AN INTRACELLULAR HEMOLYSIN OF GROUP A STREPTOCOCCI

I. Influence of Sonic Energy and pH on Hemolytic Potency

JOHN H. SCHWAB

Department of Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina

Received for publication June 23, 1955

Two hemolytic products of group A streptococci have been studied by many investigators. Todd (1938) was the first to establish the distinct nature of these hemolysins, which are referred to as streptolysin O (oxygen labile) and streptolysin S (requiring serum for production).

In two reviews Bernheimer (1948, 1954) has summarized the distinguishing properties of these hemolysins and the various techniques for their production. Therefore, only a few facts pertinent to these studies will be mentioned here. If resting group A streptococcal cells are suspended in serum (Weld, 1934), or in a solution containing ribonucleic acid, maltose, and certain salts (Bernheimer, 1949), a highly potent streptolysin S preparation is obtained in the suspending medium after a short incubation period. Bernheimer reported that, rather than being an extraction of a preformed product, the production of the hemolysin is induced by the suspending medium. He further noted that only a very small degree of hemolytic activity was obtained in the extracts of sonic-disrupted streptococcal cells. Streptolysin S can also be obtained in the culture filtrate by adding 20 per cent serum or 1.0 per cent ribonucleic acid to the culture medium. These various preparations of streptolysin S probably yield identical products (Herbert and Todd, 1944; Bernheimer, 1949) but, in lieu of conclusive proof, that prepared with nucleic acid is referred to as S′ (Bernheimer, 1949).

Streptolysin O activity is observed in the culture filtrate, and no hemolysin with properties of this material is detectable in sonic-disrupted cells, as is reported by Schwab (1955). Both of these streptolysins, therefore, are distinctly extracellular products.

The studies recorded here are concerned with a previously unreported hemolysin of group A streptococci which is an exclusively intracellular product that apparently is released only upon disruption of the cell. The comparative properties of this intracellular hemolysin and the previously defined extracellular hemolysins, streptolysin O and streptolysin S′, will be the subject of the second paper in this series (Schwab, 1955).

MATERIALS AND METHODS

Culture media and cultivation of organisms. Group A streptococci were grown in beef heart infusion medium made as described by Todd and Hewitt (1932). Either fresh beef heart or beef heart for infusion (Difco) was used. No difference in the production of hemolysin was noted when calculations were based on amount of cell material obtained. Twenty g of proteose peptone (Difco) were added per L of infusion and the pH adjusted to 7.9. Just prior to inoculation glucose-phosphate buffer was added, giving the following concentration per L of broth: dextrose, 3 g; $NaHCO_3$, 2 g; NaCl, 2 g; $Na_2HPO_4 \cdot 12H_2O$, 2 g.

A lyophilized suspension of organisms was introduced into 10 ml of this medium. After incubation for 24 hr. 0.2 ml was transferred to a 10-ml tube of medium and incubated for 8 hr. A flask of medium was then inoculated with 0.4 ml per 100 ml of this vigorously growing culture, and incubated for 15 hr. All cultures were grown at 35 C.

Organisms. The organisms were maintained by lyophilization. Strain C203U, type 3, which produces streptolysin O but no streptolysin S, and Blackmore strain, type 11, which produces streptolysin S but not streptolysin O, were kindly provided by Dr. Allen Bernheimer. Strain D-58, type 3, which is an extremely 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.

Buffer solutions. 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.

Extraction of cells. The cells were removed by

centrifugation from the 15-hr culture and washed twice with two volumes of cold saline. They were then suspended to about a 10-fold concentration in phosphate buffer pH 6.5. The initial cell suspension contained approximately 1.0 mg cell nitrogen per ml. This suspension was subjected to sonic disintegration in a Raytheon 9-kc sonic oscillator for periods of from 5 min to 8 hr. It was then centrifuged in a Spinco no. 40 rotor at 20,000 rpm for 30 min; the resulting supernatant was filtered through a Selas O2 bacterial filter. The filtrate was stored in sealed ampules at −70 C. It is essential that the temperature be maintained at 4 C or below throughout the extraction procedure, and that as little time as possible elapse between collecting the cells and freezing the extract.

