THE CHARACTERISTICS OF M PROTEINS PURIFIED BY
COLUMN CHROMATOGRAPHY WITH
HYDROXYAPATITE FROM ACID EXTRACTS OF
STREPTOCOCCUS PYOGENES OF TYPES
1, 3, 6, 12 AND 17

K. L. VOSTI

*Division of Infectious Diseases, Stanford University Medical Center,
300 Pasteur Drive, Stanford, California 94305, USA*

PLATES XLI-XLIII

THE M protein of a strain of *Streptococcus pyogenes* (Lancefield group A) is the principal factor responsible for the ability of the organism to resist phagocytosis. In addition, an M protein is antigenic and can stimulate type-specific, protective immunity in the infected or immunised host. Review articles have delineated the difficulties encountered in the isolation and investigation of these proteins in acid or alkaline extracts of whole cells or isolated cell walls (Lancefield, 1962; Fox, 1974). Only a few studies have included molecular weight and related determinations on these proteins (Fox and Wittner, 1965, 1969; Johnson and Vosti, 1968; Beachey, Alberti and Stollerman, 1969; Vosti, Johnson and Dillon, 1971; Straus and Lange, 1972). Although various schemes for purification have been utilised, purified preparations have usually contained non-type-specific antigens which could not be separated from the type-specific protein.

We have reported previously that fractionation with ammonium sulphate of acid extracts obtained from a single strain of *S. pyogenes*, M-type 12, followed by column chromatography with hydroxyapatite, resulted in the separation of two fractions containing proteins that were of different sizes and serological activity (Johnson and Vosti, 1968; Vosti *et al.*, 1971). One fraction contained the smallest proteins. These were restricted to type-specific antigens and molecular weights of about 32 000 daltons. The other fraction contained the larger proteins which had a molecular weight around 48 000 daltons. The latter fraction contained non-type-specific as well as type-specific antigens. The immunisation of rabbits with either fraction of proteins resulted in the development of opsonic antibodies. Although differing in antigenic properties, the amino acids composing these two fractions were present in similar proportions and both contained N-terminal alanine.

The present study was prompted first by our previous success in obtaining a low molecular-weight fraction with M-type 12 protein activity that was free

Received 10 Nov. 1977; accepted 2 Mar. 1978.

of non-type-specific antigens, and second, by the lack of information on the characteristics of different M-type proteins. Purified M proteins of the types 1, 3, 6, 12 and 17 were recovered from acid extracts of whole cells by fractional precipitation with ammonium sulphate followed by column chromatography with hydroxyapatite. Some physical, chemical and serological properties of these M proteins are reported.

MATERIALS AND METHODS

Organisms. Strains of *Streptococcus pyogenes*, Lancefield group A, M-types 1, 3, 6, 12 (Shaw) and 17 were used. These cultures were isolated originally from throat swabs or tonsillar tissue, were freeze-dried and revived by growth at 35° to 37°C in Brain Heart Infusion Broth (BHIB) (Difco Laboratories, USA) containing a small amount of defibrinated sheep blood. Small samples of the blood broth were stored at -20°C and thawed when needed to produce starter cultures for batch growth of the different strains. Cells for each acid extraction were cultured in 12 flasks each containing 8 litres of BHIB. After growth for 16-18 h, without agitation, at 35° to 37°C, the bacteria were harvested with the aid of a refrigerated centrifuge incorporating a continuous-flow attachment.

Purified M protein. Acid extracts were prepared by immersing in boiling water for 10 min. tubes containing the cells from 96 litres of broth in 0.2M HCl (7 ml per g wet weight cells). The extracts were then brought to pH 7.4 with 0.2M NaOH in Na₂HPO₄ (1% w/v). The proteins that precipitated at 33-60% saturations with ammonium sulphate were re-precipitated and then dialysed against the starting buffer (0.01M sodium phosphate, pH 6.7). Then 20-35 mg of protein in 10-12 ml of buffer were layered on to a column of hydroxyapatite (diameter 2.1 cm, height 6 cm) (Bio-Rad Laboratories, USA). After the sample was applied, the column was washed with the 0.01M phosphate buffer until the ultra-violet absorption (280 nm) of the effluent had returned to the baseline. The adsorbed proteins were eluted with 0.1M followed by 0.3M sodium phosphate buffer, pH 6.7. Individual tubes were saved for further study or the tubes containing protein were combined to form three pools of the proteins eluted by the 0.01, 0.1 and 0.3M buffers. The pools from six to eight column runs were combined, dialysed for 24 h at 4°C against 0.1M ammonium carbonate, pH 8.3 and freeze-dried. The dry powder was stored at 4°C in a desiccator over phosphorus pentoxide.

