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IMMUNOCHEMISTRY OF GROUP A STREPTOCOCCAL M PROTEINS

BY

David C. Straus

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy

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LIST OF ABBREVIATIONS

1.	PMN	Polymorphonuclearcytes
2.	FPF	Fibrinogen precipitating factor
3.	DEAE cellulose	Diethylaminoethyl cellulose
4.	CM (CMC) cellulose	Carboxymethyl cellulose
5.	HCl	Hydrochloric acid
6.	PHA	Passive hemagglutination
7.	AGN	Acute glomerulonephritis
8.	g/liter	Grams per liter
9.	g	The force of gravity
10.	rpm	Revolutions per minute
11.	NaOH	Sodium hydroxide
12.	N	Normal
13.	M	Molar
14.	mm	Millimeters
15.	U.V.	Ultraviolet
16.	nm	10^{-9} meters
17.	µgram	10^{-6} grams
18.	T.L.C.	Thin-layer chromatography
19.	FDNB	Fluorodinitrobenzene

20.	DNP	Dinitrophenyl
21.	KOH	Potassium hydroxide
22.	mg	Milligram
23.	ml	Milliliter
24.	cm	Centimeter
25.	SDS	Sodium dodecyl sulfate (sodium lauryl sulfate)
26.	TEMED	N:N:N':N'-tetramethyl-1:2-diamino-ethane
27.	mA	Milliamps
28.	µl	10^{-6} liters
29.	Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
30.	NaCl	Sodium chloride
31.	Vt	Total volume
32.	Vo	Void volume
33.	Ve	Elution volume
34.	RE	Rabbit erythrocytes
35.	PRE	Pyruvic aldehyde treated rabbit erythrocytes
36.	FPRE	Formaldehyde pyruvic aldehyde treated rabbit erythrocytes
37.	M_n-FPRE	M_n-protein coated formaldehyde pyruvic aldehyde treated rabbit erythrocytes (where n = type number)
38.	MBA	N,N-methylene-bis-acrylamide

39.	meq	Milliequivalents
40.	BBS	Borate buffered saline
41.	IEP	Immunoelectrophoresis
42.	IgG	Immunoglobulin G
43.	HNB	2-hydroxy-5-nitrobenzyl bromide
44.	NTSM	Non-type-specific-M-region
45.	TSM	Type-specific-M-region

I. INTRODUCTION

The mechanisms of cell and tissue injury induced by group A streptococci, both suppurative and nonsuppurative, have been extensively examined (31,32). There are still, of course, many aspects of these fastidious organisms that are still a puzzle to the microbiologist. The common suppurative streptococcal infections have, for the most part, been readily controlled following the development of effective antibiotics. In contrast, important nonsuppurative streptococcal infections, such as rheumatic fever and nephritis, have been growing in importance and have made study of the virulence factors of the group A streptococci a must, from the clinical as well as from the research point of view.

M protein of group A streptococci, one of the organism's many surface antigens (33,120), has long been recognized as the most important virulence factor of the bacterium (6,17,33,55,57,105). It is a phagocytosis-inhibitor, and its absence or loss from the cell wall of the microorganism, greatly reduces the pathogenicity of the bacterium as well as its ability to survive in normal mammalian blood. The M protein has, for many years, also been the basis for the serological typing of the group A streptococci. Purified M protein preparations have been shown to cause clumping

of platelets and leukocytes (5). M protein isolates also lysed polymorphonuclearcytes (PMN's) and retarded the migration of PMN's in in vitro systems. Similar toxic effects have been seen as a result of a complex of M protein and antibody to M protein. In 1959, a fibrinogen-precipitating factor (FPF) of Streptococcus pyogenes was described (49). The identity of FPF with purified M protein was later proposed (47,48) and expounded upon (43).

Since its original description by Lancefield in 1928 (57), M proteins have been of considerable interest to bacteriologists everywhere. The localization of the M protein on the streptococcal cell has been studied in the whole organism as well as in isolated cell walls. These studies have shown that the M protein is indeed bound to the cell wall (2,29,30,46,85,113). Further evidence for the localization of the M protein on the cell wall is found in the observation that some proteolytic enzymes such as trypsin and chymotrypsin, remove the M antigen from living and dead cells (58.62). Trypsin digestion has also been shown to destroy the immunospecificity of the M proteins when they are in a purified state (26).

Although the M proteins are located on the streptococcal cell wall, and are probably linked to underlying cell wall structures by primary chemical bonds (33,104), they are also produced by group A streptococcal protoplasts or L-forms which are completely without

cell walls. In L-form cultures, M proteins can be found in the medium, indicating that although these proteins are normally found fixed to the cell wall, this is not the site of their synthesis.

The removal of the M antigen from the streptococcal cell wall and its subsequent purification have always been an area of intense debate for workers in this field. By far, the most commonly used method for the extraction of M proteins has been acid hydrolysis (57). Acid extracted M proteins, however, have been reported to elicit poor precipitating antibody responses. Whole cells are often used for immunization of animals to obtain a potent precipitating antiserum to M protein, but absorption with heterologous organisms to remove nonspecific cross-reactions generally results in an extremely low yield of the desired specific antibody. It has been proposed that the harsh acid treatment used for its extraction might impair the integrity of the M proteins released. Therefore, various other procedures have been applied to this problem and have met with varying degrees of success. For example, various workers have employed alkaline buffers for extraction at a temperature of 37 C to avoid the harsh treatment of acid hydrolysis (38). However, alkaline buffer extraction at 37 C yielded only 10% to 15% of the amount of M protein obtained by hot acid extraction; a considerable amount of M protein was found to remain on the cell walls following these ex-

tractions (26). Other investigators have proposed that by using physical rather than chemical methods to release M proteins, a better immunogen could be isolated. Sonic oscillation has been the physical method employed by some of these workers for affecting the release of streptococcal cell wall antigens (7,10,76,95). While this procedure of sonic oscillation appears to yield a higher molecular weight M protein preparation, it has been criticized because of the possible local development of high temperatures during the process. Another method has been developed which involves extraction by the enzymatic action of a phage associated lysin which has yielded satisfactory preparations of M proteins (50,56,73). This procedure is less harsh than others, and may provide M antigens in a form that is more comparable to the "native" M protein, as it is found on the streptococcal cell wall.

Streptococcal M proteins have consistently been difficult to purify. Various procedures including fractional precipitation with ammonium sulfate solutions, chromatography with diethylaminoethyl-Sephadex (DEAE-Sephadex) (4), carboxymethyl cellulose (CM) (18,20, 21,,23,25,26,27,40,101), hydroxyapatite columns (45,109), both DEAE and CM (66,103) celluloses, and precipitation with acid (78, 103) have been employed in M antigen purification. From the purified preparations so far obtained by any of these methods, the fol-

lowing general characteristics of the M protein have been determined: it is antigenic (18,24), and it belongs to the class of acid stable proteins (105); it is resistant to boiling for a short period of time in an extremely acid environment (105), and it is destroyed by the proteolytic enzymes trypsin and chymotrypsin (58,102). An isoelectric range of about 5.3 to 5.7 has been established for M antigens of several different serologigal types (64).

The role of the M proteins in the disease process is only partly identified. It does not seem to be primarily toxic for normal animals (36,42,50,51,67,74,88,94,97). M protein isolates have been injected into normal children and adults by the usual parenteral routes without evidence of harmful effects (21,22,89). On the other hand, adrenalectomized rats, when injected with sonically disrupted streptococci containing M protein, showed toxic reactions to the antigen (94). M proteins of group A streptococci released from the cell by sonic oscillation were toxic to these rats in quantities of 1 mg./100 gram rat. Death usually occurred within two hours. However, in this experiment, one of the toxic factors for the abnormal experimental animals was associated with the protein fraction, from which M antigen was prepared by sonic oscillation, but it was not present in M proteins released by HCl at 100 C. In man, immunizing injections have been done subcutaneously or intramuscularly. There

are conflicting reports as to whether M proteins are capable of causing some kind of disease in man. Schmidt found them to be usually harmless in children and military personnel (88,89), as did other workers (83). A review of the literature examining the prospects of vaccination of humans described the formation of cold abcesses in patients injected with purified M protein isolate (98), and these authors suggested that the skin reactions observed were comparable to those induced by endotoxins of gram-negative bacteria. The problem of the purity of M protein preparations and their possible contamination with cross-reactive antigens of cardiac tissue raised serious questions about the safety of such vaccinations for use in humans (52,98). However, recent studies have shown that purified M protein used to vaccinate children did not contain any antigens in common with the heart tissue (20). Skin testing with a partially purified M protein has been used by Lawrence in the studies of cellular transfer of streptococcal reactivity (67). From the results reported, it was concluded that M proteins injected intradermally were nontoxic in humans who had not been previously exposed to these antigens. Recently, however, it was reported that three cases of rheumatic fever appeared among 21 siblings of rheumatic patients after vaccination with a type specific group A streptococcal M protein preparation (71). This rate is highly significant in contract with the

observation that only five cases of rheumatic fever were seen in 447 similar streptococcal infections in nonvaccinated siblings. Although it was suggested that the streptococcal vaccination could have altered the tissue responses of the children to subsequent streptococcal infections, no information on the degree of purity of the vaccinating antigen was given. Therefore, it could not be known whether the M protein employed contained extraneous cross-reactive antigens or other toxic materials that could have predisposed the three children to rheumatic fever.

In the early studies of M antigens, these proteins have been shown to be serologically active in precipitin tests, although it was commonly thought that these proteins were haptens capable of inducing antibody formation only in their native state on the bacterial cell wall. Subsequently however, Stamp and Hendry succeeded in preparing a solution of a type specific M protein, which when injected into mice, was shown to protect the mice against the homologous virulent strain (97). Hirst and Lancefield confirmed this report on the group A streptococcus type 3 organism and expanded it to other types (42). The preparation of M proteins has improved tremendously since Lancefield's work in 1939, so that now recent workers have obtained high titered antisera in rabbits immunized with as little as 1.0 mg of M protein incorporated either in saline or Freund's adjuvant (18, 24,38,45,50,66,103,109).

The methods for evaluating group A streptococcal type specific antibodies in man are almost as varied as the many procedures used to purify the type specific proteins. Precipitin and agglutination reactions are generally considered unsatisfactory for the demonstration of type specific antibodies in man. One of the best tests for the demonstration of protective antibodies in man is the bactericidal test (41,61,72). This test depends on the phagocytosis and destruction of streptococci by human phagocytes, after opsonization by type-specific anti-M antibodies. This procedure is difficult, time consuming and often inconclusive. Stollerman and Ekstedt have detected anti-M antibodies by studying streptococcal long-chain formation with 4-hour cultures grown in vitro in the sera of patients following streptococcal infections. Their results were shown to be consistent with the determination of protective antibody by the usual bactericidal tests (100). Kohler (54) has shown that the long-chain test is a reliable and reproducible method for the detection of protective M protein antibody in experimental animals. His data show that long-chain indices above 2.0 indicate the presence of protective antibody. Various other procedures used to demonstrate anti-M protein antibody are passive hemagglutination (PHA) (111), agglutination of latex particles (75), mouse protection tests (18), complement fixation (8), and a micro-complement fixation assay (121). The time fol-

lowing recovery from a streptococcal infection, when bactericidal antibodies become demonstrable in man, may vary from two weeks to several months. Once these antibodies have been produced, they can usually be detected for at least one or two years, and in some individuals are still present after 10 to 30 years (61). It is generally agreed that reexposure in man with the same serological type of group A streptococci, accounts for continued antibody production and thus reinfection rarely, if ever, occurs (114).

The importance of group A streptococci as a factor in the development of both rheumatic fever and glomerulonephritis is well documented. However, despite intensive investigations, the mechanism by which the M protein of these bacteria are involved in the pathogenesis of these sequelae diseases has not yet been established. The possibility of whether the antigen-antibody reactions involve components of the streptococcal cell wall or membrane has been actively researched. In such studies, the M antigen has been of special interest. This review restricts comments to the work involved with M-proteins only. At present, the predominance of cases of post-streptococcal nephritis follow infections of type 12 group A streptococci. Since other nephritogenic streptococcal types, such as 49, 55 and 57, have been recently isolated, the importance of recognizing and identifying the distribution of cross-reactive or common antigens

among all nephritogenic streptococci becomes evident. The narrow specificity of nephritogenic streptococcal types (44,99) would seem to eliminate the possibility that any membranous material is related in any way to the streptococcal group A and C membrane antigens cross-reactive with the heart (122). However, some investigators have demonstrated a cross-reaction between streptococcal membrane antigens and human and mammalian glomerular basement membranes which could act as the etiological agent in post-streptococcal AGN (70,122). Using fluorescent antibody techniques, localization of M antigen has been observed in the glomeruli of mouse kidneys following intravenous injection of partially purified M proteins (52,74). Although type 12 M antigen might possibly have been expected to localize in the kidneys more than M proteins of non-nephritogenic types, this was not the case. However, recent findings that type 12 M protein-complexed with gamma globulin, was localized in the glomeruli of human patients with acute glomerulonephritis (AGN) (90) and in the glomeruli of rats that developed nephritis after exposure to nephritogenic streptococci (68,110), points to a possible role played by the M-antigen in the pathology of AGN. However, since not all type 12 streptococci induced nephritis in the rat (110), it is of great importance to establish the nature of the chemical and biological differences between the M proteins of nephritogenic and non-nephritogenic type 12 organisms.

The purpose of this thesis is to investigate and examine the chemical and immunochemical similarities among the various M proteins, with the long range goal of determining the nature of the type-specific determinant. The proposed experiments are of importance in defining the role of the amino acid composition as it relates to this type-specific determinant, which is of utmost importance in vaccine development. Thus, hopefully, the information elucidated by this research would aid in the synthesis of a chemically defined pure vaccine, a material which could be free of all microbiological products responsible for the sequelae diseases associated with the group A β-hemolytic streptococci.

II. MATERIALS AND METHODS

A. Organisms. The following strains of group A streptococci were employed in this experimental procedure:

Type	Source	Strain or Identification Number
1	Abbott Laboratories	T1/195/1P2
2	Dr. W. R. Maxted	10005
4	Dr. W. R. Maxted	R68/1115 Matt
6	Dr. E. V. Potter	SS 596
11	Dr. W. R. Maxted	100068
12	Hektoen Institute	Hektoen
13	Dr. W. R. Maxted	SS 31
24	Dr. E. V. Potter	Brassilia
25	Dr. W. R. Maxted	100088
29	Dr. E. V. Potter	1759
48	Dr. W. R. Maxted	100075
55	Dr. S. Bergner-Rabinowitz	2004
56	Dr. S. Bergner-Rabinowitz	SS-743
57	Dr. E. V. Potter	2015403
61	Dr. W. R. Maxted	R69/2714

The following partially purified M proteins were obtained from Dr. Arthur Hirata, Abbott Laboratories, North Chicago, Illinois.

Type	Identification Number
3	3515-210P
4	3407-200P
5	3515-103P
6	3515-101P
12	3407-242P
14	3515-165P

The following strains, all M protein producers, were obtained from Dr. W. R. Maxted at the Streptococcus and Staphylococcus Reference Library in London, England. Two of these strains are "Voorhout" (107) variants or organisms nontypable by their M proteins. Strain R66/3630 gl. is an original $T_{12}M_{12}$ strain lysogenic and SOR-negative (116). Strain R66/3630 matt was isolated from original strain 3630 gl. T_{12} M Voorhout, nonlysogenic, SOR-positive. Strain 41448/var. was found to be identical with Voorhout variant; T_{12} M Voorhout, nonlysogenic and SOR-positive.

Type	Source	Identification Number
12	Dr. W. R. Maxted	R66/3630 gl.
12	Dr. W. R. Maxted	R66/3630 matt
12	Dr. W. R. Maxted	41448 var.

Dr. Elizabeth V. Potter is in the Department of Microbiology at Northwestern University and Dr. S. Bergner-Rabinowitz is from the Streptococcal Reference Laboratory, Government Central Laboratories, Ministry of Health, Jerusalem, Israel.

B. Passing streptococci through mice

1. Preparation of granular Mucin (type Difco 1701-W). Five grams of Mucin was placed in a mortar, ground with the pestle and moistened thoroughly with a small amount (15 ml) of distilled water. The mixture was allowed to stand for 30 minutes and then the mass was again

mixed with more water until it was free of lumps. Water was then added slowly (up to 100 ml) to make a smooth suspension. The mixture was autoclaved at 15 pounds per square inch for 15 minutes. When the mixture cooled, the pH was adjusted to 7.3 with sterile NaOH. The mixture was then allowed to settle, to precipitate out any large particles before using. The Mucin was then placed in tubes and incubated at 37 C to determine its sterility. The Mucin was employed for injection of streptococci in a packed cell to Mucin mixture of 1:1 on a volume basis.

2. Passing streptococci through mice. A tube of sterile Todd-Hewitt broth was inoculated with the desired group A streptococcal type specific organism and was allowed to grow overnight at 37 C. The supernatant was discarded and 0.5 ml of Mucin media was added to 0.5 ml of live cells. With a large needle (trocar), the cells were sucked up from the tube into a sterile syringe. Before leaving for the day, two mice were injected with 1.0 ml of the mixture, intraperitoneal (IP), after the skin had been cleansed with 70% ethanol. The next morning, if the mice were not dead, they were sacrificed and their spleens sterilely removed. The spleen was placed in a sterile homogenizer, along with 1.0 ml of sterile Mucin and the contents thoroughly ground. The homogenizer was then held horizontally and the suspension was removed with a sterile tuberculin syringe. One-half

the volume of the suspension was injected intraperitoneally (IP) into each of two mice, and the last drop was inoculated onto a sheep blood agar plate to see if the organisms had been filtered by the spleen and, if they had, whether or not the culture was pure group A streptococci. The IP injections of the type specific organisms were continued until the streptococci were capable of killing the mice within six hours after the inoculation. When the mice had died within six hours, the spleens were removed and homogenized and the suspension was streaked on a sheep blood agar plate and incubated overnight at 37 C. No attempt at determining a lethal dose (LD) was made. Pure β-hemolytic streptococcal colonies were isolated and transferred into a tube of Todd-Hewitt broth and grown overnight at 37 C. The virulent group A streptococci were stored in the freezer at -40 C.

C. Conditions of culture. The bacteria were grown in a modified Todd-Hewitt medium (108) consisting of (per liter): 30 grams Todd-Hewitt broth media and 6 grams yeast extract. One one-liter starter culture in a four-liter transfer flask was incubated in the stationary position at 37 C overnight. This culture was then inoculated into a 14-liter Biokulture Fermentor (Fermentation Design Inc., Allentown, Pennsylvania), containing sever liters of the above modi-

fied Todd-Hewitt broth. The culture was aerated with filtered compressed air at the rate of 2000 ml per minute with a gyration speed of 400 rpm for eight hours. Then one liter of a sterile 10% dextrose and 8% $NaHCO_3$ solution was added to the culture and aeration was decreased to 100 ml per minute and the gyration speed was reduced to 200 rpm. The cells were grown for 18 hours and were heat killed at 56 C for one hour. Plates streaked with this culture remained sterile, indicating adequate killing. The cells were harvested in a Sharples centrifuge operating at 3,500 x g. Average yields were between 2.5 and 3.0 grams wet-weight/liter of medium.

D. Preparation and isolation of cell walls. One hundred grams of cells were washed five times with distilled water and were then resuspended in distilled water at a concentration of 10% wet-weight by volume. The 10% suspension of cells was disrupted in a Gifford-Wood Eppenbach micro-mill by the use of glass beads, 0.1 mm in diameter. Sixty ml of beads for 100 ml of cell suspension, a gap opening of 0.03 mm and approximately 8000 rpm and 30 minutes running time, were used. The cell walls were harvested, after removal of the glass beads through a coarse sintered glass filter, by centrifugation at 34,000 x g. One hundred grams of cell wall was then homogenized with approximately 500 ml of distilled water in the Ser-

val Omni-Mixer. The dispersed cell walls were centrifuged in the B-20 refrigerated centrifuge at 2000 rpm for ten minutes. Precipitates were discarded, and the supernatant was transferred to 90 ml cellulose tubes and centrifuged in a Model L-Ultracentrifuge at 20,000 rpm for 20 minutes. The supernatant obtained was used for membrane recovery and the packed cell walls were washed one additional time in distilled water.

E. RNase treatment and acid extraction of cell walls. Washed cell walls were placed in a one-liter flask in sufficient 0.1 M phosphate buffer pH 7.0 to make a 20% suspension. RNase (1.0 mg per 100 ml suspension) was added while stirring and the suspension was incubated at 37 C for four hours. Centrifugation was then performed in the Model L-Ultracentrifuge at 20,000 rpm for 20 minutes which allowed for the recovery of the cell walls. The walls were washed two times by homogenizing in a Serval Omni-Mixer in 200 ml of 0.1 M phosphate buffer, containing 0.02% sodium azide to prevent microbial contamination. Each wash was followed by centrifugation at 20,000 rpm for 20 minutes. The walls were dialyzed against distilled water for three days at 4 C with daily changes of the outer liquid.

The procedure of Lancefield and Perlman (64) was used to remove the M protein from the cell walls. One gram of the washed cell walls

was placed in 100 ml of 0.01 N HCl, pH 2. This suspension was immersed in a boiling water bath, stirred for 10 minutes and then cooled in an ice bath. The pH was then adjusted to 7.0 with 0.1 N NaOH. Centrifugation at 2000 rpm for 20 minutes in a refrigerated B-20 centrifuge, precipitated the walls and allowed for decantation of the clear supernatant. This extraction was repeated two additional times. All the supernatants were combined for ammonium sulfate fractionation. The final sediment was dialyzed against distilled water for three days at 4 C and then lyophilized.

F. Removal of proteins from the whole cells. The collected killed cells were washed once with saline and twice with distilled water. The extraction procedure used was described by Lancefield and Perlman (64). One hundred grams of wet-weight cells were placed in 500 ml of saline that had its pH adjusted to 2.0 with 1.0 N HCl. The cell suspension was immersed in a boiling water bath, stirred for 10 minutes and then cooled in an ice bath. When the cell suspension had achieved room temperature, the pH was adjusted to 7.0 with 1.0 N NaOH. Centrifugation at 3000 rpm for 30 minutes in a refrigerated centrifuge (Model B-20, International Equipment Company), separated the cells, allowing for the decantation of the clear supernatant. This hot acid extraction was performed three times. Aliquots of

each acid extraction were saved for comparisons of the substances in each fraction or any changes produced by these procedures. All of the supernatants were combined for ammonium sulfate fractionation. The final sediment was dialyzed against distilled water (with daily changes) for three days at 4 C, and then lyophilized.

G. RNase treatment of protein extracts. In a two liter beaker, the crude M protein supernatants, from whole cells, described above were treated with RNase (1.0 mg per 100 ml suspension). This suspension was incubated at 37 C for 4 hours with stirring. Any precipitate was removed by centrifugation at 10,000 rpm for 30 minutes in a B-20 refrigerated centrifuge. The supernatant was then dialyzed (with daily changes) against distilled water for three days at 4 C.

H. Spectrophotometric analysis of extracts. Extracts were evaluated for nucleic acid and protein content by reading absorption at 260 nm and 280 nm on a Hitachi Perkin Elmer 139 UV-VIS spectrophotometer. Calculations for protein and nucleic acid content were made employ- the following equation which utilized the extinction coefficient for enolase and nucleic acid:

$$\text{Protein} = 1.54\ E_{280} - 0.76\ E_{260}$$

$$\text{Nucleic Acid} = 0.064\ E_{260} - 0.037\ E_{280}$$

All extracts were evaluated before and after RNase treatment.

I. Ammonium sulfate fractionation. Solid ammonium sulfate was added slowly to the combined supernatants with continuous stirring in an ice bath in the cold room until a saturation of 30% was reached (21.2 grams/100 ml protein solution). After overnight stirring and equilibration, the precipitate was separated by centrifugation at 10,000 rpm in the refrigerated B-20 centrifuge at 0 C for 30 minutes. Additional salt was added to the 30% saturated ammonium sulfate supernatant until 60% saturation was attained. Equilibration and separation was performed as above. The sediment and the supernatant were separately dialyzed against distilled water (with daily changes) until the outer liquid was free of sulfate. The sack contents were lyophilized, weighed and saved for further purification. The calculation of the amount of ammonium sulfate to be used was performed according to the following equation:

$$x = \frac{(515 + t)(S_2 - S_1)}{10 - (2.7 + t/100\ S_2)}$$

Where x = weight in grams to be added to 100 ml of saturation S_1 to yield a solution of saturation S_2, t = temperature.

$$\text{30\% saturation at 0 C} = \frac{(515 + 0)(0.3 - 0)}{10 - (2.7 + 0)} = \text{21.2 grams/100 ml protein solution}$$

$$\text{60\% saturation at 0 C} = \frac{(515 + 0)(0.6 - 0.3)}{10 - (2.7 + 0)} = \text{21.2 grams/100 ml protein solution}$$

J. Chromatography media and column preparation.

1. Carboxymethyl cellulose (CM-70, lower pH limit approximately 4.0). Carboxymethyl cellulose powder was mixed with 0.2 N NaOH to make approximately a 20% solution and was stirred for one hour at room temperature. The solution was suction filtered on a Buchner funnel and transferred to 0.2 N HCl for one hour and then filtered. Alkali and acid washes were repeated two additional times. The suction cake was then washed with one final HCl wash (4th HCl wash) and then exhaustively with water. The CM cellulose was stirred with preparative buffer (1.0 M acetate at a pH of 4.0) for three one-hour periods and allowed to settle for 30 minutes, and the fine particles removed. The pH of the suspension was checked during the second washing, and fine particles were removed by aspiration. The cellulose was resuspended in starting buffer (0.1 M acetate buffer at a pH of 4.0) and stirred for 10 minutes and allowed to settle for 30 minutes. Filtering and washing procedures were then repeated two

additional times. Finally, the cellulose was suspended in the same acetate buffer for its use in column or batch chromatography.

