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Chemistry and End-Group Analysis on Purified M Protein of Type 12 Group A Streptococcal Cell Walls[1]

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M protein was extracted from the cell walls of streptococci by use of both acidic and alkaline buffers. These extracts were further purified by ammonium sulfate fractionation and column chromatography. Both diethylaminoethyl and carboxymethyl celluloses were employed to cover the *p*H range of 3.0 to 9.0. All of the M proteins isolated were immunologically related, but their physical and chemical properties varied dependent upon the *p*H range of isolation. Each isolate appeared to be homogeneous on the basis of immunodiffusion analysis, electrophoretic mobility, and ultracentrifugal analysis, but their amino acid analyses differed slightly. Two factors were shared by all isolates: (i) they all reacted with type-specific antisera and (ii) each seemed to have L-lysine as a single N-terminal amino acid.

Over the years, many reports have been published on M protein from streptococcal cell walls; a summary of the reports published prior to 1962 has been provided by Lancefield (13). In recent years, the major area of work has centered around the antigenicity of M protein (5, 7, 9) or its synthesis and detection (12). Although Lancefield stated that "much is known about their chemical composition . . ." (14), definitive reports on the chemistry of M protein have been sparse (5, 6) and have given only nitrogen and pentose or nucleic acid content. The purified M proteins which have been isolated to date have been shown to exist in multiple molecular forms by both immunologic (6) and electrophoretic (20) techniques.

The purpose of this report is to present data on the chemical and physical analyses of a series of M proteins isolated from type 12 group A nephritogenic streptococci.

MATERIALS AND METHODS

Preparation of crude M protein. Highly virulent (LD_{50} approximately 10^2 cells) type 12 group A streptococci were grown in 40-liter batches in a New Brunswick Fermacell in modified Todd-Hewitt broth (19). Harvested cells were disrupted and cell walls were recovered as described by Markowitz (18). Isolated cell walls were treated with ribonuclease (2), exhaustively washed, and lyophilized. M protein was extracted from 50-g amounts of walls by use of either the hot acid extraction procedure of Lancefield and Perlman (15) or the alkaline buffer system of Fox and Wittner (8).

Purification of M protein. Initial purification of the crude M protein was effected by ammonium sulfate fractionation to obtain the fraction precipitating between 30 and 60% saturation. After dialysis and lyophilization, samples were dissolved in phosphate buffer (*p*H 7.75, 0.005 M) and chromatographed on diethylaminoethyl (DEAE) cellulose columns [20 × 400 packing containing 30 g (wet weight) of cellulose]. Elution was performed stepwise with buffers of increasing ionic strength and decreasing *p*H. Fractions (10 ml) were collected on a Gilson ultraviolet (UV) scanning fraction collector. Further purification was accomplished by rechromatographing individual peaks on carboxymethyl (CM) cellulose equilibrated at 0.01 M, *p*H 2.5 glycine buffer. Columns (10 × 250 mm) containing 25 g (wet weight) of cellulose, were gradient eluted with buffers of increasing *p*H and ionic strength.

Chemical determinations. Total phosphorus was determined by the Ultramicro adaptation of the Fiske and SubbaRow procedure (4) with a model 150 Spinco spectrophotometer on samples hydrolyzed in 6 N HCl in sealed tubes at 110 C for 22 hr and on native nonhydrolyzed materials. Total nitrogen was determined by the micro-Kjeldahl method.

Hexose and hexosamine determinations were made according to the procedure outlined by Rosevear and Smith (21), methylpentose determinations according to the procedure of Dische and Shettles (3), and nucleic acid determinations according to the procedure of Barkulis and Jones (2). Quantitative total amino acid analysis was performed on a Spinco model 120 C amino acid analyzer as described by Spackman et al.

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(22). Protein estimations were made according to the procedure of Lowry et al. (17) with human serum albumin as a standard.