Polyacrylamide gel electrophoresis. Proteins were separated by electrophoresis in polyacrylamide (7.5%) disk gels as described previously (Johnson and Vosti, 1968). The molecular weights of the M proteins were determined after electrophoresis in polyacrylamide (5%) disk gels by means of sodium dodecyl sulphate (SDS) and a continuous buffer system, pH 7.1 (Vosti *et al.*, 1971). The standards used were bovine haemoglobin, trypsin, pepsin, ovalbumin and bovine plasma albumin.

Amino acid analyses. These were performed on a Beckman Model 120C amino-acid analyser (Vosti *et al.*, 1971). Aqueous samples were hydrolysed *in vacuo* for 22 h at 120°C in equal volumes of reagent grade 12M HCl. The hydrolysates were dried and dissolved in 2.0 ml of 0.2M sodium citrate (pH 2.2).

Dinitrophenylation of amino acids. N-terminal amino acids were identified by the dinitrophenylation method (Vosti *et al.*, 1971). The DNP-amino acids of M protein were determined on ascending thin-layer chromatograms by comparison with the mobilities of known DNP-amino acids. Separation was attained with chloroform-benzyl alcohol-glacial acetic acid (70 : 30 : 3, by volume), benzene-pyridine-glacial acetic acid (80 : 20 : 2, by volume) or toluene-pyridine-ethylenedichlorohydrin-0.8N ammonia solution (100 : 30 : 60 : 60, by volume). Lysine could not be distinguished from tyrosine under these conditions of chromatography.

Antisera. Antibodies were produced in randomly bred New Zealand white rabbits as described previously (Vosti *et al.*, 1971). The Biological Reagents Division of the Center for Disease Control, Atlanta, Georgia, USA, generously supplied the relevant grouping and typing antisera.

Serological techniques. Gel-diffusion tests were performed at room temperature in Petri dishes containing Noble Agar (Difco) 1% in phosphate-buffered saline, pH 7.3, with thiomersal (1 in 10 000) as a preservative.

Immunoelectrophoresis was performed on microscope slides with Noble Agar (1%) in barbital buffer, pH 8.2, ionic strength 0.075 (Harleco, USA).

Opsonic antibodies were measured by Dr Richard Facklam, Director, Streptococcal Laboratory, Center for Disease Control, Atlanta, by means of the indirect bactericidal test (Lancefield, 1957).

RESULTS

Ammonium sulphate precipitation

Fractional precipitation with ammonium sulphate provided an initial separation of proteins in the crude acid extracts. Table I shows that the majority of proteins were recovered in the twice-precipitated fraction that formed between 33–60% saturation (Bb). These fractions did not appear to contain the group antigen, but M-protein antigens were consistently recovered as were one or more cross-reactive antigens (fig. 1).

Hydroxyapatite column chromatography

Further separation by elution from columns of hydroxyapatite of the Bb fractions for each M-type resulted in the three additional fractions seen in table II. The proteins eluted with the 0.3M buffer comprised 27 to 55% of the proteins applied to the column and produced particularly intense precipitation reactions with homologous type-specific antiserum.

Antigenic analyses

The immunodiffusion reactions of the starting, twice-precipitated ammonium sulphate fractions and of the two fractions eluted from columns of hydroxyapatite are compared in fig. 1. The antigenic complexity of the starting

TABLE I
Fractional precipitation with ammonium sulphate of the protein in acid extracts of different M types of Streptococcus pyogenes (Lancefield group A)

Ammonium sulphate fractions	Percentage of precipitable protein (and intensity of precipitation reaction with homologous type-specific antiserum) for M-type				
	1	3	6	12S	17
Aa (0–33%)* (0–33%)†	14(–)	31(+)	25(–)	12(+)	9(–)
Ab (0–33%) (33–60%)	4(–)	10(+)	6(+)	4(+)	4(+)
Ba (33–60%) (0–33%)	13(–)	11(+)	12(–)	14(+)	15(+)
Bb (33–60%) (33–60%)	69(+++)	48(+)	58(+++)	70(+++)	71(+)

* First precipitation.