The amount of cell material obtained from each culture was calculated by the turbidity of an aliquot of the washed cell suspension. This was determined on a Klett-Summerson colorimeter using a no. 54 filter. The final results were obtained in terms of total cell nitrogen by reference to a standard curve relating turbidity and total nitrogen. The nitrogen was determined by micro-Kjeldahl technique.

The degree of cell destruction during the sonic disruption was followed by counting intact cells in a Petroff-Hausser bacteria counter. The rate of destruction was logarithmic up to about 60 min of sonic treatment. After 90 min 0.3 per cent intact cells remained when the number of organisms initially was on the order of 44×10^9 per ml. A plot of the log of per cent cell destruction versus time was correlated with the log of the per cent decrease in turbidity; this provided a convenient method for following the course of sonic treatment.

Determination of hemolytic activity. Rabbit red cells were collected fresh each day in 0.01 M sodium oxalate, washed three times with saline, and resuspended to 4 per cent concentration in sodium phosphate buffer pH 7.0. Six rabbits were bled alternately, 9.0 ml being taken each time. The erythrocytes of one animal were considerably more resistant to lysis by the cellular hemolysin than were those from the other rabbits.

The tests were carried out in 12-ml centrifuge tubes. The volume was made up to 5.0 ml with the above phosphate buffer, and 0.5 ml of the red cell suspension was added and mixed immediately. The tubes were incubated at 37 C for 60 min, the unlysed cells centrifuged down, and the amount of hemoglobin in the supernatant determined in a Klett-Summerson colorimeter with a no. 54 filter. The per cent hemolysis was determined by comparison with a similar concentration of red cells lysed by the addition of sufficient saponin to give 100 per cent hemolysis.

A hemolytic unit (H.U.) is defined as that volume of sample which will lyse 50 per cent of the red cells in 0.5 ml of a 4 per cent suspension in 60 min at 37 C.

The number of hemolytic units in a sample was calculated using a technique similar to that developed for hemolysis by complement and sensitized red cells (Kabat and Mayer, 1948). At least three volumes (x) of each sample were run, giving between 15 and 70 per cent hemolysis (y). A plot of the log of x vs the log of $y/1 - y$ gave a straight line with a slope for the cellular hemolysin of about 0.46, with a range of 0.43 to 0.50. The volume yielding 50 per cent hemolysis was determined by the intercept of the correct abscissa. Each calculation of the hemolytic unit, therefore, represents triplicate determinations.

RESULTS

The sonic extracts of group A streptococci were found to possess a significant degree of hemolytic activity. This is referred to here as intracellular hemolysin. Several factors have been noted which have an effect on the amount of lysin obtained in these extracts. These include: (1) nutritional factors and physical properties of the culture medium, which will be the subject of a subsequent communication; (2) the strain of group A organisms used, which is discussed below; (3) the heat lability, which has been noted under Methods. The time of treatment of cells in the sonic oscillator has the most profound influence, as is shown in figure 1. In this experiment aliquots were removed at various intervals during sonic vibration and centrifuged at 4 C at 8,000 rpm for 10 min in a Servall angle centrifuge. The sediment was resuspended in an equal volume of phosphate buffer pH 6.5, and the hemolytic activity of the supernatant and sediment determined. The activity of the sedimentable material was less than 4 H.U. per ml initially, and was completely destroyed after 40 min sonic vibration. This lysin is probably streptolysin S, on the basis of criteria described in the accompanying paper (Schwab, 1955).

The activity in the supernatant increased rapidly. After sonic vibration for 60 min less

than one per cent of the cells remained intact, and yet the activity of the extract continued to increase. This experiment was repeated several times, finally extending the period of sonic vibration up to 8 hr with the results shown in table 1.

There are several possible explanations of why the hemolytic activity continues to increase after most of the cells have been disrupted: (a) elution of strongly adsorbed lysin molecules which are inactive when associated with the cell debris, or dissociation of a soluble lysin-inhibitor complex; (b) breakdown of mitochondria or comparable structures; (c) destruction of an inhibitor.