2. Diethylaminoethyl cellulose (DEAE-50). Approximately 50 grams of DEAE-50 powder was placed in a four liter beaker with distiller water and allowed to settle. The fines were removed by aspiration and the settled DEAE-cellulose was suction filtered on a Buchner funnel. The filter cake was suspended in two liters of 1 N NaOH, and stirred for one hour. The fines were removed and the cellulose was then filtered by suction. The filter cake was resuspended in two liters of 1 N HCl, mixed by stirring for one hour and then suction filtered. The suction cake was washed with water, and the washing and filtering procedure was repeated for two additional times. The washed cellulose was suspended in preparative buffer (0.1 M phosphate buffer, pH 7.75) and equilibrated overnight with stirring in the cold room. The cellulose suspension was allowed to settle for 10 minutes and the fines were removed and the cellulose was suction filtered. The residue was resuspended in starting buffer (0.01 M phosphate buffer, pH 7.75) and was mixed by stirring for one hour. Any fines still present were removed and the washing and filtering procedure was repeated twice. Finally, the cellulose was resuspended in the same phosphate buffer for its use in column or batch chromatography.

3. Column preparation.

a. DEAE-cellulose column. A column containing 150-175 ml of DEAE packing (approximately 35-40 grams wet-weight of DEAE or 5.0 grams dry-weight of DEAE with a capacity for 750 mg protein or 0.7 meq/gram cellulose) and measuring 20-25 mm in diameter by 400-500 mm in length, was employed. It was prepared by filling the column with 0.01 M phosphate buffer, pH 7.75, and then adding the DEAE cellulose suspension. The excess buffer was allowed to flow out, and after the column was packed, additional buffer was run through the column to insure equilibration. The buffer range employed was the same as described below in the DEAE portion of the batch purification. Fractions were collected on a Fractomat automated fraction collector (Buchler Company, Fort Lee, New Jersey) and scanned at 280 nm with recording on a Gilson Recorder - R.P.

b. Carboxymethyl cellulose (CM) column. A column measuring 20-25 mm in diameter and 400-500 mm in length was employed. One hundred fifty ml (approximately 50 grams wet-weight of CM cellulose or 8.0 grams dry-weight with a capacity for 750 mg protein or 0.7 meq/gram cellulose) of CM cellulose suspension was packed into the column, using 0.1 M acetate buffer at a pH of 4.0 as the initial buffer. Columns were prepared as described above. The buffer range employed was the same as described below in the CM portion of the batch purification.

4. Batch procedure. The DEAE and CM celluloses were prepared as described above. The crude 30% to 60% ammonium sulfate fractionated M protein preparation was dissolved in a sodium phosphate buffer (pH 7.75, 0.01 M) and chromatographed in a stepwise batch elution process (buffer of increasing molarity and decreasing pH) on diethylaminoethyl cellulose (65). Fractions were collected at every interval of 1.0 unit pH decrease from an initial pH of 7.75 until a final pH of 4.0 was achieved; six fractions were collected. Each fraction was examined for group and type activity by immunodiffusion (103). Further purification was accomplished by rechromatographing in a stepwise elution procedure (sodium acetate and phosphate buffers of increasing pH and constant molarity) on carboxymethyl (CM) cellulose. Fractions were collected at every interval of 0.5 pH unit increase from an initial pH of 4.0 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. Batch purified M proteins were subjected to two CM cellulose purifications.

K. Trichloroacetic acid (TCA) precipitation. The washed whole streptococcal cells were extracted by two different techniques. The first technique was the standard hot acid (HCl) extraction technique of Lancefield and Perlman (64). For complete M protein removal, the

cells were extracted three times at pH 2.0, at 95 C. An aliquot of each extract was saved for analysis. The second procedure utilized hot TCA instead of HCl. In this procedure, 10 ml of 60% TCA were added to a test tube containing 6 grams (wet-weight) of group A streptococcal cells. The tube and its contents were placed in a boiling water bath for 20 minutes and then the tube was cooled immediately in an ice bath. After 15 minutes in the ice bath, the cells were sedimented by centrifugation at 4 C for 10 minutes at 4000 rpm in an IEC Model HN-S centrifuge. The precipitate was discarded and the supernatant fluid which represented the crude M protein extract was utilized.

The crude M protein extracts obtained by hot HCl extraction were then dialyzed against distilled water (with daily changes) for three days and lyophilized. The crude, lyophilized extracts were then processed in either of two ways. In one procedure, lyophilized M protein extract was treated with 60% TCA (6 ml TCA/ 100 mg lyophilized protein) and placed in a boiling water bath for 20 minutes. In the second procedure, initial purification of the combined HCl extracts was effected by ammonium sulfate to obtain the fraction precipitating between 30% and 60% saturation (63). After dialysis and lyophilization, the protein preparation was treated with 60% hot TCA as above. After all TCA extractions, M proteins were extracted five

times with ethyl ether (three volumes of ether per original volume of TCA solution) and the precipitate was then dialyzed for three days versus distilled water (with daily changes). The fractions were lyophilized and stored at room temperature until needed. Fractions were examined before and after TCA precipitation for group and type activity by Ouchterlony immunodiffusion.

L. Criterion for determining purity of protein isolates.

1. Chemical means. Total hexose determinations were performed using the anthrone reaction (originally introduced by Dreywood) as described in Williams and Chase (119). Two ml of the anthrone reagent (0.4 grams anthrone in 100 ml of concentrated sulfuric acid) was pipetted into the bottom of 16 x 150 mm test tubes in a water-bath at 10 to 15 C. One ml of the sample (10 to 50 µg of carbohydrate) was then carefully layered above the reagents. Blanks and standards of glucose or dextran were also included. Each tube was vigorously shaken while immersed in the cold water bath (10 to 15 C) until thoroughly mixed. The tubes were brought to room temperature and heated at 90 C for 16 minutes. They were subsequently cooled to room temperature and the blue color read in a spectrophotometer at 625 nm. Hexosamine determinations were performed according to the procedure outlined by Rosevear and Smith (84). The samples

were hydrolyzed overnight with 2 N HCl. Samples containing about 0.5 µmoles of free hexosamine in a total volume of 2 ml were set up in three test tubes (two duplicate tubes of sample and one sample blank). A reagent blank was set up using 2 ml of water. Standards of 0.5, 1.0, 1.5 and 2.0 µM in 2 ml volumes were also set up. One ml of 2 M NaCl was added to each tube followed by 3.0 ml of fresh 4% acetylacetone in aqueous 1.25 N Na_2CO_3. The tubes were mixed, covered with marbles and heated at 90 C in a water bath for 90 minutes. The tubes were removed and placed in an ice bath. The tubes were maintained at 0 C while 10 ml of absolute ethanol were added and the tubes were again mixed. To all tubes except the sample blanks, 1 ml of the para-dimethylaminobenzaldehyde solutions (1.6 grams para-dimethylaminobenzaldehyde in 30 ml absolute ethanol and 30 ml concentrated HCl) was slowly added and the solution was mixed with a stirring rod. One ml of H_2O was added to each of the sample blanks and mixed. The tubes were then placed in a 30 C water bath for 60 minutes and read at 530 nm against distilled water blanks. Hexosamine was also calculated from the recoveries of the model 120 C Spinco Amino Acid Analyzer.

Methylpentose (rhamnose) determinations were made according to the procedure of Dische and Shettles (14). To a one ml ice-cold solution of methylpentose (containing 3-10 µgrams of this substance),

4.5 ml of chilled sulfuric acid (6 parts concentrated H_2SO_4 and one part distilled water) was added slowly, with constant shaking in an ice bath to prevent a rise in temperature. The tubes were then transferred to a water bath at room temperature for 10 minutes, and then to a vigorously boiling water bath. After being heated for exactly three minutes, the tubes were placed in a water bath at room temperature, 0.1 ml of a 3% cysteine hydrochloride solution was added, and the contents of each tube were thoroughly mixed. After two hours, the absorption was measured in a spectrophotometer at 396 and 430 nm. The principle of the method depends on the absence of methylpentose absorption at 430 nm and the finding that the hexoses, pentoses and glucuronic and galacturonic acids have symmetrical absorption curves such that their absorption at 396 nm and at 430 nm is the same. Hence, subtraction of the latter value from the former corrects for all absorption except that due to methylpentose.

Nucleic acid determinations were made by examining the U.V. absorption spectra of each protein preparation at 260 and 280 nm on a Hitachi Perkin Elmer 139 UV-VIS spectrophotometer. Quantitative total amino acid analyses were performed on a Spinco model 120 C Amino Acid Analyzer as described by Spackman, et al. (96). Tryptophan content was determined by the procedure of Barman and Koshland (3). In this method, 5 mg of protein to be analyzed was incubated for 16 to 20 hours at 37 C in 1 ml of 10 M urea which had been ad-

justed to pH 2.7 with concentrated HCl. The urea solution was cooled to room temperature and approximately 5 mg of 2-hydroxy-5-nitrobenzyl-bromide (HNB-bromide) in 0.1 ml of dry acetone was added (by gravity) from a 0.1 ml pipette kept below the surface of the protein solution. The protein solution was stirred vigorously during this addition, using a magnetic stirrer. The mixture usually remained clear, but occasionally a slight precipitate (usually HNB-hydroxide) formed which was removed by centrifugation. The labeled protein was separated from excess reagent on a column of coarse Sephadex G-25 (23 x 1.1 cm) which had previously been equilibrated with 0.18 M acetic acid (pH 2.7). One ml fractions were collected at flow rates of approximately 100 ml/hour and the tubes (usually 1 to 4 ml at elution volume) were pooled. The protein in the pooled protein fractions was precipitated by addition of 50% TCA to a final concentration of 5% TCA. Complete precipitation usually occurred in 30 minutes. The precipitate was collected by centrifugation (4,000 x g for 30 minutes), washed twice with 5 ml of an ethanol-HCl wash (2 ml of concentrated HCl to 98 ml of 95% ethanol) and finally dissolved in a 1 ml of 11.2 M HCl. A one ml aliquot of the HCl solution was then adjusted to a pH greater than 12 by addition of 2.5 M NaOH and then diluted (if necessary) to a final volume of 2.5 ml. The concentration of HNB groups in this saturated solution was deter-

mined by spectrophotometric assay at 410 nm with the use of an extinction coefficient of 18,000 M^{-1} cm^{-1}.

Protein was determined by a modification of the Lowry technique (69). One ml of a solution containing 50 ml of 2% Na_2CO_3 in 0.1 N NaOH and 0.5% $CuSO \cdot 5H_2O$ in 1% sodium tartrate (prepared fresh daily) was added to 0.2 ml samples containing 5-100 µgrams protein. Samples were mixed well and permitted to stand at room temperature for 10 minutes. The Folin-Ciocalteau reagent (Aloe Scientific), diluted 1 part to 1 part water immediately before use, was rapidly pipetted into these tubes and mixed and incubated for 30 minutes. Absorbancy was read at 750 nm for 5 to 25 µgrams of protein. Higher concentrations were read at 500 nm in a Hitachi Perkin Elmer Model 139 Spectrophotometer. The same procedure was used for protein standard determinations employing human serum albumin. The standard curve and all determinations were done in duplicate. Protein determinations were also calculated from the recoveries of the Model 120 C Spinco Amino Acid Analyzer.

2. Physical Means. Chromatography, column and thin layer, were used as criteria for purity. Columns of DEAE and CM cellulose, as well as Sephadex G-200, were employed. Thin-layer chromatography (TLC) was also used for the detection of N-terminal amino acids. Samples were hydrolyzed in 5.7 N HCl (constant boiling) in sealed

tubes at 110 C for 22 hours. Thin-layer silica gel sheets (Eastman #6061) were run in three solvent systems; chloroform:benzyl alcohol: acetic acid (70:30:3 v/v), n-propanol:34% ammonium hydroxide (67:33 v/v), and 1.5 M phosphate buffer pH 6.0 (138.0 grams NaH_2PO_4 + 17.0 grams Na_2HPO_4 in one liter), in one or two dimensions. Chromatograms were viewed under ultraviolet light as well as incandescent light. DNP-amino acids were readily discernable by their yellow color.

Polyacrylamide gel electrophoresis was performed using the disc method of Ornstein and Davis (12) as modified by Sargent (86). The gels were run at a pH of 8.3 and 300 µgrams of protein was run on each gel. The stock solutions used to made the gels were prepared as follows: Solution A - 48 ml of N hydrochloric acid, 36.3 grams of Tris buffer, 0.46 ml of N:N:N':N'-tetramethyl-1:2-diamino-ethane (TEMED) and water to 100 ml, pH 8.9. Solution B - 48 ml of hydrochloric acid, 5.98 grams of Tris buffer, 0.46 ml TEMED, and water to 100 ml, pH 6.7. Solution C - 30.0 grams of acrylamide, 0.8 grams of methylene-bis-acrylamide, 0.015 grams of potassium ferricyanide and water to 100 ml. Solution D - 10.0 grams of acrylamide, 2.5 grams of methylene-bis-acrylamide and water to 100 ml. Solution E - 4.0 mg of riboflavine in 100 ml of water. Solution F - 70 mg of ammonium persulfate in 50 ml of distilled water, prepared fresh daily. The stock buffer used for electrophoresis consisted of 6.0 grams of Tris,

28.8 grams of glycine and water to one liter. The pH of the buffer was adjusted to 8.3 and the solution was stored at 4 C. Prior to use, the stock solution was diluted two-fold with distilled water.

The running gels were prepared by mixing the stock solutions in the following proportion: 2 parts A, 4 parts C, and 2 parts distilled water/8 ml of mixture. Five parts of this mixture were added to five parts of F/10 ml of mixture and this preparation constituted the materials for the running gels. For the upper gels, 1 part B, 2 parts D and 1 part E were added to 4 parts distilled water/8 parts of mixture. The flask containing the upper gel solution was covered with aluminum foil.

Gels were polymerized in glass tubes, 2 1/2 inches long with an inner diameter of 6 mm. The glass tubes were filled with running gel to a level 1 1/2 cm from the top. The solution in each tube was layered over carefully with 1/4 inch of chilled, recently boiled water so that a discrete boundary was formed between the gel solution and the water. The water layer is essential since the polymerization process is inhibited by oxygen and will not take place on exposure to air. Gelling was allowed to proceed at room temperature for a period of 45 minutes until the first boundary was replaced by a second boundary some 3 mm below the first. The solution above the boundary represented water and oxygen-inhibited monomer. The water layer was

carefully decanted from the tube and the top of the gel carefully rinsed with upper gel solution.

About 3/8 inch of upper gel solution (spacer gel) was then added to each tube and again covered with a layer of water (1/8 inch). The tube holder was placed 3 inches from a 15 watt fluorescent lamp for 20 minutes to allow the spacer gel solution to polymerize. After carefully decanting the water, a further 0.25 ml of upper gel solution was added to each tube and about 300 µgrams of protein was mixed with the gel solution (sample gel). Polymerization was again effected by placing the tubes 3 inches from the fluorescent lamp for 20 minutes. Tubes were fitted into the upper chamber compartment, and the apparatus assembled and both chambers were filled with buffer.

A constant current of 5.0 mA per tube was applied. After placing the cathode in the upper chamber and electrophoresing for approximately one hour, at room temperature, the tracking dye (Bromphenol Blue) generally reached the bottom of the tube and the run was terminated. The gels were removed from the tubes and then stained employing either Naphthalene Black 12B or Coomassie Brilliant Blue.

a. Naphthalene Black 12B. The isolated gels were immersed in a solution of Naphthalene Black 12B (1% w/v) in 7% v/v acetic acid for one hour. The stained gels were then washed exhaustively in 7% v/v acetic acid for several days until a relatively clear back-

ground was obtained. It was usually difficult, however, to remove excess dye thoroughly from the gel by static washing. Thus, it was more efficient to employ the following electrophoretic technique for destaining.

In this destaining technique, the gels were washed briefly in 7% v/v acetic acid and placed in tubes of similar dimensions to those used for the electrophoretic run, except that the tubes were now constricted at the bottom end to stop the gel from falling through. Each tube was filled with a solution prepared as follows: An aqueous solution of riboflavin (0.5% w/v) and acrylamide monomer (6% w/v) was exposed to fluorescent light at a distance of 3 inches until the yellow color of the solution had disappeared. The polymer solution was diluted with an equal volume of 14% v/v acetic acid and was ready for use. (This solution was stored in the dark at 0 C.) The presence of this solution in the tube minimized convection currents during the subsequent electrophoresis. After filling the tubes with the polymer solution, they were transferred to the electrophoresis tank and the upper and lower compartments were both filled with 7% v/v acetic acid. Approximately 100 volts was applied for one hour until all the excess dye had migrated from the gel. Stained gels were stored in 7.5% v/v acetic acid in test tubes.

b. Coomassie Brilliant Blue. After fixation for 30 minutes at 65 C in 12.5% TCA, the gels were stained with a 0.2% solution of the Procion dye, Coomassie Brilliant Blue (Code #65-72-1; Colab, Glenwood, Illinois). Destaining was performed for 20 minutes 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. Stained gels were stored in 7.5% v/v acetic acid in test tubes.

M. Physical and chemical characteristics of protein isolates.

1. Determination of N-terminal amino acids by the fluorodinitrobenzene reaction.

a. DNP-reaction on whole cells. Using the procedure of Frankel-Conrat and Porter (28), 40.0 grams of streptococcal cells and 10.0 grams of sodium bicarbonate (10%) were suspended in 100 ml of distilled water in a round bottomed flask. To this was added 200 ml of an ethanolic solution (10%) of fluorodinitrobenzene (FDNB). The mixture was brought to pH 8.0 with 0.1 M KOH (approximately 50 ml) and was shaken for two hours at room temperature followed by centrifugation in the B-20 refrigerated centrifuge at 4000 rpm for 40 minutes. To remove any proteins hydrolyzed from the cells due to

this process, the following steps were employed. The supernatant was concentrated to about 250 ml in a rotary evaporator (Rinco VE-1000-B) at 40 C and extracted three times with ether (using approximately 50 ml each time in a separatory funnel). The ether layer was evaporated to dryness. The aqueous layer was adjusted to pH 3.5 with 6 N HCl to precipitate DNP-protein which was recovered by centrifugation in a B-20 refrigerated centrifuge at 4000 rpm for 20 minutes. The precipitate was dissolved in water, placed in a dialysis bag and dialyzed against distilled water (with daily changes) until no yellow color was found in the outer liquid. The contents of the dialysis bag were then lyophilized. The supernatant of the above solution was dialyzed and lyophilized according to the same procedure.

The DNP-cells or DNP-protein precipitates from the original reaction were washed with ether, corked and centrifuged in the B-20 refrigerated centrifuge at 2000 rpm for 20 minutes. The ether layer was evaporated in the rotary evaporator at 40 C. The cells remaining after extraction with ether were suspended in water, placed in a dialysis sack and dialyzed against distilled water. Dialysis was continued until no yellow color appeared in the outer liquid, and then the contents of the sack were lyophilized. The next step involved an acid extraction of the DNP-streptococcal cells. The total DNP-cells were suspended in 200 ml of saline. Adjustment of the pH

of the supernatant to pH 2 was made in 1 N HCl. The suspension was immersed in a boiling water bath and extracted three times as previously described for the native whole cell extraction (Section F). The supernatant was dialyzed in water (with daily changes) for three days and lyophilized. The precipitate (whole cells) was suspended in and dialyzed against water (with daily changes) until no yellow color was found in the outer liquid. The contents of this sack were then lyophilized.

b. DNP-reaction on purified M proteins. Assuming a molecular weight of approximately 20,000, 0.2 μM (4.0 mg) of each purified type specific M protein were placed in test tubes along with 0.1 ml sodium bicarbonate (10%) and 1.0 ml of an ethanolic solution (10% v/v) of FDNB. Each tube was shaken for two hours. The mixture (pH initially 7.5 to 8.0) was then acidified with a few drops of 2.0 N HCl to pH 1 to 2. The suspension was then extracted three times with ethyl ether, decanting after each extraction. This ether decantation removed ethanol, excess FDNB and dinitrophenol and left an aqueous suspension of insoluble DNP-protein.

c. Methods of isolation and identification of DNP-amino acids. Approximately 3 to 4.0 mg of DNP-protein or 30 to 40 mg DNP-cells (prepared as above) was suspended in 1.0 ml of 5.7 N glass distilled constant boiling HCl in a test tube (13 x 150 mm). Each tube was

sealed and hydrolyzed at 110 C for 22 hours. After the hydrolysis was complete, the hydrolysates were concentrated to dryness over NaOH in a vacuum desiccator. The DNP-amino acid hydrolysates were dissolved in 2% acetic acid and run over alumina, using the procedure of Turba and Gurdlach (106). Alumina columns remove 2,4 dinitrophenol, 2,4 dinitroanaline and various artifacts which are the breakdown or side reaction products of the FDNB in the preparation of the DNP-amino acids. Alumina powder, 80-120 mesh, was washed three times with 0.02 N HCl to remove the fines, washed once with water, once with 2% aqueous acetic acid, and then suspended in 2% acetic acid. A short column of 0.6 x 60 mm was then packed with acid washed alumina. The sample was dissolved in a small amount of 2% acetic acid and layered on the column. Elution with 2% acetic acid was continued until no more yellow color was observed in the column effluent. The alumina column retained DNP-amino acids in 2% acetic acid allowing the dinitrophenol to pass through. The DNP-amino acids were then eluted from the column with a 1% $NaHCO_3$ solution. This eluate was collected and concentrated to dryness using a rotary flask evaporator at 40 C. The DNP-amino acids were then solubilized by adding two to three drops of ethyl acetate and transferred to a thin layer silica gel sheet (Eastman #6061).

The thin layer silica gel sheet was activated before use by heating in an oven for 30 minutes at 100 C. The starting points were marked by pricking with a sharp pencil, 2.0 cm from the edge of the sheet. A sample volume of 5 µl was employed for unknowns as well as standards. A line was drawn across the top of the chromatogram, 10.0 cm from the starting points to mark the level to which the solvent was allowed to travel. Nineteen different DNP-amino acids were employed as standards (Mann Research Laboratories, Inc., Dinitrophenyl (DNP) Amino Acids Kit No. 418). The chromatographic sheets were developed at room temperature in the dark and in ascending procedures. They were placed in a Shandon T.L.C. Chromatotank containing the solvent at approximately a 1 cm depth. The sheets were removed from the chromatography tank when the solvent front reached the 10 cm boundary line drawn across the top. The sheets were dried at room temperature. Resolution in ascending chromatography could be accomplished in 45 to 90 minutes using the three separate solvent systems; chloroform: benzyl alcohol:acetic acid (70:30:3 v/v), n-propanol: 34% ammonium hydroxide (67:33 v/v), or 1.5 M phosphate buffer at a pH of 6.0 (138.0 grams NaH_2PO_4 + 17.0 grams Na_2HPO_4) as the first solvent system. Further fractionation employing two dimensional thin layer chromatography could be achieved if only one sample was applied at a site in a corner of a sheet, two cm from each edge. After the sheet

was developed once to a height of 10 cm, the chromatogram was turned around an angle of 90° and chromatographed with a second solvent system.

2. Amino acid composition of proteins. Quantitative total amino acid analyses were performed on a Spinco model 120 C Amino Acid Analyzer essentially according to the procedure of Spackman (96). One milligram of purified M protein was placed in a test tube (13 x 150 mm) along with 1.0 ml of 5.7 N glass distilled constant boiling HCl. The tube was sealed, under vacuum, and hydrolyzed at 110 C for 22 hours. After hydrolysis was complete, the tube was opened and the contents were taken to dryness over NaOH in a vacuum dessicator. Then 2.2 ml of sample dilution buffer (pH 2.2, 0.2 N citrate buffer) was added to the tube and 1.0 ml of the solution was placed on the short column as well as 1.0 ml being placed on the long column. 100% recovery was represented by recovery of 454 µgram total amino acids.