† Re-precipitation of the initial fraction.

– = No reaction; + = weak; ++ = moderate; +++ = strong.

TABLE II

Further separation by column chromatography with hydroxyapatite of the proteins in the Bb fractions of the ammonium sulphate precipitates

Eluate obtained from hydroxyapatite with sodium phosphate buffer	Percentage of total protein applied to column (and intensity of precipitation reaction with homologous type-specific antiserum) for M-type				
	1	3	6	12S	17
0.3M	42(+++)	44(+++)	32(+++)	27(+++)	55(+++)
0.1M	44(+)	36(-)	54(+)	61(++)	38(+)
0.01M	14(-)	20(-)	14(-)	12(-)	7(-)

- = No reaction; + = weak; ++ = moderate; +++ = strong.

material was apparent with each M type; one or more reactions were produced by most unabsorbed homologous and heterologous antisera. The fractions eluted from hydroxyapatite showed fewer reactions with antisera to heterologous types than did the starting fractions. Each of the "0.3M fractions" reacted with type-specific antisera and unabsorbed antisera to the homologous M type. In addition, the 0.3M fractions of M-types 1, 12 and 17 formed a single precipitation band with the unabsorbed type 6 antiserum. The 0.3M fraction of M-type 6 formed a single precipitation reaction with the unabsorbed type 12 antiserum; and the 0.3M fraction of M-type 3 formed a single precipitation line, which gave a reaction of complete identity, with the unabsorbed antisera of types 6 and 12.

The 0.1M fractions gave fewer reactions with heterologous antisera than did the 0.3M fractions. The 0.1M fraction of M-type 1 formed a single cross-reaction only with unabsorbed type-6 antiserum while that of M-type 6 formed a single, weak cross-reaction only with unabsorbed type-12 antiserum. In addition, the 0.1M fractions of M-types 3, 6 and 17 failed to react with type-specific antisera, but reacted with unabsorbed antisera prepared against whole cells. The 0.1M fractions of M-types 3, 12 and 17 were the only ones that formed precipitation reactions with homologous unabsorbed antisera and failed to cross-react with heterologous antisera. Finally, the 0.1M fractions of M-types 3 and 17 were of particular interest in that they failed to react with type-specific antisera but formed a single, strong precipitation reaction with unabsorbed homologous antisera.

The complexity of the antigens in the starting (Bb) and the 0.3M fractions was examined by immunoelectrophoresis (fig. 2). The antigens were compared, in respect of mobility, with human serum proteins. Distinct charge differences were apparent among antigens from the various M-types; those in M-type 6 moved primarily toward the cathode, those in M-type 1 moved toward the anode, and those in M-types 3, 12 and 17 remained near the point of origin. None of the antigens had a mobility as rapid as that of serum albumin. Similar studies, in which the mobilities of the antigens in the 0.1M and 0.3M fractions

were compared, revealed that antigens in the 0.1M fractions had mobilities near the anodic end of the bands formed with 0.3M fractions.

Polyacrylamide gels

The number and distribution of the protein bands differed among the fractions obtained with ammonium sulphate precipitation and with column chromatography for each of the M types (fig. 3).

The molecular weights of the proteins in the 0.1M and 0.3M fractions for each M-type are shown in table III. Considerable variation in the molecular weights (MW) and in the number of protein bands was seen with each fraction. The most prominent bands in the 0.3M fractions were associated with a MW of 30 000–50 000 daltons while those in the 0.1M fractions were associated with a MW of 22 000–26 000 daltons.

Amino acid composition

The distribution in the various column fractions of the amino acids that reacted with type-specific antisera are presented in table IV. The relative proportions of the different amino acids were similar for all of the fractions; glutamic acid, lysine, alanine, aspartic acid and leucine were present in the largest amounts. The basic amino acids, lysine, arginine, and histidine, represented 22–24% of the total amino acids, except in the 0.1M fraction of M-type 12S, in which the corresponding figure was 17%. Methionine, histidine and proline did not form a large proportion of the total residues, and cysteine was not recovered.

