COMPARATIVE KINETICS OF HEMOLYSIS INDUCED BY BACTERIAL AND OTHER HEMOLYSINS*

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Hemolytic substances are known to be present in the culture filtrates of a wide variety of bacteria. In some instances the hemolysins are closely associated, if not identical with the lethal exotoxins of the bacteria; in others the hemolytic and lethal activities are attributable to separate components of the culture filtrate. Notwithstanding the highly specialized character of the erythrocyte, this cell possesses for biological investigations many advantages the more important of which have been enumerated in a recent publication of Keilin and Hartree (1). Since there is evidence that the action of hemolysins is not limited to the erythrocyte but extends to other kinds of cells as well, it seems probable that information concerning the factors underlying hemolysis will contribute to knowledge of the mode of action of toxins in general. The kinetics of lysis induced by a number of hemolysins, most of them of bacterial origin, is described in this report.

In investigations of the kinetics of hemolysis it has been common practice to measure the elapsed time, t, between the addition of a lytic agent to a cell suspension and an optical change corresponding to lysis of all or of a definite fraction of the cells initially present. The reciprocal of t is then employed as a measure of rate of hemolysis. The practice is objectionable because it prevents observation of changes occurring prior to and during lysis, thereby completely concealing important differences in the kinetics of different hemolytic systems. It is desirable to follow the whole course of hemolysis. As had been done in an earlier study (2), concerned with the kinetics of lysis induced by the hemolysin of *Clostridium septicum*, the course of hemolysis was followed by measuring the rate of appearance of extracellular hemoglobin. The information concerning the rate of appearance of extracellular hemoglobin permits the hemolytic reaction to be treated in a quantitative manner analogous to that of classical biochemical kinetics.

Materials and Methods

Measurement of Rate of Hemolysis.—The method employed was the same as that described in the earlier report (2). In brief, 30 ml. of twice washed human erythro-

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cytes suspended in phosphate-buffered saline (M/12.9 NaCl and M/15 mono- and dibasic sodium phosphate, pH 7.0) were mixed with an equal volume of phosphate-buffered saline containing an appropriate quantity of hemolysin. After suitable intervals of time 5 ml. samples were removed and quickly centrifuged. The hemoglobin in the supernatant fluid was estimated colorimetrically at a wave length of 5790 A.u., and was expressed as "per cent hemolysis," 100 per cent corresponding to the hemoglobin liberated by an amount of saponin sufficient to cause complete lysis of the cell suspension. The standard red cell suspension employed in all the experiments was prepared by suspending the washed erythrocytes from 10 ml. blood in 750 ml. of phosphate-buffered saline. The cell concentration of the suspension was approximately 0.7 per cent by volume, a lower concentration than frequently employed in hemolysis experiments. The temperature of water baths was controlled manually in most cases, and the variation usually did not exceed ±0.3°C.

Streptolysin O.—Streptolysin O was prepared from 15 liter cultures of the C203S strain of *Streptococcus pyogenes*, grown according to the method of Bernheimer, Gillman, Hottle, and Pappenheimer (3). The streptolysin O was concentrated and stored in a manner similar to that described by Bernheimer and Cantoni (4).

Streptolysin S'.—The sodium salt of yeast nucleic acid has been shown by Okamoto (5) to induce the formation of a potent hemolysin in infusion broth cultures of hemolytic streptococci. The observations of Okamoto have been confirmed in this laboratory using the C203S strain of *Streptococcus pyogenes*. The hemolysin obtained by Okamoto's procedure was found to differ from streptolysin O in several respects among which were failure to be activated by cysteine and failure to be inhibited by cholesterol or by antistreptolysin O. Its properties, so far as they have been studied, agree in all respects with those of streptolysin S (6) but since complete proof of its identity with streptolysin S is lacking, it may provisionally be designated streptolysin S'. Streptolysin S' was prepared in neopeptone infusion broth containing 1 per cent sodium salt of yeast nucleic acid. No glucose was added to the medium. Cultures were incubated for 36 hours at 37°C. After centrifugation the cells were discarded and the supernatant fluid was employed without further treatment. It is probable that the supernatant fluid contained in addition to streptolysin S' small amounts of streptolysin O. The low concentration of streptolysin O found in neopeptone infusion broth cultures suggests that the hemolytic activity of the preparation obtained from medium containing the nucleate can almost exclusively be attributed to streptolysin S'.

