PREPARATION AND PROPERTIES OF A PROTEIN (R ANTIGEN) OCCURRING IN STREPTOCOCCI OF GROUP A, TYPE 28 AND IN CERTAIN STREPTOCOCCI OF OTHER SEROLOGICAL GROUPS*

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In previous studies it was reported that the protein considered to represent the type-specific M antigen of type 28 group A streptococci differs from that of other group A streptococci in being resistant to tryptic digestion (1). In the present investigation it was found that digestion of the microorganisms by trypsin was a means of releasing the active material from the bacterial cells. The protein thus obtained has been purified and its chemical and immunological properties studied. Evidence is presented to show that this protein does not have the essential characteristics of the known type-specific substances of group A streptococci. It is, therefore, referred to as the R antigen.[1]

Materials and Methods

Strains of Streptococci.—For convenience the group A strains previously considered representative of a single type on the basis of the R antigen will still be referred to as type 28 although several different serological types, each represented by an individual type-specific substance, may be included among these strains.

Strain T28 (strain Small, Griffith) was used for preparation of R antigen.[2] Other "type 28" strains in our collection were obtained from throat cultures of patients with streptococcal infections in several parts of the United States and Canada.

Strains of groups B, C, and G were available from the collection of the Rockefeller Hospital.

Other Methods.—Procedures for culturing and collecting the streptococci, the methods used for absorbing antisera and for precipitin and protection tests, as well as the procedures adopted for electrophoretic and ultraviolet absorption measurements, are the same as those described in the preceding paper (2).

EXPERIMENTAL

Preparation of R Antigen: Extraction of the Antigen from Streptococci.—Treatment with trypsin is an effective and convenient method for extracting the R antigen from bacterial

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[1] The letter chosen has no significance as to composition or properties of the antigen.

[2] This strain was obtained from Dr. F. Griffith in 1935 as the representative of provisional Type 28.

cells. Papain, streptococcal proteinase, and the lytic principle (3) in *Streptomyces albus* culture filtrates are as effective as trypsin in this respect. On the other hand, chymotrypsin, ribonuclease, desoxyribonuclease, lysozyme, hyaluronidase, and a combination of lysozyme and hyaluronidase are ineffective.[3]

The antigen may also be extracted from streptococcal cells by heating them under either acid or alkaline conditions. At pH 2 and a temperature of 100°C. the yield was much less than that obtained by tryptic digestion. At pH 7.8 small yields were obtained after 5 hours at a temperature of 37°C. By increasing the temperature to 100°C. for 10 minutes the yield of serologically active material in the supernate approached that obtained by tryptic digestion.

In the work to be described treatment with trypsin was employed as the means of separating the R antigen from the streptococcal cells. In a typical preparation the washed bacteria from 60 liters of culture were suspended in 500 cc. of sterile M/15 phosphate buffer, pH 8, containing 0.01 mg. of crystalline trypsin per cc. of suspension. Chloroform and toluene were added and the preparation incubated at 37°C. overnight. The supernatant fluid was removed and the procedure was repeated four times until the yield of the active material as determined by precipitin tests became negligible.

The digests were filtered first with filter cel on a Buchner funnel then through a porcelain bacteriological filter. The filtrate was dialyzed against distilled water at 3°C., and concentrated about 20-fold by evaporation in a cellophane sac with the aid of an electric fan. The almost colorless concentrate was frozen and dried prior to additional purification.

Purification of the Crude Extract by Further Enzymatic Digestion and Fractionation with $(NH_4)_2SO_4$.—Crystalline ribonuclease, chymotrypsin, and trypsin in final concentrations of 0.01 mg. per cc. were used in purifying the crude extract which had been redissolved in a small volume of buffer at pH 7.8. After the removal of ribonucleic acid by the action of ribonuclease (2), digestion was continued with chymotrypsin for 48 hours at 37°C., followed by treatment with trypsin and a fresh portion of chymotrypsin for an additional period of 48 hours. Dialysis against frequent changes of buffer was employed during these procedures in order to remove digestion products. Finally, the solution was dialyzed against distilled water at 3°C. for several days.

