STUDIES ON THE TYPE-SPECIFIC M-FRACTION OF THE HEMOLYTIC STREPTOCOCCUS, GROUP A

B. PRYTZ AND JAMES M. JABLON

Departments of Chemistry and Bacteriology of St. Francis Hospital and Sanatorium, Roslyn, New York

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The differentiation of the *Streptococcus hemolyticus*, group A (*Streptococcus pyogenes*), into types on the basis of its M component has led to a considerable amount of investigation on the physical and chemical characteristics of this component. It has been shown (Lancefield, 1928*a*) that the M component can be extracted from the organisms by heating with N/20 HCl, that it is alcohol soluble, that it is a protein with an isoelectric point of about 4.2, and that its antigenic activity can be destroyed by proteolytic enzymes such as pepsin, trypsin, and chymotrypsins. Later, the activity of proteolytic enzymes on the M component was confirmed (Zittle, 1939). It was also found that the M-substance was unaffected by ribonuclease, and that it contained a considerable amount of histidine and arginine but appeared deficient in tyrosine and tryptophan. The solubility of the M-protein was at a minimum at pH 3.5 in the absence of salt. However, later purification of the protein by ammonium sulfate fractionation (Zittle, 1942) removed much of the phosphorus containing constituents (nucleic acids), and such purified preparations gave an isoelectric point of pH 5.4. This was confirmed by electrophoretic studies (Zittle and Seibert, 1942) where it also was shown that the nucleic acids accompanying the M-protein were present in a salt-like, ionizable combination and that there were little other contaminants. Further work (Pappenheimer *et al.*, 1942) indicated that the molecular weight of the M-protein was 41,000 based on the ultracentrifugal sedimentation velocity, S_{20}, and on the diffusion constant, D_{20}.

There is much to learn about the antigenicity of the M-substance. Its relation to the type-specificity and virulence of the hemolytic streptococcus, group A, emphasizes the need for further investigation into the purification and characteristics of the substance. The present communication deals with a study on the M-fraction.

MATERIALS AND METHODS

Preparation of the M-extract. The usual method of preparing M-extracts from hemolytic streptococci is that of acid extraction (Lancefield, 1928*b*). The organisms are collected from a heavily growing culture by centrifugation. The supernatant is discarded, and the organisms are suspended in 0.05 N HCl in saline solution. The suspension is placed on a boiling water bath for 10–15 minutes, cooled, and centrifuged. The clear supernatant liquid containing the M-protein is then neutralized. An inactive precipitate forms during this neutralization; it is discarded by centrifugation, and the active material remains in the supernatant.

Various other methods of extraction have also been tried: sonic disintegration of the organisms, extraction in 2 per cent NaCl, and extraction at various temperatures (Zittle, 1939). None of the methods, however, has succeeded in yielding a better preparation than the original method of Lancefield.

In an attempt to obtain a more active extract, freer of contaminating substances, and at the same time less subject to the severe treatment of heating with acid, the organisms were treated with phosphate buffer, pH 7.3. The extracts obtained and tested with type-specific serum indicated an excellent yield of M-protein. The materials and methods are outlined below:

Hemolytic streptococcus, group A, type 15, was used. The organisms gave a rapid and heavy growth with a good yield of M-protein. Brain-heart broth (Difco) was used as the culture medium throughout the study. A 0.066 M Sorensen's phosphate buffer, pH 7.3, was used for extraction. A fresh culture was grown overnight in 1 L of broth. The organisms were centrifuged off, washed twice with saline, and finally suspended in 20 ml of the phosphate buffer. The suspension was placed on a boiling water bath for

10–15 min, cooled, and centrifuged. The clear supernatant liquid contained the M-protein.

A comparison was made with the usual acid extract. Both gave excellent reactions with the type-specific serum indicating a good yield of M-protein with both methods. However, the phosphate method requires no neutralization after the extraction, and only material which is soluble at a neutral pH is extracted.

One difference was observed between the acid and the phosphate extracts. The point of maximum precipitation of the acid extract was found to be about pH 5.0 (4.2–5.4), whereas that of the phosphate extract was about pH 3.5 (3.5–4.5). The difference in pH precipitation may be due to the variation in the nucleic acid content of the two preparations.