Pneumolysin, Tetanolysin, Theta Toxin, and Cl. septicum Hemolysin.—Pneumolysin was prepared from strain D39R of *Diplococcus pneumoniae* according to the method of Cohen, Halbert, and Perkins (7). For the experiments with tetanolysin, the supernatant fluid of a 24 hour neopeptone infusion broth culture of strain F/3 of *Cl. tetani* was employed. The hemolytic activity of the supernatant fluid was found to be inhibited by cholesterol, capable of being neutralized by antistreptolysin O, and augmented by the addition of cysteine. The theta toxin of *Cl. welchii* was the supernatant fluid of a 12 hour culture of the PB6K strain (8). The immunological and chemical behavior of the theta toxin was similar to that of tetanolysin. The hemolysin of *Cl. septicum* was prepared according to the method described in an earlier publication (9).

Other Hemolysins.—Tyrocidine was recrystallized from crude material obtained from Merck and Company. Saponin was the commercial variety obtained from Merck and Company. Sodium taurocholate was a product of the Pfanstiehl Chemical Company. Samples of cetyl pyridinium chloride and duponol C were kindly supplied by Dr. Rollin D. Hotchkiss of The Rockefeller Institute. A sample of dried venom of the snake, *Bothrops atrox*, was kindly furnished by Lic. Luis Bolaños of San José, Costa Rica. 60 mg. dried venom were mixed with 18 mg. lecithin in a volume of 6.6 ml. prior to studying the hemolytic action of the venom.

EXPERIMENTAL

Degree of Hemolysis as a Function of Time.—The following observations were made in order to study the hemolytic reaction as a function of time. The course of hemolysis at 20°C. was followed for several different concentrations of each lysin. In each case, per cent hemolysis was plotted against time. The shape of the hemolysis curves, usually more or less sigmoid, varied according to the kind of hemolysin used, and to some extent with the hemolysin concentration. Typical hemolysis curves of four different lysins are shown in Fig. 1. The reasons why such curves are usually S-shaped have been discussed by Ponder (10) and they need not be restated here.

The family of curves (Fig. 1) obtained when streptolysin O was used was found to be representative not only of this hemolysin but also of pneumolysin, tetanolysin and theta toxin of *Cl. welchii*. Streptolysin O differed somewhat from the other three hemolysins in the manner in which the slope of the curves depended upon concentration. This difference will be described in the next section.

The family of curves (Fig. 1) obtained when saponin was used was found to be representative also of sodium taurocholate and of cetyl pyridinium chloride. The form of the curves resembled to a considerable extent those of streptolysin O.

The hemolysis curves of streptolysin S′ (Fig.1), although similar in form to the preceding ones, differed from them in one important respect. In the case of streptolysin S′ the initiation of lysis was preceded by a relatively long induction period. The induction period was found to be a function of concentration and of temperature, and was similar in all respects to the induction period observed earlier (2) in the system involving the hemolysin of *Cl. septicum*.

The form of the tyrocidine curves differed strikingly from those of the preceding lysins. In the case of tyrocidine there occurred within 2 or 3 minutes rapid partial lysis, followed by an abrupt decrease in rate to a constant, slow rate of lysis. The two phases, rapid and slow, are clearly shown in Fig.1. The abrupt decrease in liberation of hemoglobin evidently is due to inhibition of tyrocidine by one of the products of hemolysis, for it was observed that a hemolysate obtained by laking cells with distilled water markedly inhibited

tyrocidine hemolysis. The inhibiting substance is a component of the red cell stroma since it could be shown that stromata inhibited tyrocidine hemolysis, while the clear hemoglobin-containing fluid obtained by centrifuging a distilled water hemolysate possessed little or no capacity to inhibit tyrocidine hemolysis. The existence of a dissociable complex between tyrocidine and its inhibitor can explain the slow lysis which occurs after the first 2 minutes. (See tyrocidine

