OXYGEN-STABLE HEMOLYSINS OF GROUP A STREPTOCOCCI

II. Chromatographic and Electrophoretic Studies*

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In the preceding paper (1) the materials and methods involved in the production of oxygen-stable hemolysins by Group A streptococci were described.

It was shown that although a variety of unrelated materials such as RNA, serum albumin, serum α lipoprotein, tween 40, and triton X-205 could induce the production of hemolysin from resting streptococci, a number of similarities could be demonstrated among the resulting preparations of hemolysin. All were inhibited by lecithin, trypan blue, congo red, papain, chymotrypsin, and by a β lipoprotein fraction of human serum, and none were affected by cholesterol or trypsin. On the other hand, differences were shown among the hemolysins induced by RNA, on one hand, and albumin, tween, and triton on the other. Thus, it was shown that the rates of production of the RNA and albumin hemolysins differ in both resting and growing streptococci, and that glucosamine, which is an excellent energy source for the production of RNA hemolysin, is not effective for the formation of the albumin or detergent hemolysin.

These observations raised the question of whether we were dealing with different hemolysins, or with the same hemolysin produced by different metabolic pathways, or with a single hemolysin associated with various inducing agents. The purpose of the present study was to explore further the relationship within this group of hemolytic agents by chromatography, by electrophoresis, and by procedures which could inactivate preparations of the hemolysin produced by individual members of this group of inducing agents.

Materials and Methods

The Streptococcal strain and culture medium were described in the preceding paper (1).

Preparation of Hemolysins.—

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1. RNA hemolysin: This was prepared by incubating washed streptococci with 5 mg/ml of yeast RNA (Mann Research Labs., New York) and with 1 mg/ml each of glucose, $MgSO_4 \cdot 7H_2O$, and cysteine HCl. The supernatant fluid obtained from centrifugation at 5000 RPM was used as a source of hemolysin.

2. RNA-core hemolysin: Yeast RNA was dissolved in distilled water at 100 mg/ml, and the pH of the solution was adjusted to 7.0 with 1 M NaOH. The RNA solution was incubated for 5 hours at 37°C with pancreatic ribonuclease at 0.1 mg/ml (twice crystallized, Mann Research Labs.). The mixture was then dialyzed for 24 hours against running cold water and for 6 hours against distilled water. The extent of the digestion was determined by precipitating the undigested RNA with uranyl acetate and perchloric acid, and determining the OD at 260 mμ of the supernatant solution (2). RNA-core was used to prepare hemolysin from resting streptococci, as described above for whole RNA.

3. Albumin, α lipoprotein, serum, tween, and triton hemolysins: These were prepared and titrated as described in the preceding paper (1).

Chromatography of Hemolysins on Cellulose Columns.—Diethylaminoethyl (DEAE) cellulose, type 40, (Schleicher and Schüll, Keene, New Hampshire) was used in all the experiments. The cellulose was sieved through a 100 mesh stainless steel sieve and washed according to the method of Peterson and Sober (3). Columns of 1 × 28 cm, based on sintered glass, were packed with DEAE cellulose with a gradual increase of pressure from 1 to 10 psi. The packed columns were washed overnight with 0.01 M phosphate buffer pH 7.3. The various hemolysin preparations were first passed through sephadex G-50 columns (Pharmacia, Uppsala, Sweden). The void volume of the columns was determined with hemoglobin. Both gradient and stepwise salt elution chromatography were employed. Since K ions were shown by Bernheimer (4) to stabilize RNA hemolysin, KCl buffers were used throughout. All the experiments were performed at 4°C and aliquots of 2 to 4 ml were collected from the columns by means of an automatic fraction collector. Of fractions thus obtained, estimates of protein or nucleic acid were made by determining the OD at 280 or 260 mμ, respectively, and the hemolytic activity was determined as required.

Chromatography on Glass Powder of Serum Lipoproteins and of Hemolysins Obtained by Whole Serum.—Separation of α and β lipoproteins from serum was carried out by the method of Carlson (5). Glass powder (sodium glass, Arthur H. Thomas Company, Philadelphia) was boiled for 2 minutes with a mixture of HNO_3 and HCl, followed by rinsing with distilled water. The glass column, 20 × 150 mm, was packed by gravity and equilibrated with 0.4 M carbonate-bicarbonate buffer, pH 8.6. Preparations of hemolysin induced by whole serum were mixed with 1 ml of the pH 8.6 buffer and applied to the column. After collecting all protein not adsorbed to the column, buffers of pH 9.6 and pH 9.8 were applied successively, and the respective lipoprotein fractions were eluted. The OD at 280 mμ and hemolytic activity of the various fractions were determined, as above.

