AN INTRACELLULAR HEMOLYSIN OF GROUP A STREPTOCOCCI

II. Comparative Properties of Intracellular Hemolysin, Streptolysin S', and Streptolysin O

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In a previous paper, Schwab (1955) described a hemolysin of group A streptococci which is confined to the bacterial cell and released upon breakdown of the cell structure by sonic oscillation. The studies reported here were undertaken to determine if the hemolytic activity of the cellular extracts could be ascribed to streptolysin O or S, or whether it is a distinct hemolytic product of the streptococcus.

The results indicate that the intracellular hemolysin can be readily distinguished from streptolysin O and has certain properties which differentiate it from streptolysin S. Thus, at least several strains of group A streptococci possess an endotoxin unrelated to the exotoxins which have a similar hemolytic capacity.

MATERIALS AND METHODS

The culture media and techniques for sonic disruption of the streptococcal cells and determination of hemolytic activity have been presented in detail by Schwab (1955). Group A streptococcal cells were suspended in phosphate buffer pH 6.5, ionic strength 0.15, and subjected to sonic vibration in a Raytheon 9-kc sonic oscillator for periods ranging up to 3 hr. The initial cell suspension contained approximately 1.0 mg cell nitrogen per ml. The soluble portion of this extract is referred to as sonic extract, and the hemolytic activity as intracellular hemolysin.

A hemolytic unit (H.U.) is defined as that amount of lysin which will hemolyse 50 per cent of the red cells in 0.5 ml of a 4 per cent suspension in 60 min at 37 C. The method of determining per cent hemolysis and number of hemolytic units are described in detail by Schwab (1955).

The group A streptococci were maintained by lyophilization. Strain C203U, type 3, which produces streptolysin O but not streptolysin S, and Blackmore strain, type 11, which produces streptolysin S but not streptolysin O, were kindly provided by Dr. Allen Bernheimer. A reversion of C203U to the hemolytic variety was not noted on our media. Strain D-58, type 3, which is a very good producer of streptolysins O and S, and a type 12 organism, were obtained from the University of Minnesota. D-58 was the strain usually used unless otherwise noted.

Streptolysin O was prepared and activated by the method of Todd (1938), using a culture filtrate reduced by treatment with $\frac{1}{25}$ M neutralized glutathione for 10 min prior to diluting to proper concentration with phosphate buffer. Streptolysin S' was prepared by a procedure of Bernheimer (1949). Broth, 100 ml, plus 1.0 per cent thioglycolate, 1.0 ml, was inoculated with 10 ml of a 17-hr culture of strain D-58. After incubation for 5 hr the cells were collected, washed with buffer, and resuspended in 7.0 ml of the following: ribonucleic acid (G.B.I.), 60 mg; maltose, 67.5 mg; 20 per cent KH_2PO_4 adjusted to pH 7.0 with NaOH, 0.6 ml; 2.0 per cent $MgSO_4 \cdot 7H_2O$, 1.2 ml; de-ionized water, 6.7 ml. This cell suspension was incubated at 37 C for 3 hr, centrifuged, and the supernatant filtered through a Selas O2 filter. The filtrate, containing a very potent hemolysin, was frozen at −70 C.

A cholesterol suspension was prepared by dissolving 5.0 mg cholesterol in 5.0 ml absolute methanol. This solution was poured slowly into 100 ml of boiling water and filtered. A solution of lecithin in ethyl alcohol was provided by Lederle Laboratories. This was diluted to 1.0 mg per ml in distilled water to obtain a fine suspension. Each of these, as well as normal rabbit serum and rabbit-antistreptolysin O serum, was mixed with the sonic extract at room temperature for 10 min prior to addition of buffer and red cell suspension.

The antistreptolysin O was obtained from rabbits immunized with active culture filtrate. It was standardized in terms of Todd combining

units (a Todd combining unit is that amount of serum required to neutralize one 100 per cent unit of streptolysin O).

