

## OXYGEN-STABLE HEMOLYSINS OF GROUP A STREPTOCOCCI

### I. THE ROLE OF VARIOUS AGENTS IN THE PRODUCTION OF THE HEMOLYSINS\*

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Group A hemolytic streptococci were shown by Todd (1) to produce two extracellular hemolysins, which were designated as the oxygen-labile hemolysin "streptolysin O" (SLO) and the oxygen-stable "streptolysin S."

Streptolysin O was produced by growing organisms cultivated in ordinary nutrients and, more recently, in defined media (2, 3), and has recently been obtained from resting cells under certain conditions (4). On the other hand, streptolysin S was produced when streptococci were grown in media containing serum, and could readily be "extracted" by serum from washed resting streptococci (5, 6). Later, Okamoto (7), and Bernheimer and Rodbart (8) found that the addition of yeast nuclei acid or RNA digested by pancreatic ribonuclease to growing or resting streptococci caused a considerable increase in the production of streptolysin S, and since then extensive studies on the RNA-induced hemolysin have been carried out by various investigators (9-12). These studies on streptolysin induced by RNA and by fractions obtained from it were extensively reviewed by Bernheimer (13) and by Okamoto (14). More recently Ginsburg and Grossowicz (15) showed that serum albumin, tween 40, tween 80, and triton could induce the formation of oxygen-stable hemolysin from resting streptococci when incubated with glucose,  $Mg^{++}$  and cysteine.

The relationships within the group of oxygen-stable streptococcal hemolysins are still not understood. Similarities among the hemolysins induced by the above mentioned agents have been found in their non-antigenicity (13), inhibition by trypan blue, congo red, and lecithin (13, 15), production by growing or resting cells, and absence in a certain streptococcal mutant (1, 11, 13, 15). On the other hand, some data have suggested differences among these hemolysins. Thus, the production of RNA hemolysin has been prevented by ultraviolet irradiation and is enhanced by

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glucosamine, whereas the hemolysin induced by albumin or tween has not been found to have these properties (15). Therefore, the problem of whether Group A streptococci are capable of forming one, two, or three oxygen-stable hemolysins requires further study.

Data will be presented first, on some conditions of production of hemolysin by serum proteins and, second, on similarities and differences in the production and inhibition of the hemolysin produced by these agents. The chromatographic behavior of the various forms of hemolysin and their electrophoretic behavior will be presented in the following paper.

#### *Materials and Methods*

*Culture Media and Streptococcal Strains.*—Most of the experiments reported were performed with a Group A streptococcus strain S 84 (Type 3) obtained from the State Serum Institute in Copenhagen. In some experiments strain C203S (Type 3), kindly supplied by Dr. A. W. Bernheimer of the New York University, was also used. When grown in brain heart fusion (BHI) (Difco Laboratories, Inc., Detroit) or in a synthetic medium (3, 16) the only hemolysin found in the culture supernate was SLO. However, when the medium included RNA, whole serum, serum albumin, tween 40, or tween 80, the production of oxygen-stable hemolysin could also be demonstrated.

A single hemolytic colony from a blood agar plate was transferred to BHI medium. After 18 hours of incubation at 37°C, 10 ml of culture was inoculated into 250 ml of BHI and grown overnight at 25°C, or for 5 to 7 hours at 37°C, following which the cells were removed in an angle centrifuge. The cells were washed twice with 50 ml of cold saline buffered at pH 7.4 with phosphate (0.1 M), and the cell suspension was adjusted to an optical density (OD) of 500 on the Klett-Summerson photoelectric colorimeter with a 540 filter. In experiments on the production of hemolysin by growing streptococci, the density of the culture was measured with a Beckmann spectrophotometer at 650 m $\mu$ , using 1 cm light path. The streptococcal suspension was placed in an ice bath and used within 2 hours.

*RNA Hemolysin.*—This was prepared from either growing or resting streptococci according to the method of Bernheimer (11). In experiments with resting cells, the following were used: commercial yeast RNA (Nutritional Biochemicals Corp., Cleveland), 5 mg/ml; bacto-peptone (Difco Laboratories, Inc.), 5 mg/ml; glucose, 1 mg/ml (or glucosamine, 1 mg/ml or maltose, 0.5 mg/ml); and MgSO<sub>4</sub>·7H<sub>2</sub>O, 1 mg/ml. In experiments with growing streptococci RNA was added to BHI or synthetic medium at 5 mg/ml. Hemolysin production was estimated after 15 or 50 minutes of incubation at 25°C or 4 to 7 hours at 37°C in experiments on growing cells.

