

## THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

### II. CHEMICAL AND IMMUNOLOGICAL PROPERTIES OF THE PROTEIN FRACTIONS.

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The presence of several antigenic substances in the hemolytic streptococcus has been indicated previously; two of these were non-type-specific, while a third exhibited type specificity. Of the non-type-specific substances one was species-specific and seemed to be non-protein (1); the other, a widely reactive "nucleoprotein" fraction, was an antigenic protein and showed partial cross-reactions with similar proteins from related species of Gram-positive cocci (2). The type-specific substance was demonstrated in extracts of hemolytic streptococci by the use of sera absorbed with intact bacteria of heterologous hemolytic streptococci (3). By this means the non-type-specific antibodies were removed from the sera and the remaining type-specific antibodies were detected with the precipitin test.

This report is concerned with the chemical and immunological relationships of the type-specific substance and with a review of the properties of the group reactive nucleoprotein for purposes of comparison.

Since it was highly desirable to determine the nature of the type-specific substance, designated as M, attempts were made to isolate and purify it. With sera absorbed as described, several kinds of extracts were tested for the presence of the type-specific substance. It was found that dried bacteria pulverized in a ball mill, and extracted with N/100 NaOH in the cold, yielded some type-specific substance, M, although the non-type-specific nucleoprotein, P, predominated. Similar extraction with 0.85 per cent NaCl, instead of N/100 NaOH,

yielded a somewhat greater proportion of M than of P. However, the separation of the two substances from each other was too difficult to make either of these extracts a profitable source of M. HCl extracts, as previously described (3), yielded the largest amount of M and the smallest of P. While it was obvious that a procedure which involved heating in a boiling water bath for 15 minutes with N/20 HCl was a dubious method of obtaining material for chemical analysis, it was hoped that some idea of the nature of the type-specific substance might be gained and thus better methods of extraction be devised. The HCl extracts, therefore, were the main source of M. N/20 to N/40 HCl gave maximum yields of M, while N/1 HCl destroyed all the serologically active materials, and dilutions of N/80 and greater yielded only small amounts of M.

#### *Methods.*

Antibacterial sera were prepared by intravenous injections first of killed then of living cultures. The plan of immunization was a series of four daily injections followed by a rest period of 3 days. The first series consisted of four daily injections of 1 cc. of heat-killed culture, the second series consisted of 2 cc. doses, the third of 0.5 cc. doses of living culture, the fourth of 1 cc. doses of living culture the fifth of 2 cc. doses of living culture. If the precipitin titer of the test bleedings, taken 7 days after the last injection, was unsatisfactory, another series of three injections of 5 cc. each of living culture was given. This last series was repeated until a satisfactory titer was obtained, or until the animal was discarded if the immune response continued inadequate. Sometimes animals which did not yield satisfactory sera by this method were given a rest period of 6 weeks to 2 months. They were then further immunized either with one series of 2 cc. doses of killed culture followed by series of 5 cc. doses of living culture as before, or else with series of 10 cc. to 50 cc. of killed culture concentrated to 2 cc. volume.

It was usually not easy to produce antisera of sufficient potency to give satisfactory immune precipitates with the type-specific substance. Some rabbits yielded potent antisera in 6 weeks, while others of the same group of animals responded only moderately. Often it was necessary to continue immunization for several months before obtaining antisera of the high titer necessary for work with the precipitin test. Usually a high agglutinin titer was reached without difficulty. This may depend on the fact that small amounts of antibody in the serum are more readily detected by agglutination than by precipitation, while a much higher concentration of antibody is necessary if the object is the detection of small amounts of antigen. Since the latter is the case in the study of substances obtained from bacteria, a potent agglutinating serum is not sufficient but a pre-

cipitating serum of high titer must be obtained even though considerable time is required for its production.