Zone Electrophoresis of Hemolysin on Agar.—Glass plates 18 × 18 cm were coated with 1.5 mm layer of a 1 per cent agar (Ionagar, Oxoid Division, Consolidated Laboratories, Chicago Heights, Illinois) dissolved in 0.01 M veronal buffer, pH 8.6. After the agar hardened, a second layer of agar (1.5 mm) was poured on the hardened agar and allowed to solidify. The second layer was poured around a mold consisting of a vertical sheet of metal with 1 cm long indentations in the bottom edge, which was allowed to rest in a vertical position on the first layer of agar, to provide slots for the application of the solutions to be examined. Electrophoresis was carried out at 40 v/cm for 4 to 8 hours at 4°C. Following electrophoresis, the agar block was immersed for 30 minutes in cold saline buffered with 0.05 M PO_4, pH 7.0, to increase the salt concentration of the medium. The agar plates were then blotted with filter paper, and a layer of melted agar (1 per cent) containing RBC in suspension (1 per cent) was poured on the agar slab and allowed to solidify. After incubation for 2 to 3 hours at 37°C, zones of hemolysin appeared in the agar RBC layer.

In each run a 10 per cent solution of dextran was included to determine the extent of the

electroosmotic effect. At the end of the run the part of the agar block which contained the dextran was immersed in 95 per cent ethanol, which caused precipitation of the dextran as a white zone. The migration of the albumin and serum hemolysins was also determined by staining with amidoblack B or ponceau S. Tween hemolysin was identified by staining with Sudan black B. RNA was stained with 10 per cent pyronin in acetone, and triton X-205 with $BiNO_3$ in KI (6).

RESULTS

A. Chromatography of Hemolysins on DEAE Cellulose.—

1. Albumin Hemolysin. Gradient Salt Elution.—

Preparations of albumin hemolysin from human, horse, or rabbit serum albumin, usually containing approximately 2000 hemolytic units/ml, were examined by chromatography on DEAE cellulose in 0.01 M phosphate buffer pH 7.3, with elution by increasing concentration of KCl in a linear gradient.

Fig. 1 *A* shows that protein (as judged by absorption of ultraviolet light at 280 mμ) was eluted in concentrations of KCl between 0.1 M and 0.35 M, the highest concentration of protein being at 0.18 M. Hemolytic activity was found in one group of fractions of the eluate, and was most concentrated in the fractions obtained at 0.22 to 0.27 M KCl. The total yield of the hemolytic activity eluted from the column was only 10 to 15 per cent of the activity of the material applied to the column. Attempts to increase the yield of hemolysin by the use of other adsorbents were not successful. These included epichlorohydrin-triethanolamine (ECTEOLA) cellulose, aminoethyl cellulose and hydroxyapatite.

2. Fractionation of Albumin Hemolysin.

Because the peak of activity of albumin hemolysin did not coincide with the peak of OD at 280 mμ, preparations of albumin hemolysin were fractionated by adsorption to DEAE cellulose and elution by stepwise increase in molarity of KCl in order to see whether a fraction of albumin relatively more strongly bound to the adsorbent contained a relatively greater amount of the hemolytic material. Fractions of the eluate obtained in this manner were examined for OD at 280 mμ and for hemolytic activity. Typical results of such an experiment are shown in Fig. 2, where it can be seen that the fractions obtained with increasing molarity of the eluant showed progressive decrease in the amount of protein eluted, but progressive increase in hemolytic activity. The lowest amount of protein and the highest yield of hemolysin were obtained on eluting with 0.25 M KCl. Treatment of this fraction with alcohol-ether yielded material giving considerable opalescence in aqueous suspension. In a different approach to the same question, fractions of fresh serum albumin were compared in their effectiveness in the induction of hemolysins in resting streptococci. The fraction of albumin eluted at 0.25 to 0.3 M KCl, under the same conditions, induced the formation of larger amounts of hemolysin than did the fraction eluted at 0.175 M, although the OD (280 mμ) was substantially less.

3. Chromatography of RNA Hemolysin on DEAE Cellulose.

RNA hemolysin (5000 hemolytic units/ml) was examined by gradient salt elution chromatography on DEAE cellulose, under similar conditions to those used for albumin hemolysin. Fig.

1 *B* shows that the peak of material giving UV absorption at 260 mμ was obtained at 0.55 M KCl. In this case also hemolytic material was recovered in varying concentrations in fractions of the eluate, the highest concentrations being in the fraction eluted from the column at 0.6 to 0.7 M KCl.