The rate of hemolysis was determined by a technique described by Bernheimer (1947). The per cent hemolysis was obtained at various time intervals by removing aliquots from a lysin-red cell suspension. The rate of hemolysis was calculated from the slope of the straight portion of the curve obtained by plotting per cent hemolysis against time.

The phosphate buffers were made by dilution of 5 M stock solutions of mono- and dibasic sodium phosphates to an ionic strength of 0.1. They were made isotonic by addition of 3.5 g NaCl per L. Borate-buffered saline solutions were made up as given in Gortner (1949), the boric acid-KCl-NaOH and sodium borate-HCl mixtures being used. The veronal buffer was Michaelis "universal" buffer (Gortner, 1949).

RESULTS

The effects of various reagents known to affect hemolysins. Each of the hemolysin preparations, O, S′, and the sonic extract, was diluted to yield comparable hemolytic activity. One hemolytic unit of streptolysin O was completely inhibited by 2.5 Todd combining units of rabbit-anti-streptolysin O. Streptolysin S′ and the intracellular hemolysin were not affected by this antibody even in concentrations up to 62 combining units per hemolytic unit. Cholesterol in a concentration of 2.5 μg per hemolytic unit completely inhibited streptolysin O, but had no effect on the other hemolysins in concentrations up to 25 μg per hemolytic unit. The addition of $\frac{1}{25}$ M neutralized glutathione 10 min prior to dilution of streptolysin S′ and the intracellular lysin had no significant effect on these lysins.

In contrast to the above reagents, calcium ions, lecithin, and normal rabbit serum had a profound effect on both streptolysin S′ and intracellular lysin. Various concentrations of these reagents were used with one H.U. of each hemolysin, as this yields the most sensitive test for inhibition. The results are summarized in table 1. There appear to be no significant differences in the behavior of these hemolysins; thus, no reagent tested so far can specifically differentiate between streptolysin S′ and the intracellular hemolysin.

The comparative kinetic properties of the hemolytic systems. The effect of temperature was determined by the technique described by Bernheimer (1947). A plot of the log of the rate of hemolysis at various temperatures against the reciprocal of the absolute temperature yielded a straight line with the slope independent of the hemolysin concentration. This is in harmony with the results reported by others for streptolysins S and O as well as other hemolysins (Cinader and Pillemer, 1950; Bernheimer, 1947), and indicates that these systems follow Arrhenius' equation relating rate of reaction to temperature, as many enzyme systems do.

The energy of activation for each system may be calculated from the slope of the above plot, using the relationship $m = (-E/2.3R)$. These calculations are shown in table 2. The values obtained with streptolysin S′ are very close to that reported by Cinader and Pillemer (1950). It should be pointed out that the streptolysin S used in each of these studies was prepared by different techniques, and that used by Cinader and Pillemer was relatively pure. The difference in the values for the hemolysins is of doubtful significance.

The relationship of rate of reaction to concentration of hemolysin was also determined by a method of Bernheimer (1947). The rate of hemolysis for both intracellular lysin and streptolysin S′ was calculated at various concentrations of hemolysin and found to be directly proportional to lysin concentration for both.

Effect of species of red blood cell on hemolytic activity. Lysins S′, O and intracellular lysin were tested for relative hemolytic activity on red cells

TABLE 1

Effect of various reagents on hemolytic activity of streptolysin S′ and intracellular hemolysin

Reagent	Concentrations Used per H.U.	Concentration Giving Complete Inhibition of 1 H.U.	
		Streptolysin S′	Intracellular hemolysin
Lecithin (Lederle) (mg)	0.01–0.2	0.1	0.15
$CaCl_2$ (M)	0.01–0.04	0.02	0.04
Normal rabbit serum (ml)	0.01–0.05	0.05	>0.05
Rabbit-anti streptolysin O (Todd combining units)	25–65	>65	>65
Cholesterol (μg)	2.5–25	>25	>25