Effect of enzymes on the M-protein. Investigations on the effect of enzymes on the M-protein indicate that it is affected only by proteolytic enzymes (Lancefield, 1928*b*; Zittle, 1939). Pepsin, trypsin, and chymotrypsin rapidly inactivate it and destroy its ability to react with the type-specific serum. Ribonuclease, on the other hand, did not affect its specific activity. Because of the peculiar nature of the M-protein, it was thought advisable to extend the study to include the effects of additional enzymes.

There is present in the euglobulin fraction of human serum or plasma a factor which has proteolytic activity when activated. This factor is present as a zymogen—i.e., in an inactive state. Interest has been shown in this factor because it can be activated by streptococcal fibrinolysin or streptokinase. Antibodies to streptokinase have been found both in streptococcal infections and in rheumatic fever. The serum factor has been called the "lytic factor" (Milstone, 1941) or plasmin (Christensen, 1945) and has been shown to be the active principle in the lysis of human fibrin clots in the antistreptokinase or antifibrinolysin test (Tillett, 1938). It was thought of interest to see whether the lytic factor when activated by the streptococcal fibrinolysin could inactivate the M-protein.

TABLE 1

Effect of lytic factor and streptokinase on M-protein activity

M-Extract	Saline	Lytic Factor	Streptokinase	Incubation Time	Reaction with Specific Antiserum
ml	*ml*	*ml*	*ml*	*hours*	
0.5	0.5	—	—	2	+
0.5	0.25	0.25	—	2	+
0.5	0.25	—	0.25	2	+
0.5	—	0.25	0.25	2	−

The lytic factor was prepared from human serum by precipitation with acetic acid (Milstone, 1941). The precipitate was dissolved in 0.066 M phosphate buffer, pH 7.3, to one-third the original volume of serum. The activity of the lytic factor was tested with streptokinase prepared from a culture of *Streptococcus hemolyticus*, group A, type 26, an organism used here for streptokinase preparations. In testing the lytic factor and the streptokinase for activity, it was observed that the plasma clot dissolved in the presence of both factors but not in the presence of one or the other factor alone.

Hemolytic streptococcus, group A, type 15, was grown and the M-extract prepared by the usual method of Lancefield. The extract when tested for activity with a type-specific antiserum gave a strong positive reaction. The extract was then incubated with the lytic factor with and without added streptokinase after which it was retested with the type-specific serum. The results are shown in table 1. From the results obtained, it is evident that the M-protein has lost its ability to react with the type-specific serum probably due to its susceptibility to the action of proteolytic enzymes.

We have also confirmed the fact that pepsin as well as trypsin inactivates the type-specific M-fraction. Exhaustive treatment with crystalline ribonuclease[1] has no effect on the activity. With the view in mind that the M-substance could be a glucoprotein or a mucoprotein, the effect of lysozyme on the extract was tried. This enzyme was found to have no effect on the specific activity of the M-substance.

Chemical analysis. The crude first extract of the organisms contains a mixture of proteins, carbohydrates, and nucleoproteins or nucleic acids. An acid extract and a neutral phosphate extract prepared as described above were compared by chemical analysis for carbohydrate with the anthrone reagent (0.2 per cent in conc H_2SO_4), for ribonucleic acid with the orcinol re-

[1] Kindly furnished by Dr. M. R. McDonald of the Carnegie Institution of Washington, Cold Spring Harbor, New York.

agent (McRary and Slattery, 1945), for deoxyribonucleic acid with the sulfhydryl reagent (Dische, 1944), and for protein with a modified phenol reagent (Lowry *et al.*, 1951). Giving the neutral phosphate extract a value of 1, the following ratios of the various constituents were found in the two preparations:

	Neutral Phosphate Extract	*Acid Extract*	
Carbohydrate	1	4	
Ribonucleic acid	1	1.6	
Deoxyribonucleic acid	1	6	
Protein	1	2.1	
M-activity on antiserum	1	3	(estimated)

It is evident not only that considerably more contaminating material is extracted by the acid treatment but also that a more active preparation is obtained by this method.