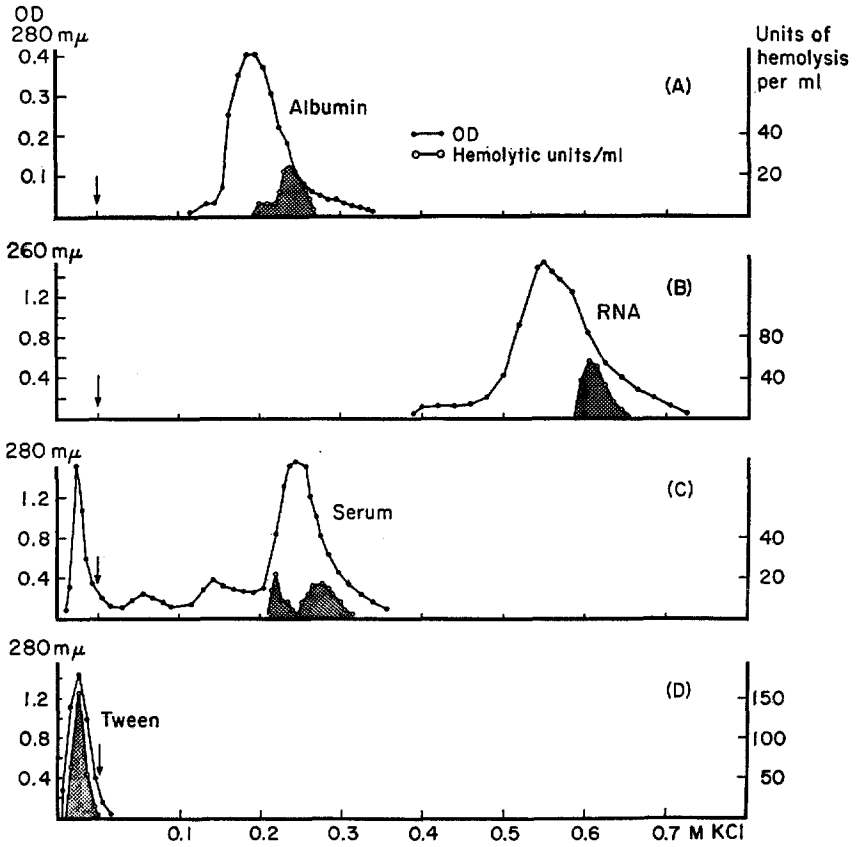

FIG. 1. Chromatography of several of the oxygen-stable streptococcal hemolysins on DEAE cellulose in 0.01 M PO_4, pH 7.2. The vertical arrow indicates the beginning of elution with KCl in linear gradient increase of concentration. The molarity of the KCl is shown on the abscissa. *A*, albumin hemolysin; *B*, RNA hemolysin; *C*, "serum hemolysin;" *D*, tween 40 hemolysin.

These data suggested that, as in the case of the albumin hemolysin, the RNA hemolysin might be associated with some fraction of the RNA. Since RNA-core (obtained by digestion with pancreatic ribonuclease) had been shown by Bernheimer (7) to be more active in inducing hemolytic activity than the total RNA, chromatography was performed with hemolysin prepared with RNA-core. The results were quite similar to those obtained with whole RNA, in that

the peak of hemolytic activity was in the eluate at 0.65 M KCl, along the descending limb of the peak of RNA. In order to confirm this observation, RNA-core hemolysin was fractionated by stepwise increase of salt concentration for elution from the DEAE column. As can be seen in Fig. 3, the material eluted

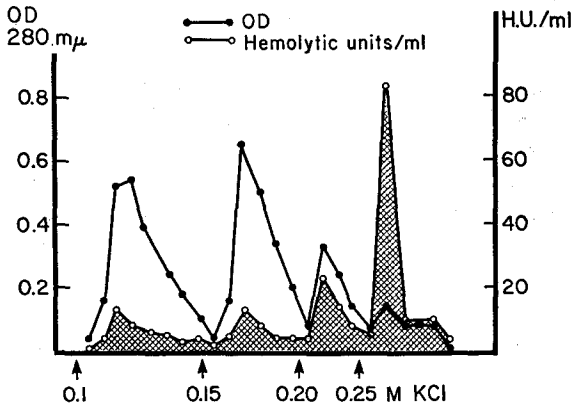

FIG. 2. Fractionation of human serum albumin hemolysin. Adsorption as in Fig. 1. Stepwise elution by increasing concentrations of KCl, in steps as shown.

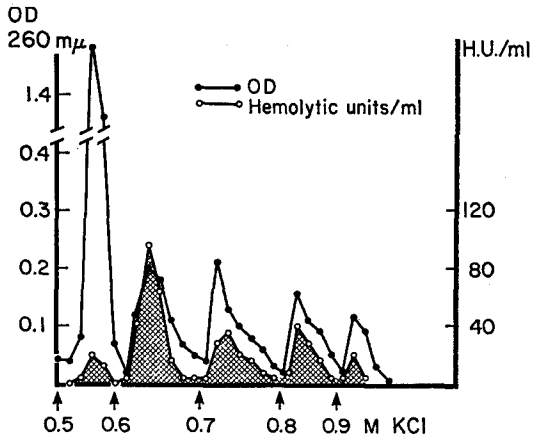

FIG. 3. Fractionation of RNA-core hemolysin, as in Fig. 2. Stepwise elution by appropriate concentrations of KCl, as shown.