3. Molecular weights.

a. Sodium dodecyl sulfate (SDS). The use of SDS-gel electrophoresis to determine molecular weights was originally conceived by Shapiro in 1967 (91). SDS molecular weight determinations were performed here on cellulose and TCA purified M proteins according to the procedure of Hedges (37). The solutions contained: Solution A - (stock solution) 31.2 grams $NaH_2PO_4 \cdot H_2O$, 154.4 grams $Na_2HPO_4 \cdot 7H_2O$, 8.0 grams SDS and distilled water to a final volume of 4 liters.

Solution B - (tank buffer) 1.0 liter of stock solution A and distilled water to a final volume of 4 liters. Solution C - (sample incubation buffer) 0.9 grams SDS, 1.0 ml mercaptoethanol, 50.0 ml stock solution A and distilled water to a final volume of 100 ml. Solution D - (sample dialysis buffer) 50 ml stock solution A, 0.02 ml mercaptoethanol, 0.1 grams SDS and H_2O to a final volume of 200 ml. Solution E - (sample running buffer) 0.5 ml glycerol, 0.03 ml Bromphenol Blue (0.1% aqueous), 0.05 ml mercaptoethanol, 0.25 ml sample incubation buffer C, and 0.25 ml of distilled water. Solution F - 5.5 grams of acrylamide, 0.15 grams of bis-acrylamide and distilled water to a final volume of 25 ml. Solution G - (catalyst) 0.10 grams of ammonium persulfate and distilled water to a final volume of 10 ml, prepared fresh daily.

Twenty milligrams of each protein standard was placed in a separate vial containing 4.0 ml of sample incubation buffer C and incubated for 2 hours. Proteolytic enzymes were incubated at 6 C. All other proteins were incubated at 37 C. Samples were used immediately or were quick frozen in liquid nitrogen for future use. Quantities were scaled down for smaller amounts of protein. For investigation of small molecular weight compounds (23,000 or less) and proteolytic enzymes, dialysis was necessary to get clear bands in electrophoresis after incubation. Such samples were dialyzed against sample dialysis buffer D for 2 hours with one buffer change.

The running gels were prepared by combining and swirling (not shaking) the stock solutions in the following proportion: 15 ml B, 13.5 ml F, 0.045 ml TEMED and 1.4 ml of G was added just prior to the casting of the gels. For the upper gel solutions, the stock solutions were mixed in the following proportions: 2.0 ml F, 2.0 ml B, 0.35 ml G and 5 µl TEMED.

Gels were polymerized in glass tubes, 2 1/2 inches long with an inner diameter of 6 mm. The glass tubes were filled with running gel solution to a level 1 1/2 cm from the top, overlaid with water, and allowed to polymerize for 20 minutes. Following the polymerization of the running gels, the water overlay was removed and the top of the running gel was washed with the upper gel solution. The well portion of the gel was then formed by adding 3/8 inch of upper gel solution (well gel) to each tube and again covering with a layer of water (1/8 inch). After 20 minutes, the well gel polymerized. After carefully decanting the water, 50 µl of sample running buffer E and 10 µl of sample from each incubation mixture were mixed and placed in the separate wells. A second preparation of the upper gel solution (cap gel) was carefully added to each well, on top of the sample until it was level with the top of the cell plate. 20 minutes was allowed for polymerization.

A constant current of 7 mA per tube was applied. After placing the anode in the upper chamber and electrophoresing for 1.5 hours at

room temperature, the tracking dye reached the bottom of the tube and the run was terminated. The gels were removed from their respective glass tubes and a marker needle was inserted in the gel at the leading edge of the visible tracking dye. The gels were then placed in 20% sulfosalicylic acid for 12 hours to remove SDS and to set the proteins. The gels were stained, after fixation for 30 minutes at 65 C in 12.5% TCA, with a 0.2% solution (45 ml 0.2% aqueous Coomassie Brilliant Blue, 45 ml absolute ethanol and 10 ml glacial acetic acid) of the Procion dye Coomassie Brilliant Blue (Code #65-72-1; Colab, Glenwood, Illinois). Destaining was accomplished by two changes of a destaining solution composed of absolute alcohol: glacial acetic acid and water (25:10:65) at 65 C for 30 minutes each. Final destaining with 10% acetic acid at 65 C for 30 minutes completed the procedure, and the gels were stored at room temperature in 10% acetic acid.

The measurement and calculation of the molecular weight in SDS gels were made as follows. With a gel in a large petri dish of 10% acetic acid, using 20 x 20 inch linear graph paper on a light box, gel mobilities were measured for all proteins. The relative mobility of each protein was calculated according to the following formula:

$$\text{Relative mobility} = \frac{\text{Distance from the bottom of the well gel to the leading edge of the protein band.}}{\text{Distance from the bottom of the well gel to the leading edge of the tracking dye.}}$$

Relative mobilities of the standards (Human serum albumin 69,000, egg albumin 43,000, pepsin 35,000, trypsin 23,800, and lysozyme 14,400) were plotted against their molecular weights on semilog paper, with mobility plotted on the linear axis and molecular weight plotted on the logarithmic axis. Standards formed a straight line. Molecular weights of the unknowns were read from the graph, knowing the relative mobilities of the unknown samples.

b. Sephadex gel chromatography. Sephadex molecular weight determinations were performed according to the procedure outlined by Pharmacia Fine Chemicals, Inc. (81). Sephadex G-200 superfine gel was soaked in the running buffer for three days at room temperature and washed extensively. A column 45 cm x 2.0 cm (inner diameter) was filled with a Tris-HCl 0.2 M NaCl buffer. Tris 0.1 M (12.11 grams/liter) and NaCl 0.2 M (11.69 grams/liter) were dissolved in 2/3 of buffer volume using distilled deionized water. One N HCl was added adjusting the pH to 8.0 before the final volume was reached. Sodium azide was added (0.2 grams/liter) to give an antimicrobial growth concentration of 0.02%. The volume was then carefully measured to determine the total bed volume ($V = \pi r^2 h$). The column was then packed with the gel slurry (approximate packing time was one hour) and equilibrated with the running buffer by gravitational flow overnight. Using upward flow adaptors, the direction of the column

was reversed. The void volume (Vo) of the packed column was determined by multiple passage of Blue Dextran 2000 (1 mg/ml). The following proteins were used to determine the elution volume (Ve) of proteins of known molecular weight: Human γ-globulin (m.w. 160,000), Bovine serum albumin (m.w. 57,000), egg albumin (m.w. 43,000), trypsin (m.w. 23,800) and lysozyme (m.w. 14,400). Separation of a mixture of these standards during passage through the column was repeated until the elution columns were constant. The molecular weights of the unknown proteins were calculated by plotting K_{av} values against molecular weights of semilog paper. K_{av} values were determined by the following formula:

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}, \text{ where}$$

Ve = elution volume of the protein
Vo = void volume of Blue Dextran 2000
Vt = total bed volume

The M proteins were passed through the column twice to determine their average molecular weights. This procedure is based on the pioneering work of Andrews (1) and Whitaker (115).

4. Peptide mapping.

a. Chymotryptic digestion. Five mg of purified M protein was dissolved in 5 ml of a 0.08 M Tris, 0.1 M $CaCl_2$ buffer. The pH of this solution was then adjusted to 7.8. This mixture was then added

to a sterile vacutainer tube (B-D Company) which contained 50 units (92) of insoluble α-chymotrypsin attached to carboxymethyl cellulose, from bovine pancreas (Sigma Chemical Company, No. C-7260). The reconstitution of the enzyme was done as follows:

1) Suspend the dry preparation in water to a concentration of 5 to 10 mg/ml.
2) Allow the suspended enzyme to stand one hour at room temperature. Shake or mix occasionally during this swelling period.
3) Filter or wash several times with water and/or buffer to remove the borate buffer salts or "hydration acids" in which they are supplied.
4) Resuspend in the appropriate buffer to prepare the enzymes for use. The enzyme-protein mixture was rotated for three hours at room temperature to insure complete peptide formation. The enzyme was then spun out and the peptide mixture was then taken down to dryness over NaOH overnight. The enzyme particles were then recycled for reuse.

b. Trypsin digestion. Five mg of purified M protein was dissolved in 5 ml of a 0.046 M Tris, 0.0115 M $CaCl_2$ buffer. The pH of this solution was then adjusted to 8.1 with 0.1 N NaOH. This mix-

ture was then added to a sterile vacutainer tube which contained 50 units (93) of the insoluble enzyme trypsin attached to polyacrylamide, from bovine pancreas (Sigma Chemical Company, No. T-3257). The reconstitution of this enzyme was done as above. The enzyme-protein mixture was rotated for 3 hours at room temperature to insure complete peptide formation. The enzyme was then spun out and the peptide mixture was then taken down to dryness over NaOH overnight. The enzyme was also recycled for reuse.

c. Fingerprinting. Peptide mapping (fingerprinting) of tryptic and chymotryptic digests was performed by the combined electrophoretic-chromatographic procedure of Katz, et al. (53) with several variations. The dried digests were dissolved in 0.2 ml of distilled water. 0.05 ml was frozen to be examined later for type specificity. 0.15 ml was applied to a full sheet of Whatman paper No. 3 MM in an area with a diameter of 1 cm. High voltage electrophoresis (HVE) was performed first in a Savant electrophoresis tank (model LT48A) in a pyridine-acetate buffer, pH 3.5. This buffer was prepared using 6.6 ml of pyridine, 66 ml of acetic acid and the remaining volume in distilled water up to 3 liters. The HVE was at 1000 volts for 90 minutes using a Savant 5 kilovolt power source, with a current draw of between 75 and 150 mA for a full sheet of paper. The chromatography was done in a Chromatocab (model A-300, Sci. Manufacturing

Industries, Oakland, California), employing n-butanol:acetic acid: pyridine and water (30:20:6:24) as the solvent system. Chromatography was done in the descending method for 24 hours. The chromatograms were then dried and developed in a 1% ninhydrin in acetone solution and then placed in a 100 C oven for 5 minutes.

5. Amino acid sequencing. Ten mg of the cellulose purified M12 protein Fraction V was subjected to amino acid sequencing employing the Edman procedure (15). Sequencing was done on a JAS-47K automated sequencer and JEOL Gas chromatograph (JEOL, Cranford, New Jersey).

6. Determination of $_{280}E_{1\%}$ of M proteins. M proteins were dried overnight over P_2O_5 in a vacuum dessicator. A 1 mg/ml solution of purified M protein was prepared using borate buffered saline (BBS) as the diluent. The optical density (OD) of this solution was read at 280 nm on a Hitachi Perkin Elmer 139 spectrophotometer. Dilutions of 1:2, 1:3 and 1:6 were made and the $_{280}OD$ were recorded. Calculations of $_{280}E$ were based on a 1% concentration.

N. Antigenic Analyses.

1. Antigenicity of M-proteins in rabbits.

a. Whole cell vaccination. For the production of antibody against the type specific M proteins, whole heat killed cells in saline (10^8 organisms/ml) were used as a vaccine. The immunization

schedule employed 0.2 ml of the vaccine injected intravenously into the rabbits (New Zealand Whites) on days 1, 3, and 5. On days 12, 19, and 26, 0.5 ml of the vaccine was given intravenously. On day 56, 1 mg (dry weight) of each purified M protein type injected, was dissolved in 1.5 ml of saline, incorporated into 1.5 ml of complete Freund's adjuvant and injected via the intramuscular route into the respective rabbits. Immune sera was collected from the marginal ear vein by vacuum with the aid of a rabbit ear bleeder (Bellco, Vineland, New Jersey) on the 15th, 22nd, 29th and 59th days. Animals were exsanguinated on the 62nd day by cardiac puncture. Pre-immune rabbit serum served as a control. Serum was separated from the whole blood collected after clotting for 18 hours at 4 C by centrifugation. These sera were absorbed with whole cells of all other different group A streptococcal types available to remove cross-reacting activity and with trypsin-treated and pepsin-treated whole cells to absorb anti-group A activity. The effects of trypsinization were followed by fluorescent antibody techniques to assess loss of M-protein. The cells pepsinized were similarly assayed to assess total loss of extracellular coat proteins. Fluorescent goat anti-rabbit immunoglobulin G (IgG) was prepared according to the procedure of Clark and Shepard (11).

b. Purified protein vaccination. Each purified isolated protein was also used as an immunogen in the preparation of specific

anti-M antisera. An amount of 3 to 5 mg (dry weight) of M protein in 1.5 ml of saline was incorporated into complete Freund's adjuvant composed of the following: 3 ml of Bayol F, 1.5 ml of Falba, 0.1 to 0.2 mg of Mycobacterium tuberculosis (var. hominis, $H_{37}Ra$) cells, and the antigen in saline. The procedure for immunization (for each rabbit) was as follows: Day 1, 1 ml intradermally into the toe pads; Day 28, an equal dose intramuscularly; Day 35, trial bleed. If antibody response was poor, another single intramuscular injection was given and the animal was exsanguinated 5 to 7 days later. Each animal thus was immunized with 1,000 to 2,500 µgrams of protein.

c. Sera from Center for Disease Control. Specific group A antisera and specific anti-M antisera (types 1, 2, 3, 4, 5, 6, 11, 13, 14, 24 and 29) were supplied by Center for Disease Control (CDC, Atlanta, Georgia).

2. Evaluation of sera produced against the M proteins.

a. Passive hemagglutination studies. The passive hemagglutination procedures were done according to a modified procedure of Hirata and Brandriss (39). Rabbits (weighing 2 to 3 kilograms) were bled by cardiac puncture (20 ml) with a syringe containing an equal volume of sterile Alsievers solution. 60 ml of Alsievers solution was placed in a 250 ml flask to which the rabbit blood mixture was added. This flask was allowed to stand at 4 C overnight. On the

second day, the contents of the flask were poured into plastic centrifuge tubes. The tubes were spun in the refrigerated B-20 centrifuge for 10 minutes at 1500 rpm at 4 C. This procedure yielded about 10 ml of packed rabbit cells (RE). The packed RE were then mixed with 10 volumes of 0.11 M PO_4 buffer at a pH of 7.2. Centrifugation was performed again as above to pack the cells. This washing procedure with 10 volumes of the phosphate buffer was repeated five times. After the last washing, the RE was resuspended as an 8% suspension in 0.11 M PO_4 at a pH of 7.2. These washed cells must be used within seven days. The 8% suspension of RE was then placed in a 500 ml Erlenmeyer flask along with 125 ml of 3% pyruvic aldehyde (diluted in 0.11 M PO_4). This solution was mixed on a magnetic stirrer for 18 hours, at low speed and room temperature. On the third day, the suspension was filtered, through 4 x 4" gauze to remove debris, into plastic centrifuge tubes. The tubes were spun in the B-20 refrigerated centrifuge for 10 minutes at 1500 rpm at 4 C.

The supernatant was poured off and the pellet washed five times as before. After the last washing, the pyruvate aldehyde treated rabbit erythrocytes (PRE) were diluted with the phosphate buffer to an 8% suspension. In a 250 ml flask containing the PRE was added 125 ml of a 3% formaldehyde solution (diluted with PO_4 buffer). This solution was mixed 18 hours overnight as above. On the fourth day,

the suspension was again filtered through gauze to remove debris and centrifuged in the B-20 for 10 minutes at 1500 rpm. The cells were then washed five times with 10 volumes of 0.11 M PO_4 buffer and spun down. The cells were then resuspended as a 10% formaldehyde treated PRE in phosphate buffer (FPRE). These cells were stable at 4 C for two months. The cells were then dispensed in aliquots and quick frozen in liquid nigrogen. Stored in the freezer, these cells were stable for one year. Coating of the FPRE with the respective antigens was begun on the fifth day. 1 ml of 10% FPRE was placed in a 12 ml glass, conical graduated centrifuge tube. The cells were washed one time with 0.1 M acetate buffer at a pH of 3.6. The cells were then spun down at 4 C for 15 minutes at 1000 rpm. The cells were resuspended in 8.9 ml of acetate buffer at a pH of 3.6. The contents of the tube were incubated for one hour at 37 C. Next, 1 ml of 1 mg protein/ml in 0.1 M acetate buffer, pH 3.6, was added to the tube and the tube was rotated overnight at 37 C. The next morning (sixth day), the cells were washed five times with 0.11 M PO_4 buffer at a pH of 7.2. After the last washing, the cells were resuspended to a 1% concentration with 0.11 M PO_4 as M_n- FPRE, where n = type number. The cells were placed in small vials, labeled and quick frozen in liquid nitrogen. The cells were stored at -20 C within three minutes.

The hemagglutination procedure was performed using the microtitre apparatus (Cooke Engineering Company, Alexandria, Virginia). One drop (dropper and loops are calibrated to deliver 0.025 ml) of diluent (prepared by mixing equal volumes of gelatin -- 2 mg/ml phosphate buffer -- and rabbit serum albumin -- 1 mg/ml phosphate buffer) was delivered into each well with a dropper. The antisera was serially diluted with loops out to the eleventh well. The twelfth well served as a control. One drop of antigen (M-FPRE) was then added to each well, and the wells were mixed by gently tapping the plates. The plates were then sealed and were incubated for five hours at room temperature. The results were read using a reverse view mirror.

b. Streptococcal long chain formation. Examination of the streptococcal long chain forming abilities of the respective antisera was performed using the procedure of Stollerman and Ekstedt (100). 0.3 ml of Todd-Hewitt broth was placed in a 9.0 x 100 mm (outside diameter) test tube along with 0.05 ml of antisera and 0.1 ml of a 10^{-2} dilution in fresh Todd-Hewitt broth of an overnight culture. Tubes containing normal antisera from rabbits were included in all experiments as controls. The tubes were closed with sterile rubber stoppers and rotated at 37 C for 4 hours. At the end of this, hanging drop preparations were made and the number of cocci in 50 chains were counted.

c. Agar gel analyses. Ouchterlony immunodiffusion (79) was performed to determine whether a protein preparation possessed either type or group specificity. It was also used as a tool to determine the degree of cross-reactivity among the different type specific protein preparations. Slides were incubated overnight.

Immunoelectrophoresis was performed according to the method of Scheidegger (87) in a 1.5% Ionagar agar gel (Colab, Chicago Heights, Illinois) with a Gelman immunoelectrophoresis chamber for 120 minutes at 36 mA and 100 volts in 0.1 M barbital buffer (pH 8.6). Antigen concentrations ranged from 0.5 to 2.0 mg/ml. Antisera troughs were filled with type specific antisera prepared in rabbits against either purified protein preparations or against whole cell vaccines. The slides were incubated for 48 hours to allow for adequate diffusion of the immunoglobulins. Two saline washes (24 hours each) were used to halt the reaction and wash out any non-specific protein. These were followed by exhaustive rinsing with distilled water to remove all excess salts. The slides were dried at room temperature and stained for 30 minutes in a 0.1% solution of Napthalene Black 12B (Allied Chemical and Dye Corporation, New York, New York) in a methanol:water:glacial acetic acid mixture (50:50:20). A solution of methanol:glacial acetic acid and water (70:10:20) was used to destain the slides.

O. Reagents. The Todd-Hewitt broth used in all experiments, as well as the Yeast Extract, was obtained from Baltimore Biological Laboratories, (BBL), a Division of Bioquest in Cockeysville, Maryland. Chromatographically purified RNase was obtained from Kock-Light Laboratories in Colnbrook, England. The trichloracetic acid, ammonium sulfate and dextrose were purchased from Mallinckrodt Chemical Works, St. Louis, Missouri. All reagents for the amino acid analyzer were obtained from Bio-Rad Laboratories, Richmond, California. TEMED, acrylamide, N,N-methylene-bisacrylamide (MBA) and riboflavin were each purchased from Eastman Organic Chemicals. DEAE and CM celluloses and Bovine albumin were obtained from Sigma Chemical Company, St. Louis, Missouri. The Sephadex G-200, along with the Blue Dextran 2000 were purchased from Pharmacia (Uppsala, Sweden). Egg albumin and ammonium persulfate were purchased from Fisher Scientific Company, Fair Lawn, New Jersey. Trypsin (for molecular weight determinations) was obtained, along with lysozyme, from Worthington Biochemical Corporation, Freehold, New Jersey. Pepsin (for molecular weight determinations) was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. Dialysis tubing was purchased from Union Carbide, Chicago, Illinois. Napthalene Black 12B was obtained from Allied Chemical, Morristown, New Jersey. 2,4-dinitrofluorobenzene was obtained from Pierce Chemical Company, Rockford, Illinois.

Activated alumina was received from Matheson, Coleman and Bell, Norwood, Ohio. Alsievers solution was made in our laboratory according to the following protocol: 41.0 grams of glucose, 9.0 grams of sodium chloride, 16.0 grams of trisodium citrate and 1.1 grams of citric acid were dissolved in two liters of double distilled water. The solution was dispensed into 100 ml screw top bottles and these were sterilized by autoclaving at 15 pounds per square inch for 15 minutes.

III. RESULTS

A. Chemical data.

1. Carbohydrate determinations. Table I represents the various analyses on the carbohydrate content of the various protein preparations. M proteins obtained by a 30 to 60 per cent ammonium sulfate fractionation of an acid extract of whole cells or cell walls were shown to be 99% free of carbohydrate. M proteins purified by CM cellulose chromatography and RNase, DEAE and CM cellulose chromatography and RNase, and a hot trichloroacetic acid precipitation were essentially free of carbohydrate except for a trace of methylpentose.

2. Quantitative total amino acid analyses. Tables II, III, IV, V and VI represent the quantitative total amino acid analyses performed on the various type specific protein preparations purified by different methods. Table II represents the amino acid analyses of M proteins from ten different strains of group A streptococci, purified by a 30 to 60 per cent ammonium sulfate fractionation. Table III represents the total amino acid analyses of six various type specific M proteins purified by a 30 to 60 percent ammonium sulfate fractionation of RNase treated cell walls followed by CMC column chromatography. Table IV represents the total amino acid analyses on a pH 6.0 fraction of three different M proteins purified by DEAE

TABLE I. CARBOHYDRATE DETERMINATIONS ON M PROTEIN PREPARATIONS.*

Method of Isolation	ug carbohydrate/ug protein		
	Methylpentose	Hexoseamine	Total Hexose
30% to 60% ammonium sulfate fractionation of acid extract of whole cells	3.0/310 (1.0)**	0.9/310 (0.29)	1.9/310 (0.61)
30% to 60% ammonium sulfate fractionation of acid extract of cell walls	2.5/350 (0.7)	0.9/350 (0.26)	1.5/350 (0.42)
CMC column chromatography on 30% to 60% ammonium sulfate fractionation of acid extract from cell walls (RNase treated)	2.2/400 (0.55)	---	---
DEAE and CMC batch chromatography on 30% to 60% ammonium sulfate fractionation of acid extract from whole cells (RNase treated)	1.5/454 (0.33)	---	---
TCA precipitation of 30% to 60% ammonium sulfate fractionation of acid extract from whole cells	2.0/406 (0.49)	---	---

* All values are averages of three determinations.
** Per cent.

TABLE II. AMINO ACID ANALYSES ON AMMONIUM SULFATE PURIFIED M PROTEIN PREPARATIONS (IN MICROMOLES PER CENT).*

	Type									
Amino Acid	1	4	6	12**	24	29	57	12a	12b	12c
lysine	8.9	8.6	7.2	11.1	4.3	8.6	9.9	9.5	9.8	9.5
histidine	0.3	1.5	1.7	2.4	1.4	0.1	1.4	1.4	0.8	1.2
arginine	4.7	4.2	2.9	7.5	4.9	2.3	4.8	4.7	3.2	2.4
aspartic acid	10.0	12.8	10.3	10.7	10.4	11.1	10.5	10.8	12.0	11.5
threonine	5.2	6.3	5.6	5.3	6.5	6.1	5.7	5.7	5.8	5.9
serine	6.4	4.5	7.0	5.4	4.9	5.3	4.3	4.9	4.8	5.2
glutamic acid	19.4	15.0	13.5	13.0	14.3	14.9	13.8	13.1	14.5	15.5
proline	0.3	2.6	3.5	2.6	3.1	2.3	4.0	3.4	4.3	3.5
cystine (1/2)	--	--	--	--	--	--	--	--	--	--
glycine	8.2	7.2	8.9	9.2	8.1	10.2	7.7	8.2	7.0	7.6
alanine	11.0	12.0	12.0	9.1	11.8	12.3	11.4	11.5	12.7	15.0
valine	5.8	6.7	7.3	7.2	8.4	7.3	7.9	7.7	7.2	4.9
methionine	--	1.0	0.7	0.1	--	1.0	0.1	--	--	0.1
isoleucine	4.5	5.1	5.0	4.4	6.1	5.7	5.8	5.2	5.3	5.5
leucine	10.3	7.9	7.1	6.4	7.7	8.3	7.1	7.1	7.3	7.4
tyrosine	1.6	1.3	2.6	2.1	1.5	1.6	1.7	2.9	1.5	1.3
phenylalanine	2.6	2.6	2.8	2.8	3.2	2.8	3.3	2.9	3.0	3.0

* All proteins except 12** extracted from whole cells.
** Extracted from purified cell walls.