The following was the usual method of preparing extracts of the type-specific substance. The bacteria from 18 liters of plain broth culture were suspended in 200 cc. of hot  $N/20$  HCl, made by diluting  $N/1$  HCl with 0.85 per cent NaCl solution, and heated for 10 minutes in a boiling water bath, then cooled and centrifuged. After removal of the supernatant fluid, a second extraction was made similarly with 200 cc. of fresh solvent. In all, six extractions were made before the bacterial residue was discarded. The volume was usually decreased to 100 cc. and then to 50 cc. for the later extractions. Preliminary experiments showed that, within reasonable limits, the yield of M was independent of the volume. The first two or three extracts contained the majority of M; but sufficient amounts were still present in the later extracts to make their preparation worth while. The supernatant fluids were combined and neutralized with  $N/1$  NaOH. The precipitate which formed on neutralization was inactive serologically, hence was discarded. Crystals of sodium acetate (about 20 gm. per liter) were added to the water-clear, slightly yellowish supernatant fluid together with 3 or 4 volumes of 95 per cent alcohol. Most of the M was brought down in the resulting precipitate, while the non-type-specific C substance, probably carbohydrate in nature, was largely left in the supernatant fluid. A few reprecipitations with alcohol served to separate this material quite completely from the type-specific M. After standing in the ice box overnight, the alcoholic precipitate was removed by centrifugation and the supernatant fluid was saved for purification of the non-type-specific C. The precipitate was freed from alcohol by evaporation at  $55^{\circ}\text{C}$ . and then redissolved in 15 cc. or 20 cc. of saline; it was then centrifuged. Although most of the alcoholic precipitate was insoluble, precipitin tests showed very little less M in solution than there was in the original extract. Washings from the insoluble precipitate were added to the main solution and the insoluble part finally discarded. The M substance was reprecipitated with three volumes of 95 per cent alcohol and redissolved in a smaller volume of salt solution than before. This process was repeated five or six times. Crystals of sodium acetate were added at alternate precipitations to insure the presence of electrolytes. Only a small fraction of the second alcoholic precipitate was insoluble in salt solution; and after one or two more alcoholic precipitations all of the precipitate was readily soluble in salt solution. Further purification was effected by precipitating with a few drops of 10 per cent acetic acid. The precipitate was redissolved in salt solution by the addition of a drop of NaOH, and the process repeated several times in spite of some loss of active material. Micro-Kjeldahl determinations on the acetic acid supernatant fluids showed some purification since the removal of nitrogenous material was greater than the removal of M. After three reprecipitations with acetic acid, the precipitate was washed three times with distilled water. The test for chlorides was negative in the second and third wash waters. The precipitate was then dehydrated by washing three times with redistilled alcohol, and finally three times with

redistilled acetone. It was then filtered on hard filter paper and dried in a desiccator. The yield from 45 liters of broth culture was, in one instance, 18 mg. of a fine white powder.

The properties of the 18 mg. lot of M were as follows:<sup>1</sup>

1. It gave a precipitate with copper sulfate.
2. It gave a violet biuret test which was not very strong.
3. With Millon's reagent it gave a white precipitate which remained on heating and did not become colored. This precipitate was not crystalline.
4. With the xanthoproteic test it gave a faint color on heating with  $\text{HNO}_3$  which deepened slightly on the addition of  $\text{NH}_4\text{OH}$ .
5. The Molisch test was positive but not strong.
6. The percentage of total nitrogen was 14.6; while only a trace of amino nitrogen was present, too small a quantity to determine.
7. The acid equivalent was 552.

Earlier tests on less purified material showed that M withstood autoclaving for 20 minutes at 15 pounds pressure when the solution was neutral or acid ( $\text{N}/20$  HCl), but not when the reaction was alkaline ( $\text{N}/20$  NaOH).

*Experiment 1.*—Precipitin tests with the purified material were made against a number of homologous and of heterologous antibacterial sera to test its specificity. These results are recorded in Table I.

In all experiments, the final volume of precipitin tests was 0.5 cc. Each tube contained 0.1 cc. of serum. Controls of antigens alone and of sera alone were uniformly negative. The tests were read after 2 hours at  $37^\circ\text{C}$ . and again after standing overnight in the ice box. The overnight readings are recorded in all tables.

The extract still reacted with two homologous sera at a dilution of 1–300,000. With four heterologous sera, it was negative in all dilutions; with three heterologous sera, it gave weak reactions; and with one heterologous serum, it gave strong cross-reactions. These cross-reactions were not due to the C substance, for they were not observed with Serum Q696, known to be very potent in C antibodies; and, as will be shown later, the cross-reactions were not present after tryptic

<sup>1</sup> The author is greatly indebted to Drs. Heidelberger and Goebel for this analysis and for several others given in this paper, as well as for much helpful advice in the chemical procedure.

or peptic digestion of this extract. This cross-reaction was, therefore, due to a protein. Since this extract reacted very slightly, if at all, with potent anti-P sera, it seemed doubtful whether P impurities were responsible for the cross-reactions. Later work with the anaphylactic reaction definitely eliminated P as the cause of this non-type-