It was pointed out above that the hydrogen ion concentration for maximum precipitation of the M-protein differed in the two types of extract. The phosphate extract can be precipitated by adjusting the solution to pH 3.5, whereas the acid prepared extract can be precipitated by adjustment to a pH of 4.5–5.5.

A phosphate extract was adjusted to pH 3.5 with 0.1 N HCl and centrifuged. The supernatant was discarded and the precipitate taken up in water and dissolved at pH 7.5 by small additions of 0.1 N NaOH. This procedure was repeated five times, after which the final pH 7.5 solution was digested with crystalline ribonuclease overnight at room temperature. When the solution, after the ribonuclease treatment, again was adjusted to pH 3.5, no precipitation took place; but a precipitate appeared between pH 4.5–5.2. Apparently, a ribonucleic acid containing compound precipitates out at pH 3.5 carrying the antigenic activity with it.

Ultraviolet absorption spectrum. In figure 1 is shown the ultraviolet spectrum of the solutions used in the above experiments. The volumes of the various fractions have been kept approximately constant. It will be noticed that in the original solution (the 5th pH 3.5 precipitate redissolved at pH 7.2) there is an absorption peak at 270 mμ, denoting the presence of nucleic acids. After ribonuclease treatment, precipitation at pH 5.2, and dissolving the precipitate at pH 7.2, the absorption peak has shifted to 275 mμ—that is, more toward the protein absorption peak. It will also be noted that a new peak has appeared at 230 mμ. Upon a second precipitation at pH 5.2, this latter peak is still more pronounced. We find that this is a characteristic absorption peak

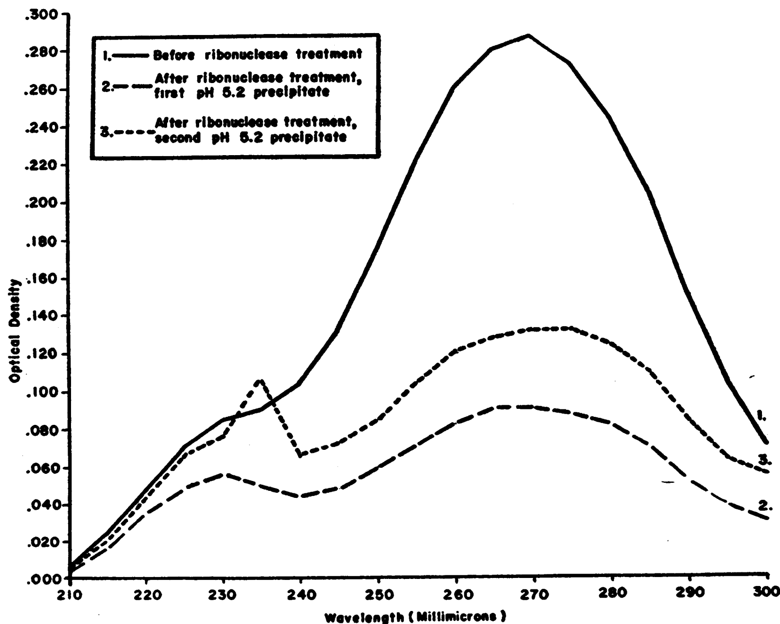

Figure 1. Ultraviolet spectrum of the M-fraction before and after ribonuclease treatment and after pH 5.2 precipitation.

TABLE 2

Chemical analysis of ammonium sulfate fractions

Precipitate from Ammonium Sulfate Saturation	P mg	Total N mg	Protein N mg	Ribonucleic Acid	Anthrone Reaction	M Activity
		per cent				
0.20	0.4	18.6	18.0	−	+	−
0.35	0.2	9.6	9.0	−	+	+
0.60	0.3	2.5	1.6	+	+	+

found present in a purified M-substance. It has been found present in numerous active, partially purified preparations. It probably denotes a carbohydrate moiety.

Curve no. 1 in figure 1 is a typical ultraviolet absorption curve of an active M-substance whether crude or slightly purified. The curve demonstrates a strong absorption between 260–270 mμ and a minor absorption peak between 225–235 mμ. Five different types of group A hemolytic streptococcus have been analyzed in this fashion, and they all have shown the same two peaks.