up to 0.6 M KCl had a large part of the UV-absorbing material (260 mμ), but very little of the hemolytic activity. The next fraction, eluted by 0.6 M KCl, had far less total UV absorbing material, but the highest concentration of hemolysin. Again, as in the case of the albumin fractions, RNA-core itself was subjected to chromatography, with stepwise elution, and the various fractions were used

to prepare hemolysin from resting streptococci. The fraction obtained at 0.6 M KCl induced the formation of by far the greatest amount of hemolysin from streptococci. As in the case of the albumin hemolysin, only a small part of active RNA-core hemolysin was recovered from DEAE cellulose, in this case, 15 to 20 per cent. (No greater yields were obtained with ECTEOLA cellulose.) Further purification of the RNA-core hemolysin obtained with the 0.6 M KCl fraction was obtained by rechromatography on DEAE cellulose. Substantial amounts of 260 mμ-absorbing material that had no hemolytic activity were eluted from the column at 0.3 M KCl. The best preparation, obtained with the 0.6 M fraction, contained approximately 2×10^6 hemolytic units/mg (based on OD determined on the whole RNA-core). Similar results to those described above were recently reported by Ishikura, who purified the RNA-core hemolysin on ECTEOLA cellulose. (For details see Ishikura, reference 8, and Okamoto reference 9.)

4. Chromatography of "Serum Hemolysin" on DEAE Cellulose and on Glass Powder.—

As shown previously, both Cohn fractions IV-1 and V obtained from human serum were capable of inducing the formation of hemolysin by resting streptococci (1). The association of hemolysin with these two fractions in normal human serum was then explored. Preparations of serum hemolysin (hemolysin induced in resting streptococci by whole human or horse serum) containing approximately 5000 hemolytic units/ml were chromatographed on DEAE cellulose and eluted by the same procedure as above. Fig. 1 *C* shows that under these conditions there were two peaks of hemolytic activity in the material eluted, the maxima corresponding to 0.22 and 0.27 M KCl. One of these peaks of hemolytic activity showed the same relation to the descending arm of the albumin peak as in Fig. 1 *A*, above; the other peak of hemolytic activity was found associated with the ascending arm of this peak. According to Peterson and Sober, protein eluted as the ascending arm of the peak due to albumin contains some of the lipoprotein fraction of human serum (3). In order to determine whether this earlier peak of hemolytic activity could be due to α lipoprotein hemolysin, chromatography of serum hemolysin was done by the method of Carlson (5), who showed that the albumin fraction of normal human serum is not adsorbed to glass powder, but that both α and β lipoprotein are adsorbed, and are eluted at pH 9.6 and 9.8, respectively.

Fig. 4 shows that on application of serum hemolysin to glass powder columns, two peaks of hemolytic activity were obtained, one due to material not adsorbed to the column at pH 8.6, and the other in the material adsorbed and then eluted at pH 9.6. No hemolytic activity was found associated with the small amount of UV-absorbing material eluted at pH 9.8 or at pH 11.0, but streptolysin-inhibiting material was found in the pH 9.8 eluate (β lipoprotein). These associations with α and β lipoproteins are in accord with the observations of Humphrey (10) and of Stollerman *et al.* (11).

5. Chromatography of Tween and Triton Hemolysin on DEAE Cellulose.— Tween 40 and triton X-205 were chromatographed on DEAE cellulose, as were

preparations of hemolysins induced by these. None of these materials were adsorbed to the column, (Fig. 1 *D*) nor were they adsorbed to carboxymethyl cellulose.

B. The Simultaneous Production of Hemolysin by Combinations of Inducers.— When streptococci were exposed to combinations of any two of the inducing agents, hemolytic activity emerged from the chromatographic column in two peaks, corresponding to the respective hemolysins as shown in Fig. 1. In experiments involving whole human serum and RNA, three peaks of hemolytic activity were found.

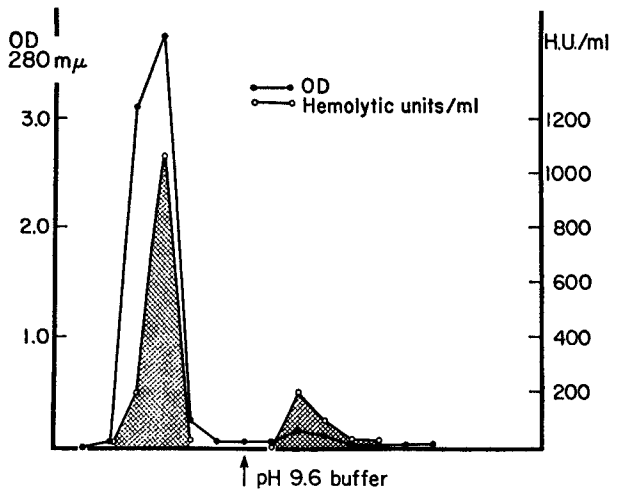

FIG. 4. Chromatography of "serum-hemolysin" on glass powder column. Vertical arrow shows the beginning of a single-step elution with carbonate buffer, pH 9.6.

C. Chromatography of Mixtures of Hemolysin with Other Inducers.—The similarity of the elution pattern of each of the hemolysins to that of its own inducing agent suggested the possibility that the differences in chromatographic behavior which had been observed reflected differences not among hemolysins *per se*, but rather differences among the inducers, to which the hemolysin(s) might be bound. Accordingly, experiments were carried out in which a preparation of a given hemolysin was incubated with another inducer, of different chromatographic characteristics, and the mixture examined by chromatography on DEAE cellulose, using stepwise elution by KCl, at 0.30 M and 0.70 M respectively, as suggested by the data of Fig. 1.