12a. R 41448/var.
12b. R 66/3630 gl.
12c. R 66/3630 matt

TABLE III. AMINO ACID ANALYSES ON M PROTEIN PREPARATIONS PURIFIED BY CARBOXYMETHYL CELLULOSE COLUMN CHROMATOGRAPHY (IN MICRO-MOLES PER CENT).

	Type					
Amino Acid	3	4	5	6	12	14
lysine	12.7	12.0	16.8	19.2	13.2	8.7
histidine	2.5	1.8	1.7	0.4	2.2	0.6
arginine	4.4	5.9	3.5	4.0	4.5	2.4
aspartic acid	11.6	10.0	13.3	14.9	11.9	15.1
threonine	4.2	2.2	7.1	7.9	2.8	5.5
serine	3.2	4.6	3.1	3.6	4.0	4.4
glutamic acid	20.7	27.0	20.8	19.9	21.2	27.4
proline	1.5	2.8	1.1	1.1	1.4	1.4
cystine (1/2)	--	0.7	--	--	--	1.1
glycine	3.2	3.0	3.8	2.8	4.0	3.4
alanine	10.9	10.3	6.5	7.2	10.9	9.5
valine	6.1	2.9	3.8	3.9	4.9	4.5
methionine	0.5	--	--	--	--	--
isoleucine	3.1	3.4	4.0	3.8	3.4	2.8
leucine	11.0	8.8	10.8	10.2	11.5	10.4
tyrosine	1.8	2.9	1.5	0.4	1.9	0.9
phenylalanine	1.8	0.7	1.3	0.5	1.6	1.1

TABLE IV. AMINO ACID ANALYSES ON pH 6.0 FRACTION V OF THREE DIFFERENT M PROTEIN PREPARATIONS PURIFIED BY DEAE AND CM STEPWISE ELUTION BATCH PROCEDURES (in mg/100 mg protein).

	Type		
Amino Acid	1	12	29
lysine	14.7	14.2	8.1
histidine	2.0	1.9	1.8
arginine	5.6	6.3	6.3
aspartic acid	11.9	11.3	13.0
threonine	4.2	4.2	5.3
serine	3.9	3.9	3.8
glutamic acid	21.8	19.6	15.4
proline	2.0	1.5	7.5
cystine (1/2)	0.3	--	0.2
glycine	2.2	2.9	8.7
alanine	6.5	8.3	6.1
valine	4.1	4.8	4.5
methionine	--	0.9	0.4
isoleucine	4.3	4.5	3.8
leucine	11.3	9.9	7.7
tyrosine	2.6	2.0	4.1
phenylalanine	2.6	2.8	3.4

and CM stepwise elution batch procedures. Tables V and VI represent the amino acid compositions of ten different M proteins whose purification has been effected using hot trichloroacetic acid in the manner described.

These data indicate that the same amino acids, glutamic acid, aspartic acid, alanine, lysine, glycine and leucine account for the major portion of these protein preparations. This value has been calculated to be between 50.2 and 74.5% of the protein total amino acid composition. These values do not change markedly even among different peaks of the same chromatographic purification, when some fractions are more strongly M reactive than others. Table VII represents the amino acid analyses on the type 12 M protein obtained by cellulose purification employing DEAE and CM. As seen in Tables IX, X and XI, the last three fractions contain the most M reactivity, and it should be noted that the total basic amino acid composition increases from Fraction I to Fraction VI while the corresponding total acidic amino acid composition decreases. This also affected the staining patterns of the disc electrophoresis as seen later in Figure 10, since Procion dyes are known to bind covalently and most actively to free amino groups (13). Tryptophan determinations employing the procedures of Barman and Koshland (3) showed that no M protein preparation contained this amino acid.

TABLE V. AMINO ACID ANALYSES OF THREE DIFFERENT TYPE SPECIFIC M PROTEINS OBTAINED AS DESCRIBED (in mg/100 mg protein).

Amino Acid	Type 1			Type 12			Type 29		
	TCA Ext.*	HCl Ext. TCA Ppt.*	HCl Ext. NH_4SO_4 Frac.* TCA Ppt.	TCA Ext.	HCl Ext. TCA Ppt.	HCl Ext. NH_4SO_4 Frac. TCA Ppt.	TCA Ext.	HCl Ext. TCA Ppt.	HCl Ext. NH_4SO_4 Frac. TCA 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	--	--	--	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	--

*Abbreviations

Ext. - Extraction of whole heat killed cells.
Ppt. - Precipitation of lyophilized protein by 60% hot TCA.
Frac. - Fractionation of the crude protein extract by obtaining the fraction that precipitates between 30% and 60% ammonium sulfate saturation.

TABLE VI. AMINO ACID ANALYSES OF SEVEN M PROTEINS OBTAINED BY FRACTIONATION WITH AMMONIUM SULFATE AND PRECIPITATION WITH HOT TRICHLOROACETIC ACID (in mg/100 mg protein).

	Type						
Amino Acid	11	13	25	48	55	56	61
lysine	13.3	17.7	10.4	12.7	14.9	14.4	10.8
histidine	1.5	1.9	1.2	1.1	1.6	1.1	1.4
arginine	4.9	5.1	3.2	4.4	3.9	5.6	4.5
aspartic acid	10.4	10.9	12.5	12.6	12.7	10.4	13.8
threonine	4.8	4.6	5.4	5.7	4.7	4.7	5.7
serine	4.4	3.8	5.5	4.1	4.2	4.5	4.5
glutamic acid	18.0	19.9	18.5	15.9	22.4	17.0	18.2
proline	4.4	4.9	3.3	4.4	4.1	3.8	3.5
cystine (1/2)	0.2	0.1	0.3	0.1	0.1	--	0.6
glycine	5.4	3.7	4.8	4.7	4.0	4.8	5.1
alanine	8.8	6.3	9.3	10.6	8.2	9.6	9.6
valine	5.5	4.3	5.8	6.1	4.5	5.9	3.8
methionine	1.4	1.2	1.6	0.8	0.3	2.0	0.9
isoleucine	3.9	3.6	5.1	4.6	3.9	4.5	5.3
leucine	7.3	6.1	6.9	6.5	4.8	7.5	6.5
tyrosine	2.1	1.9	1.9	1.9	2.4	2.0	1.9
phenylalanine	2.8	2.8	3.0	3.0	2.5	2.2	3.4

TABLE VII. AMINO ACID ANALYSES ON TYPE 12 PROTEIN PREPARATIONS OBTAINED AS DESCRIBED IN TABLE IV (in mg/100 mg protein).

	Fraction					
Amino Acid	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 (1/2)	--	--	--	--	--	--
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	5.4	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

3. Amino acid sequencing. Ten mg of Fraction V M 12 protein (purified by DEAE and CM cellulose chromatography) was subjected to automated sequencing on a JAS-47K Sequence Analyzer. Only a single amino acid, alanine, was found when this protein was subjected to the first step in automated sequencing, indicating that only one protein is present. Tentative identification has given the following sequence: Ala-Leu-Asp-Leu-Phe-Ileu-Leu-?-Val, for the nine N-terminal amino acids.

B. Physical data.

1. UV absorption spectra. The data in Table VIII are the average values of the optical densities of the various protein preparations measured at 260 nm and 280 nm and their ratios. The M proteins obtained by a 30% to 60% ammonium sulfate fractionation of an acid extract of whole cells showed approximately 10% nucleic acid contamination. A 30% to 60% ammonium sulfate fractionation of an acid extract of purified streptococcal cell walls showed a 4% contamination with nucleic acid. M proteins purified by ammonium sulfate fractionation and CM cellulose chromatography and RNase, ammonium sulfate accompanied by DEAE and CM cellulose chromatography and RNase, and ammonium sulfate fractionation coupled with hot trichloroacetic acid precipitation were essentially free of nucleic acids.

TABLE VIII. NUCLEIC ACID DETERMINATIONS ON M PROTEIN PREPARATIONS.*

Method of Isolation	260 nm	280 nm	260/280 nm	280/260 nm	**mg protein/ mg nucleic acid
30% to 60% ammonium sulfate fractionation of acid extract from whole cells	0.54	0.38	1.420	0.704	.180/.020
30% to 60% annomium sulfate fractionation of acid extract from cell walls	0.35	0.32	1.093	0.915	.230/.011
CMC column chromatography on 30% to 60% ammonium sulfate fractionation of acid extract from cell walls (RNase treated)	0.34	0.35	0.972	1.030	.290/.009
DEAE and CMC batch chromatography on 30% to 60% ammonium sulfate fractionation of acid extract from whole cells (RNase treated)	0.37	0.50	0.740	1.350	.510/.005
TCA precipitation of 30% to 60% ammonium sulfate fractionation of acid extract from whole cells	0.32	0.34	0.942	1.062	.290/.008

* All values are averages of three representative protein preparations obtained by the methods described.

** These values are calculated based on the extinction coefficients for enolase and nucleic acid.

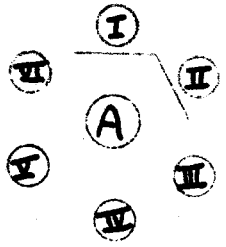
2. Chromatographic characteristics. When the 30% to 60% ammonium sulfate fraction of the M proteins acid extracted from cell walls or whole cells was applied to the CMC column at a pH of 4.0 in acetate buffer, the first peak emerging near the void volume consisted of residual nucleic acids, the group-specific antigen and other carbohydrates. At approximately a pH of 5.0, a second fraction was recorded, and this peak contained some M-reactivity and some non-specific protein. At a pH of between 5.5 and 6.0, a third peak emerged and was completely eluted by 6.5. This peak represented the M protein preparations that were used in all investigations involving CMC purified antigens.

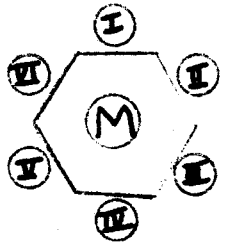
The results of the DEAE stepwise batch elution on the 30% to 60% ammonium sulfate saturated fractions showed (upon Ouchterlony analysis vs. group and type specific antisera) that the group A activity was found in the first two fractions while the M protein activity could be found in all six fractions (Table IX). When the first two fractions were rechromatographed by a stepwise CM batch elution, Ouchterlony analysis vs. group and type specific antisera indicated that the group activity and type activity were effectively separated, with the group activity being found in Fraction I while the major M-protein peaks were found in Fractions IV, V and VI (Table X). When the M-positive, A-negative fractions obtained from the prior

TABLE IX. BATCH DEAE CELLULOSE PURIFICATION OF 415 mg TYPE 12 M PROTEIN.

Fraction	Starting pH	Buffer Molarity	Eluted pH	Recovery (mg)	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+
				TOTAL 206		
				(PERCENT) 50		

OUCHTERLONY ANALYSIS



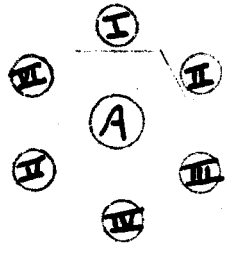


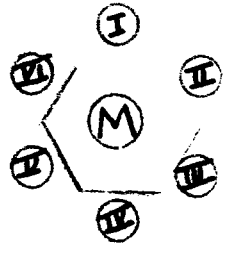
A = Anti-group A antiserum
M = Anti-type 12 antiserum

TABLE X. BATCH CM CELLULOSE PURIFICATION OF FRACTIONS I & II FROM DEAE CELLULOSE (150 mg protein).

Fraction	Starting pH	Buffer Molarity	Eluted pH	Recovery (mg)	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+
			TOTAL	75		
			(PERCENT)	50		

OUCHTERLONY ANALYSIS





A = Anti-group A antiserum
M = Anti-type 12 antiserum

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 XI). However, the major proteins peaks were found to be eluted from the CM at a pH between 5.5 and 6.5.

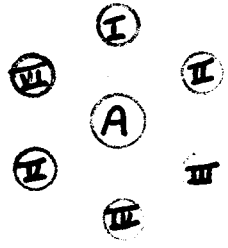
3. Molecular weights. The standard curve for direct estimation of molecular weights in the SDS-polyacrylamide gel system is represented in Figure 1. Rf and range of three determinations are plotted for each of the standards and for three determinations each of the cellulose purified and trichloroacetic acid precipitated M proteins. In this system, the cellulose purified M proteins contained two bands, with estimated molecular weights of 30,000 (band 2) and 23,000 (band 1). The stainable protein was distributed approximately equal between the two bands. The TCA precipitated proteins contained one broad band with an estimated molecular weight of 23,000.

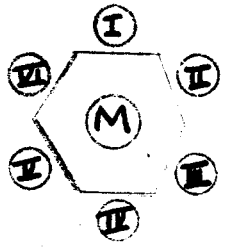
The standard curve for direct estimation of molecular weights in the Sephadex G-200 system is represented in Figure 2. The values plotted are the K_{av} and the molecular weight of the proteins. In this system, the cellulose purified M proteins contained two peaks, with estimated molecular weights of 33,000 (band 2) and 23,000 (band 1). The size of the two peaks was approximately equal. The TCA precipitated M proteins contained only one peak with an estimated molecular weight of 23,000.

TABLE XI. BATCH CM CELLULOSE PURIFICATION OF COMBINED FRACTIONS III THROUGH VI FROM DEAE CELLULOSE AND FRACTIONS III THROUGH VI CM CELLULOSE (100 mg TOTAL).

Fraction	Starting pH	Buffer Molarity	Eluted pH	Recovery (mg)	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+
				TOTAL 39		
				(PERCENT) 39		

OUCHTERLONY ANALYSIS





A = Anti-group A antiserum
M = Anti-type 12 antiserum

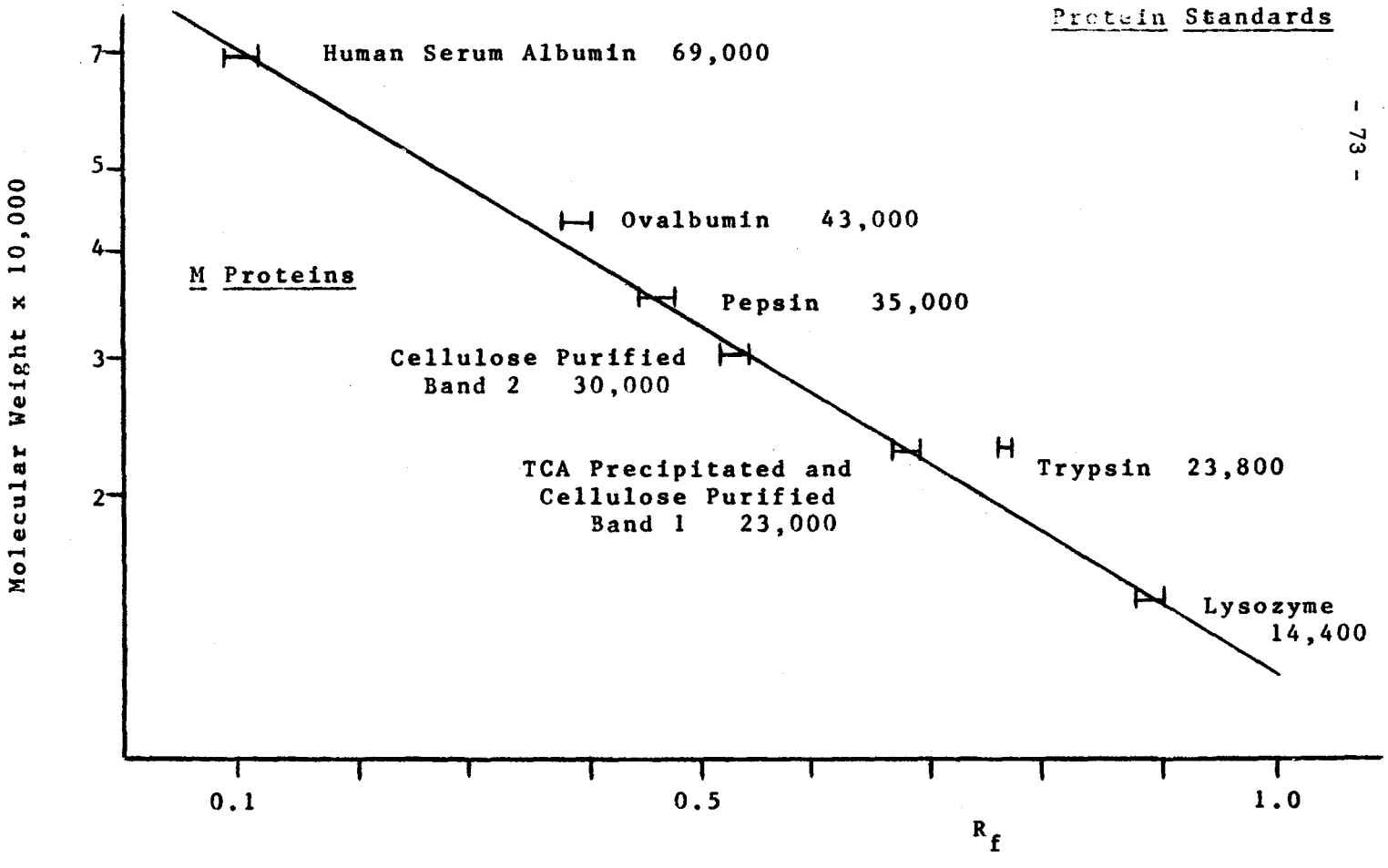


Fig. 1. Estimation of molecular weights by electrophoresis of proteins in SDS-acrylamide gels.

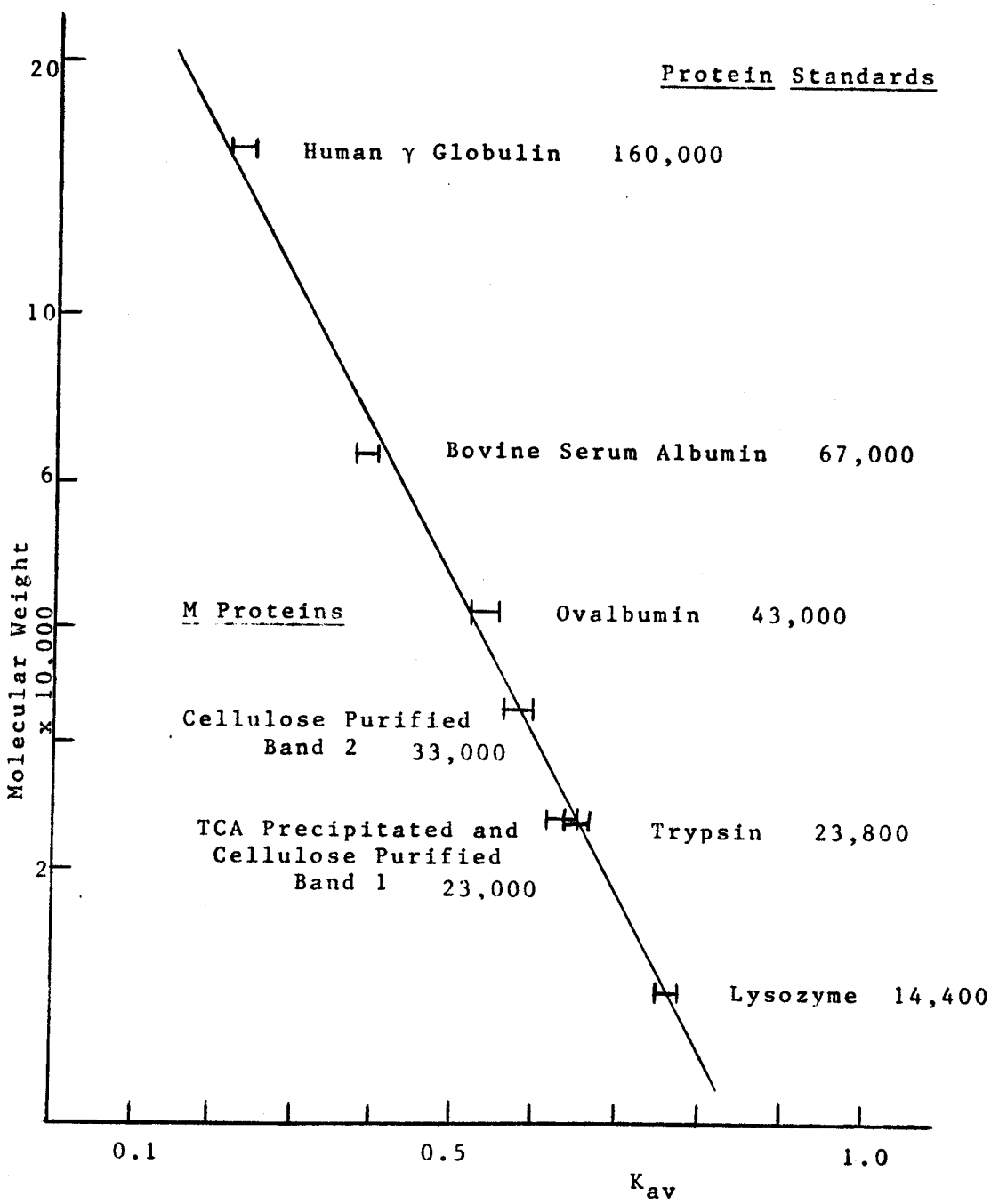


Fig. 2. Estimation of molecular weights by chromatography of proteins on Sephadex G-200.

4. Peptide mapping. Tryptic hydrolysates of the DEAE and CM cellulose purified M_1, M_{12} and M_{29} proteins were "fingerprinted." The tryptic maps of M_1, M_{12} and M_{29} indicated 42, 41 and 37 peptides respectively. This reflects and is consistant with the high concentration of lysine and arginine in M_1 and M_{12}, and the lower concentrations of the same amino acids in the M_{29} preparation (Table IV). Figure 3 represents the peptide map of the DEAE and CM batch purified M_{12} protein after chromatographic and electrophoretic separation of the tryptic digest. Figure 4 represents the peptide map of the M_{29} cellulose purified protein. The photographs show that the two peptide maps are quite similar. The peptide map of the M_1 protein showed a similar pattern while yielding more peptides than either the M_{12} or M_{29} protein.

Chymotryptic hydrolysates of the DEAE and CM cellulose purified M_1, M_{12} and M_{29} proteins were also "fingerprinted." The chymotryptic maps of M_1, M_{12} and M_{29} indicated 9, 8 and 12 peptides respectively. This reflects the higher concentration of tyrosine and phenylalanine in the M_{29} protein and the somewhat lower content of these same amino aicds in the M_1 and M_{12} preparations. Figure 5 represents the peptide map of the DEAE and CM batch purified M_{12} protein after chromatographic and electrophoretic separation of the chymotryptic digest. Figure 6 represents the peptide map of the M_1 cellulose

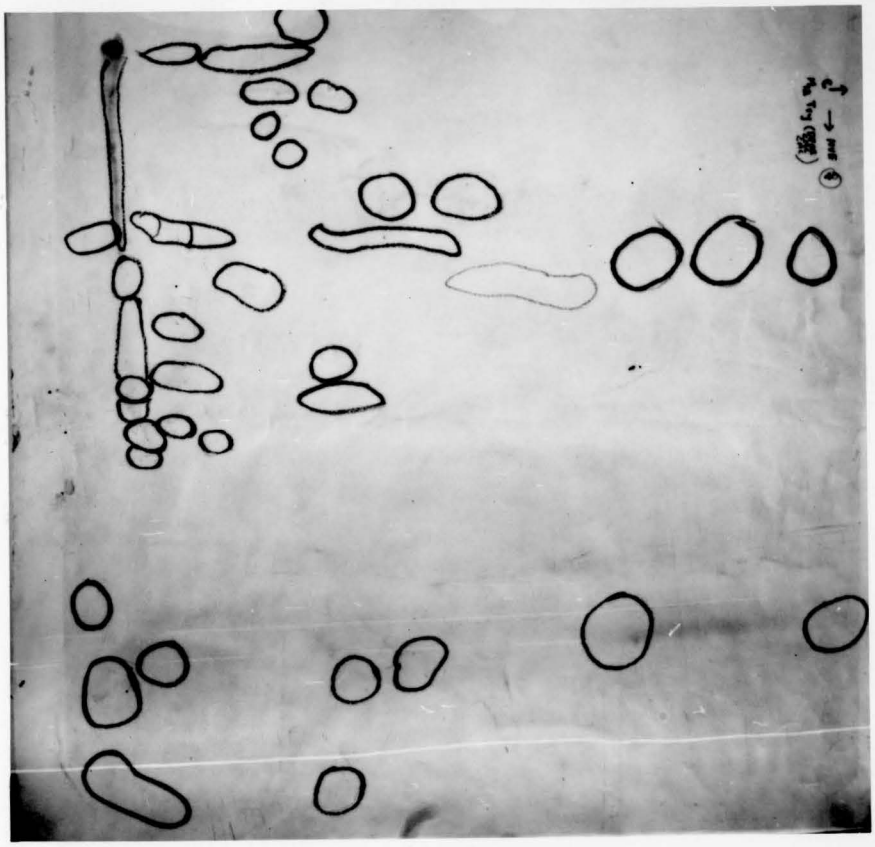


FIGURE 3. Peptide map of the DEAE and CM "batch" purified M_{12} protein (Fraction V) after chromatographic and electrophoretic separation of the tryptic digest. Forty-one peptides were observed. Chromatography was performed in the direction horizontal and to the right while high voltage electrophoresis was run vertical and downward.