The same phenomenon has been found to take place in alcohol precipitated preparations. The active M-substance was precipitated by 80 per cent alcohol. In order to insure complete elimination of accompanying polysaccharides, the alcohol precipitation was repeated five times, and the final solution, after dialysis against tap water overnight, showed the same two characteristic peaks.

Alcohol precipitation of an M-extract yields a precipitate which is not completely soluble in water at pH 7.5. The alcohol-precipitated, water-insoluble fraction when dissolved in alkali or acid shows the same type of ultraviolet absorption curve as the original solution, indicating that a denaturation of the protein has taken place presumably with loss of antigenic activity.

When the M-substance is precipitated with ammonium sulfate, the 225–235 mμ peak is brought out very clearly. A solution of an extract was precipitated with 0.6 saturated ammonium sulfate, centrifuged, and the precipitate dissolved in water and dialyzed against running tap water for 22 hours. The dialyzed solution was adjusted to pH 3.5 with 0.1 N HCl, centrifuged, and the precipitate redissolved in water by adjustment to pH 7.0 with 0.1 NaOH. This solution was divided into three parts, and solid ammonium sulfate added to 0.2, 0.35, and 0.6 saturation, respectively. After centrifugation, the precipitates were dissolved in water and dialyzed as before. The resulting solutions were adjusted to pH 7.2 with 0.1 N NaOH. Table 2 gives the chemical analysis of the three fractions.

It will be noticed in table 2 that there is no M-activity in the precipitate from the 0.2 saturated ammonium sulfate fraction. In a partially purified M-extract, the active principle is precipitated between 0.2–0.35 saturated ammonium sulfate, whereas in a crude M-extract an 0.6 saturated ammonium sulfate is required for complete precipitation of the active M-principle.

Figure 2 shows the ultraviolet spectrum of the same three solutions. It will be observed here that there is a sharp peak at 225 mμ and a fairly broad peak at 280 mμ, which is the protein peak. Apparently most of the contaminating nucleic acids have been eliminated by ammonium sulfate fractionation.

The M-substance is adsorbed completely on

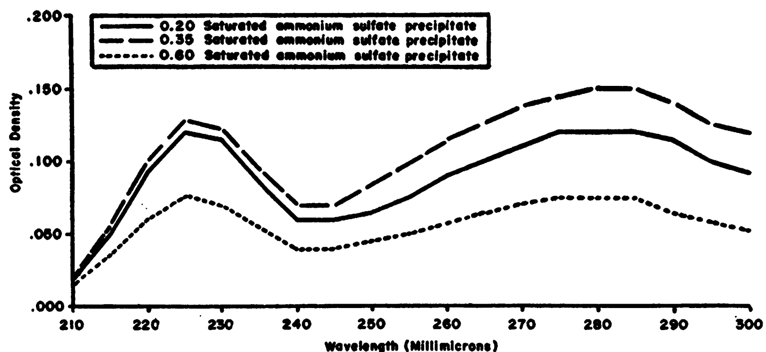

Figure 2. Ultraviolet spectrum of ammonium sulfate precipitated fractions of the M-substance.

acid washed, activated charcoal and cannot be eluted with 0.06 M phosphate buffer, pH 7, 0.01 N, 0.05 N, or 0.1 N NaOH or with 0.05 N HCl either in hot or cold solutions.

The M-substance is usually considered not to be of exogenous character. However, the active principle can be recovered from the spent broth by ammonium sulfate precipitation. The range in which it is precipitated lies between 0.2–0.6 saturation. The recovery is small, and the nature of the contaminating impurities is such that the difficulties involved in the purification of such recovered preparations are large. That the M-substance could be demonstrated in culture filtrates of the hemolytic streptococcus, group A, was shown earlier (Olarte, 1948).

