

PREPARATION AND PROPERTIES OF TYPE-SPECIFIC M ANTIGEN
ISOLATED FROM A GROUP A, TYPE 1
HEMOLYTIC STREPTOCOCCUS

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Studies of the type-specific M antigens of group A streptococci have been continued. In this paper methods for the purification of the M protein of a type 1 strain are described and the chemical and immunological properties of the purified antigen are reported.

Materials and Methods

Strain and Culture Medium.—A type 1 strain (T1, SF130 Griffith (1)) rendered highly virulent by mouse passage was used. An 18 hour blood broth culture of this organism kills mice regularly in doses of 10^{-7} to 10^{-8} cc. For mass cultures Todd-Hewitt broth (2) prepared with neopeptone and beef heart and sterilized by filtration through porcelain filters was used without enrichment. Other media were enriched with rabbit blood.

Enzyme.—A crystalline preparation of ribonuclease, free of proteolytic activity, was used in the purification procedures.

Electrophoretic Technique.—The electrophoretic studies were carried out at 0.5°C. in the apparatus described by Longworth (3). Prior to electrophoresis a 1 per cent solution of the protein was dialyzed at 5°C. for 2 to 3 days against large volumes of the same buffer employed in the analysis. The mobilities were computed from the descending patterns by measuring the bisecting ordinate of the refractive index gradient curve, and referred to 0°C.

Ultraviolet Absorption Spectra.—In the measurement of the ultraviolet absorption spectra a Beckman quartz spectrophotometer, model DU, was used. The protein was dissolved in M/15 phosphate buffer, pH 8, in a concentration of 1 mg. per cc.

Immunological Techniques.—Antisera were prepared and absorbed as previously described (4, 5). Precipitin tests were performed with the capillary tube technique (6). The procedure for passive protection tests is described in the individual experiments.

EXPERIMENTAL

In the past, various attempts have been made without success to isolate the M antigen by methods less likely to denature the protein than extraction in a boiling water bath at pH 2. In this study extraction at a lower temperature was again tried. At 37°C., however, it was necessary to extract the bacteria repeatedly over a longer period of time, and the preparation was not superior to that obtained with a shorter exposure at the higher temperature. The latter procedure was, therefore, used.

¹ Kindly supplied by Dr. M. McCarty.

Occasionally in some preparations a poor yield of the M antigen was obtained if the bacteria were removed by centrifugation before the extracting fluid was neutralized. It was thought that this might be due either to adsorption of the protein on the heavy suspension of bacterial cells or to precipitation of the material at low pH values in the presence of the ribonucleic acid liberated by hydrolysis of the streptococci. This difficulty was overcome by adjusting the suspension to pH 7.5 before centrifugation, although it was realized that cellular components other than the M protein might become soluble at the higher pH.

Preparation of Type 1 M Antigen

Extraction.—The streptococci were grown in 60 liter lots and collected in a Sharples centrifuge with an average yield of 14 to 16 gm. of bacteria (dry weight). In a representative experiment the washed bacteria from two such lots were suspended in 800 cc. of saline. The pH was adjusted to a value between 2 and 2.5 by adding *N* HCl. The temperature of the suspension was brought to 95°C. in a boiling water bath and maintained at this temperature for 10 minutes. The suspension was cooled and adjusted to pH 7.5 with *N* NaOH. After centrifugation the supernatant fluid was removed and the bacterial sediment washed once with saline at pH 7.5.

Extractions using successively smaller volumes were repeated several times. Precipitin tests showed that the first two extracts contained most of the M antigen. The combined supernates were filtered first with filter cel through paper on a Buchner funnel, and then through a porcelain bacteriological filter (Coors No. 3).

Concentration and Digestion with Ribonuclease.—The M protein in combination with the large amount of ribonucleic acid was precipitated by acidifying the filtrate to pH 2 with 6 *N* HCl. This precipitate was dissolved in a minimal amount of phosphate buffer of pH 8.