TABLE 2

Summary of calculated values of energy of activation for various streptolysins

Hemolysin	Energy of Activation (Average Values)		Temp. Range	Reference
	Calculated	Reported by others		
	cal	*cal*	*C*	
Intracellular lysin	21,800	—	20–35	
Streptolysin S	18,200	14,600	15–30	Bernheimer, 1947
		17,900		Cinader and Pillemer, 1950
Streptolysin O	—	21,400	20–30	Bernheimer, 1947
		43,000	0–10	Bernheimer, 1947

of the following species: rabbit, sheep, horse, man, chicken, dog, mouse, and guinea pig. The number of hemolytic units was determined with each species of red cell. Each experiment was run on one day. All red cells were collected in Alsever's solution and in the first experiment all were the same age (24 hr). The experiment was repeated in part four times, varying the age of the red cells. From this data it was hoped that a clear distinction between the lysins would be obtained on the basis of relative susceptibility of the red cells. The results are summarized in table 3.

As seen in table 4, the ratio of hemolytic activity with sheep and rabbit cells is always greater than 1 with streptolysin S′, and is always 1.0 or less using intracellular lysin. This provides a practical means of distinguishing these two lysins. Furthermore, sheep cells, and to a lesser extent rabbit cells, become increasingly more susceptible to lysis by hemolysin S′ as they age in Alsever's solution under aseptic conditions. This increased susceptibility of red cells is not observed with the hemolysin in the sonic extract. This could reflect a basic difference in the mechanism of action of streptolysin S′ and the intracellular lysin.

TABLE 3

Relative susceptibility of erythrocytes from 8 species to hemolysis by streptolysins

Source of Red Cells	Ratio of H.U. of Lysin for Various Species of Red Cells Compared to Rabbit Red Cells		
	Streptolysin O	Streptolysin S′	Intracellular lysin
Rabbit	1.0	1.0	1.0
Sheep	0.94	1.2	0.93
Man (O)	0.9	0.85	0.68
Horse	1.1	1.4	1.87
Chicken	<0.1	0.47	0.25
Dog	1.03	0.5	0.5
Mouse	<0.2	1.9	2.1
Guinea pig	1.2	0.94	0.96

TABLE 4

Relative hemolytic activity of streptolysin S′ and intracellular lysin on sheep and rabbit erythrocytes

Exp. No.	Lysin	Ratio H.U. Sheep/ Rabbit	Age of Red Cells	
			Rabbit	Sheep
			hr	*days*
1	Streptolysin S′	2.8	2	56
	Intracellular	0.97		
2	Streptolysin S′	1.6	24	9
	Intracellular	1.0		
3	Streptolysin S′	1.5	48	2
	Intracellular	0.78		
4	Streptolysin S′	1.2	24	1
	Intracellular	0.89		

Mouse and chicken erythrocytes are very resistant to lysis by streptolysin O in contrast to the other hemolysins tested. Wallace and Howard (1953) compared the resistance of red cells of various species to streptolysin O and, with the exception of human and guinea pig cells, the results in table 3 agree basically with theirs.

Production of intracellular hemolysin by variants not producing streptolysin S and streptolysin O. Strain C203U, which did not produce streptolysin S, Strain Blackmore, which did not produce streptolysin O, and the D-58 strain usually used in

these studies, which is a good producer of both streptolysin S′ and streptolysin O, were grown under identical conditions. The washed cells from 200 ml of broth culture were suspended in phosphate buffer to a concentration of 0.94 mg cell nitrogen per ml. Each cell suspension, in a volume of 10 ml, was subjected to sonic vibration for 30 min, and the hemolytic activity determined—yielding the following results in hemolytic units per ml: Blackmore, 23; C203U, 11; D-58, 38.

The streptolysin S′ production was also calculated for comparable cultures of strains D-58 and C203U by the technique described under Methods. The relative amount of hemolysin in the sonic extract after 30 min sonic vibration, and streptolysin S′ produced by these two strains, are shown in table 5. The fact that there is a significant amount of intracellular lysin produced by Blackmore and C203U strains is evidence for the distinction of these lysins from streptolysins O and S′. On the other hand, the fact that intracellular hemolysin varies quantitatively from strain to strain, although the amount of cell protoplasm is about the same, is evidence that this is a specific hemolysin and not an artifact resulting from the treatment.