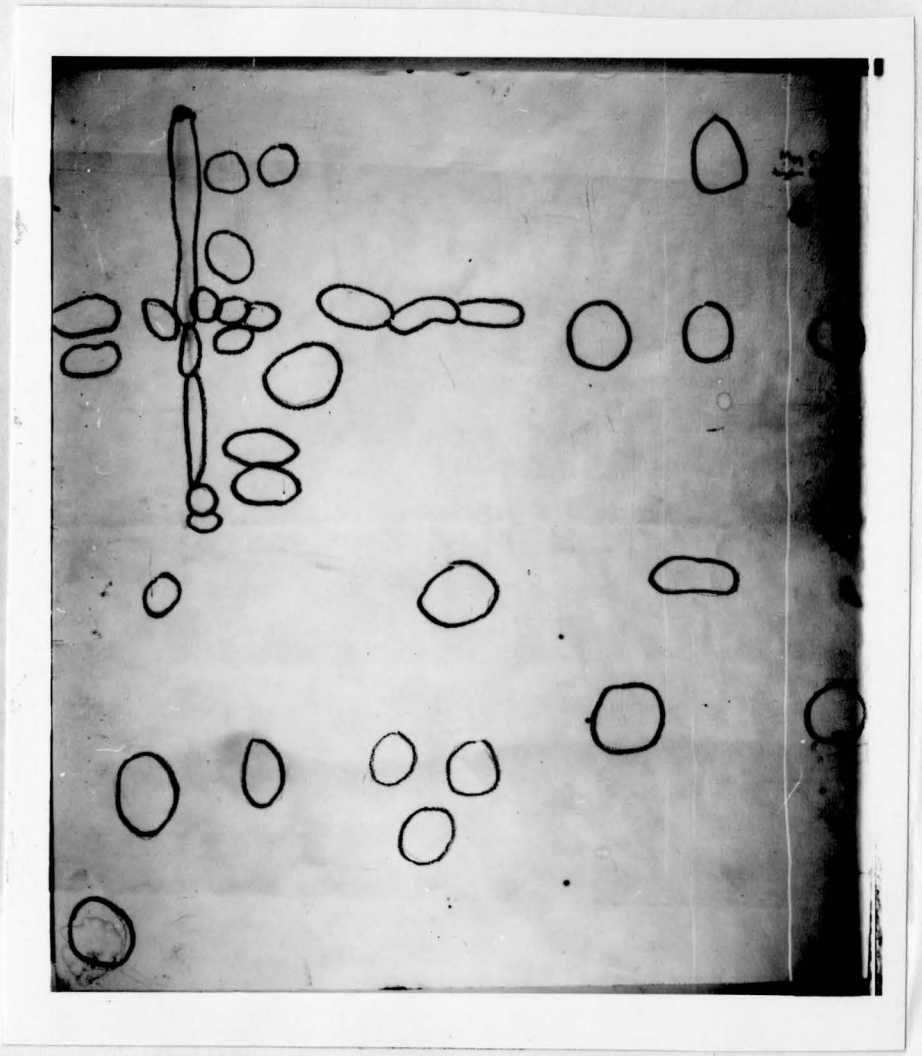


FIGURE 4. Peptide map of the DEAE and CM "batch" purified M_{29} protein (Fraction V) after chromatographic and electrophoretic separation of the tryptic digest. Thirty-seven peptides were observed here but the overall pattern is quite similar to that of M_{12}. Alignment is the same as in Fig. 3.

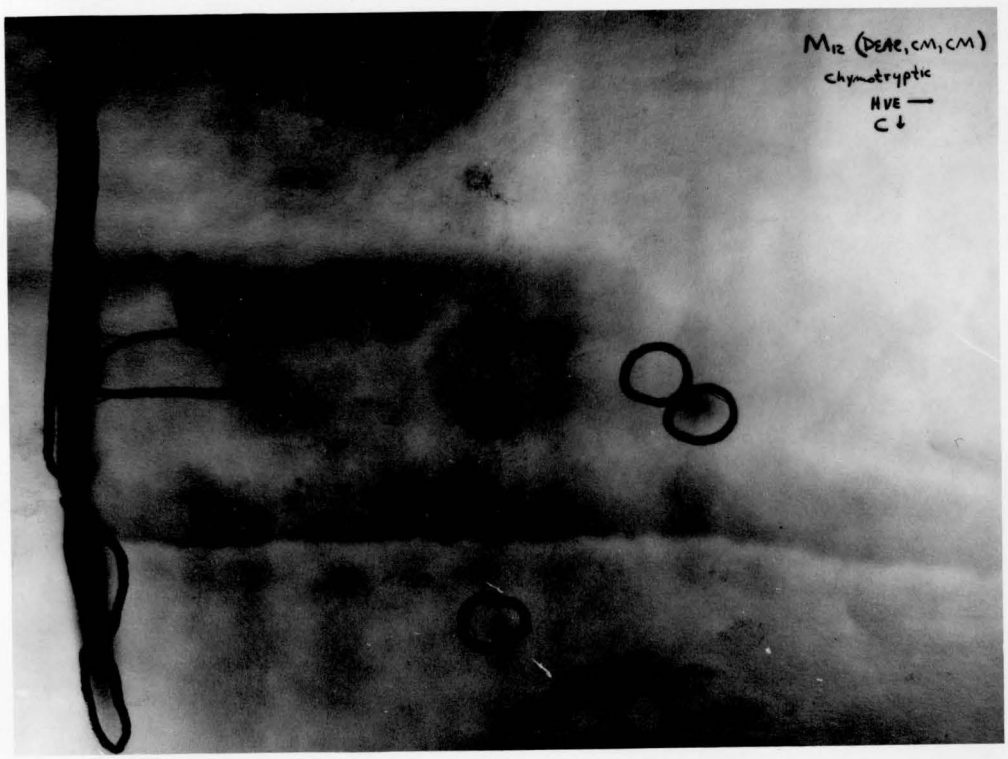


FIGURE 5. Peptide map of the DEAE and CM "batch" purified M_{12} protein (Fraction V) after chromatographic and electrophoretic separation of the chymotryptic digest. Eight peptides were observed. Chromatography was performed in the direction vertical and down while high voltage electrophoresis was run horizontal and to the right.

purified protein. The peptide map of the M29 protein showed a similar pattern while yielding more peptides than either the M_1 or M_{12} proteins.

5. Acrylic gel (disc) electrophoresis. Polyacrylamide gel electrophoresis were performed on protein preparations purified by the various methods employed in this study. The gel disc electrophoretic studies performed on all proteins except those precipitated by hot trichloroacetic acid, showed them to be alike in that they are all very heterogeneous and the mobility of their fastest moving bands were similar. Each serotype appeared to have an otherwise characteristic pattern of bands. The three Voorhout strains purified by a 30% to 60% ammonium sulfate fractionation all appeared to have similar banding patterns but were quite different as compared to the Hektoen type 12 strain purified by a 30% to 60% ammonium sulfate fractionation or the M_{12} protein purified by carboxymethyl cellulose (Figure 7). The banding patterns exhibited by types 4, 6 and 12 purified by 30% to 60% ammonium sulfate fractionation or ammonium and CMC chromatography were dissimilar (Figure 8). Figure 9 represents the gel disc electrohporetic studies on group A streptococcal M proteins purified by a 30% to 60% ammonium fractionation and CMC column chromatography. They demonstrate, once again, the usual heterogeneity of banding as well as a distinct pattern for each serotype.

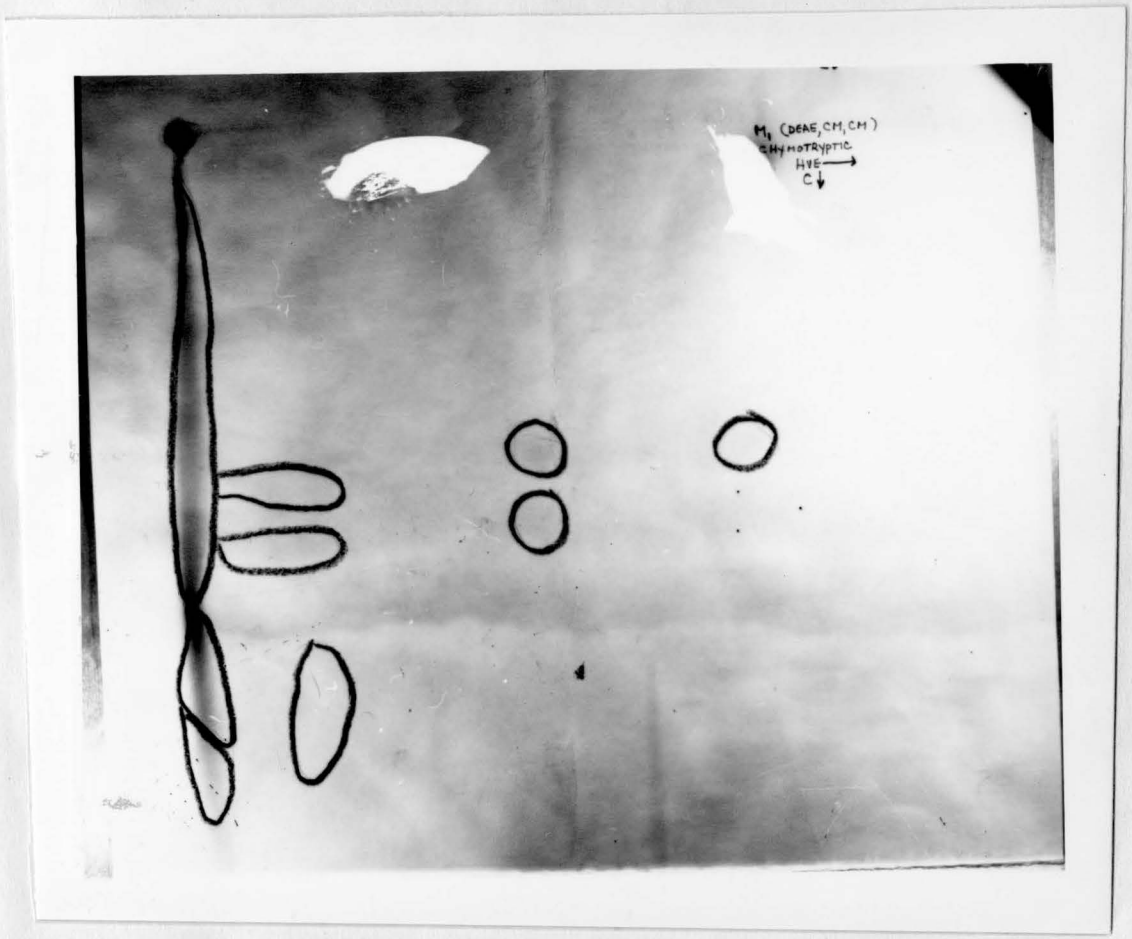


FIGURE 6. Peptide map of the DEAE and CM "batch" purified M_1 protein (Fraction V) after chromatographic and electrophoretic separation of the chymotryptic digest. Nine peptides were observed here but the overall pattern is quite similar to that of M_{12}. Alignment is the same as in Fig. 3.

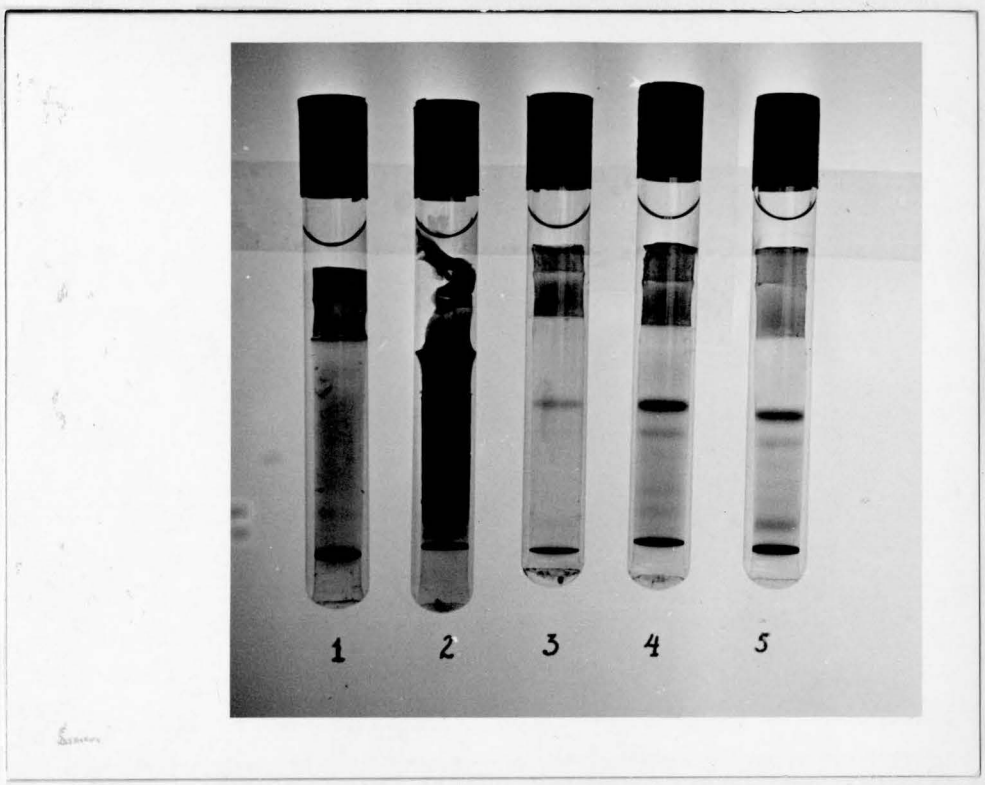


FIGURE 7. Disc electrophoretic studies performed on various strains of type 12 group A streptococcal M protein purified by ammonium sulfate fractionation alone or accompanied by CMC column chromatography. Each disc electrophoresis employed 300 µg of protein and was run at a pH of 8.3. The proteins were stained with Buffalo-black. The proteins in gels 1, 3, 4 and 5 were purified by ammonium sulfate fractionation. Gel 2 was purified by the additional method of CMC column chromatography. Gels 3, 4 and 5 represent related or Voorhout strains and show very similar banding patterns. The proteins represented in gel 1 were obtained in exactly the same manner as the Voorhout strains but exhibited a different banding pattern. The proteins purified by CMC chromatography are seen to contain many components that are not visible in the ammonium sulfate-purified proteins.

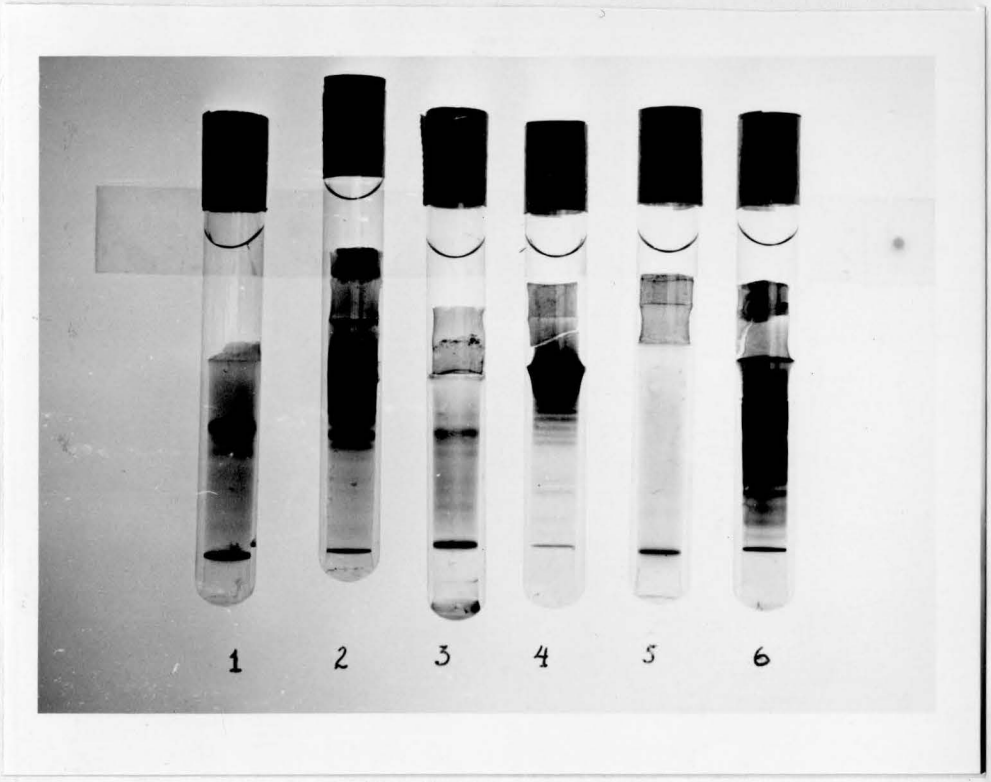


FIGURE 8. Disc electrophoretic studies on types 4, 6 and 12 group A streptococcal M proteins purified by ammonium sulfate or ammonium sulfate and CMC column chromatography. The conditions for electrophoresis and staining were as in Fig. 7. The odd-numbered gels represent ammonium sulfate-purified proteins, and the even-numbered gels represent proteins purified by the additional step of CMC chromatography. Gels 1 and 2 represent type 4 M protein. Gels 3 and 4 represent type 6, and gels 5 and 6 represent type 12. As in Fig. 7, it can be seen that the CMC-purified proteins contain many components not visible in the proteins purified by ammonium sulfate fractionation. These findings were duplicated on immunoelectrophoretic analysis wherein gels 1, 3 and 5 showed the type-specific protein to be associated with the fast-moving band and in gels 2, 4 and 6 the type-specific proteins were distributed throughout the gel.

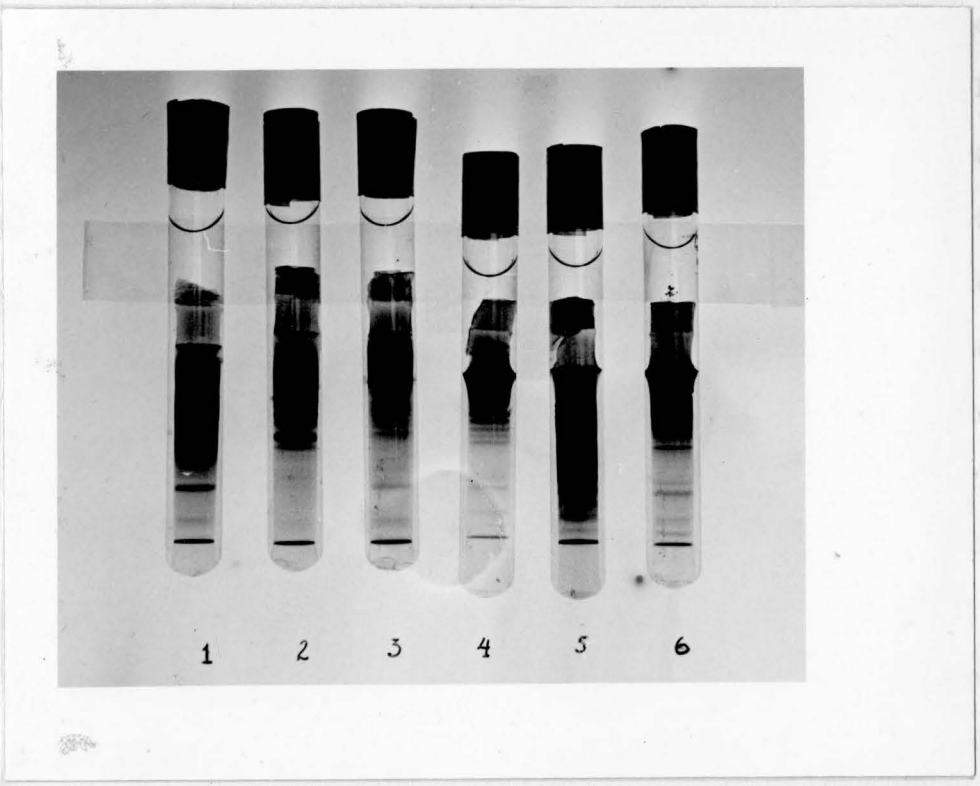


FIGURE 9. Disc electrophoretic studies on group A streptococcal M proteins purified by a 30% to 60% ammonium sulfate fractionation and CMC column chromatography. They represent, from left to right, types 3, 4, 5, 6, 12 and 14. The conditions for electrophoresis and staining were as in Fig. 7. The heterogeneity of each preparation is evident as well as the distinct patterns of banding.

Figure 10 represents the disc electrophoreses of the six fractions from the final CMC purification performed on the type 12 M proteins (Tables VII and XI). The heavily concentrated protein areas in gels 4, 5 and 6 seem to reflect an increase in the basic amino acid content (lysine and arginine) as well as an increase in M-reactivity (Table XI). 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 titre of M protein activity and these fractions were considered to be the most pure protein preparations. The banding pattern for the M proteins after exposure to hot 60% trichloroacetic acid is altered dramatically as seen by a vast reduction in the number of bands upon polyacrylamide gel disc electrophoresis. Figure 11 represents the acrylamide gel disc electrophoresis of three different M proteins purified by a 30% to 60% ammonium sulfate fractionation and treatment with hot TCA. Each gel shows only one major protein band with some trailing afterward.

The disc electrophoretic patterns of the aliquots of the three times acid extracted preparations of whole cells is shown in Figure 12. A distinct change is evident in the mobilities of the individual extracts as compared one to the other and to a mixture of the complete extract. These protein preparations showed decreasing concen-

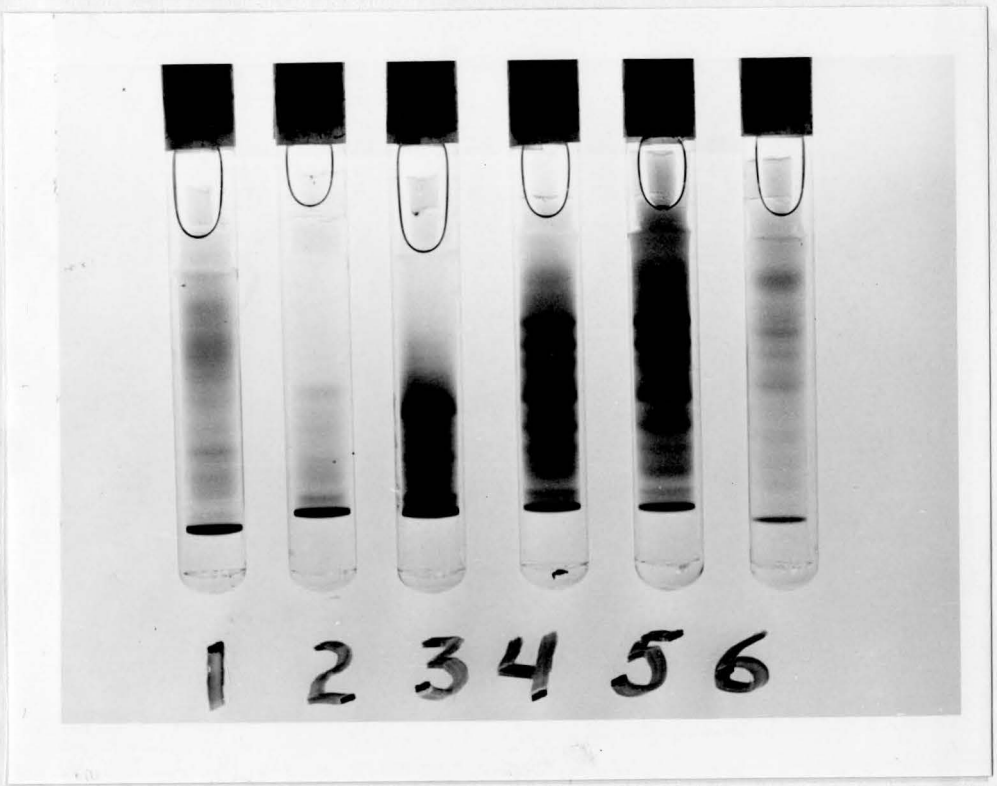


FIGURE 10. Disc electrophoretic studies performed on six M_{12} protein preparations purified by DEAE and CM stepwise elution batch procedures. Each disc electrophoresis employed 300 µg of protein and was run at a pH of 8.3. The proteins were stained with the Procion dye Coamassie Blue. The heavily concentrated protein areas seem to reflect an increase in the basic amino acid content (lysine and arginine).