Ribonuclease was added at a final concentration of 0.001 mg. per cc., and the solution dialyzed in cellophane tubing during digestion at 37°C. against *m*/100 phosphate buffer of pH 8. Chloroform was used as a preservative.

A large proportion of the yellow pigment present in the original material appeared in the dialysate during the first 2 hours of digestion with ribonuclease. In the absence of the enzyme the coloring matter did not go through the membrane. After digestion for 5 hours, dialysis was continued for several days against large volumes of phosphate buffer at 3°C. to eliminate digestion products. It was found that about 90 per cent of the ribonucleic acid was removed. Increasing the concentration of the enzyme or prolonging the period of digestion resulted in no further decrease in the concentration of ribonucleic acid.

Fractionation with $(NH_4)_2SO_4$.—The majority of the M protein present in the dialyzed material was precipitated between 0.33 and 0.6 saturation with $(NH_4)_2SO_4$, both at pH 6 and at pH 8. Several reprecipitations were carried out. The final product, designated as lot 15, was dialyzed salt-free at 3°C., and then frozen and dried.

Immunological Properties

Precipitin Reactions.—Precipitin tests with purified type 1 M antigen showed positive reactions at final concentrations of the antigen as low as 0.005 mg. per cc. (a dilution of 1:200,000) with absorbed type 1 anti-M sera (6). The exact titer varied with the antibody content of individual sera. Absorbed anti-M sera of other serological types were uniformly negative in

their reactions with this antigen. With some unabsorbed heterologous antisera obtained from rabbits which had undergone prolonged courses of immunization, the purified M gave a precipitate. In only three instances was the reaction marked, concentrations of 0.25 to 0.06 mg. per cc. (1:4,000 to 1:16,000) giving a positive reaction. This impurity in the antigen did not appear to be the group-specific C polysaccharide, but was probably protein in nature since, like the M antigen itself, it was digested by trypsin.

Antigenicity in Rabbits.—Two rabbits were immunized, each with a total of 45 mg. of type 1 M antigen (lot 15) injected intravenously. Daily doses of 5 mg. were given for four courses of 2 or 3 days each, separated by rest periods of 5 to 8 days.

Antibodies were demonstrable after 14 days, and increased somewhat with further immunization. The bleedings of rabbit R76-80 gave consistently stronger reactions than those of R76-81 (Table I *a*). Approximately half of the crude extracts prepared from 45 different types of group A streptococci gave weakly positive precipitin reactions indicating that the purification of the type 1 antigen used for immunization was not complete and that antigenic impurities were still present. No reaction was obtained with partially purified C polysaccharide specific for group A.

A single absorption of the R76-80 serum with streptococci of a heterologous type (type 4) eliminated cross-reactions almost entirely, without reducing the titer for the homologous antigen. The reaction of the purified M substance with this absorbed serum was then compared with that of a serum prepared in the usual way by immunization with the intact type 1 microorganisms. The results of these precipitin tests are presented in Table I *a*.

Protective Antibodies.—In previous experiments it was shown that protective antibodies were formed in the sera of rabbits immunized with partially purified M antigens (7). Similar results were obtained with the serum of rabbit R76-80 immunized with the more highly purified type 1 M preparation used in this study (Table I *b*). This serum protected mice against at least 1,000 lethal doses of a virulent type 1 culture, and gave no evidence of protection against two heterologous strains.

On the other hand the serum of rabbit R76-81 failed to protect mice against the homologous strain. This failure may have been related to a lower M antibody content indicated by the weaker precipitin reactions obtained with this serum as compared to serum R76-80.