N-terminal amino acids were determined by use of fluorodinitrobeńzene according to the procedure of Frankel-Conrat and Porter (10). The dinitrophenyl (DNP) reaction was performed both on isolated proteins and on the cell walls. This reaction was run in 10% sodium bicarbonate at a controlled *p*H of 8.0 and with *p*H uncontrolled. During the course of the reaction on the cell walls, protein was released which was recovered. The DNP-walls were extracted with either hot acid or alkali to release the DNP-protein. All DNP-proteins were hydrolyzed according to the same procedure (10) to recover the DNP-amino acids.

C-terminal amino acids were determined by hydrazinolysis according to the procedure of Akabori et al. (1).

Physical determinations. Sedimentation velocities were obtained in a Spinco model E ultracentrifuge. Free-moving boundary electrophoresis was run in an Aminco electrophoresis apparatus, with comparative runs on cellulose acetate and acrylic gel (disc) electrophoresis (11).

Column chromatography with columns of DEAE cellulose and CM cellulose was employed in purification procedures. Eluted peaks from one column were rerun individually on a second column. All reactions eluted at the same fixed ionic strength and *p*H were considered to have the same physical properties. Column elution employed decreasing *p*H and increasing ionic strength for the DEAE cellulose and increasing *p*H and ionic strength for the CM cellulose.

For thin-layer chromatography, silica gel sheets (Eastman no. 6061) were run in either chloroform-benzyl alcohol-acetic acid (70:30:3, v/v) or *n*-propanol-34% ammonium hydroxide (67:33, v/v). Both one- and two-dimensional chromatograms were run. DNP derivatives were visible as yellow spots; other amino acids were detected with 1% ninhydrin in *n*-butyl alcohol.

Alumina columns were used according to the procedure of Turba and Gundlach (23) for the removal of 2,4-dinitrophenol, 2,4-dinitroaniline, and various artifacts from the DNP-amino acids prior to thin-layer chromatography.

UV absorption curves were run on a Beckman DK-2 spectrophotometer; absorption at fixed wavelengths was determined in a Beckman DU spectrophotometer.

Immunochemical determinations. Double-diffusion agar analysis and immunoelectrophoresis were performed as previously described (16).

Specific anti-M antiserum was a gift from E. N. Fox. Specific group A antiserum was a gift from J. Hahn. Rabbit anti-M was prepared according to the procedure of Markowitz (18). Each purified isolate 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 Freund's adjuvant according to the following protocol: 3 ml of Bayol F, 1.5 ml of Falba, 1 to 2 mg of *Mycobacterium tuberculosis* (var. *hominis*, $H_{37}R_a$) cells, and the antigen in saline. The procedure for immunization was as follows for each rabbit: 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 was immunized with 1,000 to 2,500 μg of protein.

RESULTS

Cell wall protein extracts. Yields of killed cells from the Fermacell were in the range of 200 to 225 g (wet weight) from which the cell walls were obtained. Traces of membrane material were removed by repeated water washings and fractional centrifugation. Samples of walls were hydrolyzed at this point and indicated less than 0.1% ribonucleic acid (RNA); these same walls were free from RNA after ribonuclease treatment. Table 1 shows the recovery of the protein from the cell wall after extraction and initial fractionation. Approximately 12% of the total dry weight of the cell walls was acid-extractable protein; alkaline extraction yielded only 9% recoveries. Of this total, 20 to 30% was precipitable at 60% ammonium sulfate saturation. An indication of the nature of the total proteins which are extractable from the walls can be seen in Table 2. These results indicate that both the whole cell walls and the extractable protein are low in tyrosine content, which accounts for the low extinction coefficient of the recovered proteins and also explains why the Lowry protein determinations were somewhat lower than the Kjeldahl nitrogens. The cell wall residue showed the main amino acids already known to exist in the wall matrix (lysine, glutamic acid, and alanine).