*Serum Hemolysin.*—Serum hemolysin was prepared from growing streptococci according to the method of Todd (1), or from resting cells according to the method of Weld (5). Serum prepared from freshly drawn human, horse, or rabbit blood was used in the experiments at concentrations of 30 or 50 per cent. In the resting cell system the mixture of streptococci and serum was shaken at 37°C for 10 minutes, and the hemolytic activity was determined in the supernate.

*Albumin and Tween Hemolysin.*—Human, horse, or rabbit albumin (fraction V) (Pentex, Inc., Kankakee, Illinois, American Red Cross) was used at 30 mg/ml, or tween 40 or 80 (Atlas Chemical Company, Wilmington) at 4 mg/ml.

*Inhibition of Hemolysin.*—To ascertain that the hemolytic activity obtained by the various methods was not due to streptolysin O, samples of culture supernates were incubated for 5 minutes at 37°C with 10 mg/ml of lecithin (vegetable, Nutritional Biochemicals Corp.) or

with 100  $\mu\text{g}/\text{ml}$  of trypan blue, both of which are known to inhibit RNA and albumin hemolysin but not SLO (13, 15).

*Determination of Hemolytic Activity.*—One hemolytic unit of hemolysin was defined as the amount, in a 1 ml sample, which will hemolyse 50 per cent of a human or rabbit red blood cell suspension (final concentration 1 per cent) after 30 minutes of incubation at 37°C. The degree of hemolysis was determined by reading the OD of hemoglobin released, using a Klett-Sumerson colorimeter.

*Serum Protein Fractions.*—Human Cohn fractions were kindly supplied by the American Red Cross and by Mr. J. Smolens of the Philadelphia Serum Exchange of the Children's Hospital of Philadelphia. The serum fractions were freshly dissolved in buffered solutions for use.

## RESULTS

### A. Factors in the Induction of Oxygen-Stable Streptococcal Hemolysin by Serum Proteins.—

#### 1. The Role of Various Fractions of Serum Proteins.—

It was previously shown (15) that glucose,  $\text{Mg}^{++}$ , and cysteine were required for the production of these hemolysins, and that in the presence of these, both the supernatant protein and the precipitate obtained on dialyzing serum *vs.* acetate buffer  $\mu = 0.01$  pH 5.2 could induce the production of hemolysin by washed resting streptococci, as shown in Table I.

In order to identify the macromolecular components of serum involved, fractions obtained from human serum by Cohn's method were tested for their capacity to induce the formation of hemolysin by washed streptococci. The fractions were dissolved in buffered saline and were used at concentrations proportional to those in which they exist in normal human serum (17).

Table I shows that Cohn fraction V, and to a lesser extent fraction IV-1, yielded relatively large amounts of hemolysin. When, in other experiments, the various fractions were used on an equal weight basis (10 mg/ml of each) fraction IV-1 caused the production of more hemolysin than did fraction V, while all the other fractions yielded only 10 to 15 per cent of the values of albumin.

#### 2. The Role of Lipids in Hemolysin Production.—

Herbert and Todd (6) showed that the hemolysin-producing capacity of normal human serum is completely abolished following delipidation by ether, a finding which suggested a role of lipids in this reaction. Since it has been shown that even the most purified preparations of serum albumin are associated with a variety of fatty acids (19), the role of lipids in hemolysin production was investigated by delipidation and by proteolysis with trypsin, respectively.

Delipidation was carried out by extracting 5 per cent solutions of Cohn fractions V and IV-1 with 20 volumes of ethanol-diethyl ether (3:1) at 25°C. After three to eight extractions (until no opalescence appeared in the saline suspension of the solids of the extract) the precipitated protein was dissolved in buffered saline, and the extract was evaporated *in vacuo* and resuspended in the buffer. The pooled extract and the remaining protein, which was readily soluble in saline solution, were then used to induce hemolysin production in streptococci. Tryptic digestion of the proteins was done with similar solutions of fractions IV-1 and V using crystalline trypsin (Worthington Biochemical Corporation, Freehold, New Jersey) at 3 per cent of substrate for 6 hours at 37°C. Application of the material on sephadex G-50 (Pharmacia, Uppsala, Sweden) indicated that 65 per cent of the fraction V and 50 per cent of the Fraction IV-1 were digested to the point of not being excluded by the sephadex.