Experiments were undertaken to find out whether the purified M antigen would absorb the protective antibody from an antibacterial serum. 40 mg. of purified type 1 antigen (lot 15) was added to 8 cc. of type 1 antibacterial serum diluted with an equal quantity of saline and the mixture kept at 3°C. for 18 hours. A precipitate formed and was removed by centrifugation. Small amounts of precipitate continued to form during the next 48 hours in the

cold. As shown in Table II *a* only traces of anti-M precipitins were demonstrable in the absorbed serum as compared to an unabsorbed control.

TABLE I *a*
Anti-M Precipitins in the Sera of Rabbits Immunized with Purified Type 1 M Antigen
M Precipitin Reactions

Antiserum	Time read	Type 1 M antigen, lot 15 1 mg./cc., diluted 1:							
		2	4	8	16	32	64	128	256
<i>Rabbits immunized with purified type 1 M antigen, lot 15 (bled 33rd day)</i>									
<i>(1) R76-80</i>									
<i>(a) Unabsorbed*</i>									
	2	++	++	++	++	++	+	±	-
	18	+++	+++	+++	++	++	+	+	±
<i>(b) Absorbed with streptococci of heterologous type</i>									
	2	++	++	++	++	++	±	-	-
	18	+++	+++	++	++	++	+	+	±
<i>(2) R76-81</i>									
<i>Unabsorbed*</i>									
	2	+	+	±	±	±	-	-	-
	18	+++	++	++	++	++	+	±	±
<i>Rabbit immunized with type 1 streptococci</i>									
<i>Serum absorbed with streptococci of heterologous types until it contained only anti-M antibodies</i>									
	2	++++	+++	+++	++	+	±	-	-
	18	++++	++++	+++	+++	++	++	+	+

Readings were recorded after incubation at 37°C. for 2 hours and again after refrigeration at 4°C. for 18 hours.

- indicates "no reaction."

± to ++++ indicate degree of reaction.

* See Table I *b* for passive protection tests with these sera.

The protective antibody content of the absorbed and unabsorbed lots of antibacterial serum was also compared (Table II *b*). 0.06 cc. of unabsorbed serum protected mice against infection with a dose of 10^{-4} cc. of the homologous culture, and 0.5 cc. of serum protected against 10^{-3} cc. of culture. With the absorbed serum, on the other hand, the smaller amounts afforded little or no protection, and it was necessary to use as much as 0.5 cc. of ab-

sorbed serum to protect mice against even the smallest test dose employed (10^{-6} cc.).

From these experiments it was concluded that purified type 1 M antigen absorbed type 1 protective antibodies as well as inducing the formation of

TABLE I b
Protective Properties of Antisera from Rabbits Immunized with Purified Type 1 M Antigen
Passive Protection Tests with Type 1 Streptococcus

Mouse No.	Antiserum	Dose of culture, cc.					
		10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-7}	10^{-5}
	Rabbits immunized with purified type 1 M antigen, lot 15 (bled 33rd day)						
1	(1) Rabbit R76-80, unabsorbed	D2	S	S	S		
2		D2	S	S	S		
3		D2	D3	S	S		
4		D2	D2	S	S		
1	(2) Rabbit R76-81, unabsorbed	D2	D2	S	D6		
2		D2	D2	D3	D3		
3		D2	D2	D2	D2		
4		D2	D2	D2	D2		
1	No serum (virulence controls)			D2	D3	D2	S
2				D2	D3	D2	D2
3				D2	D2	D2	D2
4				D2	D2	D2	D2
Colony counts					720	65	4

The type 1 strain had been passed through a total of 119 mice. Swiss mice of the Rockefeller Institute stock, 18 to 22 gm. in weight, were used. 0.5 cc. of whole serum was injected intraperitoneally on the day before the infecting dose. Serial dilutions of a 16 hour blood broth culture were prepared in broth so that 0.5 cc. contained the dose indicated. Four mice were injected intraperitoneally with each dose and observed for 2 weeks.

The numeral following D indicates day of death. S indicates survival.

For colony counts serial dilutions of culture were plated in blood agar pour plates at the time of the test.