TABLE I.  
*Precipitin Reactions.*  
*Purified Type-Specific M Substance Prepared from HCl Extract.*

Serum			Antigen. HCl extract from Strain S43, Type S60. Final dilutions 1:		
No.	Prepared against		19,000	75,000	300,000
	Strain	Homologous type			
Q868	S60	S60	+++	+-	+
Q865	"	"	+++	++	+
		Heterologous type			
Q311	S23	S23	-	-	-
Q611	"	"	+	+	±
Q612	"	"	+	0	0
Q613	"	"	-	-	-
Q244	"	"	-	±	-
Q245	"	"	-	-	-
R266	S39	"	++*	++±	+
Q696	New York 5	Scarlatinal	-	-	-
Normal			-	-	-

\*Test made at a different time: dilutions only estimates.

In all tables ±, +, ++, +++, +++++ indicate degrees of precipitation; - indicates a negative reaction; 0 indicates that test was not made.

specific reaction and necessitated the assumption of another non-type-specific protein in the antigenic complex of the hemolytic streptococcus; this fraction was present only in certain strains, and antibodies for it were only found in occasional antibacterial sera. The anaphylactic reactions which confirmed the existence of this somewhat hypothetical substance, designated as Y, are given in a subsequent paper.

In spite of the evident fact that the HCl extract was not entirely freed of non-type-specific fractions by the methods used for its purification, certain information in regard to its nature was acquired by the study of the material in this state. During the preparation and early tests it had become apparent that the type-specific substance behaved more like a protein than a carbohydrate: it was precipitated by the ordinary protein precipitants, including picric acid; and preparations, purified as described, contained 14 per cent nitrogen. The effect of removal of the  $\text{NH}_2$  group on the serological specificity of M was, therefore, determined by treatment with nitrous acid.

TABLE II.

*Precipitin Reactions.**Effect of Removing  $\text{NH}_2$  Group from Purified Type-Specific M Substance.*

Serum: * Q868, against Strain S60, Type S60		
Antigen: HCl extract of Strain S43, Type S60		
Final dilutions 1:	Treated with $\text{HNO}_2$ for 1 hr.	Blank control not treated with $\text{HNO}_2$
13,500	++	++++
27,000	+±	++++
54,000	+	+++
108,000	±	+±
216,000	±	+

\* 0.1 cc. in each tube.

The same series of antigen dilutions tested against normal rabbit serum was completely negative.

*Experiment 2.*—Samples of “purified” M were treated with nitrous acid for 10 minutes and for 1 hour. After neutralization they were tested for M by means of the precipitin reaction.

Such treatment for 10 minutes caused a considerable reduction in the amount of precipitate with immune serum, and treatment for 1 hour reduced the amount very greatly, as shown in Table II. This experiment gave considerable indication that the type-specific M was a protein.

The effect of tryptic and of peptic digestion was also determined. A typical result is given in Experiment 3.

*Experiment 3.*—In this experiment, 2 per cent trypsin (Fairchild's) was allowed to act for 18 hours at 37°C., on concentrated M, in a solution sufficiently alkaline to give a permanent pink color with phenolphthalein. A control was included with the same amount of enzyme previously inactivated by heating in a boiling water bath for 10 minutes. Toluene was used as a preservative. After the incubation period, the digest and its control were neutralized with HCl and then heated for 10 minutes in a boiling water bath.

A similar digestion experiment was performed with pepsin in solutions made sufficiently acid with HCl to turn Congo red paper blue. After incubation, the digestion mixtures were neutralized with NaOH and heated to destroy the enzyme. Precipitin tests with these digests and with their controls are recorded in Table III.

TABLE III.

*Precipitin Reactions.**Effect of Tryptic and of Peptic Digestion on the Type-Specific M Substance.*

Serum: * Q868, against Strain S60, of homologous Type S60				
Antigen: HCl extract of Strain S43, Type S60				
Cc.**	Tryptic digest	Control with heated trypsin	Peptic digest	Control with heated pepsin
0.05	—	++±	—	++±
0.025	—	+++	—	+++
0.012	—	+++±	—	+++±
0.006	—	+++	—	+++
0.003	—	++	—	++
0.0015	—	++	—	±

\* 0.1 cc. in each tube.

\*\* Dilution of antigen unknown.