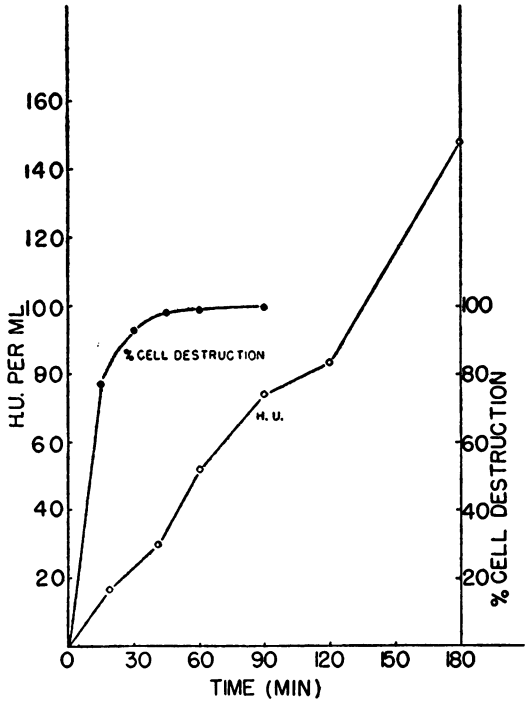

Figure 1. The per cent cell destruction and hemolytic units of intracellular lysin released, during treatment of streptococcal cells with sonic oscillation.

TABLE 1

Effect of extended sonic oscillation on hemolysin released from group A streptococci

Time of Sonic Treatment	H.U. per ml
0 min	4.0
1 hr	43.5
2	95
3	143
4	154
5	182
6	191
8	235

TABLE 2

Effect of further sonic oscillation on release of hemolysin from sediment and supernatant, separated after 30 min sonic treatment of cell suspension

Time of Sonic Vibration	H.U. per ml of Extract	
	Sediment	Supernatant
min		
0*	4	0
30	2	20
90	28	41

* Whole cell suspension.

The following experiment was designed to help clarify this observation. A cell suspension was prepared in the usual manner and subjected to 30 min of sonic vibration. It was then centrifuged at 20,000 rpm for 20 min in the Spinco 40 rotor, and the sediment resuspended in an equal volume of phosphate buffer pH 6.5. The sediment and supernatant were then separately subjected to a further 60-min sonic vibration. The hemolytic activity of each of these samples at 0, 30, and 90 min was determined, and the results are seen in table 2. After 30 min nearly all of the activity is in the supernatant. An increase in soluble intracellular hemolysin is obtained after an additional 60 min from both the supernatant and the sediment. The best interpretation seems to be that the increase in activity is due to the elution of lysin from the cell debris and further dissociation of soluble complexes yielding more active material.

Controls consisting of sterile broth gave no evidence of hemolytic activity after subjection to sonic vibration for 2 hr. Evidence that this hemolysin is not a non-specific product resulting from the effect of sonic energy on cell protoplasm is provided in experiments comparing the amount of lysin extracted from various strains of group A streptococci. In these experiments three strains of group A organisms were grown and harvested under identical conditions. The suspensions of each organism in phosphate buffer were adjusted to contain the same concentrations of cell nitrogen, and were then subjected to sonic vibration for 60 min. The results are shown in table 3 under the heading of "no acid." There was

TABLE 3

Relative production of intracellular hemolysin by 3 strains of group A streptococci, and effect of acid treatment on the hemolytic potency

Strain	H.U. per mg Cell N*	
	No acid	Acid-activated
D-58	88	115
C203U	8	14
Blackmore	48	28

* Obtained after 60 min sonic vibration.

TABLE 4

Relative amounts of streptolysins obtained from 100 ml of 15-hr broth culture

Lysin	No. of Hemolytic Units
Streptolysin O	13,300
Streptolysin S′	7,900
Intracellular hemolysin	2,950
Acid-activated intracellular hemolysin	3,250

a quantitative variation of intracellular hemolysin among strains.