See Table I a for precipitin reactions with these antisera.

these type-specific antibodies in rabbits. As far as its immunological properties were concerned the protein isolated, therefore, did not appear to have been degraded.

Chemical and Physical Properties

Elementary Chemical Analysis.—On microchemical analysis this preparation was found to have the following composition: C = 50.71 per cent, H =

7.49 per cent, N = 16.24 per cent, S = 2.46 per cent, and P = 0 (corrected for ash content of 3.94 per cent).

Ultraviolet Absorption Spectrum.—In the ultraviolet, the type 1 M antigen has a maximum absorption at a wave length of 276 m μ and a minimum at 255 m μ . The curve shown in Fig. 1 is characteristic of proteins. The lack of appreciable absorption at 260 m μ indicates that this protein does not contain nucleic acid. This result is in accord with the chemical findings that this substance is phosphorus-free.

TABLE II a
Antibacterial Serum
Absorption of Precipitins with Purified Type 1 M Antigen
M Precipitin Reactions

Type 1 antibacterial serum, diluted 1:2	Time read	Type 1 M antigen, lot 15 10 mg./cc. diluted 1:								
		1	2	4	8	16	2048	4096	8192	
(1) Unabsorbed	18 hrs.	++++	++++	++++	++++	++++	+++	++	+	±
(2) Absorbed with purified type 1 M antigen, lot 15*	18	—	—	—	—	—	—	—	—	—
	48	±	±	±	±	±	—	—	—	—
	72	+	+	+	±	±	—	—	—	—

This serum was from rabbit R76-44, immunized with heat-killed streptococci, group A, type 1. This strain (T1) had received a total of 140 passages through mice.

* Serial dilutions of this absorbed serum were tested against unabsorbed serum, R76-44, and normal serum. An excess of antigen was demonstrated by precipitin reactions positive with serum R76-44 at least as high as the 1:128 dilution.

See Table I a for the technique used in these precipitin reactions, and Table II b for passive protection tests with these sera.

Electrophoretic Properties.—In this study electrophoretic analysis was used to follow the progress of the purification of the antigen and also to characterize the purified antigen. In the early stages of fractionation, samples were withdrawn from the electrophoretic cell at the end of each experiment, and the different components of the mixtures tested serologically.

The electrophoretic patterns shown in Fig. 2 were obtained in experiments carried out in a 0.02 N diethylbarbituric acid–0.02 N sodium diethylbarbiturate–0.08 N sodium chloride buffer of pH 7.8 at a potential gradient of 6 volts per cm. These patterns show several steps in the purification of the type 1 M antigen. Those of the ascending boundaries are of particular interest. In the case of the partially purified preparation, Fig. 2 a, the active component appears only as a shoulder on the low mobility side of the main peak, whereas in the patterns of Figs. 2 b and 2 c the faster portion has been eliminated. The boundary designated as M is that of the active material and has

TABLE II b
Antibacterial Serum
Absorption of Protective Antibodies with Purified Type 1 M Antigen
 Passive Protection Tests with the Homologous Type 1 Strain

Mouse No.	Dose of antiserum	Type 1 antibacterial serum							
		Unabsorbed						Absorbed with purified type 1 M antigen	
		Dose of culture (type 1), cc.							
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸
	<i>cc.</i>								
1	0.5	D1	S	S	S	S	S	S	S
2		D1	D1	S	S	S	S	D4	S
3		D1	D1	S	S	S	S	D2	S
4		D1	D1	D1	S	S	S	D1	D2
1	0.25			D1	S	S	S	S	S
2				D1	S	S	S	D1	S
3				D1	S	S	S	D1	D4
4				D1	S	S	S	D1	D3
1	0.12			D2	S	S	S		D1
2				D1	S	S	S		D1
3				D1	S	S	S		D1
4				D1	S	S	S		D1
1	0.06			D1	S	S	S		S
2				D1	S	S	S		D2
3				D1	S	S	S		D1
4				D1	D1	D1	S		D1
1	0.03			D1	S		S		
2				D1	S1		S		
3				D1	D1		D4		
4				D1	D1		D2		
		10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸				
1	No serum (virulence controls)	D1	S	D3	S				
2		D1	D1	D1	S				
3		D1	D1	D1	D3				
4		D1	D1	D1	D2				
	Colony counts		734	95	14				