TABLE III

Molecular weights of proteins in hydroxyapatite column fractions determined by electrophoresis in polyacrylamide disk gels with an SDS continuous-buffer system

Eluate obtained from hydroxyapatite with sodium phosphate buffer	Molecular weights of proteins of M-type				
	1	3	6	12S	17
0.3M	...	...	...	...	90 000
	...	72 000	72 000	72 000	...
	63 000	...	...	63 000	63 000
	...	50 000	50 000	...	...
	48 000	...	48 000	...	48 000
	45 000	...	...	...	...
	32 000	...	...	31 000	...
	...	...	23 000	...	...
0.1M	...	...	...	100 000	...
	...	...	67 000	...	...
	...	50 000	...	...	...
	48 000	...	47 000	48 000	48 000
	...	...	40 000	42 000	...
	31 000	...	34 000	32 000	34 000
	24 000	26 000	22 000	...	24 000
	...	...	...	...	...

Molecular weights of dominant bands are in bold type.

TABLE IV

The rank order of amino acids in protein fractions eluted from columns of hydroxyapatite

Rank order of amino acids in the protein fraction of the stated M-type (eluted with 0.3M or 0.1M sodium phosphate buffer)											
1 (0.3M)		3 (0.3M)		6 (0.3M)		12S (0.3M)		12S (0.1M)		17 (0.3M)	
Glu	159*	Lys	154	Lys	175	Glu	151	Lys	136	Lys	166
Lys	136	Glu	144	Asp	130	Lys	135	Glu	136	Glu	148
Ala	111	Ala	114	Glu	124	Ala	118	Ala	120	Asp	148
Asp	107	Leu	105	Leu	124	Asp	101	Asp	103	Leu	126
Leu	100	Asp	102	Ala	85	Leu	97	Gly	93	Ala	70
Arg	85	Val	75	Thr	85	Gly	83	Leu	90	Arg	68
Gly	76	Gly	70	Arg	60	Arg	78	Val	77	Gly	63
Val	59	Arg	67	Val	54	Val	66	Thr	58	Ser	62
Thr	53	Thr	60	Gly	51	Ser	58	Ser	53	Ile	42
Ser	53	Ser	53	Ser	49	Thr	57	Ile	49	Val	41
Ile	43	Ile	47	Ile	43	Ile	48	Arg	32	Thr	36
Tyr	7	Tyr	3	Tyr	17	Tyr	2	φAla	28	Tyr	27
φAla	7	φAla	2	φAla	1	φAla	2	Tyr	23	φAla	1
His	1	His	1	His	1	His	2	His	1	His	1
Pro	1	Pro	1	Pro	1	Pro	2	Pro	1	Pro	1
Met	1	Met	1	Met	1	Met	2	Met	1	Met	1

* Expressed as residues per 1000.

N-terminal analyses

The fractions reacting with type-specific antisera were examined for their N-terminal amino acids. Most fractions contained more than one DNP-amino acid. The most prominent amino acids in the 0.3M fractions of M-type 1 and M-type 3 proteins were alanine and either lysine or tyrosine; that of M-type 6 was lysine or tyrosine, and that of M-types 12 and 17 was alanine. Alanine was the most prominent amino acid in the 0.1M fraction of M-type 12S.

Opsonic antibodies

Serum samples were usually obtained from the rabbits every 2 weeks and they were tested by gel diffusion for the presence of precipitating antibodies to the immunising fractions. The greatest response was observed in sera collected 9–11 weeks after immunisation and these sera were used in the opsonisation tests. As seen in table V, the sera from 12 of 16 rabbits immunised with the 0.3M fractions contained opsonic antibodies for the immunising strain. The sera of only five of 20 rabbits immunised with 0.1M fractions contained opsonic antibodies. None of seven rabbits immunised with the 0.01M fractions produced opsonic antibodies.

DISCUSSION

The characteristics of purified M proteins recovered from some of the 57 recognised M-types of *S. pyogenes* (Lancefield group A) have been studied by Lancefield (1962), Fox (1974) and Widdowson, Maxted and Pinney (1976). A

FIG. 1.—Analyses by double diffusion in agar of the starting (33–60%) ammonium-sulphate fraction and of the 0.3M and 0.1M hydroxyapatite column eluates for M-types 1, 3, 6, 12 and 17. A1, A3, A6, A12 and A17 represent absorbed type-specific antisera; U1, U3, U6, U12 and U17 represent unabsorbed antisera to whole-cell vaccines.