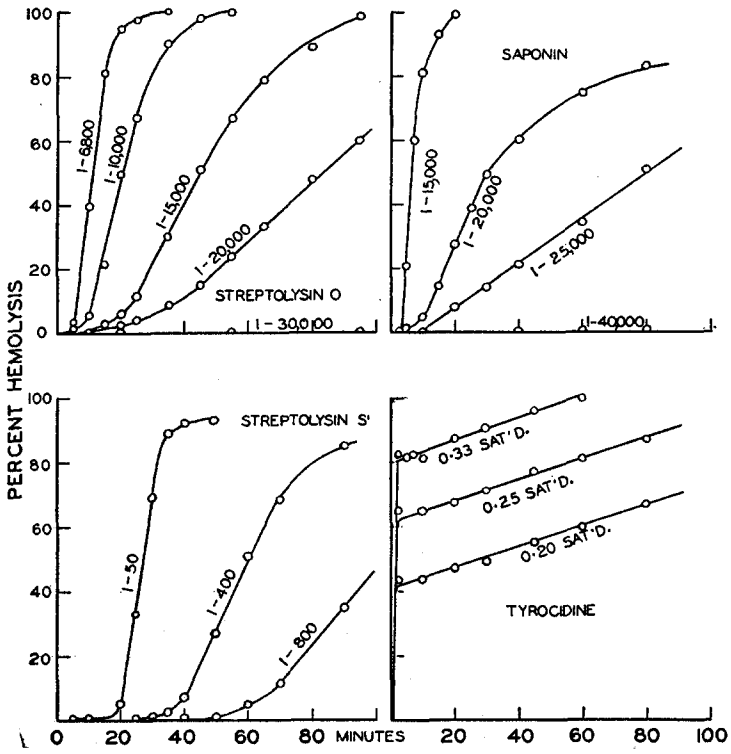


FIG. 1. Course of hemolysis induced by various concentrations of streptolysin O, streptolysin S', saponin, and tyrocidine, all at 20°C.

curves of Fig. 1.) Duponol C and the mixture of *atrox* venom and lecithin yielded curves which resembled those of tyrocidine although the decrease in rate of lysis induced by the *atrox* venom-lecithin mixture was less abrupt than that observed in systems in which tyrocidine and duponol C were the lytic agents.

Rate of Hemolysis as a Function of Concentration of Lysin.—The manner in which rate of lysis depends upon concentration of lysin is revealed by analysis of the curves shown in Fig.1 and of other curves similar to them. Inspection of the curves shows that part of each is linear. The slope of the linear part is the maximum rate of liberation of hemoglobin induced by each concentration of

each of the lysins studied. Not only are the slopes readily measurable but they are analogous to the rates employed in kinetic studies of reactions catalyzed by