The effect of ribonucleic acid on the production of intracellular hemolysin. If the intracellular lysin is related to streptolysin S′, one would expect the production of this lysin to be affected by exposure of streptococcal cells to ribonucleic acid. Strain D-58 cells were collected and suspended in the RNA, maltose, and salt solution at 37 C for 3 hr, as described above for production of streptolysin S′. These cells were then washed and subjected to sonic vibration for 30 min. Controls consisted of an equivalent number of cells in phosphate buffer pH 6.5. The sonic extract of the RNA-treated cells contained 38 hemolytic units per ml compared to 25 units in the control extract. The hemolytic activity of the RNA extract had properties of the intracellular lysin rather than S′ in regard to effect of Na and K ions and relative activity on sheep and rabbit erythrocytes (see below). This experiment is difficult to interpret. The difference is not as great as one would expect if the hemolytic activity of the sonic extract is due to streptolysin S′, in view of the vast effect RNA has on the production of this lysin. A difference in stability afforded by the two types of suspending media may account for the results observed.

TABLE 5

Relative production of streptolysin S′ and intracellular hemolysin by two strains of group A streptococci

Strain	H.U. per mg Cell N		Ratio S′/Intracellular Lysin
	Intracellular lysin	Streptolysin S′	
C203U	11	3.5	0.33
D-58	38	520	13.5
Ratio D-58/C203U	3.5	150	

Effect of pH and buffer ions. Streptolysin S′ and the intracellular lysin were compared as to the effects of pH and various buffers on their activity. Each lysin was diluted to give approximately 40 per cent hemolysis with a pH 7.0 phosphate buffer. The per cent hemolysis was then determined at various pH values using the following isotonic buffers: veronal, phosphate, borate-HCl, and borate-KCl (see Methods). The 4 per cent rabbit red cell suspension was made in 0.9 per cent NaCl. The results are shown in figures 1 and 2. The pH values given were determined with an aliquot of each tube after addition of red cells, using a Beckman glass electrode pH meter.

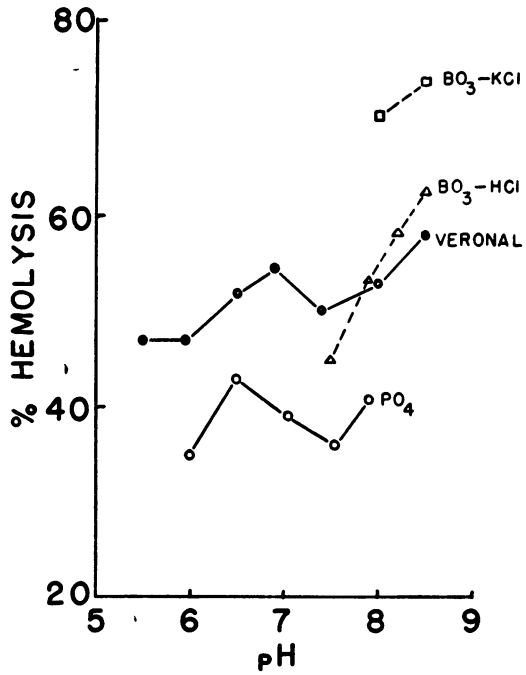

Figure 1. The effect of pH and various buffers on hemolytic activity of the intracellular hemolysin.

Each point represents an average of at least 3 experiments and each determination was run in duplicate.

This method of determining the influence of pH on activity was used because quantities yielding approximately 50 per cent activity are most sensitive to small changes in environmental factors. The curves shown in figures 1 and 2 are highly reproducible. The values of per cent hemolysis are a fair reflection of hemolytic activity; since the relationship of activity to concentration for these hemolysins is nearly linear between 10 to 60 per cent hemolysis.