FIG. 2.—A comparison by immunoelectrophoresis of the mobilities of antigens in the Bb (33–60%) and 0·3M hydroxyapatite column eluates of M types 1, 3, 6, 12 and 17 with those of human serum proteins (HS). Antisera in the troughs were antihuman serum (AHS), and unabsorbed antisera (U1, U3, U6, U12 and U17) to whole cell vaccines of M-types 3, 6, 12 and 17.

FIG. 3.—Separation by electrophoresis in polyacrylamide disk gels of the proteins in the starting (33–60%) ammonium-sulphate fraction (AS) and of the peak tubes of the 0.01M, 0.1M, and 0.3M hydroxyapatite column eluates for M types 1, 3, 6, 12 and 17.

TABLE V

The occurrence of opsonic antibodies in the sera of rabbits immunised with the proteins in fractions obtained by column chromatography with hydroxyapatite

Eluate obtained from hydroxyapatite with sodium phosphate buffer	Number of rabbits forming opsonins/number immunised, for M-type							Total
	1	3	6	12S	12B	12-126/4	17	
0.3M	2/2	1/2	3/4	1/1	2/2	1/1	2/4	12/16
0.1M	0/4	0/4	1/4	1/1	2/2	1/1	0/4	5/20
0.01M	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/7

major complication of attempts to purify M proteins has been the presence of cross-reactive antigens. The association of non-type-specific antigens with purified M proteins is not an artefact of acid extraction, as such antigens have been observed in purified M proteins released by treatment of whole cells or cell walls with hydroxylamine at pH 10 (Cunningham and Beachey, 1975), nonionic detergents (Fischetti *et al.*, 1973), pepsin (Beachey, Campbell and Ofek, 1974), and cyanogen bromide (Vosti and Williams, 1976). The almost uniform occurrence of non-type-specific antigens among the different preparations of purified M proteins has raised the possibility that the cross-reactive antigens may represent antigenic determinants on the molecules of M protein, rather than contaminating antigens. This concept is supported by the co-purification of both the type- and non-type-specific antigens, and supported even more strongly by the co-precipitation of these different antigens by antisera against either the type- or non-type-specific antigens (Vosti *et al.*, 1971; Widdowson, Maxted and Pinney, 1971; Vosti, 1975).

In the present studies, we confirmed our previous findings with purified type-12 M protein, but failed to recover fractions of M protein free of cross-reactive antigens from single strains of M-types 1, 3, 6 and 17. These observations suggest that type-12 M protein is probably not randomly hydrolysed by hot-acid extraction. In addition, they suggest that type-12 M protein is hydrolysed differently from other M proteins, as the latter did not yield fractions devoid of cross-reactive antigens, although the methods remained uniform. Others have shown that it is possible with other techniques to derive fractions relatively free of these cross-reactive antigens. Thus by partial degradation with pepsin, Cunningham and Beachey (1974) were able to reduce significantly, but not completely, the non-type-specific moiety of alkaline-extracted and purified type-24 M protein while still retaining the opsonic inhibitory and immunogenic properties.

In addition to the M proteins and cross-reactive antigens, the acid extracts obtained from M-types 3 and 17 in the present study contained what appeared to be additional type-specific antigens that did not possess the accepted properties of M proteins. These antigens were separable from M protein by column chromatography. They reacted with unabsorbed antisera against whole-cell

vaccines of the homologous M type, but not to three heterologous M types; they failed to react with absorbed type-specific antiserum, and failed to stimulate opsonic antibodies. Similar antigens have been described by Wilson and Wiley (1963) in certain strains of M-type 17. Further characterisation of these type-specific antigens has not been completed, and it is not possible to decide what relationship, if any, they have to M protein.