In Table I, it can be seen that when RNA hemolysin was incubated with fresh albumin, or albumin hemolysin with fresh RNA, and the mixtures were examined, hemolytic activity was in each case found in the eluates obtained

both at 0.30 M and at 0.70 M KCl. When albumin hemolysin was incubated with tween, hemolytic activity was found both in the unadsorbed material and in the 0.3 M KCl eluate, and when this hemolysin was incubated with triton, all the hemolysin detectable was recovered in the unadsorbed fraction. Finally, when RNA hemolysin was incubated with tween, hemolysin was found both in the column volume and in the 0.7 M KCl eluate. Typical results of such experiments are shown in the lower part of Table I. These results suggested that a hemolytic moiety could be transferred from one inducer to another. Further data on this

TABLE I

Chromatographic Elution Patterns of Various Preparations of Streptococcal Hemolysin

Preparation of hemolysin	Inducer added to hemolysin	Hemolysin applied to column	Hemolysin recovered from column		
			In column volume (unadsorbed)	Eluted at 0.3 M KCl	Eluted at 0.7 M KCl
		units	*units*	*units*	*units*
Triton hemolysin	—	1600	1320	0	0
Tween hemolysin	—	1200	1110	0	0
Albumin hemolysin	—	2000	0	200	0
RNA hemolysin	—	1900	0	0	365
Albumin hemolysin	RNA	2000	0	50	100
Albumin hemolysin	Tween	4000	411	27	0
Albumin hemolysin	Triton	1000	200	0	0
RNA hemolysin	Albumin	1800	0	66	188
RNA hemolysin	Tween	4000	265	0	138

Incubation for 1 minute at 25°C, then applied to DEAE cellulose column in 0.01 M PO_4 buffer, pH 7.2.

Concentration of hemolysins or inducers, in the incubation mixtures: albumin hemolysin or albumin, 20 mg/ml; RNA hemolysin or RNA, 5 mg/ml; tween hemolysin or tween, 6 mg/ml; triton hemolysin or triton, 4 mg/ml.

point were obtained in experiments on electrophoretic migration of hemolysins and their inducers.

D. Electrophoresis of the Hemolysins on Agar.—The preparations of hemolysin were subjected to zone electrophoresis on agar plates as described above. In Table II it can be seen that the rate of migration of the RNA hemolysin to the anode was approximately twice that of the albumin hemolysin. Serum hemolysin showed two zones of hemolysis; one with the rate of migration of albumin hemolysin and the other with about one-half that rate. On the other hand, tween and triton hemolysin were carried towards the cathode to a position quite near that of dextran, indicating very little, if any, electrophoretic mobility of these hemolysins. When the agar blocks were stained with amidoblack B (in the case of albumin and serum hemolysin), pyronin (RNA hemolysin),

Sudan black (tween hemolysin), and $BiNO_3$ + KI (6) (triton hemolysin), it was found that in each case the position and shape of the spot of hemolysin coincided with the position and shape of the spot giving the stain of the corresponding inducer.

1. Electrophoresis of Mixtures of Hemolysins with Other Inducers.—Because of the clear differentiation of migration rates among the hemolysins, experiments were done to seek evidence of transfer of hemolytic activity from one inducer to another, by analogy with the data obtained by chromatography. Using high ratios of detergent to albumin or RNA, 10 mg of tween or triton was incubated with 5 mg of albumin hemolysin or 1 mg of RNA hemolysin, and the

TABLE II

Electrophoretic Migration of Hemolysins and Derived Hemolysins in Agar

Preparation of hemolysin	Inducer added to hemolysin	Rate of migration toward anode* of material giving stain for:			
		Hemolysin	Protein	RNA	Detergent
		cm/hr.	*cm/hr.*	*cm/hr.*	*cm/hr.*
Albumin hemolysin		1.3	1.3		
Serum hemolysin		0.75, 1.3	0.75, 1.3		
RNA hemolysin		2.4		2.4	
RNA-core hemolysin		2.4		2.4	
Tween 40 hemolysin		−0.2			−0.2
Triton hemolysin		−0.2			−0.2
Albumin hemolysin	Triton	−0.2	1.3		−0.2
RNA hemolysin	Tween	−0.2		2.4	−0.2

* Relative to position of dextran, at end of run.
Electrophoresis at 40 v/cm.

mixtures were examined by electrophoresis. On subsequent staining of the agar blocks both for the inducers and for hemolytic activity, the stain for each of the inducers was found in the same position as that described above. However, as can be seen, in the lower part of Table II, the hemolytic activity was found not at the position of the albumin or RNA, but at the position of the tween or triton.