The active material was next precipitated two or three times at 0.7 saturation with $(NH_4)_2SO_4$ at pH 8 followed by six reprecipitations at pH 6. Usually no precipitate was obtained until after 0.5 saturation was reached, and precipitation of the largest amount of serologically active material occurred at about 0.6 saturation with $(NH_4)_2SO_4$. With some preparations a pigmented precipitate could be removed at 0.58 saturation leaving more than half of the active protein in the supernatant fluid. After dialysis against distilled water at 3°C., the final product was frozen and dried.

It is possible that this material contained as an impurity some of the trypsin used in the initial separation of R antigen from the streptococci. Tryptic activity, however, was no longer demonstrable. The other enzymes employed in the early part of the purification procedures were probably eliminated or present in negligible amounts in the final preparation.

Properties of Purified R Antigen

Elementary Chemical Analysis.—Microchemical analysis of the purified R antigen showed the following percentage composition: C = 51.82, H = 7.6, N = 14.68, S = 1.04, P = none.

Ultraviolet Absorption Spectra.—In Fig. 1 the ultraviolet absorption spectrum of a typical preparation, No. 28H, is shown. The different preparations

[3] Most of the purified enzymes were prepared by Dr. M. McCarty. Streptococcal proteinase was obtained through the courtesy of Dr. S. D. Elliott.

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of R antigen gave essentially identical curves typical of proteins, with a maximum absorption at 280 mμ and a minimum at 254 mμ.

Electrophoretic Properties.—Electrophoretic analysis was used to follow the purification of the streptococcal antigen. In the pattern of the starting material, Fig. 2 *a*, the component designated as R is that of the antigenic material. In a 0.02 N sodium diethylbarbiturate—0.02 N diethylbarbituric acid—0.08 N sodium chloride buffer of pH 7.8, this protein has a mobility of $-3.9_2 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}. The fast component, N, in patterns 2 *a* and 2 *b*,

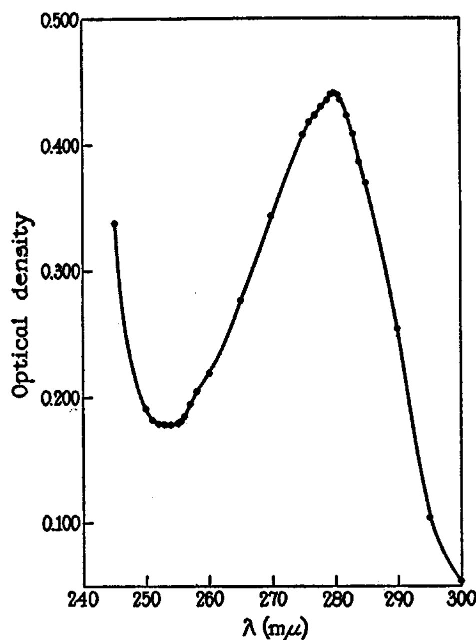

FIG. 1. Absorption spectrum of a 0.1 per cent solution of R antigen in phosphate buffer of pH 8.0.

has a mobility similar to that of nucleic acid. As the fractionation with ammonium sulfate proceeded, these impurities were removed. In pattern 2 *c*, which is that of the purified antigen, the R peak represents 95 per cent of the material that is present. For further characterization the electrophoretic measurements were extended over a pH range of 3 to 9. The composition of each of the monovalent buffers used for these measurements is given in column 1 of Table I. Although only one moving boundary was present at most pH values, two peaks of about equal areas were observed in an acetate buffer of pH 4.6 and 0.1 ionic strength. They were resolved after migration for 4 cm. at a potential gradient of 6 volts per cm. Tests on material withdrawn

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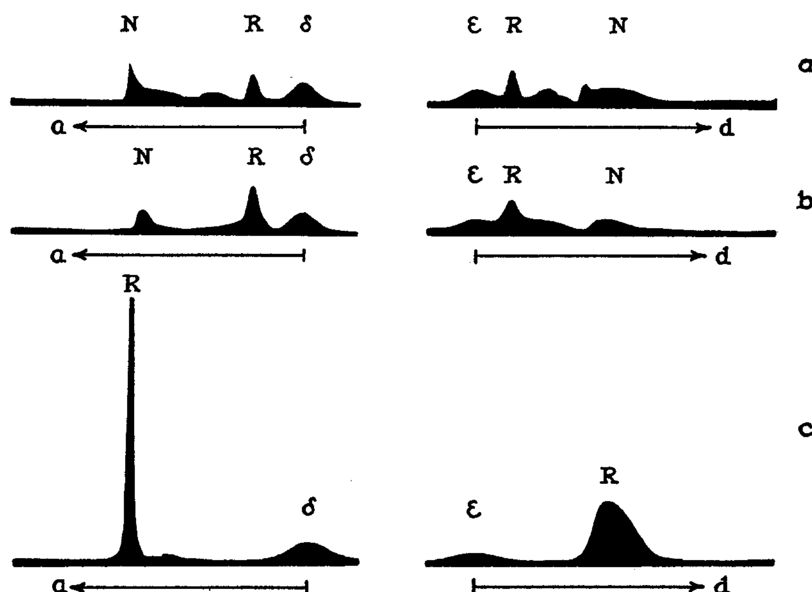