The molecular weights of the major proteins in our purified preparations of M protein from types 1, 3, 6, 12 and 17 were between 32 000 and 63 000 daltons, with some variation between the different M types. The properties of forming a precipitate with type-specific antibodies and of stimulating the formation of opsonic antibodies in rabbits were restricted to column fractions containing primarily proteins with molecular weights of 32 000 daltons or greater. This finding was consistent with the observations of others, who used different purification methods for types 6, 12 and 24 (Fox and Wittner, 1965, 1969). We did not identify small molecules of protein such as those recently found by Fischetti *et al.* (1976) in their studies with detergent-released type-6 M protein. They isolated proteins that formed precipitin reactions with type-specific antiserum, but that failed to absorb opsonic antibodies from type-specific antiserum.

In the present study, the amino acid compositions of the various M proteins of types 1, 3, 6, 12 and 17 were similar. The five amino acids present in the greatest amounts in the M proteins were glutamic acid, lysine, alanine, aspartic acid and leucine. These findings confirmed our previous results with M protein obtained from another strain of M-type 12 (Vosti *et al.*, 1971), and were similar to those reported as a result of studies with purified acid-extracted M proteins obtained by means of different purification schemes from M-types 3, 4, 5, 6, 12 and 14 (Fox and Wittner, 1969; Lange, Lee and Merdinger, 1969; Straus and Lange, 1972).

Our previous study (Vosti *et al.*, 1971) suggested that M protein of type 12 had N-terminal alanine, and lesser amounts of glycine, leucine and lysine were also found. In the present study the presence of N-terminal alanine was confirmed in a second strain of M-type 12. Under the conditions used for identification of the DNP-amino acids, we were unable to distinguish lysine from tyrosine; however, analyses of the other M types revealed an apparent variation in the N-terminal amino acid; alanine and lysine-or-tyrosine were present in M-types 1 and 3, lysine-or-tyrosine was present in M-type 6, and alanine was present in M-type 17. Although alanine or lysine-or-tyrosine or both may have appeared dominant in individual fractions, each occurred together with several other amino acids in all fractions. These results differ from those of Straus and Lange (1972), who identified only N-terminal alanine in purified M proteins of types 3, 4, 5, 6, 12 and 14. We used alumina columns as described by these authors, but failed to alter the complexity of our analyses. The occurrence of more than one N-terminal amino acid in purified preparations made from acid-extracted M proteins does not necessarily indicate the presence of contaminating proteins, as the cleavage of proteins by acid hydrolysis is not specific. Our findings are consistent with those discussed by Myoda,

Wiley and Bruno (1973) who also encountered more than one N-terminal amino acid in alkali-extracted M protein and its acid hydrolysate.

Differences in antigenic reactivity of M proteins are unlikely to be resolved by analysis of either their total amino acid compositions or their N-terminal amino acids. Analysis of the sequences of amino acids in the proteins might determine whether M proteins have common regions that could account for shared antigenic determinants and variable regions that determine their type-specific properties. A possible complication is raised by recent studies suggesting that regions responsible for binding type-specific precipitins may not be the same as those responsible for binding type-specific opsonins (Cunningham and Beachey, 1975; Russell and Facklam, 1975; Fischetti *et al.*, 1976).

SUMMARY

Purified M proteins were recovered from acid extracts of *Streptococcus pyogenes*, M-types 1, 3, 6, 12 and 17, by elution from columns of hydroxyapatite of the proteins precipitated with ammonium sulphate. M protein free from non-type-specific antigens was recovered only from M-type 12. Although similar fractions were not recovered from M-types 1, 3, 6 and 17, purified preparations containing a single cross-reactive antigen were obtained. In addition to the M proteins associated with cross-reactive antigens, type-specific antigens that did not stimulate opsonic antibodies were isolated from the acid extracts of M-types 3 and 17. Further study of the purified M proteins revealed molecular weights that ranged from 32 000 to 63 000 daltons, total amino acid compositions that were similar, and N-terminal amino acids that were variable.

The author thanks Mrs Nancy Sterngold and Mrs Pamela Thomas for their skilful technical assistance, and Mrs Isabelle Smith for her help in the preparation of the manuscript. This work was supported by Grant AI-06964 from the National Institutes of Health, United States Public Health Service.