Protein fractionations. The 60% ammonium sulfate fraction of the acid-extracted cell walls showed a strong precipitin band on microprecipi-

TABLE 1. *Comparison of acid and alkaline extraction of type 12 group A nephritogenic streptococcal walls*

Type of extraction	Dry wt of cell walls (g)	Protein in total extract (g)	Protein in 30–60% ammonium sulfate precipitate (g)
Alkaline	40.0	1.5 (4%)	0.09 (6%)[a]
	20.0	1.6 (8%)	0.08 (5%)
	40.0	2.7 (7%)	0.19 (7%)
	40.0	1.6 (16%)	0.12 (7%)
Acid	10.0	0.60 (6%)	0.12 (20%)
	24.5	2.00 (8%)	0.40 (20%)
	19.0	1.56 (8%)	0.38 (24%)
	30.5	5.00 (16%)	1.40 (28%)
	10.0	1.68 (17%)	0.54 (32%)
	10.0	1.60 (16%)	0.12 (7%)

[a] Percentage of total extract.

tin testing with our antisera as well as with typing serum from the National Communicable Disease Center and that provided by Dr. Fox. A weak but positive precipitin line was also obtained versus group A antisera. The alkaline-extracted protein produced stronger M activity at equivalent concentrations and was negative for group A polysaccharide. To purify these materials further, the 60% ammonium sulfate fractions were subjected to gradient elution from *p*H 7.75 DEAE cellulose columns. The *p*H range from 6.1 to 5.7 produced a uniform peak which was recovered (Fig. 1). Several minor peaks at *p*H 7.0, 5.0, and 3.0 were also recovered. The main M protein activity was shown to reside in the *p*H 6.0 range and amounted to approximately 50% of the protein applied to the column. This material was further fractionated by gradient elution from a *p*H 2.5 CM cellulose column. Again, the *p*H range of 5.7 to 6.1 produced a uniform peak which was recovered. This peak again amounted to 50% of the protein applied; however, on recycling on a DEAE cellulose column this peak gave quantitative recoveries in a single peak. From 10 g of lyophilized cell walls, about 25 mg of purified M protein could be recovered via the acid extraction procedure; about 7 mg was recovered from an equal weight of walls by alkaline extraction. Acid-extracted M protein generally was eluted nearer to a *p*H of 6.0, whereas the alkaline-extracted material was eluted nearer to a *p*H of 5.7.

TABLE 2. *Comparison of amino acid composition of whole cell walls and extracted protein (in micromole per cent)*

Amino acid	Multi-washed ribo-nuclease-treated cell walls	Three times acid-extracted protein	Wall residue
Lysine	9.8	7.6	15.0
Histidine	1.4	1.6	0.4
Arginine	3.3	3.8	1.4
Glucosamine	+	±	0.1
Cysteic acid	0.1	0.3	0.1
Aspartic acid	8.1	12.0	3.6
Threonine	3.8	4.6	1.4
Serine	2.6	2.5	0.9
Glutamic acid	12.9	13.9	14.5
Proline	2.9	4.1	1.4
Glycine	6.7	8.7	3.2
Alanine	23.4	12.0	47.7
Valine	6.7	9.3	2.7
Methionine	1.4	0.1	0.2
Isoleucine	5.3	6.5	2.3
Leucine	6.7	8.2	3.2
Tyrosine	1.7	1.1	0.4
Phenylalanine	2.9	3.3	1.4
Alloisoleucine	0.2	0.5	0.1

Chemical, physical, and immunological data. Table 3 presents some of the amino acid analyses of the various M protein-rich fractions obtained via column chromatography on the DEAE and CM celluloses.