Although there is no sharp pH optimum, the activity of these hemolysins is definitely greater at about pH 6.5 and in the range of 8.0 to 8.5, especially with borate buffers. These are essentially the same results Cinader and Pillemer (1950) reported for streptolysin S.

The lack of a sharp pH optimum may be due to the complex nature of the substrate one must employ. With each lysin the buffer ions used have a marked effect, as is true with many enzyme systems. The greatest difference between the hemolysins is the activity in the two borate buffers relative to phosphate. Streptolysin S′ gives the least activity in a borate-KCl buffer and considerably greater activity in a borate-HCl buffer. The opposite results were observed with intracellular hemolysin.

This difference in activity in borate buffers is due to the relative effect of K and Na ions on the activity of the two lysins, as is shown in figure 3. In this experiment each lysin was diluted in phosphate buffer to give approximately 50 per cent hemolysis at pH 7.0. A volume of 2.0 M KCl or NaCl was added to each tube prior to addition of red blood cells, to give final values of from 0.02 to 0.12 M of the added cation. These cations yield the opposite effect on the activity of the two hemolytic preparations tested. The Na and K are not antagonistic, since addition of equivalent amounts of the two cations yielded an activity even lower than that with either alone. This provides a second practical method for distinguishing between the hemolysins prepared by these two techniques. Streptolysin S′ always gives less activity in the presence of added K ions than with a comparable concentration of Na ions. These ions have an inverse effect on the intracellular hemolysin. The maximum distinction is obtained with a final concentration of added cation of 0.04 M.

Both hemolysin preparations are in essentially the same suspending medium since the streptolysin S′ is in a final dilution of 1:5,000 in phosphate buffer when the red cells are added. The

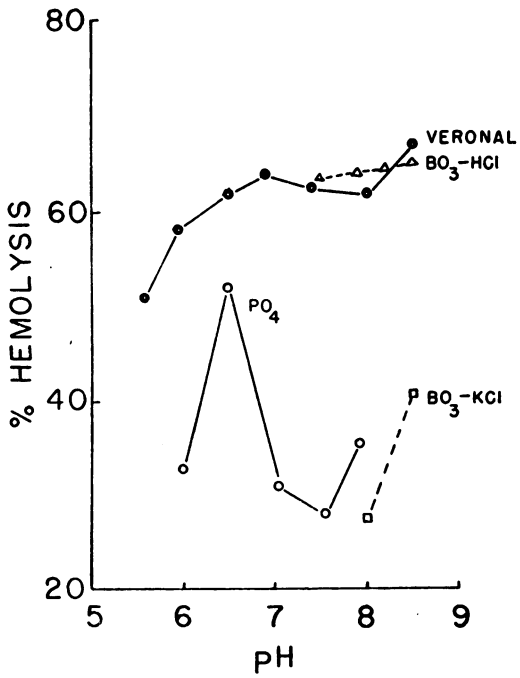

Figure 2. The effect of pH and various buffers on the hemolytic activity of streptolysin S′.

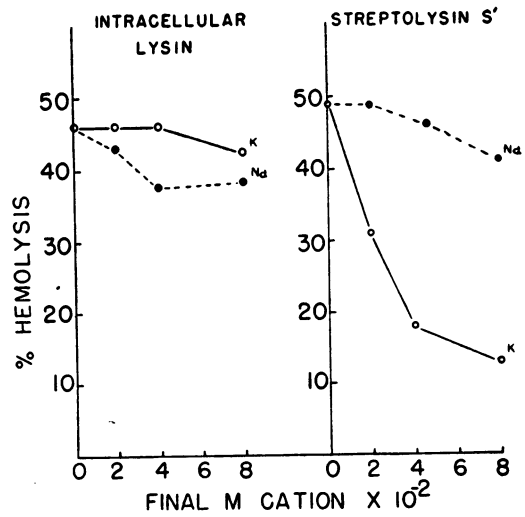

Figure 3. The relative effect of Na and K ions on the hemolytic activity of intracellular lysin and streptolysin S′.