E. A Hypothecated Hemolysin-Carrier Complex: Experiments on the Inactivation of Hemolysins by Alterations in the Hemolytic Group or the Carrier Molecule.—The experiments described above suggested that this group of streptococcal hemolysins may exist as a carrier-hemolysin complex, the carriers being the molecular species which can function as inducers, and the hemolytic moiety being transferable among the carriers. In order to examine this hypothesis experiments were performed with the purpose of affecting either the hemolytic group or the carrier.

1. The Inactivation of the Hemolysin by Enzymes Capable of Destroying the Hemolytic Group.—

The hemolysins induced by RNA, albumin, serum, tween 40, or triton X-205 have all been found to be destroyed by papain, chymotrypsin, and by streptococcal proteinase (1, 7). On the other hand, trypsin and pepsin were not effective. In the present study, experiments were carried out on the effect of papain and chymotrypsin on the hemolytic activity of hemolysins prepared by transfer from RNA hemolysin or albumin hemolysin to tween or triton. It was found in each case that the hemolysin was inactivated by these enzymes regardless of the carrier molecule employed in producing the hemolysin or that to which the hemolytic activity was transferred.

2. Inactivation of the Hemolysins by Conditions Affecting the Integrity of the Carrier Molecule.—

(a) Effect of pH on RNA and albumin hemolysin and on tween hemolysin derived from them:

RNA hemolysin and albumin hemolysin were exposed to a wide range of pH by maintaining solutions of these for 30 minutes at 25°C to the following buffers, each at 0.2 M: acetate buffer at pH 3.0 and 5.0, phosphate buffer at pH 7.0 and carbonate buffers at pH 9.0 and 11.0. Following incubation, 0.5 M of phosphate buffer at pH 7.4 was added to all the tubes to restore the pH to approximately 7.4, and the hemolytic activity of the preparation was then determined.

Fig. 5 *A* shows that the hemolytic activity of the albumin hemolysin was markedly reduced at pH 3.0 but was unaffected at pH 11.0. On the other hand, RNA hemolysin was markedly affected at pH 11.0 but was stable at pH 3.0. Tween hemolysin showed little effect on exposure either to pH 3.0 or 11.0. In the second part of the experiment, RNA hemolysin and albumin hemolysin were each incubated with tween 40, and the reaction mixtures were each passed through columns of DEAE cellulose as described above. In each case the unadsorbed material had hemolytic activity. These preparations of tween hemolysin, derived from RNA hemolysin and albumin hemolysin, respectively, were exposed to the same range of pH levels as those used in the first part of the experiment. As can be seen in Fig. 5 *B*, there was no loss of activity of these derived hemolysins at either extreme of pH.

(b) Effect of alcohol-ether on RNA and albumin hemolysin: It had been previously shown (1, 12) that both the serum and the albumin hemolysin could be destroyed by treatment with certain organic solvents. Also, serum and albumin treated with these solvents failed to induce hemolytic activity from washed streptococci. Because of the implication of a role of lipids in the induction or binding of hemolysin, the effect of alcohol-ether on the RNA hemolysin and albumin hemolysin was studied.

One ml aliquots of RNA hemolysin (5000 hemolytic units/ml) and albumin hemolysin were chilled to 1°C. Five ml of ethanol–diethyl ether 2:1 chilled to −20°C were then added to the corresponding hemolysin preparation. The precipitate obtained was removed by centrifuga-

tion, subjected to vacuum to remove the solvent, and resuspended in saline. Both the resuspended precipitate and the supernate were assayed for hemolytic activity.

It was found that following treatment with alcohol-ether the albumin hemolysin lost 98 per cent of its activity. On the other hand, the activity of RNA hemolysin precipitated by alcohol-ether was unaffected. In fact, treatment of RNA hemolysin with ethanol-ether proved to be an excellent method of concentrating this hemolysin.

(*c*) *Effect of a ribonuclease from Aspergillus niger on RNA hemolysin and on albumin hemolysin:* Because of the interest in the possible role of lipids in the

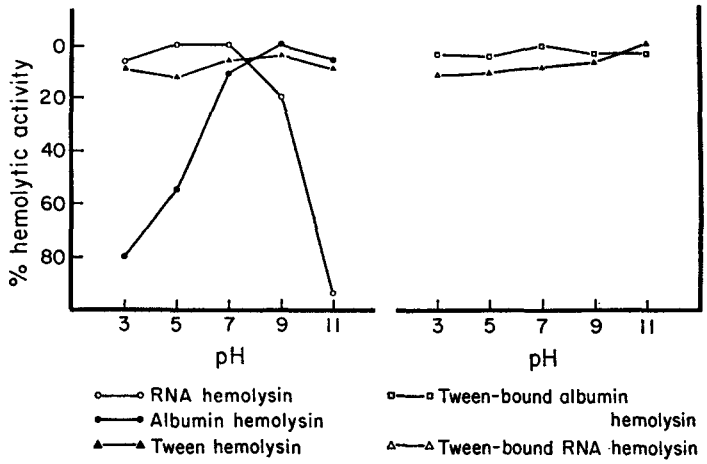

FIG. 5. Effect of pH on the activity of some native and derived hemolysins. Left side, original RNA hemolysin, albumin hemolysin, and tween hemolysin. Right side, hemolysins derived by transfer from RNA hemolysin and albumin hemolysin to tween.