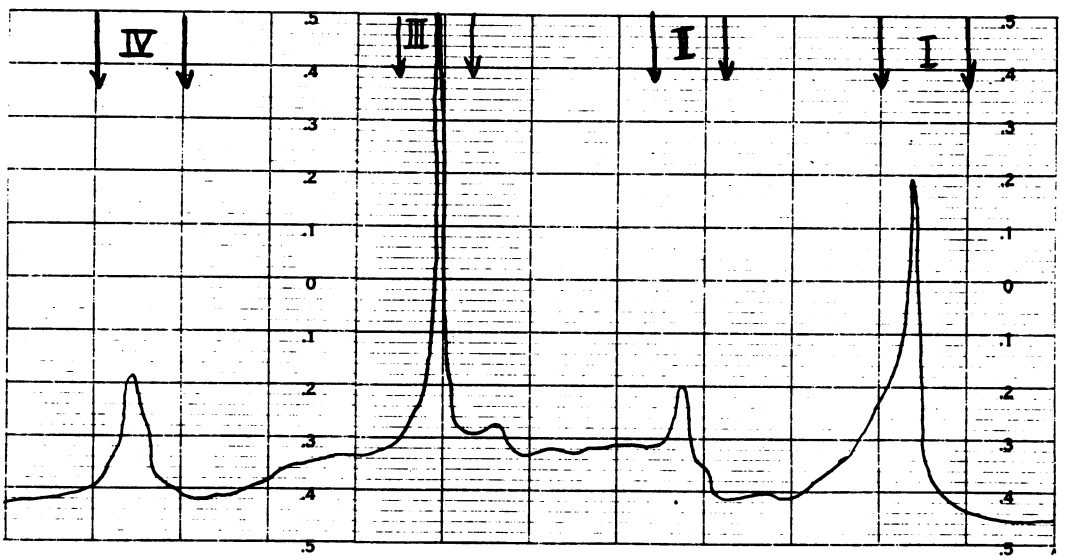

FIG. 1. *Chromatogram of the precipitate from 60% ammonium sulfate fractionation of M protein, released by alkaline extraction, on a 23-g DEAE cellulose column, with 0.005 M, pH 7.75 phosphate buffer as the initial eluting solvent. Peak I was the nonabsorbed fraction eluted at pH 7.75. Peak II was eluted at pH 7.0, and peak III represents the main M protein fraction eluted in the range of pH 6.0. Fraction IV was in the range of pH 5.0, and an additional peak V (not shown) was eluted at a pH of 3.0.*

TABLE 3. *Amino acid analyses on purified M protein preparations (in micromole per cent)*[a]

Amino acid	*p*H 3	*p*H 5	*p*H 7	*p*H 5.7–6.1
Lysine	8.2	8.7	11.7	12.6
Histidine	1.3	1.5	1.3	1.8
Arginine	2.9	3.1	4.3	4.4
Aspartic acid	13.8	13.4	11.3	11.4
Threonine	4.6	4.9	4.3	3.3
Serine	2.7	3.6	2.5	4.4
Glutamic acid	18.0	15.9	15.2	18.5
Proline	3.4	4.3	3.2	1.5
Cystine (½)	0.4	0.2	0.3	0.1
Glycine	7.0	7.5	7.2	5.8
Alanine	12.5	11.8	12.4	13.2
Valine	7.7	9.2	6.5	4.8
Methionine	0.2	0.1	0.5	0.05
Isoleucine	5.6	5.3	4.4	3.5
Leucine	8.0	6.7	7.9	11.0
Tyrosine	0.9	1.2	1.7	1.5
Phenylalanine	2.5	2.3	3.0	1.6

[a] Each determination is an average of six different preparations. Samples are the eluates from DEAE and CM cellulose columns at the *p*H indicated.

As can be seen, the main shift with increments in *p*H was the increase in the basic amino acid content with a concomitant reduction in the acidic amino acids. Each of the *p*H ranges (i.e., 3.0, 5.0, or 7.0) yielded nonuniform peaks by all analyses, with multiprecipitating bands on immunoelectrophoresis.

Another observation seems to indicate that as the degree of homogeneity and purity of the isolate increases the content of sulfur-containing amino acids decreases.

Both the acid- and the alkaline-extracted material gave similar amino acid analyses, with six amino acids (lysine, arginine, aspartic acid, glutamic acid, alanine, and leucine) accounting for 70% of the total molecule. The high concentration of basic amino acids is also consistent with the great sensitivity this protein shows to tryptic digestion.

Both the acid- and the alkaline-extracted DEAE and CM cellulose isolates (*p*H 5.8) were negative to carbohydrate and phosphorus analyses, and their UV analysis produced an almost flat curve in the range of 283 to 255 nm, with a 280 to 260 ratio of 1.1. The low tyrosine content of 1.5% is in accord with this finding.