TABLE 6

Relative stability at 4 C of intracellular lysin and streptolysin S′ in phosphate buffer pH 6.5

Time at 4 C	Intracellular Hemolysin				Streptolysin S′			
	Undiluted		1:20		Undiluted		1:100	
	H.U./ml	Per cent left	H.U./ml (undiluted)	Per cent left	H.U./ml	Per cent left	H.U./ml (undiluted)	Per cent left
days								
0	158	100	158	100	1,130	100	1130	100
1	85	54	110	70	1,000	88	770	68
6	35	22	47.5	30	760	67	260	23
7	18.5	12	27	17	700	62	156	14

intracellular hemolysin is likewise diluted in phosphate buffer.

Stability of hemolysins. The hemolytic activity of streptolysin S′ and of the sonic extract is completely destroyed at 56 C for 30 min. At 37 C the activity is reduced to ½ in 15 min. Both have about the same stability at 4 C, when adjusted to comparable concentrations and suspended in phosphate pH 6.5 buffer. When diluted to contain about 10 H.U. per ml, and held at 4 C, the activity is about 70 per cent of the original after 24 hr and about 25 per cent after 72 hr.

Table 6 shows typical observations extending over 7 days at 4 C. It is interesting to observe that the sonic extract is more stable when diluted 1:20 than when undiluted. This could be explained on the basis of an inhibitor occurring in relatively low concentrations. Besides the factor of concentration, the stability of the cellular hemolysin depends upon the period of treatment in the sonic oscillator. Extracts treated for shorter periods are considerably less stable than those obtained after more prolonged sonic treatment. This is not accounted for on the basis of lysin concentration.

Effect of sonic oscillation on streptolysin S′ and streptolysin O. The influence of sonic energy on streptolysins O and S′ was determined in the following experiment: 10 ml of an unreduced 15-hr broth culture filtrate at pH 6.4 was subjected to sonic vibration for 2 hr. Aliquots were removed at 0, 1, and 2 hr, reduced with ½₅ M glutathione, and tested for hemolytic activity. The results are shown in table 7. The initial concentration of 154 H.U. per ml was reduced 57 per cent after 2 hr. The streptolysin S′ was diluted 1:10 in pH 6.5 phosphate buffer to contain 93 H.U. per ml. After 4 hr of sonic vibration this activity was reduced 22 per cent.

TABLE 7

The effect of sonic oscillation on streptolysin S′ and streptolysin O

Time of Sonic Oscillation	Streptolysin O (Unreduced Culture Filtrate)	Streptolysin S′ (1:10 in Phosphate Buffer pH 6.5)
hr	*H.U./ml*	*H.U./ml*
0	154	93
1	91	90
2	66	80
3	—	76
4	—	73

These results contrast with those observed for intracellular lysin (Schwab, 1955). The hemolytic activity of the sonic extract in phosphate buffer increased with extended sonic vibration. This cannot be presented as a distinguishing feature, since the suspending media in each case are not comparable. The observations may be a reflection of an association of the lysin with another factor not present in each of the preparations.

DISCUSSION

The studies reported here comparing the properties of the intracellular hemolysin, streptolysin S′, and streptolysin O indicate quite clearly that the hemolytic activity in the sonic extracts of group A streptococci is not due to streptolysin O. This is obvious from the failure of cholesterol, specific antibody, and reducing agents to affect the activity of the intracellular hemolysin in concentrations which have a profound effect on streptolysin O.

Establishing the distinction or identity of the intracellular lysin and streptolysin S′ is more difficult, since there is no specific agent affecting the activity of either. Lecithin, Ca ions, and normal rabbit serum give comparable inhibition

of the two hemolytic preparations; stability and kinetic properties, such as the critical thermal increment, are not significantly different. They are also similar in the demonstration of a lag phase, before observable hemolysis begins, which is dependent on temperature and the lysin concentration.

On the other hand, the relative activity of the sonic extract and lysin S′ on sheep and rabbit red cells quite clearly distinguishes the activity of these two preparations. The increasing susceptibility of sheep cells to streptolysin S′ as they age also distinguishes this lysin from the intracellular hemolysin, and possibly reflects a distinct substrate upon which they act.