FIGS. 2 *a*, *b* and *c*. Electrophoretic patterns of a 1 per cent solution of R antigen in sodium diethylbarbiturate-sodium chloride buffer of pH 7.8 and 0.1 μ at different stages of purification. Electrophoresis was carried out for 3600 seconds, Fig. 2 *a* and *b*, and for 10,800 seconds, Fig. 2 *c*, at a potential gradient of 6 volts per cm.

TABLE I

Mobilities of R Antigen in Buffer Solutions of Ionic Strength 0.1

Buffer	pH	Electrophoretic Mobility × 10⁵
0.1 N HCl–0.5 N glycine	3.02	5.44
0.02 N NaAc–0.1 N HAc–0.08 N NaCl	3.91	1.85
0.1 N NaAc–0.1 N HAc	4.64	−1.37
0.1 N NaAc–0.01 N HAc	5.64	−3.2
0.02 N NaCac–0.004 N HCac–0.08 N NaCl	6.79	−3.77
0.02 N NaV–0.02 N HV–0.08 NaCl	7.82	−3.92
0.1 N NaV–0.02 N HV	8.60	−4.28

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

from the electrophoresis cell indicated that both boundaries represented serologically active material. From mobility measurements in buffers of 0.1 ionic strength an isoelectric pH of 4.5 was computed (Fig. 3).

In the ultracentrifuge a 1 per cent solution in 0.1 ionic strength acetate buffer of pH 5.3 sedimented as a single boundary with a sedimentation constant at 20°C. of 3.1×10^{-13}. The pattern, after centrifugation for 3 hours at 51,000 R.P.M., is shown in Fig. 4. In diffusion studies of this material with the

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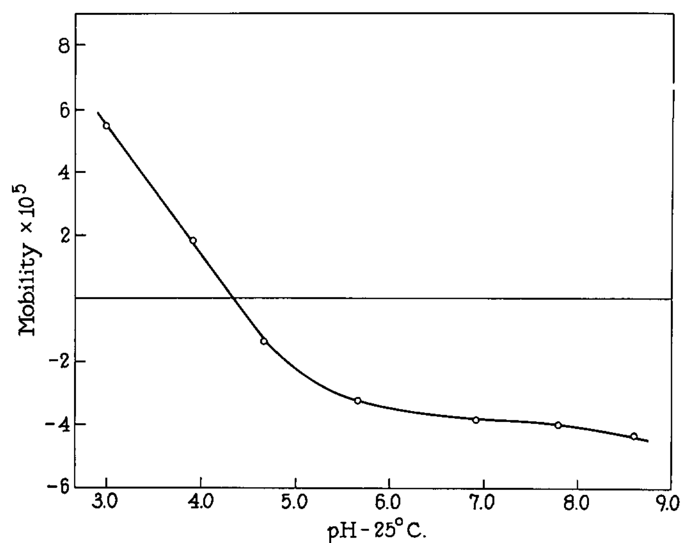

FIG. 3. Mobilities of R antigen as a function of pH.

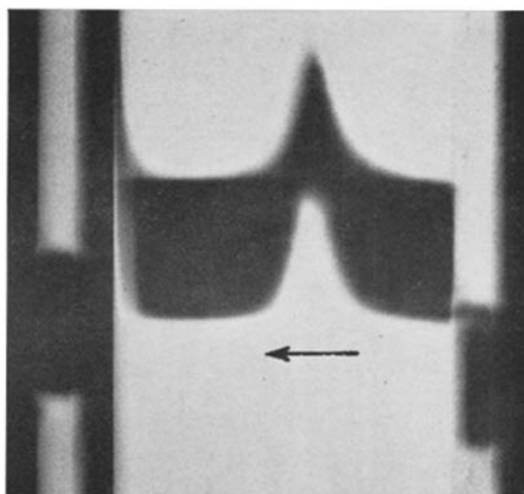

FIG. 4. Sedimentation pattern of R antigen.

aid of the Gouy method (4) deviations from ideal behavior were observed and consequently no attempt was made to estimate the molecular weight of the protein.