Electrophoretic studies. A study of the electrophoretic behavior of the M-substance has been carried out from time to time on various preparations. A paper electrophoretic technique was used (Kunkel and Tiselius, 1951). In all cases a veronal buffer, pH 8.6, with an ionic strength of 0.025 has been used. If the experiment was carried out overnight at room temperature, 200 v were used with an initial current consumption of 0.2 ma which increased during the run to about 1 ma. Occasionally 400 v were used for 4–6 hours with an initial current consumption of 2 ma which increased to 3 during the run.

After the experiment was terminated, the paper was dried in an oven at 100–105 C and immersed in 0.1 per cent bromphenol blue in alcohol containing 10 per cent $HgCl_2$ for 15–20 minutes, after which the paper strips were washed in 4 per cent acetic acid, dried, and exposed to NH_3 vapors.

Difficulties were usually experienced in locating the protein spots on the paper. Concentrated solutions of the M-extract had to be used, and even then only very weak blue spots were found. The protein component migrated toward the positive pole (anode), but only very little migration was found to take place. In purified preparations the migration overnight measured from 0.5–1 inch only. Crude, impure extracts showed several diffuse spots on each paper strip, but one component was always found near the starting line. The slow migration may be caused by the association of the M-protein with nucleic acids, or it may be a natural property of the substance just like the gamma globulin of serum migrates very little and slowly on a paper strip.

M-extracts from five different types of hemolytic streptococcus, group A, did not show any differences in their nature.

In one case where the M-substance was run in duplicate, the spots were located with bromphenol blue, and the corresponding places on the second strip which was not stained were cut out and eluted with 0.06 M phosphate buffer, pH 7.2. No M-activity could be recovered under the conditions of the experiment.

Amino acid analysis. A number of extracts of various types of hemolytic streptococcus, group A, were analyzed for their amino acids content with the use of paper chromatography. Neutral phosphate and acid extracts, prepared as described above, were precipitated at pH 3.5, centrifuged, the supernatant discarded, and the precipitate redissolved in water by small additions of 0.1 N NaOH to pH 7.0. This was repeated three times, and the last pH 7.0 solution was dialyzed overnight against distilled water in the cold. It was then reprecipitated once more at pH 3.5, centrifuged, the supernatant discarded, and the precipitate dissolved with 0.1 N NaOH in a minimum amount of water to pH 7.0. This final solution was used for the electrophoretic studies mentioned above. One-half of each of the remaining solutions was hydrolyzed in 6 N HCl by autoclaving at 15 pounds pressure for 20 minutes, and the other half of the solutions was autoclaved similarly but in an alkaline medium. The resulting hydrolyzates were evaporated to dryness *in vacuo* over conc H_2SO_4 and the amino acids extracted with 95 per cent alcohol, evaporated to dryness, and the residue taken up in a minimal amount of water. No differences were found between the original hydrolyzates and the alcoholic extracts with the exception that the latter were easier to apply on the paper since one or two applications sufficed, whereas 7–10 superimposed applications had to be made with the hydrolyzate as such with drying of the spot between each application.

Whatman no. 1 filter paper was used with the descending technique. The solvent consisted of 80 parts *n*-propyl alcohol, 10 parts glacial acetic acid, and 10 parts water. The chromatograms were developed overnight whereupon the paper was removed, the solvent front marked, and the paper strip air dried. The dry paper was sprayed with 0.2 per cent ninhydrin in 95 per cent alcohol containing 0.5 ml glacial acetic acid per 100 ml

reagent. The sprayed paper was hung in the dark for the colors to develop, and the ninhydrin spots marked. Known amino acids were run simultaneously with the unknown solutions in such a fashion that every second strip contained the hydrolyzate and the alternate strips contained the known amino acids.

Extracts from five different types of *Streptococcus hemolyticus*, group A, were used: types 15, 30, 31, 33, and 34. Acid and phosphate extracts were compared. Alanine was found only in the phosphate extracts of all five types while the following amino acids were found in both extracts: histidine, glycine, glutamic acid, threonine, tyrosine, and phenylalanine. In addition, isoleucine was found in types 15 and 31; leucine in 30; norleucine in 15, 31, 33, and 34, but absent in 30; and valine was found in 15, 31, and 33. Lysine was found in the phosphate extracts only of types 31, 33, and 34, and methionine in phosphate extracts only of 15, 30, and 33. The differences observed here are probably not significant since we are dealing with impure preparations.