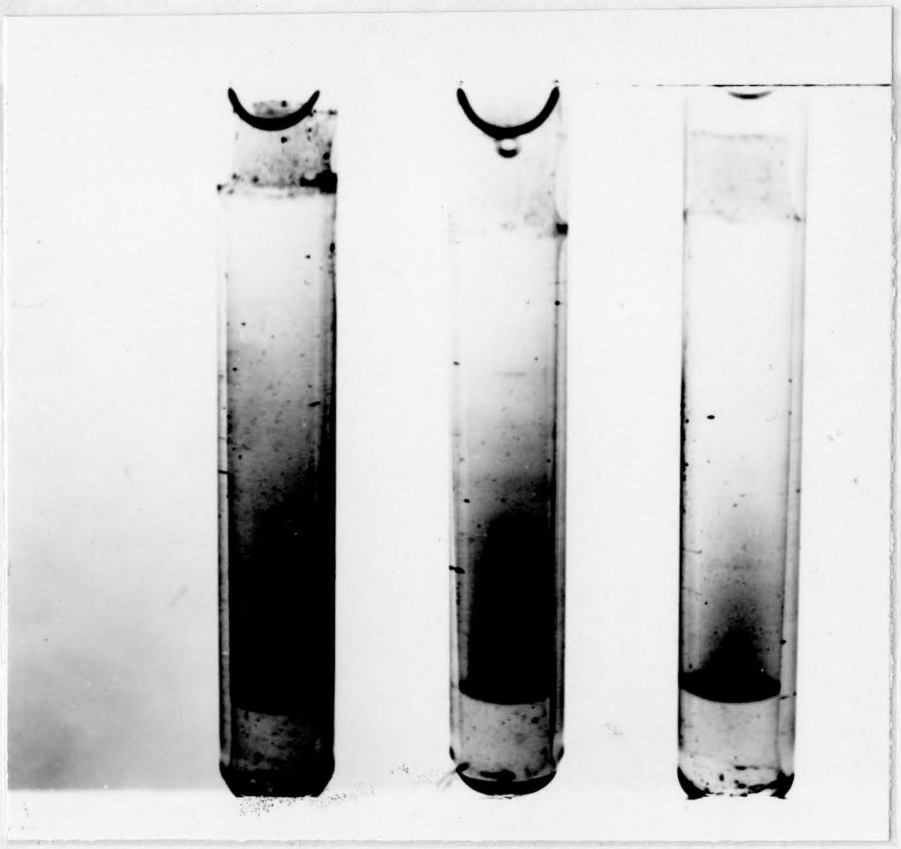


FIGURE 11. Disc electrophoretic studies performed on the three different type specific M-proteins extracted from whole cells and purified by ammonium sulfate fractionation and 60% TCA precipitation. Each disc electrophoresis employed 300 mg of protein and was run at a pH of 8.3. The proteins were stained with the Procion dye Coomassie Blue. The gels represent M-proteins, types 1, 12 and 29 from left to right.

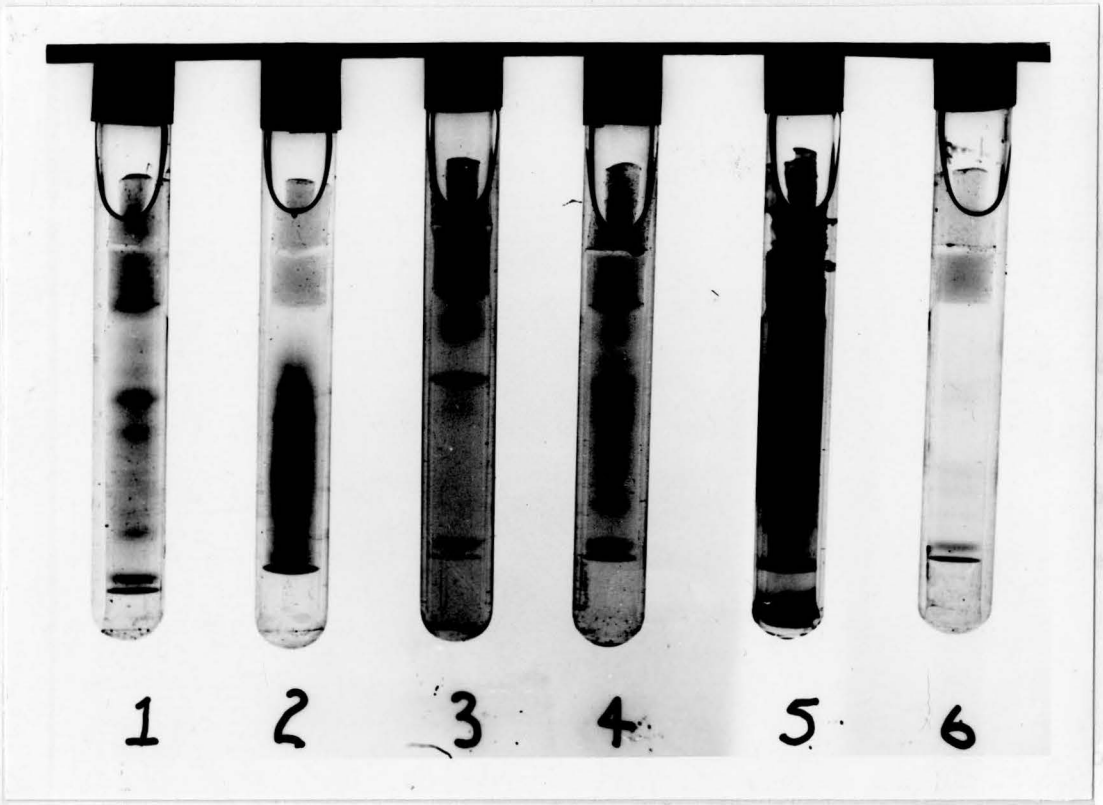


FIGURE 12. Disc electrophoresis on the three times hot acid extracted M_4 protein. Gels 1, 2 and 3 are the three respective extracts. Each disc contains 300 µg of protein and was run at a pH of 8.3. Gel 4 is the combined extracts, gel 5 is the 30% to 60% ammonium sulfate fraction and gel 6 is the hot TCA precipitated M protein. The gels were stained with Procion dye Coomassie Blue.

trations of ammonium (amide nitrogen) on amino acid analysis. That is, each subsequent extraction mixture contained less amide nitrogen than its predecessor. Once again, the hot TCA treated proteins showed less banding than any other preparation.

6. Extinction coefficient $_{280}E_{1\%}$. The low values of tyrosine content were reflected in the determination of the extinction coefficient for each M protein (Table XII). In general, TCA precipitated M proteins had the highest extinction coefficient with an average of 5.60. CMC column purified M protein had an average $_{280}E_{1\%}$ of 3.58 with the DEAE and CMC batch purified proteins having the lowest extinction coefficients at an average of 3.40.

C. End group analyses. The FDNB reaction was first performed on the 30% to 60% ammonium sulfate fraction of the M protein extracted from the type 12 Hektoen strain to substantiate the results of Lange et al. (66) DNP-alanine proved to be the only detectable amino acid spot in the 0 to 30% ammonium sulfate fraction as well as in the 30% to 60% fraction. The same result was obtained when the FDNB reaction was performed on whole cell or whole cell wall preparations. This result was then confirmed on all other M proteins examined where in each case, the N-terminus amino acid was found to be L-alanine (Tables XIII, XIV and XV).

TABLE XII. EXTINCTION COEFFICIENTS FOR M_1 PROTEINS PURIFIED BY VARIOUS TECHNIQUES.[a,b]

Method of Isolation	Undiluted	1:2	1:3	1:6	Average $^{280}E_{1\%}$
CMC column chromatography on 30% to 60% ammonium sulfate fractionation of acid extract from cell walls (0.88 mg protein per ml BBS) (RNase treated)	0.310	0.160	0.11	0.05	3.58
DEAE and CMC batch chromatography on 30% to 60% ammonium sulfate fractionation of acid extract from whole cells (RNase treated - 1 mg protein/1 ml BBS)	0.34	0.18	0.10	0.06	3.40
TCA precipitation of 30% to 60% ammonium sulfate fractionation of acid extract from whole cells (1 mg protein/ 1 ml BBS)	0.62	0.30	0.16	0.09	5.60

[a] All OD's read at 280 nm.

[b] All values were an average of three M_1 protein preparations.

TABLE XIII. N-TERMINAL AMINO ACID ANALYSES ON AMMONIUM SULFATE PURIFIED M-PROTEIN PREPARATIONS.

Type	Identification No.	N-terminal amino acid
1	T1/195/1P2	L-alanine
4	R68/1115 Matt	L-alanine
6	SS 596	L-alanine
12	Hektoen	L-alanine
24	Brassilia	L-alanine
29	1759	L-alanine
57	2015403	L-alanine
12a	41448/var.	L-alanine
12b	R66/3630 gl.	L-alanine
12c	R66/3630 matt	L-alanine

TABLE XIV. N-TERMINAL AMINO ACID ANALYSES ON AMMONIUM SULFATE AND CELLULOSE PURIFIED M-PROTEIN PREPARATIONS.

Type	Identification No.	N-terminal amino acid
3*	3515-210P	L-alanine
4*	3407-200P	L-alanine
5*	3515-103P	L-alanine
6*	3515-101P	L-alanine
12*	3407-242P	L-alanine
14*	3515-165P	L-alanine
1**	T1/195/1P2	L-alanine
12**	Hektoen	L-alanine
29**	1759	L-alanine

* Purified by a 30% to 60% ammonium sulfate fractionation plus CMC column chromatography.

** Purified by a 30% to 60% ammonium sulfate fractionation plus DEAE and CMC batch chromatography.

TABLE XV. N-TERMINAL AMINO ACID ANALYSES ON AMMONIUM SULFATE AND TCA PRECIPITATED M-PROTEIN PREPARATIONS.

Type	Identification No.	N-terminal amino acid
1	T1/195/1P2	L-alanine
11	100058	L-alanine
12	Hektoen	L-alanine
13	SS 31	L-alanine
25	100088	L-alanine
29	1759	L-alanine
48	100075	L-alanine
55	2004	L-alanine
56	SS-743	L-alanine
61	R69/2714	L-alanine

To evaluate the efficiency of the alumina binding of free amino acids, attempts were made at elution and quantitation. It was established that 2% acetic acid would elute the amino acids from the alumina. The amino acid analyses of the acetic acid (2%) eluates of the alumina columns showed constant recovery (75% to 80%) of all the free amino acids. Unexpectedly, however, the per cent recovery of free L-alanine did not deviate noticeably from the percent recovery of the other free amino acids. After the FDNB reaction, most of the lysine was shown to have disappeared as the ε-DNP derivative. The concentrations of aspartic acid and threonine were also found to be diminished, and it was discovered that these two amino acids bind to the alumina column and can only be eluted with 1.0 N HCl. The two by-product artifacts from DNP reaction were separable on the amino acid analyzer. One ninhydrin-positive compound that appeared was coincident with the arginine peak, and the other, a ninhydrin-negative compound, was coincident with the tyrosine peak.

D. Immunochemical determinations. Although it could be established that antisera could be type specific reacting with only a single M protein, the protein itself was not necessarily a single homogeneous material. In most cases, the M protein was more or less a family of proteins, all of which could react with its specific typing serum.

This phenomenon was seen under many experimental conditions. Immunodiffusion studies on the protein preparations showed M-reactivity could exist at any location along the entire length of the gel contiguous to the heavily concentrated protein bands (Figures 13, 14 and 15). Figure 13 represents disc electrophoretic separations on M proteins type 3, 6 and 12 purified by a 30% to 60% ammonium sulfate fractionation accompanied by a CMC column chromatographic isolation. Figure 14 represents immunoelectrophoretic (IEP) separation performed on the final six fractions isolated from the second CMC chromatographic fractionation on the type 12 M protein (Table XI). This photograph shows that M activity could be seen in many areas of the electrophoretic separation in Fractions IV, V and VI. This was demonstrated in another fashion as seen in Figure 15 where Fraction V of the type 12 M protein was subjected to disc electrophoresis and fractions were isolated by transverse sectioning of the gels (50 mm in length) into ten pieces. Each slice was aligned next to a trough and an unstained whole gel was 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 were serologically identical. The most important observation here is, regardless of the number of bands in the disc electrophoresis, only one line was obtained on Ouchterlony

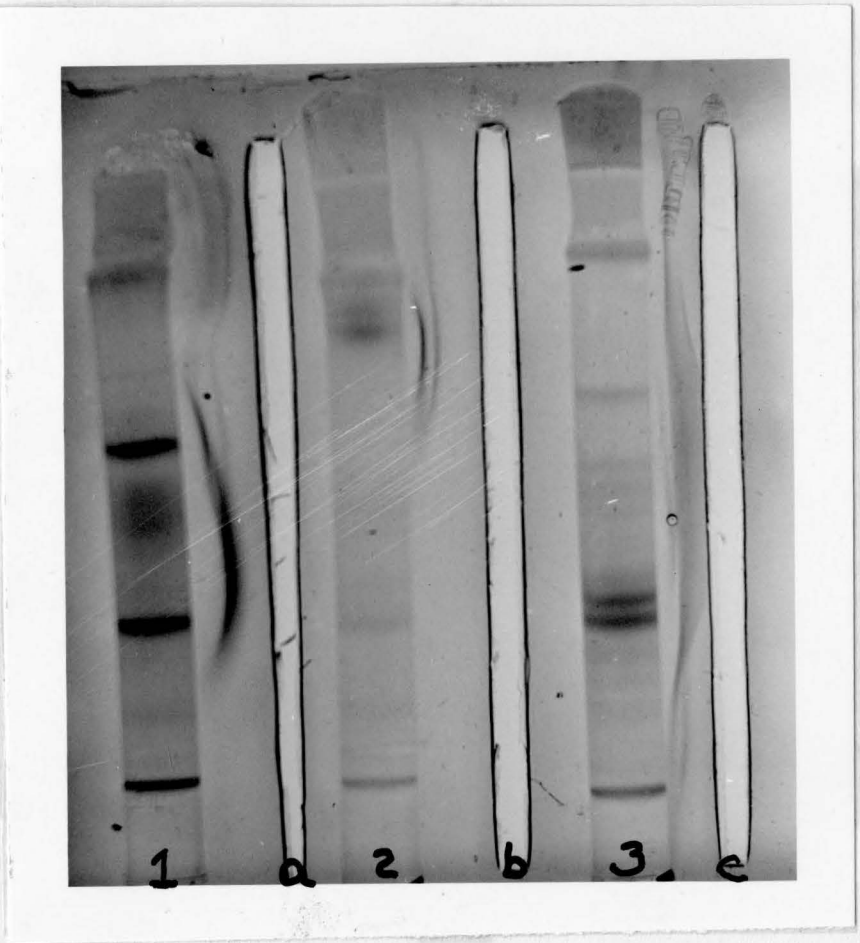


FIGURE 13. Disc electrophoretic separations were run in duplicate on CMC purified types 3, 6 and 12 M proteins. Conditions were as in Fig. 7. Three gels were stained as specified, and three were layered on an agar-coated slide next to troughs containing the respective antisera. The figure is a composite where after diffusion had occurred (24 hr.) the unstained gels were removed and the respective stained gels placed in position for photography. Type 3 M protein and its antisera are identified by 1a: types 6 and 12 as 2b and 3c, respectively. No evidence of cross-reaction between types was noted.

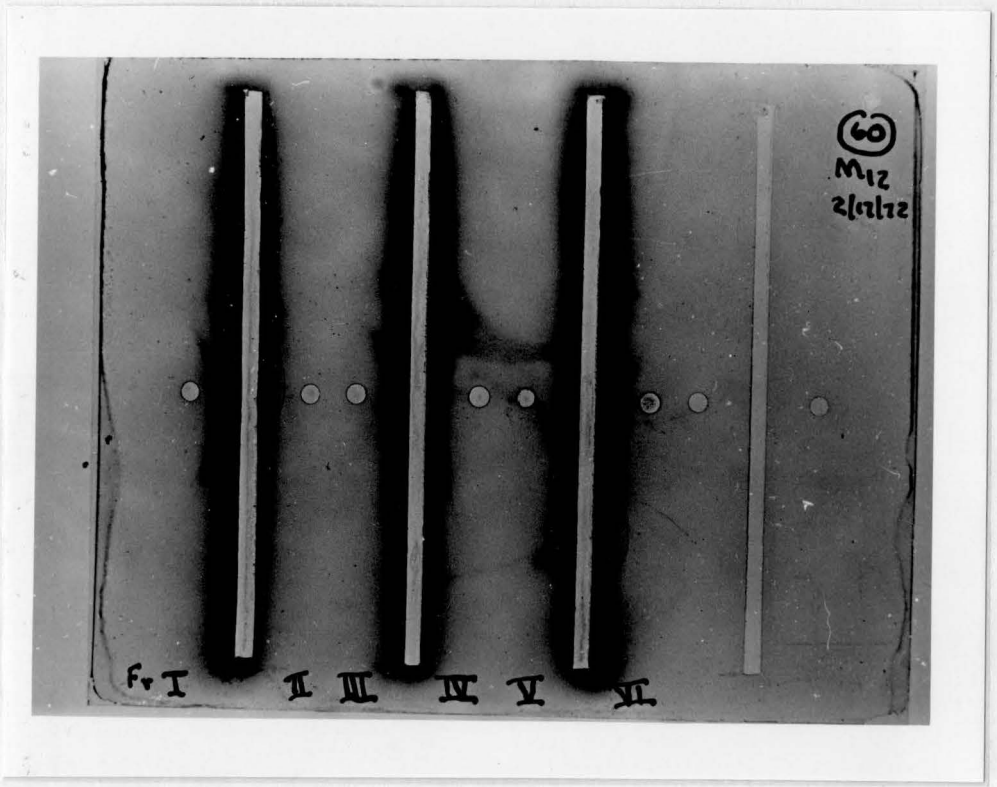


FIGURE 14. Immunoelectrophoresis performed on six M_{12} protein preparations purified by DEAE and CM stepwise elution batch procedures. 300 µg of protein was placed in each antigen well and was run at a pH of 8.6. The slide was stained with the dye Napthalene Black 12B.

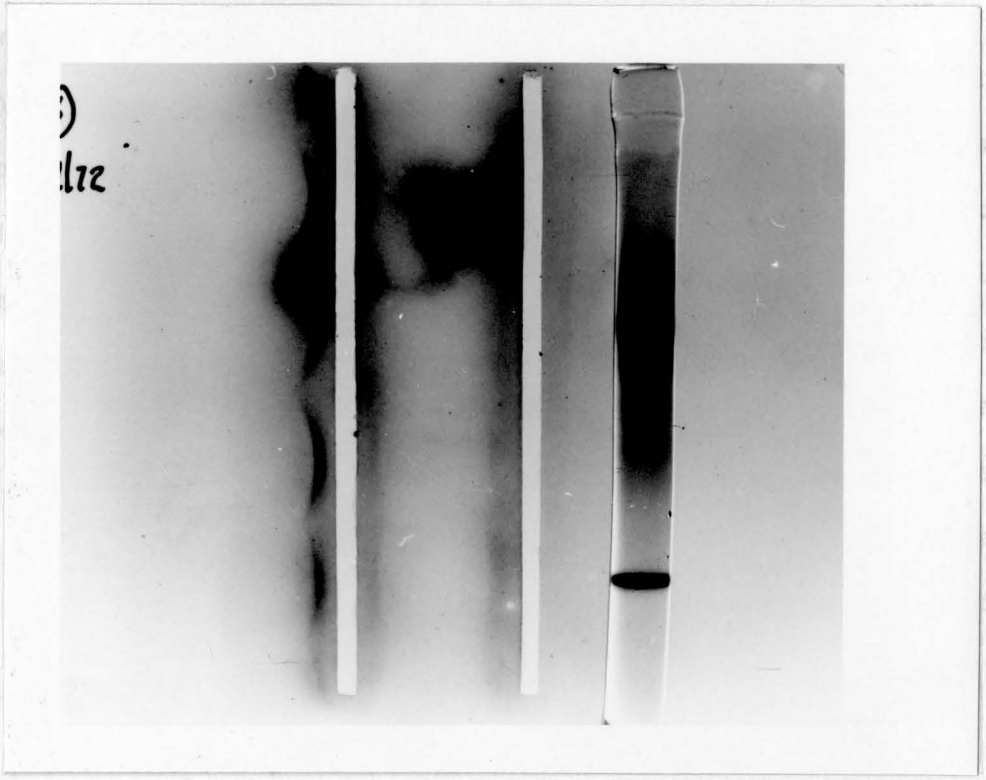


FIGURE 15. Gel disc electrophoretic separations were run in triplicate on M_{12} proteins purified by DEAE and CM. The conditions were as in Fig. 7. One gel was sliced into ten sections and aligned next to a trough containing the respective type specific antisera. Another unstained gel was laid next to another trough containing the same antisera. The unstained gel was removed (24 hr.) and the respective stained gel was placed into position for photography. The gel in this photograph was stained with Buffalo Black.

analysis (Figure 16). Once again Fractions II and III gave the weakest precipitin lines whereas Fractions I, IV, V and VI were the high M containing preparations. The Ouchterlony and IEP analyses on the different M proteins purified by a 30% to 60% ammonium sulfate fractionation and treatment with 60% hot TCA showed that the one major protein band possessed all the M-activity in the protein preparation. This band was shown to react strongly with its type specific antisera as seen in Figure 17, where the M_{29} protein was subjected to disc electrophoresis in duplicate. One gel was stained (Figure 11) 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.

Proteolytic enzymes such as trypsin and chymotrypsin were shown to completely destroy the immunospecificity of the M proteins. This destruction appeared to begin as soon as the M proteins were exposed to the enzymes, although final degradation was not complete until after 120 minutes of exposure time. Figure 18 shows the results after 5 mg of DEAE and CM cellulose purified M_{12} protein has been exposed to insoluble trypsin (50 units) for three hours. Aliquots were removed at 0 time, 30 minutes, 60 minutes, 120 minutes and 180 minutes and examined for M-reactivity by immunodiffusion.

FIGURE 16. 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.

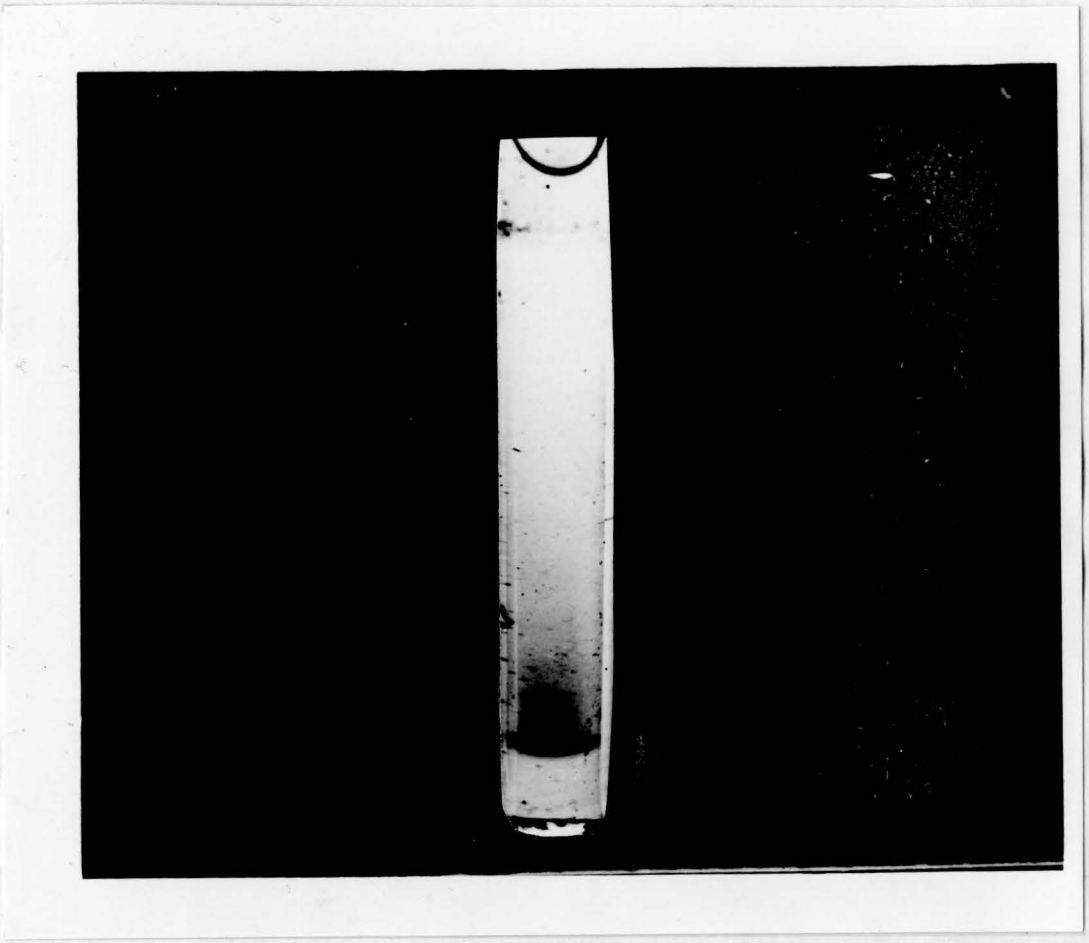


FIGURE 17. A disc electrophoretic separation was run in duplicate on the M_{29} protein represented in Figure 11. One gel was stained (Figure 11) and one was layered on an agar-coated slide next to a trough containing the respective type specific antisera. The figure is a composite where after diffusion had occurred (24 hr.) the unstained gel was removed and the respective stained gel placed in position for photography.