The experimental conditions presently utilized for the release of maximum hemolytic activity involve using an 8-fold concentration of washed streptococcal cells (approximately 0.75 mg cell N per ml) in a volume of 10 ml in the cup of the sonic oscillator. The relative amount of hemolysins obtained from 100 ml of a 15-hr culture of the D-58 strain of streptococci is shown in table 4. The activity of the intracellular hemolysin is about ⅓ that of streptolysin S′ produced by the method described above. These values do not represent the total amount of either streptolysin S′ or intracellular lysin produced by the cells.

Momentary exposure of the extract to acid, followed by neutralization, results in an increase in hemolytic activity.

Figure 2 illustrates the effect of decreasing pH on the degree of acid activation. Aliquots of the sonic extract were exposed to increasing amounts of HCl for 2 min, then readjusted to the original pH of 6.5 by the addition of an equivalent amount of NaOH. The number of hemolytic units of this acid-activated extract was then determined as described under Methods. There is a loss of activity as the pH is lowered to about 4.4. At an exposure to a pH of 4.2 the hemolytic activity increases, and continues to do so until it reaches a maximum activation at about pH 2.5. This level of activation is maintained to a pH of about 1.0; greater acidity than this results in destruction of the hemolytic capacity of the sonic extract.

Maximum activation occurs within 1 min at optimum acid exposure and, as shown in table 5, the degree of activation decreases as the exposure to a lowered pH is extended beyond 5 min.

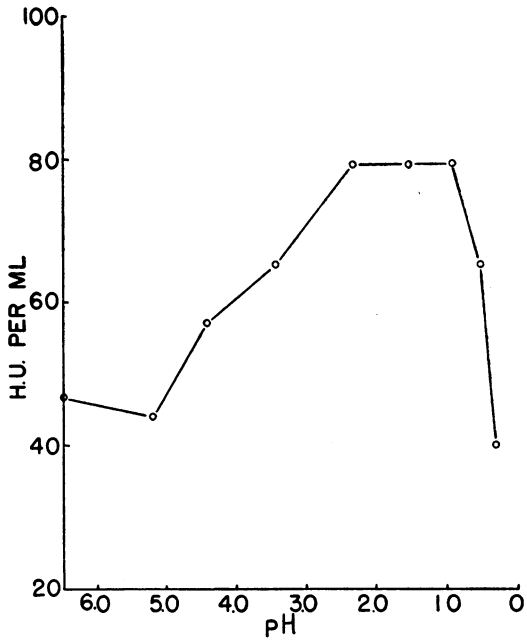

Figure 2. The influence of the exposure of sonic extract to various degrees of acidity for 2 min, followed by neutralization and determination of the hemolytic activity of the extract.

TABLE 5

Effect of time of exposure to pH 2.0 on hemolytic activity of sonic extract

Time at pH 2.0	H.U. per ml
min	
0	44
1	183
5	186
10	162
20	149
30	127
60	47

TABLE 6

*Effect of acid activation on hemolytic activity of sonic extract as hemolysin is inactivated at 4 C or at 40 C**

Time at 4 C	H.U. per ml of Extract		Time at 40 C	H.U. per ml of Extract	
	No acidification	Acid-activated†		No acidification	Acid-activated†
days			*min*		
0	92	120.5	0	75	144
1	60	89.5	15	27	70
5	20.5	50	30	13	48
14	10	47.5	60	0	29
23	5	38	90	0	19

* These experiments were not done with the same lot of sonic extract.

† Just prior to testing pH is lowered to 2.0 with 0.1 N HCl for 2 min, then neutralized with equivalent NaOH and hemolytic potency determined.

As the potency of the intracellular hemolysin deteriorates upon standing, the increase in activity after exposure to acid becomes relatively greater. This increase may be up to 7-fold greater than the activity of the non-acidified sonic extract. Table 6 compares the hemolytic activity, with and without acid activation, as the sonic extract is held at 4 C and at 40 C. The same relationship is observed as the intracellular hemolysin is destroyed at higher temperatures over a shorter period of time. This experiment also illustrates the relative stability of the precursor of acid activiation as compared to the very labile hemolytic activity of the non-acidified sonic extract.

This increased activity is not due to a specific action of the acid, since increasing the pH to about 11 by addition of NaOH will also enhance hemolytic potency in a similar fashion.