Effect of Proteolytic Enzymes on Purified R Antigen.—Further evidence as to the chemical nature of the purified R antigen was obtained by testing the effects of proteolytic enzymes on solutions of the antigen. Despite its resist-

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ance to trypsin, chymotrypsin, papain, and streptococcal proteinase the susceptibility of R antigen to digestion with pepsin agreed with the evidence so far presented that this substance is protein in nature.

Immunological Activity

Antigenicity.—Antibodies were readily produced, and usually in high concentration, when rabbits were immunized with purified R antigen. Four different preparations of R antigen, including the most highly purified, were employed. In rabbits given a total of 25 to 40 mg. in 5 mg. doses, injected intravenously two or three times a week, precipitins developed 14 days after the first injection. When less than 25 mg. of R antigen was used, the response was unsatisfactory.

The precipitin reactions of these antisera were highly specific, giving no cross-reactions with any of the other antigens tested. The precipitin reactions of the purified antigen were similarly specific, giving no reactions with antisera which did not contain R antibodies.

R antigen gave marked precipitin reactions not only with antisera prepared with the purified solution but also with antibacterial sera made by immunizing rabbits with heat-killed cultures of any of the R-containing strains available for study. Five different group A strains were used for immunization. Specific precipitates were obtained in final concentrations of R antigen as low as 0.005 mg. per cc. (about 1:200,000 dilution). In comparison with other precipitin reactions with streptococcal proteins, the amount of precipitate obtained was exceptionally large.

The results of an experiment illustrating specific absorption of these precipitins are given in Table II. Absorption with purified R antigen was carried out by the method of optimal proportions (5) and was complete, as shown by tests of the absorbed serum with serial dilutions of the antigen. Streptococcal cells of R-containing strains also absorbed R precipitins; strains lacking this antigen failed to do so.

With all these antisera positive precipitin reactions were obtained, not only with solutions of the purified R antigen but also with unpurified tryptic digests of the streptococci, as well as with extracts derived from the bacteria by heating them at pH 2 or at pH 8 at a temperature of 100°C. When crude extracts were tested with antibacterial sera, it was necessary to employ antisera which had been specifically absorbed so that they contained only R antibodies. Purified R antigen, on the other hand, owing to its highly specific reactions, could be tested with unabsorbed antibacterial serum.

R antigen was demonstrated in extracts prepared by these three methods from all the type 28 strains which were available, a total of 18 strains.

Resistance of R Antigen to Heat.—The effect of heating solutions of R antigen at different pH values was tested by means of the precipitin reaction for comparison with the effect of heating the intact cells containing the antigen.

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Aliquots of a solution in water containing 4 mg. per cc. of the purified R antigen were treated as follows: One was adjusted to pH 2 with N HCl; a second was adjusted to pH 8 with phosphate buffer; and a third was kept as an untreated control. The first two were immersed in a boiling water bath for 15 minutes. Other aliquots similarly prepared were heated for 30, 45, 60, and 120 minutes at 100°C.; and one of each was autoclaved at 20 pounds' pressure for 20 minutes. All preparations were then brought to pH 7.8 and a final concentration of 1 mg. of R antigen per cc. of solution.

TABLE II

Effect of Absorption with Purified R Antigen on Precipitin Reactions of Antibacterial Sera

R Precipitin Reactions

Antisera	Purified R antigen 10 mg./cc. diluted 1:							
	2	4	128	256	512	1024	2048	4096
Group A serum								
(1) Prepared with strain C649A								
Unabsorbed	++++	++++	+++	++	++	++	+	±
Absorbed with R antigen	−	−	−	−	−	−	−	−
(2) Prepared with strain C510								
Unabsorbed	++++	++++	++++	+++	++	++	++	+
Absorbed with R antigen	±	−	−	−	−	−	−	−
Group C serum								
Prepared with strain B337								
Unabsorbed	+++	++++	+++	+++	++	++	+	±
Absorbed with R antigen	−	−	−	−	−	−	−	−

Purified R antigen was prepared from the group A strain, T28. Strain B337, used for preparing the group C serum, is Griffith's strain Radford designated by him as type 21.