The purified recycled peak (*p*H 5.7 to 6.1) from either the acid or the alkaline extraction produced a homogeneous peak on electrophoretic and ultracentrifugal analysis, with a single line of identity in agar analysis at concentrations to 1 mg/ml as well as a single line on immunoelectrophoresis. Indeed, though these materials proved to be similar in many respects, their behavior in an ultracentrifuge showed a consistent difference: the acid-extracted material had sedimentation coefficients in the range of $S_{20,w}$ 1.0 to 1.1, whereas the alkaline-extracted material gave a value of 1.7*S*. This finding is consistent with the report of Fox (8).

When these materials were employed in an immunization schedule, the rabbit antisera obtained showed single lines in agar analysis for both the challenging immunogen and other preparations. These antisera were all type-specific and did not react with neutralized acid extracts of other known types.

End-group analysis. Having established the purity of the preparations via the physical and immunochemical criteria, further chemical indications of purity were sought. Figure 2 shows the effect of the reaction of fluorodinitrobenzene on the whole cell wall preparations. As can be seen, the same single DNP-amino acid was detected in all of the samples. After removal of the dinitrophenol and aniline, samples were run on two-dimensional chromatographs which permitted the identification of this derivative as diDNP-lysine. Figure 3 shows some DNP-amino acid standards versus the DNP-amino acids found in the protein released during the preparation of the derivative, as well as in the ammonium sulfate fractions from the hot acid extraction of the DNP-walls. Apparently the bulk of the protein comes down at 30% saturation. Some of these materials were placed on DEAE cellulose columns, but no distinct separations were effected. Further, identification of various fractions was difficult because none of the DNP-proteins tested could be shown to have any reactivity versus any of the type-specific antisera. Only a complete amino acid analysis would indicate a relationship to any of the isolated M proteins. Figure 4 shows the results on 0.1 μM samples of the various M proteins isolated from the cellulose columns. Again, only a single amino acid derivative was evident, regardless of which of the isolates was run. Although other criteria proved some of these samples to be heterogeneous, this technique indicated only a single N-terminus amino acid. The results of the hydrazinolysis reaction, to determine the C-terminus amino acid, on six of the same samples are given in Table 4. Only two samples of the highly purified M protein gave single C-terminus reactions, although these do not agree. Alanine or aspartic acid may be the C-terminus or close to the linkage site of the M protein to the cell wall.