The effect of a temporary alteration of pH on the sonic extract may represent a reversal of an inhibitor-lysin complex since the degree of acid activation is always parallel to the non-acidified hemolytic activity, as is shown in table 3. The degree of acid activation of the sonic extract varies from strain to strain and, in the case of one strain (Blackmore), there is destruction of hemolysin by exposure to a pH of 2.0 for 2 min. This would indicate that there is some specific condition required before the enhancing effect of a pH change can be observed.

DISCUSSION

The results presented here indicate that a significant amount of hemolytic activity can be obtained from the streptococcal cell. The method of preparing this intracellular hemolysin suggests that it is distinct from the hemolysins observed in the suspending medium. Schwab (1955) presents some evidence to support this idea.

The significance of the increased hemolytic activity observed after a temporary alteration of the pH of the sonic extract remains obscure. This influence of pH may be interpreted as a dissociation of a lysin-inhibitor complex. Such an observation is not without precedent: Landsteiner (1909) has shown that deterioration of a staphylococcal toxin could be reversed by heating or acid treatment, which dissociated an inhibitor-toxin complex. Sonic vibration might also be functioning in this manner, as suggested above. These observations may also be interpreted as being a non-specific, mild hydrolysis of some protein constituent of the cell. This seems less likely, since the degree of acid activation obtained varies with the strain of organism used—although the total cell protoplasm has approximately the same concentration.

The fact that the activity of the sonic extract continues to increase with prolonged sonic oscillation may indicate that the lysin is a structural component of the bacterial cells. The material released from the cellular debris can be made more active by further dissociation by sonic energy. Whether the energy of sonic waves is peculiar to the release of this hemolysin has not yet been determined.

There is no evidence that any product having the properties of the intracellular lysin is released prior to cellular breakdown. Thus, it fits one criterion of an endotoxin. Streptolysins O and S′, on the other hand, are produced with no appreciable cell destruction and are not present in detectable amounts in sonic extracts.

SUMMARY

Extracts of sonic-disrupted group A streptococcal cells contain a significant amount of intracellular hemolysin or endotoxin which is probably distinct from streptolysins O and S. The evidence indicates that this lysin is a product of the streptococci rather than an artifact and is probably a part of the cell structure. Extended

treatment in the sonic oscillator increases the amount of hemolysin released from the cell.

The hemolytic activity of the sonic extract can be increased by a temporary alteration of pH. This may be due to a dissociation of a lysin-inhibitor complex.

REFERENCES

BERNHEIMER, A. W. 1948 Properties of certain rapidly acting bacterial toxins as illustrated by streptolysins O and S. Bacteriol. Revs., **12**, 195–202.

BERNHEIMER, A. W. 1949 Formation of a bacterial toxin (streptolysin S) by resting cells. J. Exptl. Med., **90**, 373–392.

BERNHEIMER, A. W. 1954 *Streptococcal infections*. Edited by M. McCarty. Columbia Univ. Press, New York, N. Y.

HERBERT, O. AND TODD, E. W. 1944 The oxygen-stable hemolysin of group A hemolytic streptococci (streptolysin S). Brit. J. Exptl. Pathol., **25**, 242–254.

KABAT, E. A. AND MAYER, M. M. 1948 *Experimental immunochemistry*. Charles C Thomas, Springfield, Ill.

LANDSTEINER, K. 1909 Ueber das Verhalten des Staphylolysins beim Erwärmen. Z. Immunitatsforsch., **1**, 439.

SCHWAB, J. H. 1955. An intracellular hemolysin of group A streptococci. II. Comparative properties of intracellular hemolysin streptolysin S′, and streptolysin O. J. Bacteriol., **71**, 100–107.

TODD, E. W. AND HEWITT, L. F. 1932 A new culture medium for the production of antigenic streptococcal hemolysin. J. Pathol. Bacteriol., **35**, 973–974.

TODD, E. W. 1938 The differentiation of two distinct serological varieties of streptolysin, streptolysin O and streptolysin S. J. Pathol. Bacteriol., **47**, 423–445.

WELD, J. T. 1934 Toxic properties of serum extracts of hemolytic streptococci. J. Exptl. Med., **59**, 83–95.