It can be seen in Table II that delipidation of both albumin and  $\alpha$  lipo-protein markedly decreased their capacity to yield hemolysin. On the other hand, trypsinization caused no decrease, but actually some increase in the yield of hemolysin by both fractions. This increase may have been due to an effect of trypsin on the permeability of the streptococcus to nutrients, recently described by Fox and Stevenson (20). It can be also seen that treatment of the RNA preparation with lipid solvents did not reduce its hemolysin-inducing capacity.

TABLE I  
*The Role of Plasma Protein Fractions in Hemolysin Production*

Inducing agent*	Concentration of inducing agent	Hemolytic activity
	<i>per cent</i>	<i>units/ml</i>
Human serum . . . . .	50	2700
Low ionic strength precipitate‡ . . . . .	50, of serum	450
Low ionic strength supernate‡ . . . . .	50, of serum	2300
	<i>mg/ml</i>	
Fraction I . . . . .	3.5	13
Fraction II . . . . .	7.7	66
Fraction III . . . . .	10.0	136
Fraction III-0 . . . . .	4.2	15
Fraction IV . . . . .	11.0	84
Fraction IV-1 . . . . .	4.2	320
Fraction IV-5,6 . . . . .	1.7	90
Fraction V . . . . .	38.0	1750

\* Glucose,  $MgSO_4 \cdot 7H_2O$  and cysteine HCl added to each agent at 1 mg/ml, except in the case of whole serum where only cysteine was added.

‡ Fraction obtained by dialyzing serum against acetate buffer, pH 5.2,  $\mu = 0.01$ .

Attempts to obtain hemolysin from streptococci with the lipid extract obtained from fractions IV-1 and V, and from RNA failed. These lipid fractions strongly inhibited both the albumin and RNA hemolysin. This is in agreement with the findings of Humphrey (21) on the presence in albumin of a lipid inhibitor of streptolysin.

*B. Study of the Relationship of Hemolysin Production by RNA, Albumin, Serum, and Tween.—*

*1. The Effect of Glucose and Amino Sugars on the Production of Streptococcal Hemolysin.—*

Bernheimer and Rodbart (8) found that maltose and glucosamine were superior to glucose as energy sources for the production of hemolysin by growing streptococci and were as effective as glucose for the formation of hemolysin by resting streptococci. On the other hand, it has

been shown (15) that for the formation of albumin or tween hemolysin, glucosamine is much less effective than glucose or maltose. The role of glucose and various amino sugars in the production of streptococcal hemolysin was therefore re-examined.

Table III shows that glucosamine was a far less effective energy source for the production of albumin and Tween hemolysin than was glucose, but that this difference was not due to inhibition of utilization of glucose since the presence of glucosamine in 5-fold molar concentration, relative to glucose, did not interfere with the production of tween or albumin hemolysin.

TABLE II  
*Effect of Delipidation and Trypsinization of Fractions IV-1 and V and of RNA in Hemolysin Production*

Preparation of inducing agent	Hemolytic activity
	<i>units/ml</i>
Fraction V .....	550
Fraction V delipidated 3 times .....	50
Fraction V trypsinized .....	750
Fraction IV-1 .....	700
Fraction IV-1 delipidated 3 times .....	10
Fraction IV-1 trypsinized .....	1000
RNA mixture .....	1500
RNA mixture delipidated 5 times .....	1500

Each reaction mixture contained glucose,  $Mg^{++}$ , and cysteine, as in Table I.  
Fraction V and IV-1 each used at 20 mg/ml.

## 2. The Production of RNA, Serum and Albumin Hemolysin by Growing and Resting Streptococci.—

Bernheimer (11), showed that streptococci yielded only small amounts of RNA hemolysin over most of the logarithmic part of the growth curve, as compared with streptococci in the stationary phase. In the case of serum hemolysin, on the other hand, Weld (5) emphasized the importance of employing young cultures. In order to study further the relationship between the RNA hemolysin and the albumin hemolysin, the effect of the age of the culture on the capacity of the streptococci to yield hemolysin was studied in relation to these two inducing agents.