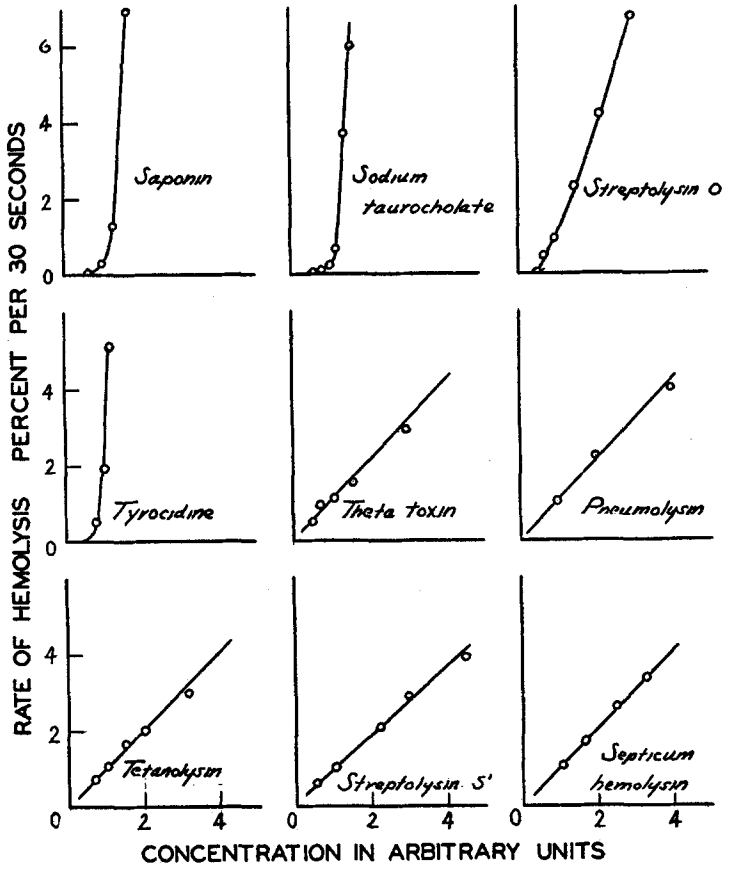


FIG. 2. Rate of lysis as a function of lysin concentration. The unit concentration is the concentration of lysin which liberates hemoglobin from the standard cell suspension at a maximum rate of 1 per cent per 30 seconds at 20°C. and at pH 7.0. The unit concentrations are equivalent to the following dilutions: saponin 1:40,000; sodium taurocholate 1:3,300; streptolysin O 1:15,000; theta toxin 1:20,000; pneumolysin 1:160,000; tetanolysin 1:300; streptolysin S' 1:400; *septicum* hemolysin 1:150. The tyrocidine curve is constructed from the curves published by Dimick (11). The curve for *septicum* hemolysin is adapted from (2).

enzymes. They are, therefore, susceptible to the same kind of analysis as that used in enzyme kinetics. In Fig. 2 the slopes of curves similar to those of Fig.1 are plotted against concentration of each of nine different hemolysins. The unit of concentration of each lysin except tyrocidine is the quantity of lysin which liberated hemoglobin at a maximum rate of 1 per cent per 30 seconds at 20°C.

For example, the unit concentration of the particular preparation of streptolysin S′ employed was 1:400, as can be verified by comparing the curves of streptolysin S′ in Figs.1 and 2. The first phase of lysis produced by tyrocidine occurred so rapidly that it could not be measured by the method employed for the other hemolysins. The necessary data were provided, however, by the published curves of Dimick (11) and from them the tyrocidine curve of Fig. 2 was constructed.

The results shown in Fig.2 indicate that theta toxin of *Cl. welchii*, pneumolysin, tetanolysin, streptolysin S′, and *Cl. septicum* hemolysin are similar in the manner in which rate of lysis depends upon concentration. In each case the rate of lysis was found to be directly proportional to the concentration of lysin. The results obtained when saponin and sodium taurocholate were studied in the same way contrast sharply with those given by the bacterial lysins. The rate of lysis induced by saponin and by sodium taurocholate was found to increase logarithmically with increasing lysin concentration. The curves for the latter two lysins (Fig.2) can be seen to be superimposable. The curve for tyrocidine (Fig. 2) based on Dimick's data, resembles those of saponin and sodium taurocholate. These results are summarized in Table I.

TABLE I

Comparison of Kinetics of Lysis Induced by Various Hemolysins

Hemolytic agent	Manner in which rate of lysis depended upon lysin concentration	Lysis preceded by a long induction period
Streptolysin S′	Directly proportional	+
Cl. septicum hemolysin	Directly proportional	+
Pneumolysin	Directly proportional	−
Theta toxin	Directly proportional	−
Tetanolysin	Directly proportional	−
Streptolysin O	Anomalous*	−
Saponin	Exponential	−
Sodium taurocholate	Exponential	−
Cetyl pyridinium chloride	Exponential	−
Duponol C	‡	−
Lecithin-atrox venom	‡	−
Tyrocidine	‡	−

* The manner in which rate of lysis depended upon lysin concentration is not a direct proportion nor does it conform to the exponential relationship shown by saponin, sodium taurocholate, and cetyl pyridinium chloride. (See Fig. 2.)