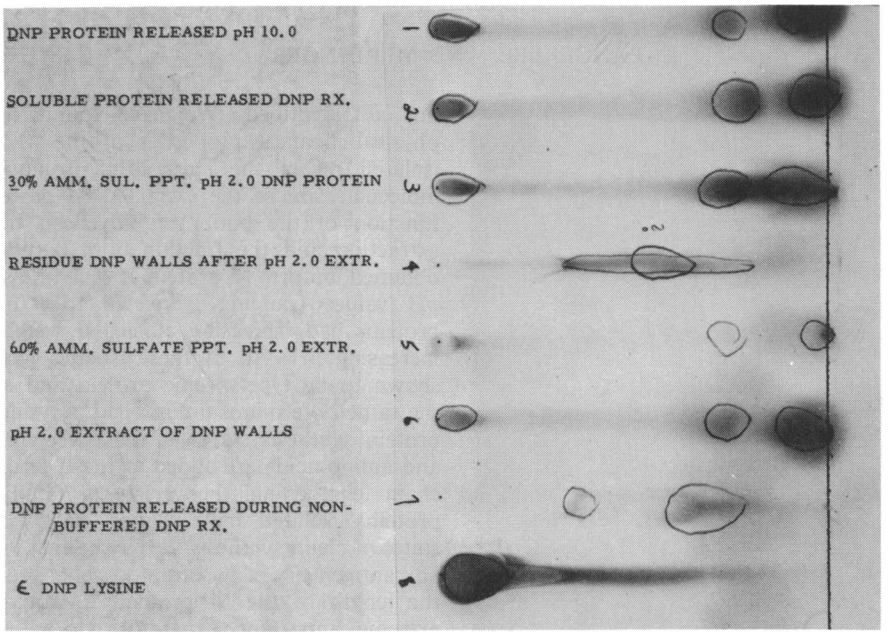

FIG. 2. *One-dimensional chromatogram (migration is left to right) of various DNP-amino acids released from the DNP-proteins extracted from the DNP-cell walls. Shown are the derivatives obtained from the protein extracted by an alkaline buffer, the protein released during derivative formation at a pH of 8.0, the result of ammonium sulfate fractionation of the acid-extracted protein, the cell wall residue after acid extraction, the acid extracted protein, the DNP-protein released during an unbuffered derivative formation reaction, and a standard of ε-DNP-lysine. The dark spots at the solvent front are a mixture of dinitrophenol and dinitroaniline. The other DNP derivative was shown to be di-DNP-lysine.*

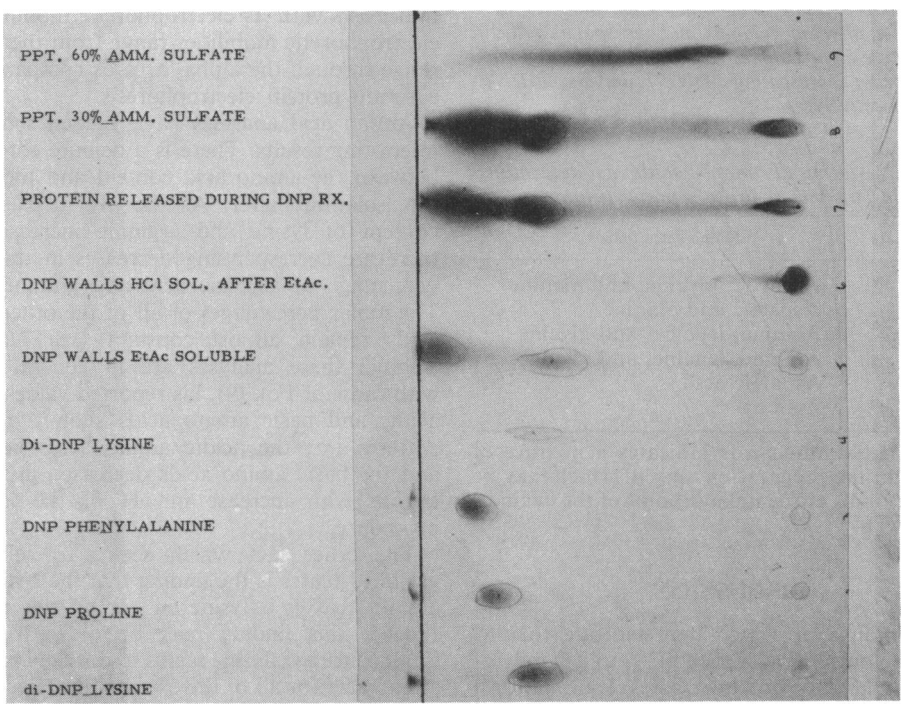

FIG. 3. *One-dimensional chromatogram (migration is right to left) of the various products resulting from the preparation of the DNP-derivative of the whole cell walls. Shown are the acid hydrolysis products from the ammonium sulfate-precipitated proteins released by hot acid after the DNP reaction, the protein released into the reaction mixture during the derivative formation, the DNP-amino acids released as a result of acid hydrolysis of the DNP-cell walls, and the three standards. The DNP derivative remaining at the origin is ε-DNP-lysine.*

