CM column at a pH range between 5.5 and 6.5. These data correspond to the multiple molecular structure of M-proteins as described by Fox (3).

The disk electrophoresis patterns of the samples of the three-time acid extraction preparations of the whole cells is shown in Fig. 4. There is a distinct change in the mobilities of the individual extracts as compared one to the other and to a mixture of a complete extract. These protein preparations showed decreasing concentrations of ammonia (amide N) on amino acid

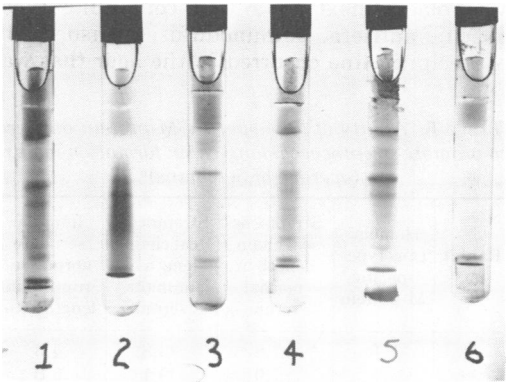

FIG. 4. *Disk electrophoresis performed on the three times hot acid-extracted M_4 protein. Gels 1, 2, and 3 are the three respective extracts. Each disk contains 300 μg of protein and was run at a pH of 8.3. Gel 4 is the combined extracts, gel 5 is the 30 to 60% ammonium sulfate fraction and gel 6 the hot acid-precipitated M protein. The gels were stained with Procion dye Coomassie blue.*

analysis. That is, each subsequent extraction mixture contained less amide nitrogen than its predecessor.

The amino acid analyses of the various types of M-protein which were purified by the DEAE and CM stepwise elution batch procedures showed that fraction V, in each case, contained the highest titer of M-protein activity and that these fractions were considered to be the most pure protein preparation. The five amino acids: glutamic acid, aspartic acid, lysine, leucine, and alanine, ranged from 50.2 to 66.2%. This concept has been reported in earlier investigations (3-5, 7, 12, 15).

Table 6 represents the data obtained upon examination of the antisera prepared by injection into rabbits of M-proteins extracted by HCl and purified by a 30 to 60% ammonium sulfate fractionation and treatment with hot acid. These antisera were shown to be type specific by Ouchterlony analysis and were seen to be capable of promoting streptococcal long-chain formation with their homologous organisms that averaged a 3.3- to 5.0-fold increase over nonimmune serum.

Figure 5 represents the acrylamide gel disk electrophoresis of three different M-proteins purified by a 30 to 60% ammonium sulfate fractionation and treatment with hot acid. Each gel shows only one major protein band with some trailing afterward. This band was shown to react strongly with its type-specific antisera as seen in Fig. 6, where the M 29 protein was subjected to disk electrophoresis in duplicate. One gel was stained (Fig. 5) and the other gel was placed next to a well containing type-specific antisera. Immunodiffusion ensued and a precipitin line occurred in the agar that was coincident to the major band in the stained gel.

TABLE 6. *Ability of type-specific M-protein antisera to promote streptococcal long-chain formation in vitro (average of 50 chains)*[a]

Rabbit no.	Homologous type-specific M-protein	Streptococcal chain length in normal serum	Streptococcal chain length in immune serum	Immune serum to normal serum chain length ratio
21	1	7.8	33.8	4.3
22	1	7.9	39.1	5.0
23	12	8.4	39.7	4.7
24	12	10.0	32.7	3.3
25	29	9.4	39.2	4.2
26	29	8.7	37.7	4.3

[a] The M proteins employed in the production of these antisera were obtained by HCl extraction of whole heat-killed cells with subsequent ammonium sulfate fractionation and hot acid precipitation.