DISCUSSION

During the extraction procedure of the antigenic active M-substance from hemolytic streptococcus, group A, a large amount of various contaminating products is also extracted: proteins, nucleic acids, nucleoproteins, carbohydrates, and others. All of these impurities have to be removed before we can decide whether the active principle is a pure protein or a conjugated protein. That the M-activity resides in a protein is evident by the fact that it is destroyed by proteinases (Lancefield, 1928*a*; Zittle, 1939). Despite considerable purification by various fractionation procedures, both types of nucleic acids as well as a carbohydrate fraction are found; the latter is found in amounts over and beyond what can be accounted for from the pentoses in ribose- and deoxyribosenucleic acids.

The ultraviolet absorption peak found at 225–235 mμ probably represents a carbohydrate fraction. This peak seems to increase as the purification proceeds, indicating that we are dealing with a protein conjugated with a carbohydrate. The only other compound of carbohydrate nature tested which shows an absorption peak in that range is pectin. This may indicate that we are dealing with a protein conjugated with a pentose in contrast to glucoproteins in which the carbohydrate moiety is a hexose.

Although no quantitative measurements have been carried out on the antigenic activity of the M-substance, it appears that most of the activity is retained as the purification proceeds. In all our preparations both ribonucleic acid and deoxyribonucleic acid were found. Zittle (1939) failed to find deoxyribonucleic acid in his preparations. Although he used solutions of the M-substance of a much higher concentration than ours, his failure to detect the presence of deoxyribonucleic acid may be explained on the basis that the diphenylamine reaction may be a less sensitive test than Dische's sulfhydryl reaction (1944).

Pappenheimer *et al.* (1942) have reported a molecular weight of the M-protein of 41,000. In view of the difficulties which we have experienced in locating the protein spot on paper either by paper chromatography or paper electrophoresis, it does not seem likely that we are dealing with a protein molecule of that size. Another fact is that as purification proceeds, it becomes increasingly more difficult to precipitate the protein at its maximum point of precipitation, and more of it stays in the supernatant solution. This would indicate that we are dealing with a low molecular weight protein or a large peptide.

In this as well as in other studies on the M-substance, only partially purified preparations have been dealt with. An amino acid analysis of such impure preparations is not conclusive, but the fact that only 6 amino acids were found common to all the types and extracts used here also might indicate that we may be dealing with a large peptide rather than a protein.

Fox and Krampitz (1954) reported that their M-protein contained a high percentage of lysine and isoleucine but was devoid of tryptophan and phenylalanine. However, they did not state what type of hemolytic streptococcus they worked with nor whether it was a partially purified preparation. We found lysine present in three different types and phenylalanine present in all types tested.

Finally, as the purification proceeds, the relative proportions of the two absorption peaks in the ultraviolet region are reversed so that the 225–235 mμ peak is increased and the protein peak at 275–280 mμ is decreased. Until quantitative studies of this ratio can be investigated, it is difficult to ascertain whether we are dealing with a protein-carbohydrate or a peptide-carbohydrate relationship.

The use of chromatography and paper electro-

phoresis in conjunction with ultraviolet studies are approaches which could be used to advantage in the purification of the M-substance, particularly with regard to the ultimate purification of this important fraction of the hemolytic streptococcus, group A.

SUMMARY

The antigenic M-substance can be extracted from hemolytic streptococcus, group A, with an acid solution as well as with a neutral, pH 7.3, phosphate buffer. Dissimilar isoelectric points are shown by such extracts.

The M-activity is destroyed by plasminogen activated by streptokinase, as well as by pepsin and trypsin. Ribonuclease and lysozyme have no effect on the antigenic activity of the M-substance.

Two absorption peaks in the ultraviolet light are observed on a partially purified preparation. One near 270 mμ indicates a protein component, the other peak near 230 mμ indicates possibly a pentose moiety.

The M-protein is negatively charged and moves toward the anode in an electric field.

Histidine, glycine, glutamic acid, threonine, tyrosine, and phenylalanine were found present in partially purified acid extracts of five different types of hemolytic streptococcus, group A.

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