The antiserum was from rabbit R76-44, immunized with heat-killed streptococci, strain T1. The serum volumes were all brought to 1 cc. (instead of the usual 0.5 cc.) The dosage is computed in terms of whole serum. With this exception the protection test was set up in the same way as that recorded in Table I b.

The homologous group A, type 1 strain used to test protection had received a total of 119 passages through mice.

See Table II a for precipitin reactions with these sera.

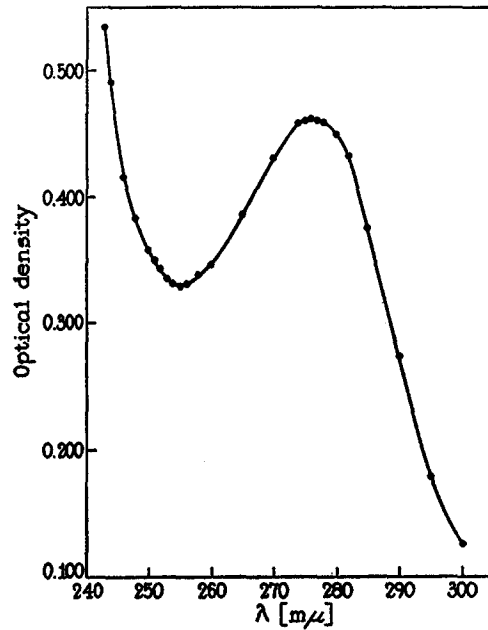
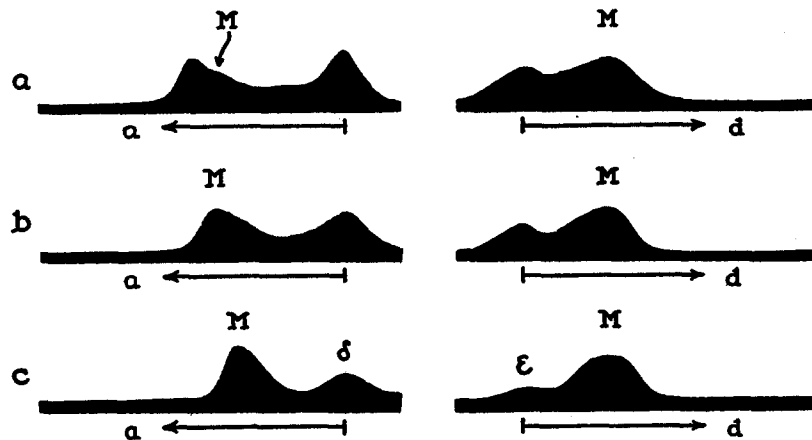


FIG. 1. Absorption spectrum of a 0.1 per cent solution of type 1 M antigen in phosphate buffer of pH 8.0.



FIGS. 2 *a*, *b*, and *c*. Electrophoretic patterns of a 1 per cent solution of type 1 M antigen at different stages of purification. Electrophoresis was carried out in a sodium diethylbarbiturate-sodium chloride buffer of pH 7.8 and at ionic strength 0.1 for 12,600 seconds at a potential gradient of 6 volts per cm.

a mobility of -3.8×10^{-5} cm.² sec.⁻¹ volt⁻¹. In the best preparation of M antigen thus far obtained the protein migrated as a single peak in the

pH range 3 to 9. It cannot be considered, however, as electrophoretically homogeneous since the boundary spreading exceeded that to be expected from an independent measurement of the diffusion coefficient. In turn, this