induction of hemolysin by serum albumin, the effect of two commercial lipase preparation (from wheat germ and from *Aspergillus niger*) (Mann Research Labs, Inc.) was studied on albumin and RNA hemolysin. In the case of the *Aspergillus* preparation, the effects were clear cut, although they were the reverse of what had been expected: albumin hemolysin was unaffected, whereas the activity of the RNA hemolysin was markedly reduced. These results suggested that some other constituent than lipase in the crude *Aspergillus* lipase preparation was responsible for this effect. The crude preparation of *Aspergillus* lipase was examined by the use of sephadex G-50 and DEAE cellulose. It was found that the early fractions of the sephadex-excluded material contained most of the RNA hemolysin–destroying factor. This fraction also contained lipase and proteolytic activity. On application of this fraction to DEAE cellulose in 0.01 M phosphate buffer, pH 7.2, the material which inactivated RNA hemolysin was

found in the column volume, whereas the proteolytic and lipolytic materials were adsorbed to the column.

In an approach to the question of the mechanism of inactivation of the RNA hemolysin by this fraction, a 2 ml aliquot of RNA-core hemolysin (2000 hemolytic units/ml) was incubated at 37°C with 0.5 ml of the fraction of the *Aspergillus* preparation thus separated by sephadex G-50 and DEAE cellulose. At 10-minute intervals, aliquots were withdrawn and assayed for hemolytic activity. In a parallel experiment, RNA-core obtained from yeast RNA by treatment with pancreatic RNAase was incubated at 37°C with the same amount of the *Aspergillus* fraction, and at 10-minute intervals aliquots were withdrawn and treated with uranyl acetate–perchloric acid reagent (2). The acid-soluble fractions obtained were examined with a Beckmann DU spectrophotometer at 260 mμ. The relation between depolymerization of RNA-core and inactivation of RNA-core hemolysin is shown in Fig. 6.

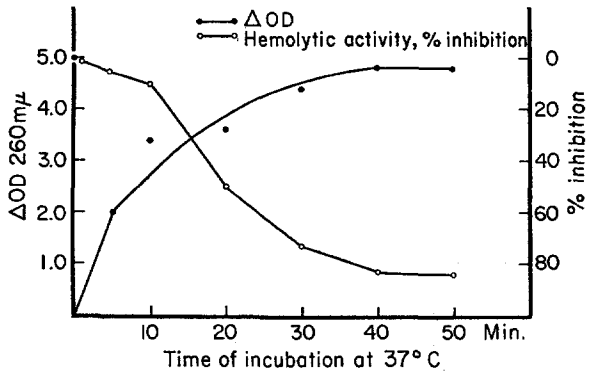

FIG. 6. Effects of a crude preparation of RNAase obtained from *Aspergillus niger*. Inhibition of RNA-core hemolysin and release from RNA-core of acid-soluble 260 mμ-absorbing material in samples obtained at 10-minute intervals of incubation at 37°C.

It can be seen that with the destruction of the hemolytic activity, there is a substantial increase in the release of acid-soluble material from RNA-core by the enzyme. These results indicated that the hemolysin-destroying factor present in the *Aspergillus* preparation is probably due to a ribonuclease having different specificity from that of the pancreatic RNAase. Indeed, such RNA-core destruction, by a ribonuclease derived from taka-diastase, was recently described by Sato and Egami (13).

The selective destruction of the RNA-core hemolysin by an RNA-core-digesting fraction, and the resistance of albumin hemolysin to this enzyme suggested that here, again, alteration of a carrier molecule was responsible for the inactivation of hemolysin. In order to test this hypothesis further, RNA-core hemolysin was incubated with triton X-205 and the resulting derived triton hemolysin was separated from the solution by placing the mixture on DEAE cellulose and collecting the column volume of buffer. The triton hemolysin thus

obtained was found not to be inactivated by the *Aspergillus* RNAase. In control preparations it was found that the presence of triton itself did not affect the digestion of RNA-core by this RNAase. Papain, on the other hand, destroyed both the original RNA-core hemolysin and the RNA-derived triton hemolysin. These results are summarized in Table III.

TABLE III

The Effect of Papain and Aspergillus RNAase on Hemolysins

Hemolysin	Enzyme added to hemolysin				
	None	Papain		RNAase	
	units/ml	*units/ml*	*per cent inhibition*	*units/ml*	*per cent inhibition*
Albumin hemolysin	1000	0	100	1000	0
RNA-core hemolysin	800	0	100	200	75
Triton-bound* albumin hemolysin	100	0	100	100	0
Triton-bound RNA-core hemolysin	100	0	100	100	0

The various hemolysin preparations were incubated for 20 minutes at 37°C with 100 mg/ml of crystalline papain (Worthington Biochemical Corporation, Freehold, New Jersey) or with *Aspergillus* RNAase prepared by use of sephadex G-50 as described above.