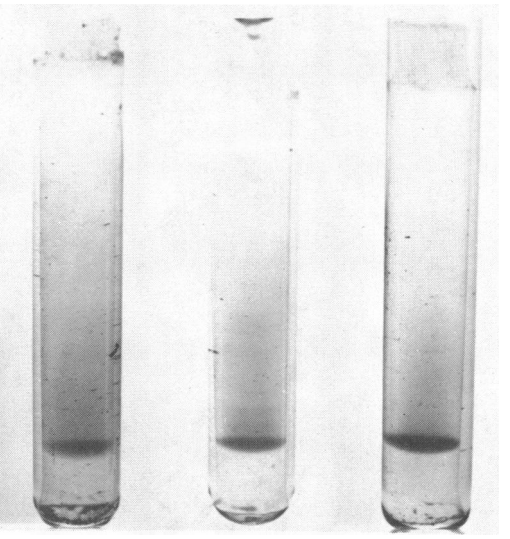

FIG. 5. *Disk electrophoretic studies performed on the three different type-specific M-proteins extracted from whole cells and purified by ammonium sulfate fractionation and 60% acid precipitation. Each disk electrophoresis employed 300 μg of protein and was run at a pH of 8.3. The proteins were stained with the Procion dye Coomassie blue. The gels represent M-proteins, types 1, 12, and 29 from left to right.*

End group analyses. The fluorodinitrobenzene reaction was performed as previously described (12, 15) on the nine different type-specific M-proteins purified by a 30 to 60% ammonium sulfate fractionation accompanied by hot acid precipitation. DNP-alanine proved to be the only detectable amino acid spot in all nine protein preparations.

DISCUSSION

A simplified method for a rapid purification, in high yield, of streptococcal M-proteins has been presented here. Comparing the trichloroacetic acid-prepared product obtained in 30% yields in 1 to 2 days time produced results more than favorable over the 4% yield of DEAE and CM-cellulose-purified fractions which involved at least 5 to 8 days time in preparation. The extensive comparative methods clearly established this procedure as a valuable new technique.

Three of the M-proteins (types 1, 12, and 29) employed here have been purified by extensive chromatography on DEAE and CM-celluloses. These same three M-proteins along with six others were purified by employing hot 60% acid and gave preparations which were quite similar to the cellulose-purified proteins in several

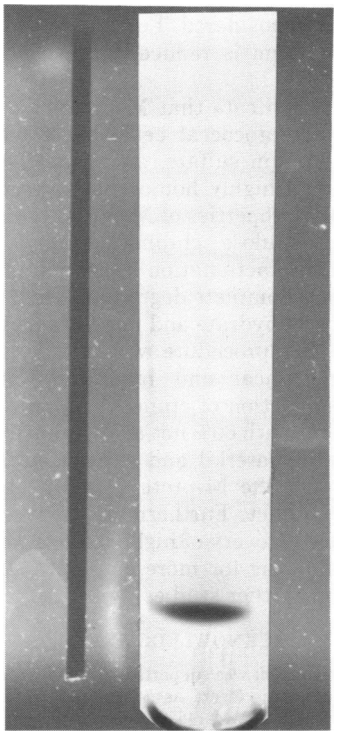

FIG. 6. *A disk electrophoretic separation was run in duplicate on the M_{29} protein represented in Fig. 5. One gel was stained (Fig. 5) and one was layered on an agar-coated slide next to a trough containing the respective type-specific antisera. The figure is a composite where after diffusion had occurred (24 h) the unstained gel was removed and the respective stained gel was placed in position for photography.*

respects. The amino acid analyses of the M-proteins extracted by HCl, fractionated by ammonium sulfate, and precipitated by acid showed them to be free of cell wall material and to be comprised mostly of glutamic acid, aspartic acid, lysine, leucine, and alanine (Table 1). These same five amino acids have already been reported elsewhere as the major constituents of cellulose-purified M-proteins (3, 12, 15, 17) as well as in the present study. Glutamic acid was also seen to be the most prevalent amino acid in all cases and this is also consistent with preexisting data.

Ouchterlony analyses have also given evidence that the protein fractions whose purifications have been described here represent highly purified M-protein preparations. Ribonucleic acid (RNA) has been shown to be eliminated by hot acid treatment. No group A carbohydrate could be detected in any of the acid-precipitated proteins at any concentration. Although the acid-extracted material and the acid-treated HCl-extracted material showed glucosamine to be present, this must be a non-group A carbohydrate since this concentration would be detectable with the grouping sera. No explanation for this material can be made at this time other than possible nonspecific adsorption of free glucosamine to the M-protein throughout the purification steps. Each type-specific protein preparation, however, gave strong precipitin reactivity with its homologous type-specific antisera regardless of its source. These M-proteins reacted with their respective type-specific sera supplied by CDC (whole cell injections), or prepared by injection of purified M-proteins, or vaccines. No evidence of cross-reactions was noted among seven of the nine types investigated, with only a weak one-way cross-reaction between type 1 M-protein and type 29 antisera. A similar observation was also noted by Wiley and Bruno (18) for type 49 antiserum and type 43 cocci.