Table III shows that digestion of M with either pepsin or trypsin completely destroyed its ability to form precipitates with homologous antibacterial sera. Later it was determined that 0.5 per cent trypsin acting for 10 to 20 minutes was sufficient to destroy this antigen.

Seibert (4) in her studies on the chemical composition of the active principle of tuberculin, states that trypsin in neutral solution does not digest whole protein readily, although it does digest some of the split products at this pH. Following this suggestion, tryptic digestion of the M substance was carried out at pH 7.0. Digestion at this reaction was, however, just as complete as it was at an alkaline reaction.

It was also shown that the negative precipitin reactions after digestion were not due to the possible inhibiting action of digestion products since the peptic digest added to undigested M did not change the range or the degree of precipitin reactions.

The digests used in Experiment 3 were also tested against the heterologous serum which gave the best cross-precipitations. Table IV shows these tests. This non-type-specific substance also was destroyed by both tryptic and peptic digestion: the digests no longer reacted with this serum, although the controls with inactivated

TABLE IV.

*Precipitin Reactions.*

*Effect of Tryptic and of Peptic Digestion on the Non-Type-Specific Substance in Purified HCl Extracts.*

Serum:* R266, against Strain S39, of heterologous Type S23				
Antigen: HCl extract of Strain S43, Type S60				
Cc.**	Tryptic digest	Control with heated trypsin	Peptic digest	Control with heated pepsin
0.05	—	+±	0	+±
0.025	0	0	—	++±
0.012	—	+++	0	++±
0.006	0	0	—	++
0.003	—	++±	0	0

\* 0.1 cc. in each tube.

\*\* Dilution of antigen unknown. The antigen preparation in this table is the same as that used in Table III.

enzyme gave good cross-precipitations. Evidently the non-type-specific substance concerned here was a protein.

Numerous tests were made with regard to the time and the concentration of enzyme necessary for digestion of these two substances. It was observed consistently during a large series of such experiments that the non-type-specific fraction was digested much more rapidly than the type-specific. A separation of these substances on this basis was attempted, but the loss of the type-specific fraction was too great to make such a method useful.

Since the digestion experiments, supported by the nitrous acid test,



indicated clearly that the type-specific M was a protein, attempts were made to separate it from its impurities by salting out, not only from HCl extracts but also from NaOH and NaCl extracts of pulverized bacteria. Ammonium sulfate was found more satisfactory than sodium sulfate for this purpose; and a certain amount of separation of the type-specific substance from the non-type-specific could be attained. The non-type-specific substance was precipitated more readily than the type-specific by the lower concentrations of the salt; but in order to remove most of the non-type-specific materials a great loss of the type-specific substance was also sustained, although full saturation with ammonium sulfate was necessary to precipitate all of the M. This method was therefore abandoned. Purification by adsorption on voluminous alumina and on kaolin was also attempted without much success.

Finally, all available material was used in immunization and in anaphylaxis experiments, and further attempts to isolate M in a pure form were suspended until more material could be collected for this purpose. The immunization experiments follow.

*Experiment 4.*—Four rabbits were injected with HCl extracts from two strains of different types; two received intravenous injections of extracts from each strain. Five daily injections were given followed by a rest of 5 days. The dose was then doubled for the next five daily injections. This plan was followed for four series of injections. Test bleedings taken after the second and fourth series showed no agglutinins and no precipitins for the type-specific M or for the non-type-specific P and C substances. One rabbit from each series was then given a rest and the two remaining animals continued with all of the available material. Each received the HCl extract from 9 liters of plain broth culture in six daily injections in each of the two succeeding series of injections. At the end of this time these two animals had been in process of immunization for 10 weeks. Test bleedings after the fifth and sixth series of injections still showed no trace of antibodies. All four animals were finally given intravenous injections of whole bacteria in the same manner ordinarily employed for producing antibacterial sera. All responded promptly with the production of antibodies at the usual time after such injections. The failure to produce agglutinins or precipitins in response to the HCl extracts was not, therefore, due to inability of these rabbits to produce antibodies.

The failure to give an immune response to intravenous injections of HCl extracts could not be considered final proof that the M substance was not antigenic. The explanation might be that the rabbits had

not received enough antigen, or that the protein had been so altered by extraction with heat and hydrochloric acid that it could no longer function as an antigen. The latter hypothesis was tested as follows.