* Preparation obtained by incubating triton with albumin hemolysin, applying to DEAE cellulose column in 0.01 M PO_4 buffer, pH 7.3, and collecting non-adsorbed material.

DISCUSSION

The data presented above indicate that the oxygen-stable hemolysins of the hemolytic streptococcus comprise a group of substances with a given hemolytic moiety which can be bound to any of a diverse group of carrier molecules, and that the hemolytic group can be transferred from one carrier to another within the group. These two interrelated conclusions were derived from three kinds of data. First, a number of properties of these hemolysins were found to agree closely with the corresponding properties of the molecular species used in the induction of the respective hemolysins. These include chromatographic behavior, electrophoretic migration, stability in solutions of extreme pH, and susceptibility to certain organic solvents and to ribonuclease. Second, it was possible in a number of cases to transfer hemolytic activity from one inducer to another. Third, in the case of a number of preparations of such derived hemolysins it was possible to show that these were not susceptible to agents or conditions which would inactivate the hemolysin from which they had been derived. It may be pointed out here that if, in studying certain physical properties of the hemolysins, one is actually observing the properties of the postulated carrier, this would explain the failure of some investigators to separate or distinguish hemolysins from corresponding inducers, in a number of cases, even

by the use of a variety of methods such as infrared spectrophotometry, zone electrophoresis, and chromatography on ion exchange cellulose (7–9).

As to the chemical nature of the hemolytic or prosthetic group, it is likely that this is at least largely a peptide, since papain, chymotrypsin, and streptococcal proteinase inactivated all of the oxygen-stable hemolysins tested, whether as original or derived hemolysins. The chemical nature of the postulated carriers presents a more complicated problem because of the diversity of materials to be considered. The compounds which have been found to be involved in the induction or binding of hemolysin fall within three groups: first, lipids, as in α lipoprotein, tweens, lecithovitellin (12), or fatty acids bound to serum albumin; second, the polynucleotides of RNA or RNA-core; and third, substituted oxyethylene polymers, as in the tweens and tritons. The only common basis for binding of a hypothetical peptide by substances in these three classes of compounds which can be suggested at present is hydrogen bonding.

In the case of serum albumin, where there is evidence that bound fatty acids are involved, hydrogen bonding of the hemolytic moiety by these would call for the accessibility of the carboxyl ends, and a necessary condition for this would be that the fatty acids be bound to the albumin at their non-polar ends, by apolar bonds. It is of interest in this connection that Peterson and Chiazze (14) have reported a high degree of heterogeneity in the chromatographic behavior on DEAE cellulose of oleic acid–bound albumin. This would suggest that it is the non-polar end of the fatty acid that is bound to the protein, leaving the carboxylic acid ends free to affect the strength of binding to the charged cellulose.

In the transfer of the hemolytic group from one carrier molecule to another, the distribution of this moiety between the carriers involved would depend on the relative affinities of the carriers for the hemolytic group and the relative concentrations of the carriers. It is not possible at present to assign relative values of average affinity of binding sites of the respective carriers for the hemolytic moiety. However, certain relative differences among the carriers have been suggested by the chromatographic and electrophoretic data of Tables I and II. These data suggest that tween and triton have greater affinity for the hemolytic moiety than do RNA and albumin, and that between the last two RNA has a greater affinity than albumin. However, it may be presumed that any of these have greater affinity for the hemolytic moiety than does the streptococcal component in its original cell-bound form (15, 16), so that these molecular species, if present in suspensions of streptococcal cells, become recognized as inducers.

These data are also of interest because of the several kinds of evidence which they present on the configurational integrity of the active site of a biologically active substance as a condition for its function, since the activity of the hemolysin was destroyed in several instances by agents which could only have acted by hydrolysis or deformation of the inducer, *e.g.* lipid solvents, RNAase, pH 3.0, pH 11.0, and since the activity of derived hemolysins was not affected by these agents, if the transfer was to a carrier which was itself not deformable by the agent.

SUMMARY

The oxygen-stable streptococcal hemolysins, which can be induced by a number of diverse substances, have been studied. Differences among these hemolysins have been found in electrophoresis, chromatography, pH stability, and susceptibility to some organic solvents and to an enzyme, RNAase. These properties have in each case been found to characterize the inducing substances as well.

In a number of instances it has been found possible to incubate one inducer with the hemolysin induced by another of these agents and then, after appropriate fractionation, to find hemolytic activity in the fraction containing the fresh inducer. These observations suggest that the oxygen-stable streptococcal nemolysins are constituted as carrier-hemolysin complexes, the carriers being the set of molecular species effective as inducers, and the prosthetic group being transferred from one carrier to another under appropriate conditions.

After transfer of the hemolytic moiety from a hemolysin molecule which is susceptible to inactivation by a given agent or set of conditions to a carrier which is not itself significantly affected by this agent, the new, derived, hemolysin has been found not to be inactivated by the agent. The hemolysins of this group can thus be inactivated by enzymatic attack on the prosthetic group, or by hydrolysis or deformation of the postulated carrier molecule.

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