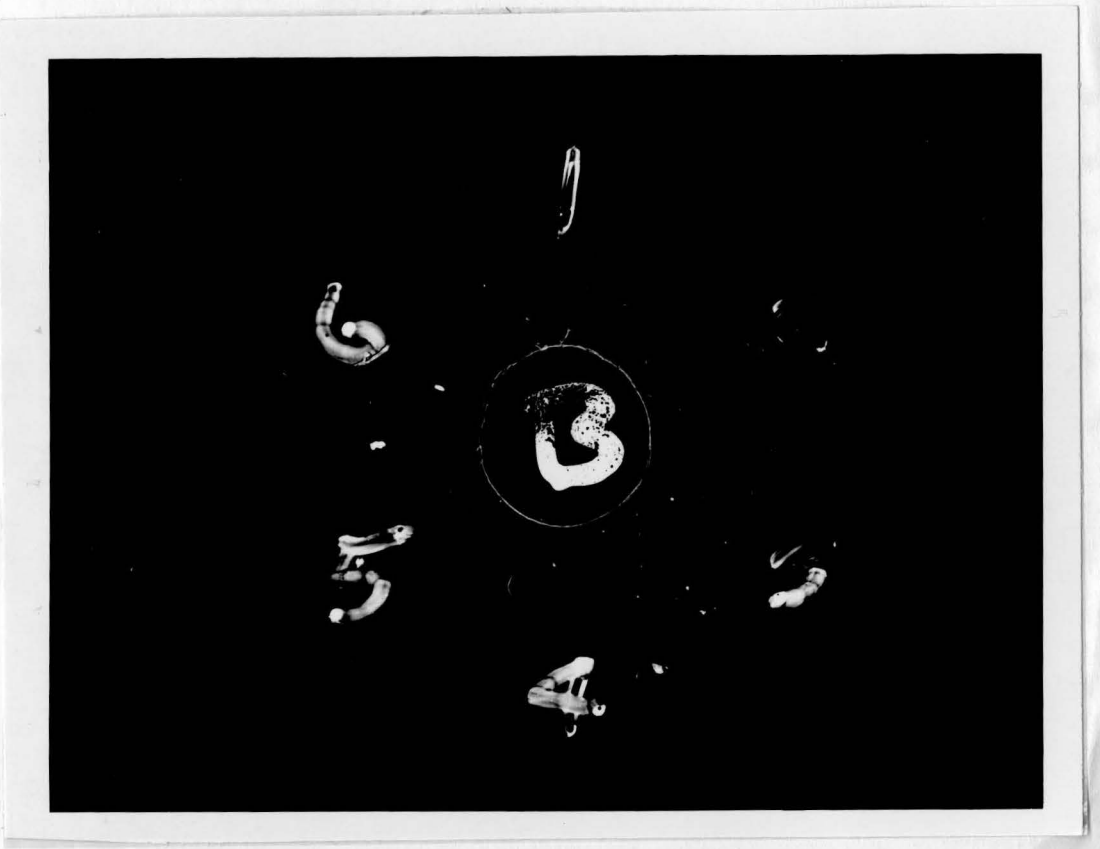


FIGURE 18. Five mgs of M_{12} protein purified by a DEAE and CM cellulose fractionation procedure were treated with 50 units of insoluble trypsin. The reaction was run for 3 hours at a pH of 8.1. The antiserum well, B, contained type specific antisera. Antigen well 1 contained the untreated M proteins. Wells 2, 3, 4, 5 and 6 represent aliquots taken at 0 time, 30 minutes, 60 minutes, 120 minutes and 180 minutes respectively.

E. Biological activity of M proteins.

1. Antigenicity in rabbits. Each purified M protein showed strong precipitin activity with its homologous serum prepared with either whole cell vaccine or purified proteins. At least two rabbits per type specific M protein were injected via the intravenous route with whole heat killed cells or intradermal and intramuscular with a purified protein preparations. As was expected, there was not only variation in the immune response of the animals to different serotypes but to the same serotype as well. Upon completion of the basic immunization schedule, all animals that had been injected with whole heat-killed cells types 1, 12 and 29 had developed good to excellent titres. During the one month "rest" periods, titres dropped to both the group A antigen as well as the M antigens. After this rest period, and the injection (intramuscular) of purified M proteins in complete Fruend's, the titre of anti-M antibody was sufficiently high to warrant exsanguination of the animals. There were considerable cross-reactions with these unadsorbed serums, when they 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. This

one-way cross-reaction between M_1 protein and the M_{29} antiserum was also reflected in the passive hemagglutination and long chain studies performed on antisera prepared against purified protein alone.

Pepsinization was seen to cause a marked decrease in the amount of coat protein on the intact cell, as followed by the indirect fluorescent antibody technique. These pepsin-treated cells proved effective in adsorbing out any group A activity from the type specific serums. 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 TCA purified M protein preparation showed strong precipitin activity against its homologous serum and no cross-reactions could be detected with the other type specific antiserums. These TCA precipitated M proteins also reacted strongly with their respective type specific antisera obtained either from the CDC or the prepared homologous typing sera.

2. Passive hemagglutination data. Table XVI represents the hemagglutination titres for each of the antisera prepared against the DEAE and CM cellulose purified M proteins, types 1, 12 and 29. These antisera were prepared by incorporating the M proteins into complete Freund's adjuvant and injecting them into rabbits, following the schedule outlined in the Methods section. Using this pro-

TABLE XVI. FINAL HEMAGGLUTINATION (H.A.) TITER OF RABBITS IMMUNIZED WITH THREE DIFFERENT CELLULOSE PURIFIED TYPE-SPECIFIC M PROTEIN PREPARATIONS IN COMPLETE FREUNDS ADJUVANT.

Rabbit #	Homologous Type Specific M Protein	H.A. Titer vs. M_1 Protein	H.A. Titer vs. M_{12} Protein	H.A. Titer vs. M_{29} Protein
12	1	1:20,480	1:128	1:640
13	1	1:51,200	1:32	1:320
15	29	1:640	1:32	1:1800
16	29	1:400	1:64	1:5120
17	12	1:32	1:5120	1:128
18	12	1:64	1:1600	1:64

cedure, it is not only possible to evaluate the potency of the prepared sera but also to quantitate the degree of cross-reactivity between the various M antigens when they are compared to the same serums. The hemagglutination titre for the M_1 protein-antiserum system was seen to be as high as 1:51,200. The M_1 antisera had a titre of 1:32 and 1:64 against the heterologous M_{12} protein, but did have a substantial titre against the M_{29} protein of 1:640. The homologous M_{12} protein system was seen to have a titre of 1:5120 and almost no titre against the heterologous M_1 and M_{29} proteins. The homologous M_{29} system had an antibody titre that was equal to the M_{12} system. This system was almost free of activity against the M_{12} protein, but did contain a strong degree (1:640) of cross-reactivity against the heterologous M_1 protein. The titre of the cross-reactivity between the M_1 and M_{29} proteins appeared to be equal in both cases (1:640). However, the degree of specificity of the homologous M_1 system was approximately ten times (1:51,200 to 1:5120) as strong as that of the M_{29} homologous system. Therefore, the cross-reactivity between M_{29} antisera and the M_1 antigen is decidedly more significant than the cross-reactivity between the M_1 antisera and the M_{29} antigens. This information was also noted in the immunochemical determinations (Ouchterlony) and in the streptococcal long chain formation studies. The M_{12} antisera appeared to be highly specific for the M_{12} antigens.

3. Streptococcal long chain formation studies. Injection of M-containing whole cells or M proteins purified by cellulose chromatography or hot TCA precipitation into rabbits resulted in production of antisera that were capable of promoting streptococcal long chain formation in vitro (Tables XVII, XVIII and XIX). The length of the group A streptococcal chains were seen to increase (in the homologous systems) from 3.05 to 6.23 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 all these antigen preparations do contain M proteins.

Table XVII represents the data obtained when long chain streptococcal formation was allowed to proceed in the presence of anti-M antibodies formed against a whole cell vaccine. The highest values of the immune serum to normal serum chain length ratios were obtained here. Tables XVIII and XIX represent the ability of type specific purified M proteins to produce antisera capable of promoting streptococcal long chain formation in vitro. Antisera produced against purified M-antigens does not seem to give as high an immune to normal chain ratio as does antisera prepared against a whole cell vaccine. Table XVIII allows for another method of examination of the one-way cross-reaction between the type 1 M protein and the M_{29} antiserum. M_1 antisera did not seem to give any appreciable increase

TABLE XVII. THE ABILITY TO TYPE SPECIFIC M-PROTEIN* ANTISERA TO PROMOTE STREPTOCOCCAL LONG CHAIN FORMATION *in vitro* (AVERAGE OF 50 CHAINS).

Rabbit #	Homologous Type Specific M-Protein	Homologous Streptococcal Chain Length in Normal Serum	Homologous Streptococcal Chain Length in Immune Serum	Immune Serum to Normal Serum Chain Length Ratio
1	12	6.6	37.3	5.65
2	12	7.9	46.7	5.91
3	29	7.7	39.1	5.08
4	29	6.1	34.4	5.60
5	1	10.9	44.6	4.09
6	1	7.3	45.5	6.23

* These M-reactive sera were produced by the intravenous injection of whole heat-killed cells and a 1 mg booster shot (one month after final whole cell injection) of cellulose purified protein.

TABLE XVIII. THE ABILITY OF TYPE SPECIFIC M-PROTEIN* ANTISERA TO PROMOTE STREPTOCOCCAL LONG CHAIN FORMATION *in vitro* (AVERAGE OF 50 CHAINS).

Rabbit #	Homologous Type Specific M-Protein	Long Chain Formation Against Type-Specific Organism	Immune Serum to Normal Serum Chain Length Ratio
12	1	1	3.05
12	1	12	1.10
12	1	29	1.13
13	1	1	4.49
13	1	12	1.00
13	1	29	1.06
15	29	1	1.55
15	29	12	1.19
15	29	29	3.31
16	29	1	1.70
16	29	12	1.08
16	29	29	2.25
17	12	1	0.94
17	12	12	4.07
17	12	29	0.90
18	12	1	1.01
18	12	12	3.32
18	12	29	0.99

* These M proteins were obtained by HCl extraction of whole heat-killed cells with subsequent ammonium sulfate fractionation and DEAE and CMC chromatography.

TABLE XIX. THE ABILITY OF TYPE SPECIFIC M-PROTEIN* ANTISERA TO PROMOTE STREPTOCOCCAL LONG CHAIN FORMATION *in vitro* (AVERAGE OF 50 CHAINS).

Rabbit #	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

* 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 TCA precipitation.

to the immune versus normal serum chain length ratio when M_{29} cells were grown in its presence but this ratio was seen to increase to 1.55 and 1.70 when M_1 cells were grown in the presence of M_{29} antiserum.

IV. DISCUSSION

A. Significance of physical data. The data presented here and obtained by others (62) indicate that the group A streptococcal M proteins is indeed a biological rarity. The classical technique employed to remove this antigen from the streptococcal cell wall, hot (95 C) acid (pH 2) extractions, does not seem to denature these proteins in any way. This hot acid solubility factor, which places the M protein in a relatively small class of such thermal stabile proteins as RNase, lysozyme and Bence Jones protein, is not its only unique property. There appear to be at least 60 serologically different M proteins, yet they all appear to have quite similar amino acid compositions as well as similar chromatographic characteristics. The single most important reason for studying the various M proteins is that these antigens are solely responsible for the protective antibodies elicited in the course of infection with a group A streptococcus. This thesis represents a comprehensive study of 23 different strains of 19 different type specific organisms. M proteins have been removed, isolated, purified and their biological and chemical characteristics compared and contrasted.

All strains examined appeared to release their M proteins under identical conditions and the great majority of these antigens were seen to appear in a 30% to 60% ammonium sulfate saturated fraction. The M protein preparations purified by CM cellulose column chromatography, DEAE and CM cellulose batch chromatography and hot TAC precipitation were shown to be essentially free of nucleic acid contamination (Table VIII). The best preparations of M protein were those obtained by DEAE and CM chromatography after RNase treatment. The ultraviolet extinction value of 1.35 for 280 nm/260 nm agreed with the 1.31 value for the same reading obtained by Fox and Wittner (26) for their cellulose purified proteins. The most common contaminants of preparations of M proteins were nucleic acid and carbohydrate. The highest reported 280 to 260 O.D. ratios for M antigens were consistently in the range of 1.34 to 1.37 (2,9,64), and Phillips and Pine in their paper evaluating methods used to purify acid- extracted M proteins (82) accepted these ratios as representing maximal nucleic acid removal. This ultraviolet absorption ratio was 1.062 for the hot TCA precipitated M protein and was in agreement with the value of 1.06 obtained for an acid precipitated M protein in a paper by Noble and Moody (78). The relatively low 280 nm/260 nm values are misleading as to the actual concentration of protein to nucleic acid because of the relatively low proportion of aromatic amino acids

in the serotypes examined (Tables II, III, IV, V, VI, VII and VIII). This idea is also reinforced by an examination of the relatively low extinction coefficients for the various M proteins at 280 nm. The values for $_{280}E_{1\%}$ ranged from 3.40 to 5.60 for the cellulose purified and hot TCA precipitated proteins.

Isoelectric pH values of 5.3 to 6.1 have been established for M antigens of several different serological types (23,64,67,80,83, 88,89,123,124). This agrees with chromatographic characteristics possessed by the M proteins examined in this work. The major M protein peak to be eluted from the CMC column came off at a pH between 5.5 and 6.0. A minor protein peak was recorded at a pH of 5.0 and this peak contained some M-reactivity and some nonspecific protein. Ouchterlony analysis on the M proteins purified by DEAE and CM batch chromatography showed that M activity could be present in any one of the final six fractions off of the last CMC fractionation. However, the major protein peaks were found to be eluted from the CM cellulose at a pH between 5.5 and 6.5.

Molecular weights were estimated by electrophoresis in polyacrylamide gels in the presence of the anionic detergent SDS as well as chromatography on Sephadex G-200. These methods allowed identification of primarily a single band of estimated molecular weight 23,000 in both the cellulose purified and TCA precipitated M protein

preparations and of a second band in the cellulose purified fraction with a molecular weight of approximately 30,000 to 33,000. These bands all possessed M-reactivity.

These molecular weights of 23,000 and 31,500 correspond well with the range (19,000 to 31,000) reported by Fox and Wittner for their "Lo-M" protein of type 12 streptococci using sedimentation equilibrium analysis (26). No proteins with molecular weights of 116,000 to 180,000 similar to those reported by these authors for their "Hi-M" protein of type 12 organisms were isolated in this study. This is probably related to the use of acid extracts in this study since Fox and Wittner reported that alkaline rather than acid extraction favored release of the larger molecular weight proteins.

Other investigators have reported on the molecular weight of the M proteins, and the results have been extremely diverse. Vosti et al. (109) estimated the molecular weight of the type 12 streptococcal acid extracted M protein using electrophoresis in polyacrylamide gels in the presence of the anionic detergent SDS. This group worked with two purified fractions of type 12 M protein, fractions "a" and"b". Their fraction "b" contained two bands, one of molecular weight 32,000 and a lesser band of molecular weight 17,000. Their fraction "a" contained three bands with estimated molecular weights of 32,000, 48,000 and 63,000. Pappenheimer et al. in 1942 (80),

using sedimentation velocity, estimated the molecular weight of a type 3 group A streptococcal M protein to be approximately 41,000. Besdine and Pine (7) were able to isolate high-molecular weight proteins having M-reactivity without acid or alkaline extraction. Treatment of a type 1 group A streptococcus with sonic vibrations released antigens which reactived strongly and specifically with absorbed type-specific antiserum. When these antigens were placed on Sephadex G-200 columns, the M reactive materials appeared in the void volumes, suggesting that the active material in each had a molecular weight greater than 300,000. It should also be added that these investigators reported that this antigen preparation was released without diminishing the total yield of acid-extractable M protein of the original heat killed cells.

One of the most important points that can be gleaned from the molecular weight data generated here by Sephadex G-200 and SDS gel electrophoresis is seen in a comparison of the hot TCA precipitated proteins and the cellulose purified proteins. The TCA precipitated proteins (which contained less amide nitrogen and appeared more homogeneous on polyacrylamide gel electrophoresis than cellulose purified proteins) possessed approximately the same molecular weight (23,000) as the cellulose purified antigens but did not contain the higher molecular weight proteins (30,000 to 33,000) seen in the latter

preparations. It would appear that the harsh acid treatment (60% hot trichloroacetic acid for 20 minutes) is sufficient to cleave all M proteins to their smallest possible molecular size, while leaving their immunospecificity intact. This is of extreme importance, because this would seem to destroy the region of the protein believed to be responsible for the non-type-specific toxic effects of purified M proteins upon platelets and leukocytes in human blood (5). This region, the non-type-specific M protein region (NTSM), was hypothesized by Stollerman (98) who believes that toxic nature of M protein vaccines is related to common antigenic determinants (NTSM) against which man becomes generally intensely hyperimmune.

The only other reported peptide mappings of M proteins were performed by Fox (26) and Vosti (45). Both of these investigators performed fingerprinting on tryptic digests of the type 12 group streptococcal M protein. Vosti isolated two M_{12} reactive fragments, "a" and "b", and compared them by peptide mapping. The maps for "a" and "b" appeared quite similar with 28 to 32 spots identified. This number of peptides was seen to be low compared to the numbers reported here and those of Fox. He reported 40 peptides for his Lo-M which was reported to have a molecular weight of 25,000 and 42 peptides for the Hi-M preparations which were seen to have molecular weights of 116,000. Fox concluded that either Hi-M is a multiple

structure of Lo-M units or Lo-M is fragmented Hi-M, and he tended to accept the multiple structure theory. The tryptic digest of the M_{12} protein preparation purified by DEAE and CM cellulose chromatography was seen to have 41 peptides and a molecular weight of approximately 23,000. This compares favorably with the data of Fox. Tryptic digests of the M_1 and M_{29} protein showed 42 and 37 peptides respectively. Figures 3 and 4 represent the peptide maps of the tryptic digests of the type 12 and type 29 M proteins. The tryptic peptide maps of M_1, M_{12} and M_{29} were all similar in appearance with the number of peptides reflecting the number of arginine and lysine residues. These maps do show sufficient homology to account both for the reported cross-reactions and a common phylogenetic origin. The peptides present in some proteins which are not present in others could account for or represent type-specific determinant peptides. Since peptide mapping of chymotryptic digests has never been reported before, these analyses can only be compared to the types performed here. Chymotryptic digests of the M_1, M_{12} and M_{29} proteins showed 9, 8 and 12 peptides respectively. Figures 5 and 6 represent the peptide maps of the type 12 and type 1 proteins. The chymotryptic peptide maps of M_1, M_{12} and M_{29} were all similar in appearance with the number of peptides reflecting the aromatic amino acid concentration. Once again, the maps do show sufficient homology to account

both for the reported cross-reactions as well as a common phylogenetic origin.

The acrylamide gel (disc) electrophoretic patterns obtained on the M proteins purified by a 30% to 60% ammonium sulfate fractionation (Figures 7 and 8), an ammonium sulfate fractionation accompanied by a CM cellulose column fractionation (Figures 8 and 9) or an ammonium sulfate fractionation accompanied by a DEAE and CM cellulose batch fractionation (Figure 10) all showed the heterogeneity of banding that has been reported by many other investigators (19,23, 35,78,109). The data support the concept that the M protein belongs, in all probability, to a family of proteins and does not exist as a single uniform molecular species. This concept was first proposed by Fox (19) for types 12, 14 and 24, and has now been extended to include the types examined here. While one is tempted to view the heterogeneity of banding as an indication of impurity of a single species, it must be borne in mind that these are all collectively M protein. The multiplicity of bands may be due to the variable C-terminal amino acid which could markedly affect their mobilities. Reports by Lange et al. (66) and Vosti et al. (109) have shown the presence of the same N-terminal amino acid, but different C-terminal amino acids (aspartic acid, glycine, alanine, leucine, serine, lysine, arginine and histidine), for the type 12 streptococcal M protein.

This is consistent with the hypothesis that all of the bands in the electrophoretic gels are M proteins that differ slightly in molecular weight or greatly in electrophoretic mobility primarily as a result of random cleavage at the C-terminal end during the initial acid extraction of proteins from the cell wall. This latter point gained more credence from the experiments involving treatment of the M protein with TCA.

The TCA precipitated M protein preparations seem to differ drastically from cellulose purified proteins as is seen in Figures 11 and 12. The banding pattern of these proteins after exposure to hot 60% TCA is altered drastically as seen by a vast reduction in the number of bands upon polyacrylamide gel disc electrophoresis. The type specific M proteins M_1, M_{12} and M_{29} were shown to give only one major band with some trailing.

The values for the extinction coefficients $_{280}E_{1\%}$ for the cellulose purified and TCA precipitated M proteins are seen in Table XII. The extinction coefficient at 280 nm is directly related to the tyrosine content of the protein preparations. The values for the extinction coefficients of the purified M proteins range from 3.40 for the DEAE and CM cellulose purified proteins to 5.60 for those obtained by a TCA precipitation. Tyrosine values (in mg/100 mg protein) were seen to range from 0.4 to 4.1 with most values between 1.5 and 2.0.

This compares favorably to extinction coefficients of some more familiar proteins and their amino acid content. Serum albumin (human) has an $_{280}E_{1\%}$ of 5.3 with a tyrosine content of 5.5 (in mg/100 mg protein) and a tryptophan content of 1.0 (in mg/100 mg protein). Tryptophan is found to have its maximal absorption at 280 nm and the tyrosine absorption maxima occurs at 275 nm; therefore at 280 nm, the importance of tryptophan to tyrosine is in the ratio of 27:7 for absorbance. This is an important fact because M proteins do not seem to contain any tryptophan. Another protein that does not contain any tryptophan is insulin. Insulin, however, does have a high tyrosine content (13.0 in mg/100 mg protein) and a correspondingly high extinction coefficient of 11.3. Chymotrypsin, which has a relatively low tyrosine content (2.7 in mg/100 mg protein) is similar to the M protein in tyrosine content, but has a very large (5.6 in mg/100 mg protein) tryptophan content. This is reflected in the high extinction coefficient of 20.0 for chymotrypsin.

B. Significance of chemical data. As previously stated, the most common contaminants of preparations of M proteins are nucleic acid and carbohydrate. Phillips and Pine (82) have reported that a single 30% to 60% ammonium sulfate fractionation removed 99% of the carbohydrate contamination and this figure agrees with the carbohydrate

content of the M proteins obtained by the ammonium sulfate fractionation of an acid extract from whole cells or cell walls (Table I). Cellulose chromatography or hot TCA precipitation was seen to effectively remove 99.7% of the carbohydrate contamination.

Methylpentose has long been a common contaminant of streptococcal M protein preparations. The values for rhamnose obtained here (0.3 to 0.5%) agree with those obtained by other workers (26). There are two possibilities for the source of this carbohydrate with one allowing speculation that a very important cell wall linkage is yet to be elucidated. The first and most unexciting possibility is that this methylpentose is simply adsorbed onto the M protein and cannot be removed during the course of purification. The second, and most interesting, is that this rhamnose content may very well represent the point at which the M proteins are attached to the cell wall. The group-specific antigen of group A streptococci (C-polysaccharide) is a multibranched polysaccharide that consists of N-acetyl-D-glucosamine and L-rhamnose in a ratio of 2:5 (32). This polysaccharide is linked to the mucopeptide by bridges of phosphate containing rhamnoside (32) and it is possible that one rhamnose molecule could be what attaches the M protein to the streptococcal cell wall. Indeed, if one were to divide the molecular weight of rhamnose (164) by the molecular weight of the M proteins (23,000), the value of 0.7% would

be obtained which is reasonably close to the rhamnose values presented here. This could indicate that there is one molecule of rhamnose attached to each M protein as a result of cleavage from the cell wall. Future work will include total digestion of the M antigens by pronase and then identification of the amino acid to which the methylpentose is attached. Information generated by such research would be extremely useful in elucidating the structural relationship between the M protein and the streptococcal cell wall.