‡ These lysins exhibited a phase of rapid liberation of hemoglobin followed by a phase of slow hemoglobin liberation. The rate of lysis during the rapid phase appeared to be an exponential function of concentration.

Streptolysin O differs from all the other hemolysins in the manner in which

rate of lysis depends upon concentration. The form of its curve (Fig. 2) differs not only from that of saponin and sodium taurocholate but also from those of the other bacterial hemolysins. This result is unexpected in view of the similarity in immunological (12) and chemical behavior known to exist between it and the other oxygen-labile bacterial hemolysins. The reproducibility of the curve for streptolysin O is demonstrated by the observation that similar curves were obtained when other lots of streptolysin O were employed, when the concentration of cysteine in the system was increased, and when gelatin was included in the system as a protective colloid. Although it seems possible that the dissimilarity between the curve of streptolysin O and those of the other oxygen-labile hemolysins may be related to factors concerned in the activation of streptolysin O with cysteine, the experiments which have been performed do not support this idea.

Rate of Hemolysis as a Function of Temperature.—Although the manner in which biochemical reactions are affected by temperature has long been of interest to biologists, a comprehensive comparative study of the effect of temperature upon lysis induced by bacterial and other hemolysins appears not to have been made.

The effect of temperature on rate of hemolysis was determined by measuring the slopes of curves similar to those of Fig. 1 obtained at a series of temperatures differing by 5°C. increments. The temperatures employed were in the range of 0–31°C., temperatures greater than 31°C. having been avoided in order to minimize thermal inactivation of the lytic agents. The six hemolysins studied and the final concentrations used were as follows: pneumolysin 1:80,000; theta toxin 1:20,000; streptolysin S′ 1:400; streptolysin O 1:60,000; tetanolysin 1:200 and 1:800. Pneumolysin, theta toxin, tetanolysin, and streptolysin O were activated with 0.5 per cent cysteine prior to diluting them to the above concentrations. The results are expressed in the form of Arrhenius plots (Fig. 3) in which log rate of hemolysis is plotted against the reciprocal of absolute temperature. The values of the critical thermal increment, μ, calculated from the Arrhenius plots, are tabulated in Table II. For comparative purposes the μ values reported by other investigators are also included in the table.

The μ values of pneumolysin, theta toxin, tetanolysin (15–30°C.), and streptolysin O (20–30°C.) were found to be in the vicinity of 22,000. The differences between the individual μ values of the four hemolysins in this temperature range appear to be of questionable significance. Streptolysin S′, however, exhibited the much lower μ value of 14,600, and this, it may be noted, is not far from that (12,700) reported for the hemolysin of *Cl. septicum* (2).

Tetanolysin and streptolysin O exhibited much higher μ values between 0 and 15°C. (40,000 and 43,000 respectively) than between 20 and 30°C. (21,200 and 21,400 respectively). In both systems the shift from the higher to the lower μ value occurred in the vicinity of 15–20°C. It may be noted that two lots of tetanolysin, one prepared in this laboratory and a second lot obtained

from another laboratory, yielded curves which were superimposable. Of interest, also, is the close agreement between the two μ values found for tetanolysin and those found for streptolysin O. It is not clear why streptolysin O