Injection of the M-proteins purified by acid precipitation into rabbits resulted in production of an antiserum which was capable of promoting streptococcal long-chain formation in vitro (Table 6). The length of the group A streptococcal chains were seen to increase from 3.3 to 5.0 times in the presence of these antisera as opposed to the chain length where they were allowed to grow in the presence of normal rabbit serum. These data indicate that these protein preparations do indeed contain M-protein. In the work by Stollerman and Ekstedt (14) involving the formation of long chains of group A streptococci in homologous antisera, they showed that their antisera were capable of promoting long chains which were 10 to 15 times as long as chains formed in the presence of normal sera. We were not able to attain that high figure of chain length increase with our sera. This is, in all probability, due to the fact that our antisera were produced against purified M-protein, whereas the sera that Stollerman and Ekstedt (14) employed was produced against whole cells. Köhler and Kühnlemund (8) recently reported that chain length ratios of 2 or greater are indicative of anti-M antibodies and compare well with data for bactericidal and mouse protection tests.

Another factor which indicates that these acid-precipitated proteins represent M-proteins is the fact that L-alanine proved to be the N-terminal amino acid in all cases. This agrees with previous reports (15, 17) and further supports the claim that all M-proteins have alanine as their N-terminal amino acid (10).

These acid-precipitated proteins do seem to differ from cellulose-purified proteins in two

important aspects: a high yield of a single banding material on electrophoresis as compared to a low yield of multiple banding material.

The banding pattern of these proteins after exposure to hot 60% acid is altered dramatically as seen by a vast reduction in the number of bands upon polyacrylamide gel disk electrophoresis (cf. Fig. 1, 4, and 5). The type-specific M-proteins M_1, M_{12}, and M_{29} were shown to give only one major band but M_{12} did show some trailing behind that band (Fig. 5). All of these bands were shown to be capable of precipitin reaction with their type-specific antisera as seen in Fig. 6.

An explanation for the reduction in banding of the M-proteins after hot-acid treatment is that treatment of the protein with hot HCl for 10 min converts only a portion of the asparagine and glutamine side chains in the protein to aspartic acid and glutamic acid while leaving the other amide linkages intact. This accounts for the multiple banding as seen in most crude M-protein extracts, as well as in the more highly purified protein preparations. However, with the hot-acid treatment, which involves a stronger acid for a longer period of time, all or almost all of the asparginines and glutamines are converted to their acid forms, causing all the M-proteins to have a similar migration in disk electrophoresis. Evidence supporting this explanation is demonstrated in Fig. 4, where the patterns of the first, second, third, and pooled extracts to that of the acid-heated M-proteins are compared. Indeed, even the amino acid analysis data further supports this conclusion, since each subsequent extract was shown to contain less recoverable ammonia than the preceeding fractions. At the same time the amino acid recoveries were not markedly different in the respective preparations. It was concluded from these data that amide nitrogen was lost from the M-proteins due to the acidic conditions, thereby effecting a net increase in the charge on the residual protein. Secondly, there is a marked difference in the amount of protein recovered as well as the ease of recovery. With the DEAE and CM cellulose procedure, fraction V (Table 4) represented a 4% recovery, whereas the total (fractions I through VI) corresponded to a 10% recovery. With the present rapid trichloroacetic acid procedures, recoveries were in the range of 30% recovery for a single homogeneous band on electrophoresis. This represents approximately a 10-fold increase of a single, homogeneous M-protein and even a threefold increase if all of the different M-proteins are considered. Furthermore, the time to prepare them is reduced approximately by 80%.

The data indicate that M-proteins extracted from the streptococcal cell wall, fractionated with ammonium sulfate, and heated with hot acid, yields a highly homogeneous preparation that has the properties of M-protein purified by extensive cellulose chromatography. Ammonium sulfate fractionation appears to be necessary to allow complete degradation and removal of group carbohydrate and nucleic acid contamination. This procedure would, therefore, be ideal for clinical and laboratory situations wherein subjection of crude M-protein extracts to treatment with 60% hot acid seems to destroy all cell wall material and extraneous antigens while leaving the M-protein and its immunospecificity intact. Furthermore, this procedure leads to the recovery of high yields of M-protein thereby allowing for more elaborate structural analyses in further studies.

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