The amino acid analyses have yielded some very interesting results. Not only is glutamic acid always the most prevalent amino acid and lysine, alanine, aspartic acid, leucine and glycine present in similar micromolar amounts, but the remainder of the amino acid compositions are also very similar (Tables II, III, IV, V, VI and VIII). Therefore, the amino acid analyses seem to indicate that all the M proteins are similar in amino acid composition. These results are in agreement with the finding of several other reports regarding amino acid composition of M proteins, types 12, 14 and 24 (references 19,66,109). Furthermore, the amino acid compositions seem to be dependent upon and reflected in the method of purification of the proteins (19,23,66). This idea is reinforced by examination of the disc electrophoretic gels represented in Figure 7. The three related strains obtained from Dr. Maxted's laboratory gave banding patterns

which varied only slightly from each other. The banding patterns exhibited by the Hektoen type 12 strains were quite different from the Voorhout strains, although the method of purification was the same. Finally, the type 12 M protein obtained from yet another strain and purified by the additional step of CMC column chromatography, gave yet a third pattern of bands, quite dissimilar to the other two. These findings seem to contrast those as reported by Fox and Wittner (23). These authors reported that each of the serotypes they had worked with (types 12, 14 and 24) had a characteristic pattern of bands easily reproducible from various batches of cells on M protein preparations.

Eight of the first nine N-terminal amino acids of the M_{12} protein were tentatively identified by automated sequencing as Ala-Leu-Asp-Leu-Phe-Ileu-Leu-?-Val. This supports the finding of Vosti that the N-terminal amino acid of the type 12 M protein is alanine (109), and also gives supporting evidence that all of the bands in Fraction V of Figure 10 are collectively M protein.

C. N-terminal analyses. Another fact that seems to relate the various protein preparations is the finding that the N-terminal amino acid is L-alanine in all of the types examined, including the M Voorhout strains obtained from Dr. Maxted. This observation confirms

and furthers the N-terminal chemistry work performed on the type 12 M protein by Vosti et al. (109) and corrects the finding as originally reported by Lange (66). In an earlier report, Lange et al. established that a single N-terminal amino acid was obtained regardless of whether the material subjected to the FDNB reaction was whole cells, cell walls, crude acid extracted proteins or highly purified M protein. The findings here further support that concept. Vosti reported L-alanine as the major DNP-amino acid identified in the type 12 protein in agreement with the findings reproted here. Vosti also reported "minor" spots of DNP-glycine, DNP-leucine and di-DNP-lysine which were not found in this investigation although the concentrations of proteins employed were equivalent. Contrasting the heterogeneity on polyacrylamide gels by 300 µgrams of M protein with the single N-terminal amino acid yielded with 5 mg of protein (a 15-fold increase in concentration), it is concluded that electrophoretic heterogeneity is due to C-terminal amino acid variability. Regardless of the above arguments, it remains that as a criterion of purity, the finding of the single N-terminus may be interpreted as an indication of only a single protein being present. Or as reported before (66) for type 12 alone and now for several other types, these results could be concluded as being due to either (1) only M proteins of all the surface antigens have a free N-terminus and that is the only de-

tectable amino acid regardless of purity of the protein, or (2) all the proteins of the extracellular coat have the same N-terminal amino acid.

D. Immunochemical determinations. Employing the selectivity and specificity of immunological techniques, it was established that each of the protein isolates represented M antigen in totality. How this related to the physical heterogeneity was clarified by the use of hot TCA. Fox (23) first reported on the multiple banding of the M protein. Other data supporting a multiple molecular structure for the streptococcal M proteins is presented in Figures 13 and 15. All bands showed M-activity in this study, as well as that of Fox (23) on disc electrophoresis and double diffusion agar analysis. The important thing observed here is, regardless of the number of bands in the disc electrophoresis, only one line was obtained on Ouchterlony analysis (Figure 16). Immunodiffusion analyses gave the first indication that a one-way cross-reaction existed between two of the streptococcal M types. A faint line of precipitin was observed between the DEAE and CM cellulose purified type 1 M protein and rabbit antisera prepared against the DEAE and CM cellulose purified type 29 M protein. This same one-way cross-reaction was further supported by the data obtained from the passive hemagglutination studies and the

streptococcal long chain formation studies. A similar observation was also noted by Wiley and Bruno (117) for type 41 antiserum and the type 43 cocci, and by Harrell et al. (34) involving type 46 antiserum and type 51 streptococci. Cross-reactions between group A streptococcal types 1 and 29 were reported by Vvedenskaya et al. (112). These cross-reactions were revealed in studying the cultures of group A, types 1 and 29, in indirect bactericidal tests with anti-streptococcal sera. Also, a common antigen was revealed between these two types by immunodiffusion and this antigen was destroyed by trypsin. The cross-reaction reported by these authors, however, was a two-way cross-reaction.

Two of the more common streptococcal antigens which might possibly contaminate the M protein preparations are the T and R antigens. However, acid extracts that contain M proteins do not contain T, for this antigen is destroyed by heat and acid (120). T antigen is usually obtained by employing tryptic extracts of whole cells. Tryptic extracts that contain T do not contain M, for the latter has been destroyed by this enzyme. T antigen was not a contaminant in the proteins prepared in this study because of the acid extraction and the fact that all proteins were trypsin sensitive (Figure 18). Lancefield and Perlman first described a R antigen in group A, type 28 streptococci (64). These protein antigens are also found in types

2, 3, 33, 43 and 48 (59,60,118). These antigens are extracted from the cocci by heat and acid and can therefore appear in acid extracts of the group A streptococci. They may be confused with M proteins and could cause errors in typing by the precipitin method, since they do not appear to be type specific. The type 28 R antigen also has been shown to occur in some strains of types 2 and 48 and even in certain groups other than A, such as B, C and G (59,64). The R antigens seem to vary in their susceptibility to digestion by proteolytic enzymes. The type 28 R antigen is susceptible to digestion by pepsin but resists digestion by trypsin (64). It is not likely that the protein preparations employed here were contaminated with R antigens because they were all trypsin sensitive.

The TCA precipitated proteins were also trypsin and chymotrypsin sensitive but did differ from the cellulose purified proteins as seen by the fact that only one major band appeared on their gel (disc) electrophoretic analyses (Figure 11). All of these bands were shown to be capable of precipitin reaction with their type specific antisera as seen in Figure 17.

E. Biological activity. By definition, proteins extracted from group A streptococci must meet important criteria to qualify as M proteins. They should be capable of reacting with type specific

antisera and they should stimulate formation of type specific antibodies that possess bactericidal, protective or long chain formation activity. In the presented experiments, these proteins meet these requirements. The immune responses presented here show that purified M protein is a relatively good immunogen when administered in limited amounts in rabbits. Examination of the passive hemagglutination data and long chain formation data indicate that the type 1 M protein and type 29 antisera do not exactly meet the requirements for M proteins in that there appears to be a one-way cross-reaction between the two. Table XVI demonstrates that the cross-reactivity between the M_1 protein and the M_{29} antiserum is ten times stronger (in comparison to the homologous system) than the cross-reactivity between the M_{29} protein and type 1 antisera. This is supported by the observation in Ouchterlony analysis that a faint line of precipitin existed between the type 1 M protein and type 29 antisera, that could not be removed by repeated whole cell absorptions. The strongest evidence for this one-way cross-reaction was seen by an examination of the data in Table XVIII. Type 29 antisera was seen to promote streptococcal long chain formation by a type 1 group A streptococci by a ratio (immune serum to normal serum chain length ratio) of 1.55 to 1.70 when the type 29 homologous system gave ratios of 3.31 and 2.25 respectively. There was no appreciable rise of this

ratio above 1.0 when the type 29 organisms were grown in the presence of anti-M_1 antiserum.

Another interesting observation that can be made from these biological data is that injection of whole cells seems to give a better anti-M response in rabbits than does injection of purified M proteins. It can be noted that the highest immune serum to normal serum chain length ratios (Tables XVII, XVIII and XIX) were observed in the antisera prepared by whole heat-killed cell injection. These values would have undoubtedly been higher had not a 30 day rest period been employed before administering a purified M protein booster with exsanguination 7 days later. Stollerman and Ekstedt (100) reported immune to nonimmune serum ratios of 10 to 15 for homologous systems employing serums produced by whole heat-killed cell injections. The question of the differences in the avidity of antisera produced by multiple injections of whole heat-killed streptococci as compared with sera prepared against purified M antigens is still a puzzle. Fox has shown (24) that both antisera are composed primarily of IgG; however, the antisera prepared against purified M proteins usually exhibits poorer precipitin capabilities as well as much lower immune serum to normal serum chain length ratios. This could have one of several possible explanations. Other surface antigens could play a role here and produce confusing results. Indeed, Ekstedt and

Stollerman (16) have shown that the combination of the streptococcal R antigen, with its appropriate antibody, amy under appropriate conditions also inhibit chain scission. This effect was not seen to be as great as that caused by the M protein-anti-M complex but nevertheless was significant. These authors concluded that "the effect of antibodies to R and T antigens would most likely be apparent with degraded variants which have lost M protein but which have retained other antigenic components." Another explanation is the possibility that more antigenic determinants are exposed when these M proteins are still in their native configuration on the outer streptococcal cell wall. As a result, the antibodies formed against the native M antigens are far more heterogeneous than those found against the purified M proteins. This heterogeneity, with respect to a variety of specific combining sites, would give a more avid antigen-antibody reaction. The above theory, postulated by Fox (24) agrees with results obtained here involving type-specific antisera against whole heat-killed cells and antisera prepared against purified streptococcal M protein.

F. Proposed M protein structure. A diagramatic representation of the findings reported here and in the literature is presented in Figure 19. Most observed data fit relatively well into this representation

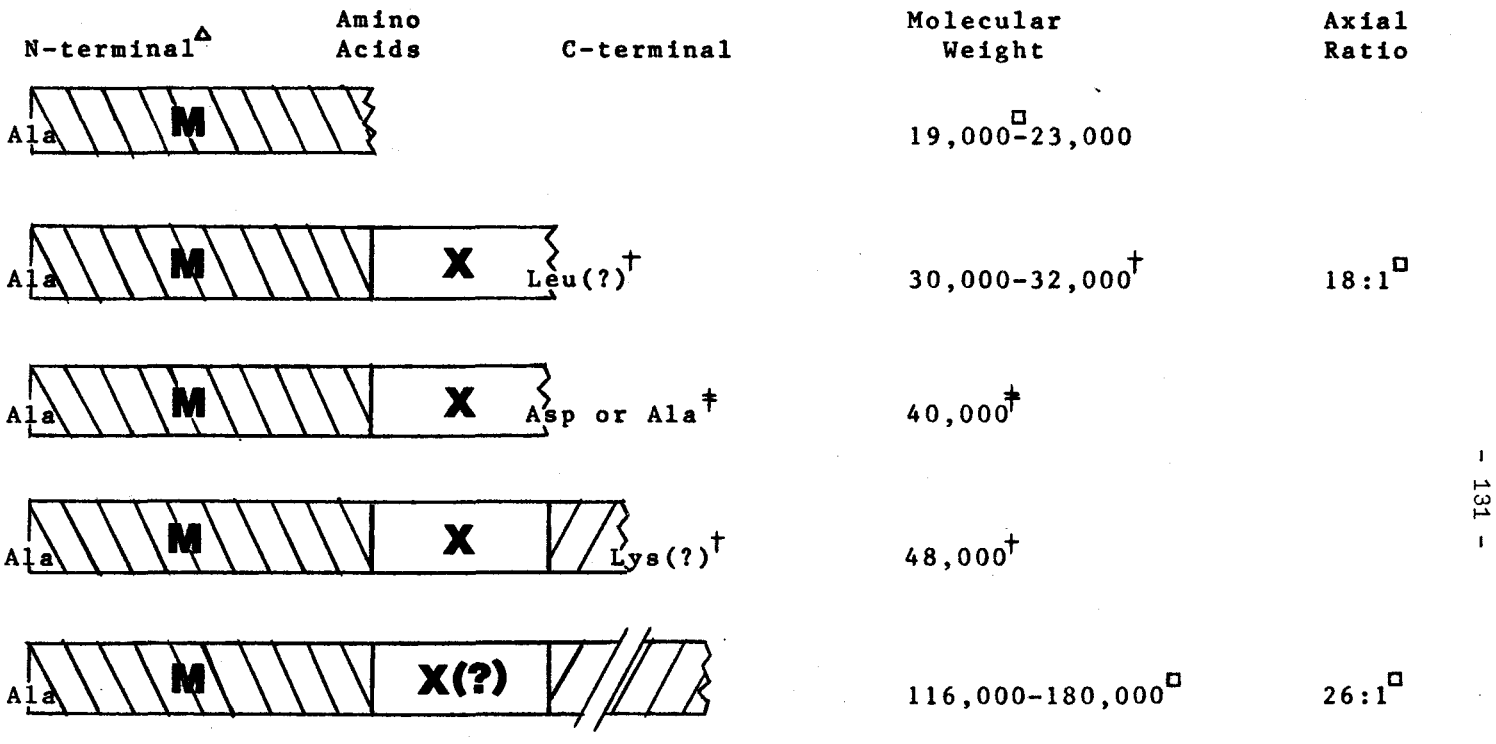


Fig. 19. Schema of proposed structure for M protein of group A type 12 streptococci. M represents type-specific and X non-type-specific (perhaps cross-reactive) antigenic determinants. □ denotes data of Fox and Wittner(26), † denotes data of Vosti et al(109), and ‡ denotes data of Lange et al(66).

△ The first eight N-terminal amino acids are tentatively identified as follows: Ala-Leu-Asp-Leu-Phe-Ileu-Leu-?-Val.

and could account for the constancy of the N-terminal and the variation in the C-terminal amino acids, as well as for the relationship of increased molecular weight and increased antigenic structure. This representation of the type 12 M protein is based on the amino acid sequencing as well as the C-terminal amino acid determinations. However, work in this report and others have indicated that many of these structural aspects will hold true for most, if not all, M protein. As presented here, most of the available data gives evidence that the M-specific and the cross-reactive antigenic determinants are on the same molecule, although there are some workers who dispute this theory (77). There are several possible explanations for the observed variations in size and antigenic comples among the M proteins. One explanation is that non-selective cleavage of the M proteins off of the cell walls occurs during the hot acid extraction procedure and the variations in molecular size reflect or are due to the variable C-terminal amino acids that have been reported. Another and less obvious explanation is that these proteins represent varying stages in the development of the nascent cell wall antigens.

G. Action of TCA on M proteins.

1. Advantages of TCA precipitation. Precipitation by 60% hot trichloroacetic acid allows for the rapid purification, in high yield,

of group A streptococcal M proteins. Purification, as used in this sense, is referring only to the physical properties of this heterogeneous, immunospecific protein antigen. The most important aspect of TCA precipitation is removal of all interfering substances, while at the same time, producing a more homogeneous protein. Comparing the TCA precipitated product obtained in 30% yields in one to two days time produced results more than favorable over the 4% yields of DEAE and CM cellulose purified fractions which involved at least five to eight days time in preparation. Ouchterlony analyses have also given evidence that these protein fractions whose purification have been described here represent highly purified M protein preparations. RNA has been shown to be eliminated by the hot TCA treatment. No group A carbohydrate could be detected in any of these precipitated proteins at any concentration. Although the TCA extracted material and hot TCA treated HCl extracted material showed glucosamine to be present, this must be non-group A carbohydrate since this concentration would be detectable with grouping antisera. No explanation for this material can be made at this time other than possible non-specific adsorption of free glucosamine to the M protein throughout the purification steps. Future work will have to be done to determine if the glucosamine detected with the M protein may represent a linkage between the cell wall and the protein chain. Each type spe-

cific protein preparation, however, gave strong precipitin reactivity with its homologous type specific antisera, regardless of source.

Purification of M proteins by precipitation with 60% hot TCA gives a marked difference in the amount of protein recovered as well as the ease of recovery over the DEAE and CM cellulose chromatographic procedures. With the DEAE and CM cellulose procedure, Fraction V (Table VIII) represented a 4% recovery while the total (Fractions I through VI) corresponded to a 10% recovery. With the present rapid TCA procedure, recoveries were in the range of 30% for a single homogeneous band on electrophoresis. This represents approximately a 10-fold increase of a single homogeneous M protein and even a 3-fold increase if all of the different M proteins are considered. Furthermore, the time to prepare them is reduced by approximately 80%.

2. Possible solution to multiple band problem. The banding pattern of M proteins after exposure to hot 60% TCA is altered dramatically as seen by a vast reduction of bands upon polyacrylamide gel disc electrophoresis (compare Figures 10, 11 and 12). An explanation for the reduction in banding of the M proteins after hot TCA treatment is that treatment of the protein with hot HCl for 10 minutes converts only a portion of the asparginine 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 TCA 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 disc electrophoresis. Evidence supporting this explanation is demonstrated in Figure 12, where the patterns of the first, second, third and pooled acid extracts are compared to that of the TCA treated M proteins. Indeed, even the amino acid analysis data further supports this conclusion, since each subsequent extract was shown to contain less recoverable ammonia than the preceding fractions. At the same time, amino acid recoveries were not markedly different in the respective preparations. It was concluded from these data that the amide nitrogen was lost from the proteins due to the acidic conditions, thereby effecting a net increase in the charge on the residual protein.

H. Peptide mapping as a tool for structure proofing of M antigens.

Peptide mapping or fingerprinting has been demonstrated to be a powerful tool in examining relatedness of different type specific M proteins. Areas of homology, as well as areas of type specificity, can be easily differentiated. Fox et al. (27) as well as the experiments

presented here have shown that tryptic digestion does not completely destroy all M activity until after at least 30 minutes of exposure time. Thus, partially degraded M proteins could then be peptide mapped with their immunospecificity still intact. Those proteins which display unique type specificity as well as cross-reactivity could then be fingerprinted. The peptides in common could be analyzed for their complete sequencing. Thus the type specific determinant could be eluted from the peptide map and identified. Of special interest will be the homologous peptides among the various types which are seen to be capable of inducing the production of "protective" antibodies. In addition, these isolated peptides can be assayed via inhibition of the hemagglutination assay employing the cross-reactive peptide in the homologous and heterologous system. This appraoch will strengthen our knowledge of the relatedness of the individual type specific strains of the group A streptococci. The greatest overall significance of such work may lie in the influence of the N-terminal amino acids upon the determination of the type specificity and the role that the primary amino acid sequence plays with respect to the cross-reactions encountered between some strains. An important aspect of this problem is to determine if areas of homology do exist between strains and therefore account for the reported cross-reactions. Noting that all protein synthesis is initiated at the N-

terminal end, allows speculation that if the type specificity resides in a gene controlling the synthesis of either the N-terminal or C-termenal end, and another gene common to all group A streptococci controls the synthesis of the remainder of the protein, the problem is reduced to one of relating amino acid sequence to function. The long range goal is to define the amino acid sequences required to produce protective antibody. If this is accomplished, a synthetic vaccine may be feasible, eliminating any danger from streptococcal products related to sequelae complications.

V. SUMMARY

Type-specific M proteins were examined to determine whether their immunological specificities were also reflected by major chemical differences. Twenty-four different type-specific strains of group A streptococci were used, including two non-M typeable strains. These M antigens, acid-extracted from whole cells or cell walls, were purified by ammonium sulfate fractionation and cellulose chromatography or hot trichloroacetic acid precipitation. These proteins were compared by immunodiffusion, electrophoretic mobilities, amino acid and N-terminal amino acid analyses. Molecular weights were determined by SDS gel electrophoresis and chromatography on Sephadex G-200. Three of the cellulose purified proteins were "fingerprinted" employing tryptic and chymotryptic digests. Biological activities of the M protein preparations were determined by examining their antigenicity in rabbits, their degree of cross-reactivity (passive hemagglutination) and their ability to induce, in rabbits, the production of antiserum capable of promoting the induction of streptococcal long chain formation *in vitro*.

Although the data did not reflect major chemical distinctiveness amongst the types examined, some interesting results did evolve.

Five important factors were observed to be shared by all cellulose purified M proteins examined: (1) glutamic acid was the most prevalent amino acid, (2) amino acid molar ratios were similar with five amino acids (glutamic acid, aspartic acid, lysine, leucine and alanine) comprising up to 70% of the proteins, (3) each had L-alanine as a single N-terminus, (4) each could react with their type specific antisera in Ouchterlony diffusion and was capable of producing antisera in rabbits capable of promoting streptococcal long chain formation in vitro, and (5) purified peaks from cellulose still showed heterogeneity while giving type-specific reactivity from multiple bands. Evidence for a one-way cross-reaction between type 1 M protein and type 29 antiserum was also presented. The molecular weights of the cellulose purified M proteins were seen to be in the range of 23,000 to 33,000 when examined by SDS gel electrophoresis and chromatography on Sephadex G-200. The tryptic peptide maps of DEAE and CM cellulose purified M_1, M_{12} and M_{29} showed 42, 41 and 37 peptides respectively and were consistent with lysine and arginine content in each protein. The chymotryptic peptide maps of these same three M proteins showed 9, 8 and 12 peptides respectively and were consistent with the aromatic amino acid content of each protein. These maps showed sufficient homology to account both for the reported cross-reactions and a common phylogenetic origin. The peptides which were

present in some proteins but were not present in others could account for or represent type-specific determinant peptides.

A simple and rapid procedure for the isolation in high yield (about a 30% recovery based on the total 30% to 60% ammonium sulfate fraction) of homogeneous purified group A streptococcal M protein was also described. The data indicated that M proteins extracted from streptococcal cell wall, fractionated with ammonium sulfate and treated with hot TCA, yields a highly homogeneous preparation that has the properties of M antigen purified by extensive cellulose chromatography. Ammonium sulfate fractionation appeared to be necessary to allow complete degradation and removal of group carbohydrate and nucleic acid contamination. These M protein preparations were shown to: (1) have similar amino acid analyses as well as the same N-terminal amino acids as their respective type-specific proteins purified by cellulose chromatography, (2) react with their respective type-specific antisera in Ouchterlony diffusion, (3) produce antisera in rabbits capable of promoting streptococcal long chain formation in vitro, and (4) give only one major band on polyacrylamide gel disc electrophoresis. Data was also presented which allows for an explanation of the hitherto described multiple banding M proteins seen on acrylamide electrophoresis. This procedure would therefore be ideal for clinical and laboratory situations wherein subjection of crude M

protein extracts to treatment with 60% hot TCA 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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ACKNOWLEDGEMENTS

The author is grateful for the technical assistance of Miss Aruna Mehta and Miss Elizabeth Stansbrough and the excellent technical guidance of Mr. Benjamin Booker. I am extremely thankful to Miss Maria Weber who taught me all I know about sterile technique and the proper handling of the group A streptococci. Sincere appreciation is extended to Mrs. Carol Blue for her patience in typing this dissertation.

Finally, I would like to extend my sincerest gratitude to my advisor and good friend, Dr. Charles F. Lange, for his guidance and encouragement, and without whom, this work would never have been possible.

ADDENDUM

The author wishes to stress that the presence, or absence, of homogeneity could not necessarily be unequivocally established by the techniques that were employed here. Additional physical techniques such as gradient polyacrylamide gel electrophoresis, cationic buffer systems, isoelectric focusing and analytical scanning isoelectric focusing could possibly have supplemented the presented information about the homogeneity of the prepared proteins. It should be recognized that even the best physical techniques have many limitations in regard to proving homogeneity. One employs a series of methods to reach a conclusion on collective data. When supplemented, as in the present study, by the strong chemical data of only a single N-terminus amino acid and the single immunoreactive protein with antisera, it was felt that a defined purity of protein was accomplished. Only complete sequence data would give unequivocal proof of purity.

Also, no attempts were made at examining the various protein preparations for any of the many secreted streptococcal products that might possibly be contaminating the isolates. With the methods employed, most of these would have been eliminated prior to the step of hot acid

extraction of the M proteins. Additionally, the majority of these products would be destroyed by the harsh hot acid treatment. It would be, however, in the best interests of the researcher and any prospective patients, to examine potential M-antigen vaccines for the presence of these products.

Approval Sheet

The dissertation submitted by David C. Straus has been read and approved by the members of the Advisory Committee listed below.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

8 May 1974

Date

Charles F. Lange

Signature of Advisory Committee Director

Advisory Committee:

1.) Dr. Charles F. Lange (Director) Charles F. Lange

2.) Dr. Edward Bermes E W Bermes

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