R precipitins were also completely absorbed from these sera with intact streptococci of group A, "type 28," strains C649A, C510, T28, and D140A, and with the group C strains B337 and B342, all of which contain R antigen. Strain T1 (group A, type 1) does not contain R antigen and did not absorb these antibodies.

Readings were recorded after incubation at 37°C. for 2 hours followed by refrigeration at 4°C. for 18 hours. − indicates no reaction, ± to ++++ indicate degree of reaction. See Methods for techniques employed.

Precipitin reactions were carried out both with antibacterial sera and with antisera from rabbits immunized with purified R antigen. Typical results are recorded in Table III. Heating at pH 2 for 15 minutes destroyed most of the activity of the antigen. Although still readily demonstrable with the potent serum shown, it could hardly be detected with the weaker serum. Longer periods of heating at this pH resulted in greater inactivation of the R antigen. Heating at pH 8, however, had little effect on its serological activity. Semiquantitative precipitin reactions carried out turbidimetrically (6) also showed only slight loss of activity in the solution heated for 15 minutes

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TABLE III

Effect of Heating Solutions of R Antigen

Precipitin Reactions

Conditions of heating R antigen			Antisera from rabbits immunized with														
			T28 streptococci*					T28 streptococci*					Purified R antigen				
pH	Temperature	Duration	Purified R antigen 1 mg./cc. diluted after heating 1:														
			8	16	32	64	128	8	16	32	64	128	8	16	32	64	128
	°C.	*min.*															
2	100	15	+++	+++	++	+	±	±	±	−	−	−	++	++	++	+	+
	100	60	+	+	±	−	−	−	−	−	−	−	+	±	±	−	−
	100	120	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	120	20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
8	100	15	++++	++++	++++	++++	+++	++	++	+++	+++	++	++++	++++	++++	++++	+++
	100	60	+++	++++	++++	+++	+++	++	+++	++	++	+	+++	++++	++++	+++	++
	100	120	+++	++	++	+	+	±	++	++	++	+	++	+++	++	++	++
	120	20	+	±	±	−	−	−	−	−	−	−	±	±	±	−	−
Untreated control			++++	++++	++++	++++	+++	+++	++	++	++	++	++++	++++	++++	++++	+++

Antisera from rabbits immunized with group C R-containing strains gave the same results as the potent T28 serum.
Controls with normal rabbit serum included with each set of antigen dilutions gave negative reactions.
See Table II for symbols.

* These antisera were from different rabbits. The sera were absorbed with heterologous group A streptococci which did not contain R antigen.

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at pH 8. Even exposure to 100°C. for 2 hours had only a moderately deleterious effect, although autoclaving for 20 minutes at 20 pounds' pressure destroyed all but a trace of activity. A further indication of the stability of this antigen under these conditions is the fact that antisera prepared with material heated at pH 8 are almost indistinguishable from those prepared with the unheated purified R antigen.

Heating a crude, but concentrated, preparation of R antigen obtained from the bacteria at pH 2 gave the same results as those obtained with the similarly treated purified R antigen.

Occurrence of R Antigen in Streptococci of Other Serological Groups.—It has been reported by Maxted that antigens serologically similar to the trypsin-resistant protein of group A, type 28 streptococci occur in some strains belonging to groups B, C, and G (7). In the present experiments, two group C strains were studied which appeared to have antigens like the R antigen described in group A strains. R precipitin reactions with an antiserum prepared with one of these strains are shown in Table II. Purified R antigen from the group A strain absorbed these precipitins from group C antisera, and the group C strains readily absorbed all R antibodies from group A antisera.

Observations on group B R-containing strains indicate some differences in the antigenicity of their R antigens, but a thorough study of these strains has not been made. No group G strains containing R antigen were found among those available for this study.