TABLE III
Mobilities of Type 1 M Antigen in Buffer Solutions of Ionic Strength 0.1

Buffer	pH	Electrophoretic Mobility $\times 10^5$
0.1 N HCl-0.5 N glycine.....	3.02	8.0
0.02 N NaAc-0.1 N HAc-0.08 N NaCl.....	3.91	4.78
0.1 N NaAc-0.1 N HAc.....	4.64	2.32
0.1 N NaAc-0.01 N HAc.....	5.64	-1.13
0.02 N NaCac-0.004 N HCac-0.08 N NaCl.....	6.79	-3.25
0.02 N NaV-0.02 N HV-0.08 N NaCl.....	7.82	-3.86
0.1 N NaV-0.02 N HV.....	8.60	-5.13

Ac = acetate; Cac = cacodylate; V = diethylbarbiturate.

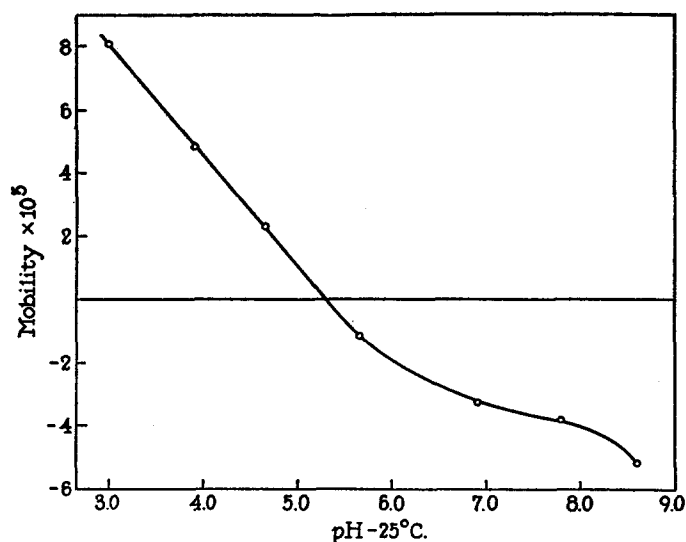


FIG. 3. Mobilities of type 1 M antigen as a function of pH.

measurement indicated some polydispersity and possibly a slight dependence of the diffusion coefficient on the concentration.²

The decrease in the areas of the concentration boundaries during purification procedures (Fig. 2 *a*, *b*, and *c*) indicates the removal of another impurity. When a sample of this component was tested for serological activity

² We are indebted to Dr. L. G. Longworth for making these measurements.

the presence of group-specific C polysaccharide was detected. This boundary in the patterns of the impure material, therefore, probably represents a polysaccharide or an electrophoretically immobile polysaccharide-protein complex superimposed on the δ and ϵ gradients. No C polysaccharide could be detected in the final preparations.

Mobility measurements were carried out over the pH range of 3 to 9 in order to determine the isoelectric pH value of the type 1 M protein. The compositions of the monovalent buffers used, glycine-hydrochloric acid, acetate, cacodylate, and diethylbarbiturate, are shown in Table III. The mobility values plotted against pH (Fig. 3) indicate that the isoelectric pH value of the type 1 M protein is 5.3.

DISCUSSION

In group A streptococci the M antigen is important not only because it determines type specificity (4, 8) but also because it is closely related to the potential virulence of these microorganisms (9-11). Of the various antigens so far isolated from the streptococcal cell, it is the only one in which this relationship has been definitely established, although probably other unknown factors are also involved. Furthermore, immunity to infection with group A streptococci both in man and in experimental animals appears to be primarily type-specific and dependent upon antibodies directed against the M antigen (5 b, 9, 10, and 12).