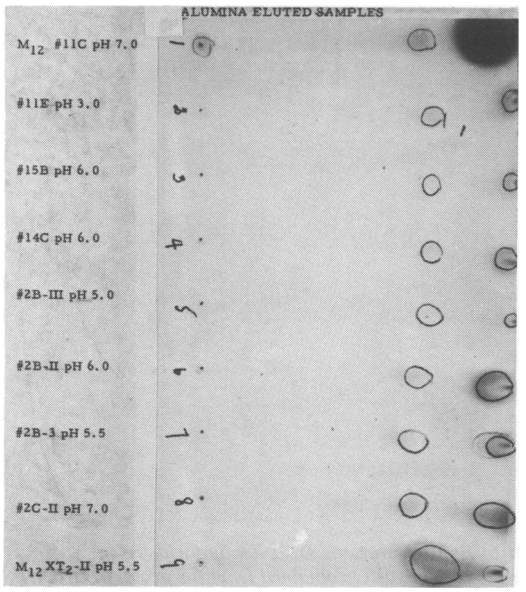

FIG. 4. *One-dimensional chromatogram (migration is left to right) of the DNP-amino acids released from the DNP-purified M proteins. Sample 1 was not run over alumina columns and shows the spot of ε-DNP-lysine at the origin, the mixture of dinitrophenol and dinitroaniline at the solvent front, and the single other spot of diDNP-lysine. The other eight samples were all run over alumina columns and the spot is evident. Each of these eight samples had a spot at the origin and one other spot representing ε-DNP-lysine and di-DNP-lysine, respectively.*

TABLE 4. *C-terminal amino acids as determined on purified M protein preparations*

*p*H of isolate	Amino acids found[a]
3.0	Aspartic, glycine, and alanine
3.0	Aspartic and alanine
6.0	Alanine, leucine, and glycine
7.0	Alanine, leucine, and glycine
5.8	Aspartic acid
5.8	Alanine

[a] Multiple amino acids indicates a mixture of proteins in the preparation, any of which may be the C-terminus at the linkage point of the chain to the cell wall.

DISCUSSION

The findings presented here indicate that M protein belongs, in all probability, to a family of proteins and does not exist as a single uniform molecular species. The M protein that is fractionated arises from a gross mixture of proteins, some of which are M proteins; the balance, as yet, are not all identified. We have found that the physical-chemical properties of the M protein isolated reflect the method of isolation. The molecular size of the extracted M protein is a function of the buffer employed in both the extraction and the fractionation. Although we obtained uniform M protein at each of a series of *p*H values (ranging from 3.0 to 9.0), these proteins had increasing molecular weights with increasing *p*H. All of these isolates have been shown to be type-specific protein and all have the same N-terminus amino acid, L-lysine. Since protein synthesis starts at the N-terminus end, and amino acids are added at the C-terminus in chain lengthening, the variety or family of M proteins isolated may in part reflect various states of chain synthesis. It may be that virulence and antigenicity of the streptococci are related to the length of the M protein. It would be of extreme importance, therefore, to be able to correlate chain length to such biological activities.

Each of the specific isolates has been shown to be homogeneous on electrophoretic, immunoelectrophoretic, and ultracentrifugal analysis. Whereas the molecular size of the M protein isolates seems to follow the *p*H (i.e., the size increases with increase in *p*H), no such correlation exists with its electrophoretic mobility. The electrophoretic mobilities range from the gamma range through the $alpha_1$ area as compared with a serum protein electrophoresis.

Amino acid analyses have yielded some very interesting results. There is a definite correlation between the amino acid content and the *p*H of the isolating buffer. As the *p*H increases, the content of lysine and arginine increases, and there are corresponding decreases in the molar percentage of aspartic acid and glutamic acid. The molar percentages of all of the other amino acids remain almost constant (*see* Table 3). Though these analyses are in general accord with those of Fox (9), his reported values for the acidic and basic amino acids show a reversed pattern; i.e., the acidic amino acids increased and the basic amino acids decreased in concentration with increase in *p*H of the isolation procedure.

The other fact which seems to relate the various isolates is the finding that the N-terminal amino acid is L-lysine in all of the isolates. Indeed, this finding may be of even greater significance as L-lysine seems to be the N-terminus amino acid for all of the external proteins of type 12 group A nephritogenic streptococci. Whether this finding relates to the typing of these organisms remains the subject of further study.

ACKNOWLEDGMENT

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