### (a) Production of hemolysin by growing cells:

BHI broth (200 ml, containing 100  $\mu$ g/ml of thioglycolic acid) was inoculated with 10 ml of an overnight culture of strain S84 grown at 25°C. The inoculated flask was incubated at 37°C. At 1 hour intervals, aliquots were withdrawn. Streptococci obtained from these samples by centrifugation were resuspended and examined for the production of hemolysin by RNA and by albumin.

It can be seen in Fig. 1 that the production of albumin hemolysin took place during the logarithmic phase of growth, maximal concentration of the hemolysin

being attained at the end of this phase. On the other hand, the concentration of RNA hemolysin reached a maximum only after the streptococci had reached the stationary phase of the growth. Similar results were obtained with strain C203 S (Type 3).

(b) *Production of hemolysin by resting cells:*

Washed streptococci were obtained from culture in BHI harvested from the stationary phase of growth. The cells were washed twice with 50 volumes of cold phosphate-buffered saline and the cells were resuspended in the buffer and adjusted to an OD of 500. One ml aliquots of this cell suspension were incubated with either (a) 4 ml of RNA and peptone, each at 5 mg/ml, and glucosamine HCl,  $MgSO_4 \cdot 7H_2O$ , and cysteine HCl, each at 1 mg/ml, or

TABLE III  
*Relative Activity of Glucose and Amino Derivatives in the Induction of RNA Hemolysin and Tween Hemolysin*

Carbohydrate	Per cent activity relative to glucose	
	RNA hemolysin	Tween hemolysin*
<i>M/270</i>		
Glucose .....	100	100
Glucosamine .....	110	12
Glucosaminic acid .....	65	0
<i>N</i> -Acetylglucosamine .....	45	10
Glucose + glucosamine ( <i>M/50</i> ) .....		100
Glucose + glucosaminic acid ( <i>M/50</i> ) .....		100
Glucose + <i>N</i> -acetylglucosamine ( <i>M/50</i> ) .....		100

\* Similar results were obtained with albumin hemolysin, and with hemolysin obtained with dialyzed serum.

(b) 4 ml of 3 per cent human serum albumin (fraction V) containing glucose,  $MgSO_4 \cdot 7H_2O$ , and cysteine HCl, each at 1 mg/ml, or (c) 4 ml of freshly drawn human serum.

At 10 minutes of incubation at 37°C, and at 20 minute intervals thereafter, 1 ml aliquots were withdrawn. The cells were removed by centrifugation and the supernate was assayed for hemolytic activity.

Fig. 2 shows that the rate of production of serum hemolysin and of albumin hemolysin by resting cells was maximal after 10 to 15 minutes of incubation at 37°C. On the other hand, the rate of production of RNA hemolysin was much slower, reaching a maximum value after 50 minutes of incubation.

When the production of the RNA hemolysin and the albumin hemolysin was carried out in the absence of cysteine, no such difference between the RNA and the albumin systems could be seen, the hemolytic activity reaching a maximum in 50 minutes of incubation in both systems. Also, the yield of both hemolysins was much lower in comparison to that produced with cysteine. This