REFERENCES

- BEACHEY, E. H., ALBERTI, H. AND STOLLERMAN, G. H. 1969. Delayed hypersensitivity to purified streptococcal M protein in guinea pigs and in man. *J. Immun.*, **102**, 42.
- BEACHEY, E. H., CAMPBELL, G. L. AND OFEK, I. 1974. Peptic digestion of streptococcal M protein. II. Extraction of M antigen from group A streptococci with pepsin. *Infect. Immun.*, **9**, 891.
- CUNNINGHAM, M. W. AND BEACHEY, E. H. 1974. Peptic digestion of streptococcal M protein. I. Effect of digestion at suboptimal pH upon the biological and immunochemical properties of purified M protein extracts. *Infect. Immun.*, **9**, 244.
- CUNNINGHAM, M. AND BEACHEY, E. H. 1975. Immunochemical properties of streptococcal M protein purified by isoelectric focusing. *J. Immun.*, **115**, 1002.
- FISCHETTI, V. A., GOTSCHLICH, E. C., SIVIGLIA, G. M. AND ZABRISKIE, J. B. 1973. Streptococcal M protein: a molecule composed of subunits. *J. clin. Invest.*, **52**, 28a.
- FISCHETTI, V. A., GOTSCHLICH, E. C., SIVIGLIA, G. M. AND ZABRISKIE, J. B. 1976. Streptococcal M protein extracted by nonionic detergent. I. Properties of the antiphagocytic and type-specific molecules. *J. exp. Med.*, **144**, 32.
- FOX, E. N. 1974. M proteins of group A streptococci. *Bact. Rev.* **38**, 57.

- FOX, E. N. AND WITTNER, M. K. 1965. The multiple molecular structure of the M proteins of group A streptococci. *Proc. natn. Acad. Sci. U.S.A.*, **54**, 1118.
- FOX, E. N. AND WITTNER, M. K. 1969. New observations on the structure and antigenicity of the M proteins of the group A streptococcus. *Immunochemistry*, **6**, 11.
- JOHNSON, R. H. AND VOSTI, K. L. 1968. Purification of two fragments of M protein from a strain of group A, type 12 streptococcus. *J. Immun.*, **101**, 381.
- LANCEFIELD, R. C. 1957. Differentiation of group A streptococci with a common R antigen into three serological types with special reference to the bactericidal test. *J. exp. Med.*, **106**, 525.
- LANCEFIELD, R. C. 1962. Current knowledge of type-specific M antigens of group A streptococci. *J. Immun.*, **89**, 307.
- LANGE, C. F., LEE, R. AND MERDINGER, E. 1969. Chemistry and end-group analysis on purified M protein of type 12 group A streptococcal cell walls. *J. Bact.*, **100**, 1277.
- MYODA, T. T., WILEY, G. G. AND BRUNO, P. N. 1973. Cross-reactions among group A streptococci. IV. Extraction, separation and purification of two protective antigens of type G cocci. *J. Immun.*, **111**, 249.
- RUSSELL, H. AND FACKLAM, R. R. 1975. Guanidine extraction of streptococcal M protein. *Infect. Immun.*, **12**, 679.
- STRAUS, D. C. AND LANGE, C. F. 1972. Immunochemistry and end-group analyses of group A streptococcal M proteins. *Infect. Immun.*, **5**, 927.
- VOSTI, K. L. 1975. Characterization of a non-type-specific antigen(s) associated with group A streptococcal type 12 M protein. *Infect. Immun.*, **11**, 1300.
- VOSTI, K. L., JOHNSON, R. H. AND DILLON, M. F. 1971. Further characterization of purified fractions of M protein from a strain of group A, type 12 streptococcus. *J. Immun.*, **107**, 104.
- VOSTI, K. L. AND WILLIAMS, W. K. 1976. Characterization of streptococcal M proteins released by treatment of whole cells with cyanogen bromide. *Clin. Res.*, **24**, 153A.
- WIDDOWSON, J. P., MAXTED, W. R. AND PINNEY, A. M. 1971. An M-associated protein antigen (MAP) of group A streptococci. *J. Hyg., Camb.*, **69**, 553.
- WIDDOWSON, J. P., MAXTED, W. R. AND PINNEY, A. M. 1976. Immunological heterogeneity among the M-associated protein antigens of group-A streptococci. *J. med. Microbiol.*, **9**, 73.
- WILSON, A. T. AND WILEY, G. G. 1963. The cellular antigens of group A streptococci. Immuno-electrophoretic studies of the C, M, T, PGP, E₄, F and E antigens of serotype 17 streptococci. *J. exp. Med.*, **118**, 527.