Lack of Correlation between R Antibody Response and Protective Antibodies; Preparation of Mouse-Virulent Strains.—None of the stock strains of type 28 (group A) were virulent for mice. In only two instances, by means of 71 and 120 mouse passages respectively, was it possible to increase the virulence of type 28 cultures (strains C510 and C649A) so that mouse protection tests could be done. Strain T28, used in the preparation of purified R antigen, remained avirulent in spite of 143 mouse passages and 10 rabbit passages. Similarly, repeated mouse passage failed to raise the virulence of the other type 28 strains in which this was attempted.

Failure of Mouse Passage to Affect Content of R Antigen.—Extracts of the virulent strains, C510 and C649A, were compared with those of the original avirulent cultures. No increase in the content of R antigen was observed, nor did rabbits immunized with mouse-passage strains show an increased R antibody response.

Loss of Group-Specific C Polysaccharide.—During the serial mouse passages it was noted that strains C510 and C649A had lost the polysaccharide characteristic of group A streptococci and that the sera of animals immunized with these cultures contained no antibodies for this substance. The other distinctive characteristics of these organisms remained unchanged and per-

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mitted the identification of the variants as derivatives of the original cultures. Subsequently, the original strain C510 has been passed through another series of mice. After 100 passages, it became highly virulent (killing mice in a dose of 10^{-8} cc. within 48 hours) without losing its ability to produce the

TABLE IV

Effect of Absorption with Purified R Antigen on the Protective Properties of C649A Antiserum

Passive Protection Tests

Mouse No.	Dose of antiserum	Agent used to absorb antiserum								
		None		Purified R antigen		Homologous streptococci (C649A)				
		Dose of infecting organism, C649A, *cc.*								
		10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
	cc.									
1	0.5	S	S	S	S	D1	D1	D2	D3	S
2		S	S	S	S	D1	D1	D2	D3	D6
3		S	S	S	S	D1	D1	D2	D2	D1
4		S	S	S	S	D1	D1	D1	D1	D1
1	0.25	S	S	S	S	D1	D1	D1	D1	S
2		S	S	S	S	D1	D1	D1	D1	D2
3		S	S	D1	S	D1	D1	D1	D1	D1
4		D1	D3	D1	S	D1	D1	D1	D1	D1

Serum doses of 0.12, 0.06, and 0.03 cc. were tested in the same way with essentially similar results to those tabulated.

Sets of 4 control mice without serum received doses of 10^{-4} to 10^{-8} cc. of culture. 19 died within 1 to 2 days, and 1 receiving a dose of 10^{-8} cc. survived.

In pour plates, colony counts averaged 400 colonies in 10^{-6} cc. of culture.

The antiserum was obtained from rabbits immunized with strain C649A which had received 120 mouse passages. Swiss mice of the Rockefeller Institute stock 18 to 22 gm. in weight were used. The serum, in 0.5 cc. volume, 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. 4 mice were injected intraperitoneally with each dose and observed for 2 weeks.

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

See Table II for precipitin reactions with these sera.

carbohydrate, C, specific for group A streptococci. Similar observations on the loss of the group-specific C polysaccharide were made originally by Wilson (8).

Mouse Protection Tests.—The virulent variants of strains C510 and C649A were used to test the protective properties of antisera which had a high content of R antibodies. In addition to antibacterial sera prepared with both the original and the virulent C510 and C649A organisms, sera were also tested

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which had been obtained by immunizing rabbits with strain T28, with the purified R antigen obtained from this strain and with intact group C streptococci. Although all these sera contained R antibodies, only those prepared

TABLE V

Effect of Absorption with Homologous and Heterologous Streptococci on the Protective Properties of C649A Antiserum

Passive Protection Tests

Mouse No.	Strain used to absorb R antibodies	Antiserum (C649A) — Dose of infecting organism, *cc.*					
		C649A			C510		
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
1	None	S	S	S	S*	S	S
2		S	S	S	S	S	S
3		D9	S	S	S	S	S
4		D1	D1	S	S	S	S
1	C649A (homologous)	S	D2	D3	D3	D6	S
2		D4	D2	D2	D2	D6	S
3		D1	D2	D2	D2	D3	D3
4		D1	D1	D1	D1	D2	D3
1	C510 (heterologous)	S	S	S	S	D4	S
2		S	S	S	D4	D2	D4
3		S	S	S	D2	D2	D4
4		D11	S	D6	D2	D2	D2

The mice were given 0.25 cc. of antiserum intraperitoneally on the day before the infecting dose of streptococci.