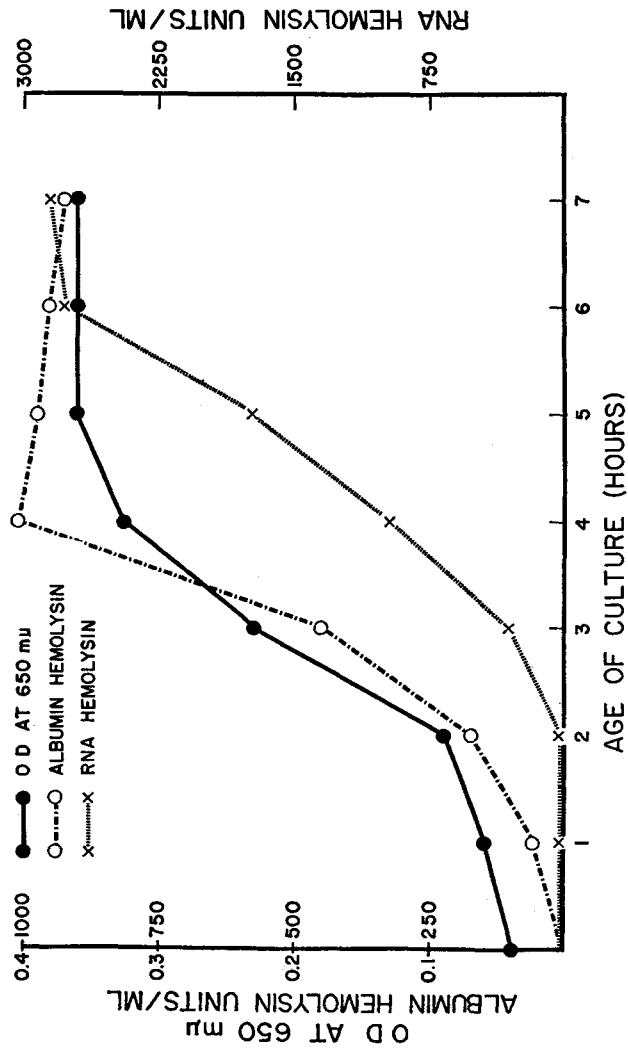


FIG. 1. Production of albumin hemolysin and RNA hemolysin in streptococcal culture during the logarithmic phase of growth.

effect of cysteine indicated that sulfhydryl compounds affect both the rate and total yield of albumin hemolysin, but that cysteine affects the yield but not the rate of formation of the RNA hemolysin. (Ascorbic acid did not produce this effect.)

The effect of cysteine on the production of albumin hemolysin was confirmed in experiments involving various concentrations of cysteine. It was found that

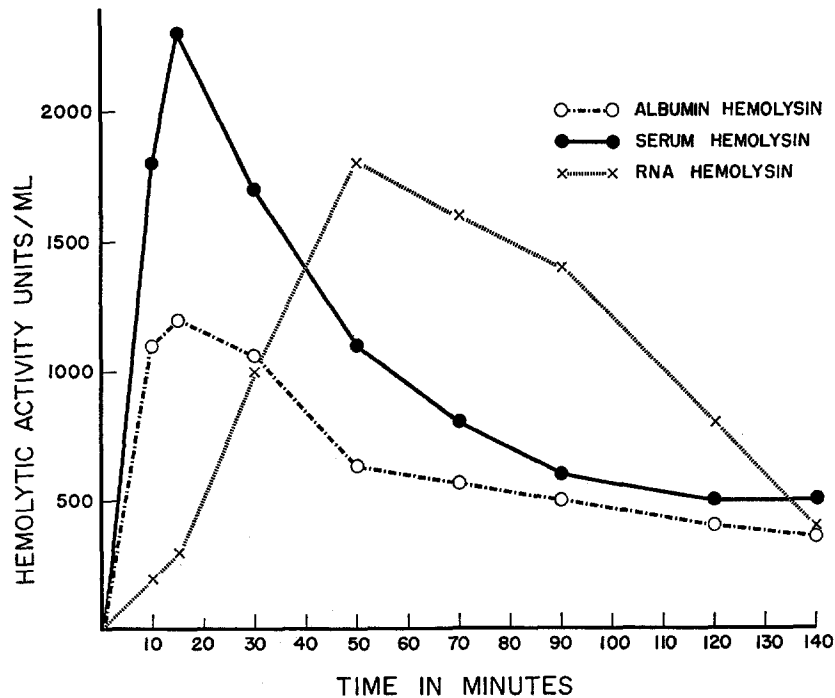


FIG. 2. Relative rates of production of serum hemolysin, albumin hemolysin, and RNA hemolysin by streptococci in the stationary phase.

with less than 100  $\mu\text{g}/\text{ml}$  of cysteine the rate of production of the hemolysin was low, 60 minutes of incubation being required for maximum hemolytic activity. On the other hand, higher concentrations of cysteine caused more rapid production of the hemolysin, and at a cysteine concentration of 500  $\mu\text{g}/\text{ml}$  to 3000  $\mu\text{g}/\text{ml}$  maximal levels were reached after 5 to 10 minutes of incubation.

### 3. Effect of Temperature on Production of the Hemolysins.—

Bernheimer (11), and Younathan and Barkulis (22) gave evidence for *de novo* synthesis of RNA hemolysin by showing that the production of this hemolysin was temperature-dependent and that there was no preformed hemolysin as indicated by disruption of streptococcal cells.



In the present study, it was found that the production of albumin and tween hemolysins was also greater at higher temperatures.

The effect of a difference in temperature on the production of the respective hemolysins was studied in connection with the question of whether the induction of the hemolysins involves a synthetic or extractive process, by dividing the incubation into two stages, as follows:

Washed streptococci were incubated for 10 minutes at 37°C with glucose, Mg<sup>++</sup>, and cysteine. Each mixture was then chilled to 10°C, and after the addition of albumin, tween, horse serum, or RNA, was divided into two parts, one for continued incubation at 10°C and the other for incubation at 37°C.