The M antigen seems to contribute to the virulence of group A streptococci by interfering with phagocytosis and thus permitting the streptococci to become established in the tissues of the host. This antigen *per se*, as isolated from the streptococcal cell, shows no primary toxicity. In many ways its relation to virulence is analogous to that of the capsular polysaccharide of pneumococcus. In mice the relationship of M antigen to virulence and protection is well established (13-15). In man the evidence from bacteriostatic studies strongly suggests that the same relationship holds true (16-19). The importance of determining the chemical nature of this component of the streptococcal cell is, therefore, obvious.

The initial step in isolating the M protein from the streptococci is heating them at pH 2. Attempts to use a less drastic procedure have been unsuccessful. After acid extraction the M antigen must be separated from other soluble components of the bacterial cell. In previous studies the amount of nucleic acid varied in different preparations, suggesting that this substance is not an integral part of the type-specific protein. With the aid of a preparation of ribonuclease, free of proteolytic activity, it was possible to remove the nucleic acid without destroying the M protein, and further purification was then accomplished by fractionation with $(\text{NH}_4)_2\text{SO}_4$.

The degree of purification achieved is indicated by a comparison of the

electrophoretic patterns of the material in the early stages of purification, in which the shoulder of the main peak represents the active M protein, with those of the final product in which all the material migrates as a single component. Although the boundary spreading of the latter indicates inhomogeneity of the material, most of the extraneous impurities have, nevertheless, been eliminated.

Immunological experiments indicate that the protein isolated is the type-specific M antigen of type 1 streptococci. Except for occasional minor cross-reactions, the serological reactivity of the material was type-specific. The purified preparation retained the most characteristic immunological properties of type-specific substances: (a) the ability to induce the formation of type-specific protective antibodies when injected into animals, and (b) the ability to absorb protective antibodies induced by immunization with homologous intact streptococci.

It is of interest to compare this protein with the T antigen previously studied by similar methods. The distinctive characteristics of M and T antigens have been tabulated in a previous paper (20). In contrast to the M antigen, the T antigen is unrelated to virulence and immunity to infection. The M antigen, therefore, is used as the basis for the serological differentiation of group A streptococci into types. The M and T antigens can also be differentiated by heating them at pH 2: The M antigen is extracted from the bacterial cell by this procedure, and is apparently uninjured by heating at this pH. The T antigen, on the other hand, is destroyed under these conditions (20, 21). Other differentiating characteristics of the M antigen are its marked susceptibility to proteolytic enzymes and its solubility in 70 per cent alcohol acidified with hydrochloric acid. Over a wide pH range the electrophoretic mobilities of these two type 1 proteins also differ: In acetate buffers of 0.1 ionic strength the isoelectric pH of the M protein is pH 5.3, that of the T antigen pH 4.5.

SUMMARY

Type-specific M antigen was extracted by heating type 1 group A streptococci at pH 2 in a boiling water bath. The protein was then purified by digestion with a preparation of crystalline ribonuclease which was free of proteolytic activity. It was further purified by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$.

Elementary chemical analysis of the preparation thus obtained showed an absence of phosphorus and a sulfur content of 2.46 per cent. In the ultraviolet the maximum absorption was at a wave length of $276 \text{ m}\mu$ and the minimum at $255 \text{ m}\mu$.

In electrophoresis experiments the preparation showed a single peak in the pH range of 3 to 9, but considerable boundary spreading was observed.

The type 1 M antigen was isoelectric at pH 5.3 in sodium acetate buffer of ionic strength 0.1.

The serological reactivity of the protein isolated was typical of type 1 M antigen. This protein induced the formation in rabbits of type-specific precipitins and protective antibodies. The absorption of type 1 antibacterial serum with the purified M antigen removed both the protective antibodies and the type-specific precipitins from the serum.

Dr. T. Shedlovsky very kindly carried out electrophoretic analysis in the early exploratory stages of this work. We are indebted to Miss Katia Altschuller for assistance with the bacteriological work and to Mrs. Joan J. Berdick for her help in carrying out some of the electrophoretic measurements.

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