Sets of 8 control mice each received 10^{-6}, 10^{-7}, or 10^{-8} cc. of cultures C649A and C510. One mouse receiving 10^{-7} cc. and 2 receiving 10^{-8} cc. of C649A culture survived. One receiving 10^{-6} cc. of C510 culture survived. The rest of the control mice (21 receiving C649A and 23 receiving C510) died within 1 to 3 days.

In pour plates the colony counts for each culture averaged 180 colonies in 10^{-6} cc.

See Table IV for symbols.

* Four mice receiving unabsorbed C510 antiserum and 10^{-3} cc. of culture died within 1 day; of 4, receiving unabsorbed serum and 10^{-4} cc. of culture, 2 survived and 2 died within 1 day; all 4 mice receiving unabsorbed serum survived 10^{-5} cc. of culture.

with strains C510 and C649A protected mice against the virulent cultures, C510 and C649A. It was of particular interest that antisera against the purified R antigen had no protective value.

To investigate further whether the protective antibodies were related to the R antigen present in the intact streptococcal cell, protective anti-bacterial serum was absorbed either with the purified R antigen or with streptococci

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of the homologous strain. As shown in Tables II and IV absorption with the purified R solution removed antibodies precipitated by this substance without affecting the protective properties of the serum. Absorption with the homologous streptococci (strain C649A) on the other hand, removed both R precipitins and protective antibodies.

In other similar absorption experiments, portions of the same C649A antiserum were absorbed with several different strains of streptococci. Strains of groups A and C which contained R antigen absorbed all R precipitins from the serum; strains of heterologous type which showed no evidence of the presence of this antigen, such as the group A, type 1 strain, T1, did not remove these precipitins. The protective properties of the serum as tested with the homologous strain, C649A, were not significantly reduced by absorption with any of these strains except the one with which the rabbit was immunized, strain C649A. These results show that strain C649A has specific antigens which are directly related to protection and are not present in any of the other strains tested.

Strain C510, however, has certain antigens partially related to those of strain C649A which are concerned with protection. Like strain C649A, strain C510 also has specific antigens peculiar to itself which are more directly related to its own protection. These relationships were demonstrated in reciprocal absorption tests (Table V). Unabsorbed C649A antiserum protected mice against infection with either of these strains. When the serum was absorbed with the homologous strain, C649A, it no longer protected against either strain; but when it was absorbed with the heterologous strain, C510, it showed unchanged protection against the homologous strain, C649A, although it had lost all protective power for the absorbing heterologous strain, C510.

In experiments not tabulated here the converse relationship was observed when C510 antiserum was absorbed with these two strains. Strain C510 absorbed the protective antibodies for both organisms while strain C649A was only able in this case to absorb those responsible for protecting against itself, leaving the protective antibodies for the homologous strain, C510, essentially unaltered. In all these sera absorbed with the two related strains, R precipitins were completely removed without affecting the protective properties of the serum which were dependent upon other antibodies.

DISCUSSION

The properties of the R antigen of (type 28) group A streptococci distinguish it from previously described streptococcal antigens. Pepsin destroys it rapidly; trypsin, on the other hand, releases it without apparent injury from the streptococcal cell. Small amounts of the R antigen can be extracted by heating the bacteria at pH 2, the amount increasing as the pH is raised,

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and a much greater yield is obtained by heating at pH 8. The material thus obtained is slightly different serologically, but only in a quantitative respect from the substance obtained as a result of tryptic digestion of the streptococci. R antigen prepared by either method has some chemical similarity to T antigens (9) but differs in that it is susceptible to peptic digestion and is easily removed from the streptococcal cells by heating at an alkaline pH.

In earlier work the R substance was thought to be the type-specific antigen of type 28 strains, although it was recognized that it differs from the type-specific M antigens of other serological types of group A streptococci in its resistance to tryptic digestion. It resembles M antigens in being present in extracts of type 28 streptococci which have been prepared in the usual way by heating the bacteria at pH 2. These extracts give apparently type-specific precipitin reactions similar to the M precipitin reactions of other types. Agglutination of these group A streptococci by antibodies apparently directed against the R antigen appears in no way unusual, and the designation by Griffith of these strains as a provisional type (type 28)[2] on this basis was fully confirmed in this laboratory by precipitin reactions with the R antigen found in acid extracts of the organisms.