TABLE IV  
*Effect of Temperature on the Production of Hemolysin by Various Inducers after Incubation of the Streptococci with the Diffusible Components of the System*

Reaction mixture*			Hemolytic activity
A	B	C	
Preincubated 10 min. at 37°C	Added to A and incubated 45 min. at 10°C	Added to A and incubated 45 min. at 37°C	
Streptococci‡	Tween 40		units/ml 30
Streptococci	Human fraction V		18
Streptococci	Human serum		8
Streptococci	RNA + peptone		15
Streptococci		Tween 40	500
Streptococci		Human fraction V	500
Streptococci		Human serum	1100
Streptococci		RNA + peptone	550

\* Concentrations of reagents: tween 40, 4 mg/ml; human fraction V, 20 mg/ml; human serum, 50 per cent; RNA, 5 mg/ml; peptone, 5 mg/ml.

‡ Plus glucose, Mg<sup>++</sup>, cysteine, as above.

When the supernates of the incubation mixtures were examined (Table IV) only traces of hemolytic activity were found if the albumin, detergent, serum, or RNA was incubated with the streptococci at 10°C, even if the cells had been previously incubated with glucose and the other diffusible components at 37°C. On the other hand, continued incubation with the inducers at 37°C caused the production of substantially larger amounts of hemolysin. These results suggest that the hemolysin which appears on incubation of streptococci with tween, albumin, serum, or RNA is not simply extracted from the organism, but is synthesized by the streptococci under conditions of such incubation.

4. *Inhibition of Albumin Hemolysin and RNA Hemolysin by Plasma Fractions.*—As another approach to the question of the relationship of the hemolysin produced by these two agents, the inhibitory effect of known plasma fractions was compared in the case of albumin hemolysin and RNA hemolysin.

To 0.5 ml portions of solutions containing approximately 500 hemolytic units per ml of albumin hemolysin or 1000 hemolytic units per ml of RNA hemolysin, were added solutions of various serum fractions, each at the relative concentrations found in human serum (18). Following incubation, the hemolysin-protein mixtures were serially diluted and assayed for hemolytic activity.

Table V shows that fraction III-0 was the strongest inhibitor of the fractions, causing 97 and 99 per cent inhibition of albumin and RNA hemolysin, respectively, at the concentrations used. Marked inhibition of both preparations of hemolysin was also caused by fraction III and to a smaller extent by fraction IV-1, which had been previously shown to be second to albumin in releasing hemolytic activity. Fractions I, II, IV, IV-5, 6, and V caused very slight in-

TABLE V  
*Inhibition of RNA Hemolysin and Albumin Hemolysin by Plasma Fractions*

Plasma fractions	Concentration	Albumin hemolysin		RNA hemolysin	
		Activity	Inhibition	Activity	Inhibition
	mg/ml	units/ml	per cent	units/ml	per cent
Control.....		350		500	
Fraction I.....	3.5	350	0	500	0
Fraction II.....	7.7	350	0	500	0
Fraction III.....	10.0	125	65	75	85
Fraction III-0.....	4.2	8	97	8	98.5
Fraction IV.....	11.0	320	9	500	0
Fraction IV-1.....	4.2	215	40	185	63
Fraction IV-5,6.....	1.7	335	5	430	8.5
Fraction V.....	38.0	350	0	500	0

hibition or none. These observations are consistent with the findings of Humphrey (21) and of Stollerman *et al.* (23) on a serum  $\beta$  lipoprotein inhibitor of streptolysin S.

Aside from the tests of the serum protein fractions themselves, alcohol-ether extracts of fraction V were tested. These were found to be highly inhibitory to both the albumin and RNA hemolysin. This finding indicates that this inhibitor is active only when separated from the protein to which it is bound.

#### DISCUSSION

The experiments described in the earlier part of this paper were undertaken to elucidate the role of serum constituents in the production of oxygen-stable hemolysin by streptococci. It was shown that the production of hemolysin by albumin or by  $\alpha$  lipoprotein required the presence of a group of diffusible substances, glucose,  $Mg^{++}$ , and cysteine. This explains the difference between these data and the results of Herbert and Todd, who found little hemolysin production by solutions of serum proteins, since these authors did not supplement the proteins with the diffusible components

of the system. Our data do, however, confirm those of Herbert and Todd (6) in showing the destructive effect of organic solvents on the hemolysin-inducing capacity of serum, in that delipidation of the fractions completely destroyed, or markedly reduced their activity, thus suggesting the role of associated lipids in the induction of hemolysin by albumin.