*Experiment 5.*—Two healthy young rabbits, weighing 1,800 gm. each, were immunized with a saline extract from Strain S43. This strain was selected because it gave a uniformly large yield of M. To the dried and pulverized bacteria 0.85 per cent NaCl solution was added in the cold; and the extract was made by shaking in the cold overnight. The smallest amount of 10 per cent acetic acid necessary to give maximum precipitation was added in the cold, and the extract again left in the ice box overnight. The precipitate, removed by centrifuging, was dissolved in a suitable small volume of saline with the aid of a few drops of N/4 NaOH. With precipitin reactions as previously described (3), the presence of M in such solutions was proved. It was similarly shown that practically all of the M had been precipitated from the original solution with the acetic acid. The object of this precipitation with acetic acid was to concentrate the M sufficiently for intravenous injections with the least harmful chemical manipulation.

Each rabbit was given such an extract from 1.5 liters of original broth culture in four daily intravenous injections. After 5 days rest, double that amount was given in three daily injections. Test bleedings 5 days later showed the presence of P antibodies but no M antibodies. One of the animals was sick and died as a result of *B. lepi-septicus* infection after two more injections. The remaining animal received three more similar series of injections. The extract from 3 liters of culture was used for the third series, the extract from 19.5 liters for the fourth series, and that from 15 liters for the fifth series. In spite of the fact that this animal received the extract from a total of 42 liters of broth culture over a period of 5 weeks, and that bleedings after the fourth and fifth series of injections showed a high titer of P antibodies, still there were no traces of M antibodies. The anaphylactic test, reported in a succeeding paper, also failed to reveal the presence of M antibodies in the serum of this rabbit.

The failure to produce antibodies in these two rabbits by the injection of a simple saline extract gave weight to the previous evidence of the non-antigenicity of M which was obtained by the failure to immunize the four rabbits in Experiment 4 with HCl extract. Thus, although the type-specific substance in the hemolytic streptococcus is a protein, nevertheless, after it has been separated from the bacterial cell, it either does not give rise to antibodies when injected into rabbits intravenously, or else may do so only under conditions not yet determined. This substance seems, therefore, to be a haptene rather than a true antigen.

Solutions of the nucleoprotein P, on the contrary, are true antigens in that rabbits are easily immunized with them. Antisera from these animals are precipitated equally well by nucleoproteins from all strains of hemolytic streptococci. This has been reported previously (2) and has been repeated and confirmed in the present experiments. Nucleoproteins from non-hemolytic streptococci and from pneumococcus also precipitate these hemolytic streptococcus anti-P sera to a certain extent. This fraction of the hemolytic streptococcus is, therefore, not only not type-specific but not even species-specific. It is possible that with appropriate methods this nucleoprotein portion could be further fractionated into a strictly species-specific part and into other parts which are responsible for the cross-reactions with other species. Such methods are not at present available.

The species-specific substance first described by Hitchcock is considered in the succeeding paper, in which there is also included a discussion of all these reactive substances.

#### SUMMARY.

The chemical and immunological characteristics of the type-specific substance (M) of *Streptococcus hæmolyticus* are considered.

1. A summary of the evidence for the protein nature of this substance follows:

(a) It is precipitated by the usual protein precipitants such as, dilute alcohol, dilute acetic acid, and picric acid.

(b) It contains 14 per cent protein nitrogen after considerable purification.

(c) It is progressively destroyed by removal of the  $\text{NH}_2$  group by treatment with nitrous acid.

(d) It is completely and readily digested by trypsin and by pepsin.

2. "Purified" extracts react in relatively high dilution with homologous antibacterial sera, but do not precipitate most heterologous antibacterial sera or sera potent in non-type-specific antibodies for the group reactive nucleoprotein P or for the species-specific probable carbohydrate C. Attempts to immunize rabbits with the type-specific protein have been unsuccessful, with simple salt solution extracts of

streptococci as well as with purified solutions. This protein seems, therefore, to have the characteristics of a haptene.

The type-specific substance (M) is contrasted with the so called nucleoprotein (P) which shows group relationships with nucleoproteins of related species and is the only fraction of hemolytic streptococcus extracts so far obtained which, after separation from the bacterial cell, is a true antigen leading to antibody production when injected into rabbits.

The occurrence of another non-type-specific protein (Y) is suggested by occasional cross-reactions of purified M with certain anti-bacterial sera. Since it has not been separated from extracts containing the type-specific M, little is known of it either chemically or serologically. The cross-reaction disappears on tryptic or peptic digestion of the extract. The fact that such extracts do not show cross-reactions with anti-P sera is evidence that this non-type-specific protein is not P.

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