In view of the sensitivity of the R antigen to heating at pH 2, it is of interest that the precipitin reaction involving the use of antigens prepared in this way was as effective as the agglutination reaction in classifying these strains together as belonging in type 28. The explanation lies in the fact that some serologically active R antigen still remains after heating for a short time at pH 2. Since the antisera have always been selected for use with antigens extracted by this method, they are of suitable strength for detecting the reduced amount of R protein which is left.

Subsequent to the designation of group A streptococci containing this antigen as type 28, occasional strains not belonging to group A were observed to give cross-agglutination and cross-precipitin reactions with absorbed group A, type 28 antisera. In Maxted's study of the serological relationships of such strains occurring in groups A, B, C, and G, he was unable to increase the virulence of any of them sufficiently for use in passive protection tests in mice.

In the present investigation two strains of group A, type 28 streptococci were made virulent by numerous mouse passages, and no relationship of the R antigen to virulence or of R antibodies to mouse protection was found. Mouse protection is generally accepted as the ultimate basis for type-classification of streptococci and questionable cases are decided by the use of this method. Since the R antigen is unrelated to protection, it does not satisfy the requirements for designation as a type-specific antigen in the group A strains in which it occurs. It is obvious, therefore, that strains of group A streptococci should not be classified as a single type merely because they

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contain this antigen. Protective antibodies which are not R antibodies have been found in antibacterial sera prepared with virulent strains. Whether or not the corresponding antigens are similar to the M antigens characterizing other serological types has not been fully explored. Current investigations, as well as Maxted's studies (10) indicate that the type-specific precipitin reactions of these strains have been obscured by the simultaneous occurrence of R precipitin reactions. Further study is necessary to establish whether group A streptococci representing more than one specific type are now classified together as type 28.

SUMMARY

A group A, type 28 protein antigen, resistant to tryptic digestion and previously considered to be a type-specific substance, was purified and its chemical and immunological properties studied.

This protein lacks the characteristic properties of a type-specific M antigen since it is apparently unrelated to virulence and does not induce the formation of protective antibodies although precipitins are readily produced. It is designated the R antigen.

The R antigen in addition to occurring in "type 28" group A strains also occurs in some strains of streptococci of other serological groups.

Protective antibodies, distinct from precipitins for the R antigen, are present in "type 28" antibacterial sera. The antigens responsible for protection have not been identified, and it is possible that several different types may be included among strains designated as type 28 on the basis of the R antigen.

The purified R antigen is phosphorus-free and has a sulphur content of 1.04 per cent. In the ultraviolet a maximum absorption was obtained at a wave length of 280 mμ and a minimum at 254 mμ.

Electrophoretically the R antigen was found to be 95 per cent homogeneous at most pH values, but at pH 4.6 the main peak separated into two peaks of approximately equal areas, both containing serologically active material. The interpretation of this finding is at present uncertain. The isoelectric pH was at 4.5 in sodium acetate buffer of 0.1 ionic strength.

The purified R antigen sedimented in the ultracentrifuge as a single boundary.

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BIBLIOGRAPHY

1. Lancefield, R. C., *J. Exp. Med.*, 1943, **78**, 465.
2. Lancefield, R. C., and Perlmann, G. E., *J. Exp. Med.*, 1952, **96**, 71.

Downloaded from http://rup.silverchair.com/jem/article-pdf/96/1/83/1184986/83.pdf by guest on 30 January 2023

3. Maxted, W. R., *Lancet*, 1948, **2,** 255.
4. Longsworth, L. G., *J. Am. Chem. Soc.*, 1947, **69,** 2510.
5. Dean, H. R., and Webb, R. A., *J. Path. and Bact.*, 1926, **29,** 473.
6. Libby, R. L., *J. Immunol.*, 1938, **34,** 71, 269; 1938, **35,** 289.
7. Maxted, W. R., *J. Gen. Microbiol.*, 1949, **3,** 1.
8. Wilson, A. T., *J. Exp. Med.*, 1945, **81,** 593.
9. Lancefield, R. C., and Dole, V. P., *J. Exp. Med.*, 1946, **84,** 449.
10. Maxted, W. R., personal communication.

Downloaded from http://rup.silverchair.com/jem/article-pdf/96/1/83/1184986/83.pdf by guest on 30 January 2023