The experiments on the rate of production of the hemolysin in the various systems were undertaken in the hope that they might shed some light on the relationship between the hemolysins thus produced. The data presented in Table IV further support the hypothesis of an active synthetic process being involved, rather than extraction, since the 37°C incubation of the resting organisms with the diffusible components including the energy source, only *before* the incubation with the macromolecular inducing agents was not a sufficient condition for the production of hemolysin by any of these inducers, as would be expected if the function of these agents was one of extraction of preformed hemolysin. Rather, the continued incubation at 37°C with glucose, Mg<sup>++</sup>, and cysteine, as well as the inducing agent, was required for the production of hemolysin by resting streptococci.

In considering the problem of the relations among serum-, albumin- and RNA-induced hemolysin, the problem is narrowed by the indications, in the data above, that serum hemolysin may simply represent albumin hemolysin plus a small contribution of  $\alpha$  lipoprotein hemolysin. The rates of production by resting cells, the effective inhibitors, the effect of cysteine on production, and the non-production by the mutant strain C203U have all been the same for induction by both serum and albumin. The problem then involves two types of inducers, RNA on one hand, and, on the other, a group of reagents effective as detergents or lipids (associated with proteins or as parts of lipoproteins). The albumin hemolysin has generally been used in this study as the type reagent for this group.

Of the experiments involving comparisons between albumin (or in some cases tween) and RNA as inducers of hemolysin production by streptococci, some have yielded data suggesting that the same hemolysin may be produced in the two systems, while the data of other experiments have indicated apparent dissimilarities. Differences between RNA hemolysin and albumin hemolysin are suggested by the differences in the kinetics of production by resting and growing streptococci, and in the effect of cysteine on the rates of production. Another difference is in the failure of the albumin system to utilize glucosamine, in comparison with the RNA system. (It should be pointed out in this connection that Bernheimer found that Group A hemolytic streptococci can utilize glucosamine as effectively as glucose; reference 11.)

However, the other types of experiments indicated similarities between albumin hemolysin and RNA hemolysin. Thus, the effect of temperature on the rate of production by resting cells and the inhibition by human serum fraction III-0 are similar in both systems. Other similarities from recent published data include the inhibition of production of both hemolysins by the same enzymatic poisons and antimetabolites (11, 15), the inhibition of both hemolysins by

trypan blue and congo red (11, 15), and the failure of production of hemolysin in same mutant strain C203U by either inducer.

The inconsistency between the apparent similarities and differences raises a possibility that we are dealing with a single hemolysin which can be formed by different metabolic pathways. On the other hand, the possibility must be considered that the hemolysins represent an active group which can be bound to different macromolecular carriers, RNA, or serum proteins. Studies on chromatographically or electrophoretically separated hemolysins which bear on this point are reported in the following paper.

#### SUMMARY

The production of oxygen-stable hemolysin in growing and resting Group A streptococci has been induced by RNA, by detergents, and by mammalian blood serum proteins, in the presence of glucose,  $Mg^{++}$ , and cysteine. Of the serum proteins, albumin and  $\alpha$  lipoprotein could act as inducers. In the case of both these serum proteins treatment with trypsin did not affect the capacity to induce hemolysin production, but removal of the bound lipids by alcohol-ether or chloroform-methanol destroyed this property.

In comparisons of the conditions of production and of activity between the hemolysin produced by RNA on one hand and albumin and detergents on the other, some data indicated similarities among the hemolysins, and others, differences. The similarities included similar degrees of temperature dependence for production and equal degrees of inhibition by serum  $\beta$  lipoprotein. Differences found among these hemolysins included differences between the rate of production of the RNA hemolysin from that of albumin or detergent hemolysin by both resting and growing streptococci, and the failure of utilization of glucosamine as an energy source for the production of albumin hemolysin, in contrast with that of RNA hemolysin. The fact that the data have in some cases indicated similarities and in other cases differences among the hemolysins raises the question of whether these are different molecular species, or a single hemolysin synthesized by the streptococci *via* different pathways of metabolism, or complexes of a single hemolytic moiety with various molecular carriers.

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