These hemolysins can also be differentiated by the uniformly inverse effect of the cations Na and K on their activity. This test, and the comparative activity with sheep and rabbit cells, provide practical means of differentiation. In addition, the production of significant amounts of intracellular hemolysin in a strain which produces very little streptolysin S′ is evidence for their distinct nature. The final proof of the relationship of the hemolysins produced by group A streptococci must await further purification and establishment of their physico-chemical properties.

Streptolysin S′ prepared by the technique described by Bernheimer (1949) was the only streptolysin S preparation used in these studies. There is no evidence to indicate that the hemolysin prepared in this manner differs in its properties from that produced by other techniques involving serum or culture filtrates from broth containing nucleic acid (Herbert and Todd, 1944; Bernheimer and Rodbart, 1948).

The properties described here do not prove the enzyme nature of the intracellular hemolysin but the following characteristics are those usually associated with enzyme action and the activity of other hemolysins: effect of temperature on activity; effect of pH and certain ions; heat lability; relationship of lysin concentration to rate of reaction; and finally, although not reported here, the influence of substrate concentration is typical of enzymes.

The relative influence of Na and K ions on hemolytic activity is probably related to the observations of Ponder (1946) on the loss of K and gain of Na during the prolytic stage of hemolysis induced by several lysins. This, in turn, may reflect an influence of the lysin on cell metabolism, which determines cation exchange, rather than a reaction of the lysin with the cell membrane. In contrast to the findings reported here, Bernheimer (1950) reported that K ions increased the heat stability of streptolysin S′; he could detect no effect of K ions on the hemolytic activity of the lysin. From the data presented, the concentration of K ion present during hemolysis is difficult to calculate—but it is apparently less than 0.01 M, which is below the concentration used in this paper. Human red cells were employed instead of rabbit, and there may be a different level of sensitivity to cation concentration between cells from various species.

A further characteristic of streptolysin S′ listed by Bernheimer and Rodbart (1948) is that this lysin is irreversibly inactivated by heat and dilute acid. Our studies confirm this; streptolysin S′ suspensions of 10 H.U. per ml were completely destroyed by a 2 min exposure to a pH of 2.4 or below. As reported in the previous paper of this series (Schwab, 1955), the hemolytic activity of the sonic extract is actually increased by such an alteration in pH for a period of 1 to 5 min. This may represent a reactivation of some lysin molecules. It is not possible at present to determine if this is a distinguishing feature of these hemolysins, since the conditions of the suspending medium are not entirely comparable. Some other factor in the sonic extract, not present in the streptolysin S′ preparation, may be playing a role in this reaction—as was suggested above for the effect of sonic energy on these lysins. Resolution of this point must await further purification of the intracellular hemolysin.

Herbert and Todd (1944) state that the production of streptolysin S in culture or by resting cells is an extraction of the preformed hemolysin from the intact cell by lipoproteins of serum or egg yolk. On the basis of several studies Bernheimer (1949) concluded that, rather than extracting the lysin, the ribonucleic acid and serum induce its formation by the cell. The studies presented in this paper indicate that, while there is a significant amount of hemolysin present in streptococcal cells grown without contact with added concentrations of ribonucleic acid or serum, this intracellular lysin is probably distinct from streptolysin S. These results are in agreement with Bernheimer's conclusions.

SUMMARY

Several properties of an intracellular hemolysin of group A streptococci have been determined. This lysin can be readily distinguished from streptolysin O by virtue of the failure of various reagents which are known inhibitors of streptolysin O to influence its activity.

The relationship of the intracellular lysin to streptolysin S is more difficult to establish, but the two preparations can be differentiated on the basis of the effect of Na and K ions and their relative activity on sheep and rabbit erythrocytes.

The influence of sonic energy and altered pH on hemolytic activity is discussed with regard to the relationship of the streptococcal hemolysins.

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