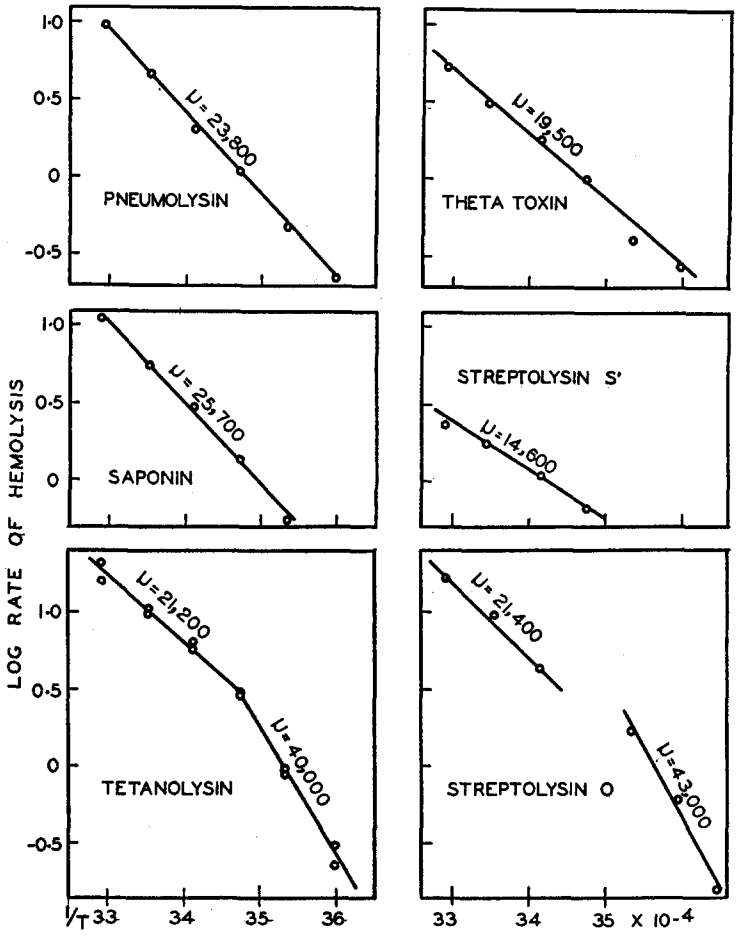


FIG. 3. Arrhenius plots showing the effect of temperature on rate of hemolysis. Temperature ranges: pneumolysin, 5–30°C.; theta toxin, 5–30°C.; saponin, 10–30°C.; streptolysin S', 15–30°C.; tetanolysin, 5–30°C.; streptolysin O, 0–10°C. and 20–30°C.

and tetanolysin each exhibited two μ values. Similar observations on the effect of temperature on other systems, however, are recorded in Sizer's review (15). It is possible that the change in μ value at a critical temperature is due to a shift in the rate-determining reaction of a catenary series of reactions leading to lysis, or, that the change in μ value is associated with a change in the molecular configuration (15) of the hemolysin. The results can also be

explained by assuming that what is called streptolysin O (and tetanolysin) is not one but two hemolysins each characterized by a different μ value. This hypothesis requires the additional assumption that below a certain temperature, one of the two hemolysins is more active than the other, and that above this temperature the other is the more active. There is no evidence in favor of any of these hypotheses.

TABLE II

Critical Thermal Increment, μ, of Rate of Hemolysis Induced by Various Lysins

Hemolytic agent	μ	Source
Ammonia	29,000	Arrhenius (13)
Acetic, propionic, and butyric acids	26,000	Arrhenius (13)
Saponin (5–20°C.)	27,000–32,000	Calculated from results of Ponder and Yeager (14)
Saponin	25,700	Fig. 3 of this paper
Hot water	64,000	Arrhenius (13)
Heat	60,400	Wilbrandt (21)
Vibriolysin	26,000	Arrhenius (13)
Cl. septicum hemolysin	12,700	Bernheimer (2)
Streptolysin S′	14,600	Fig. 3 of this paper
Streptolysin O (20–30°C.)	21,400	Fig. 3 of this paper
Tetanolysin (15–30°C.)	21,200	Fig. 3 of this paper
Pneumolysin	23,800	Fig. 3 of this paper
Theta toxin of *Cl. welchii*	19,500	Fig. 3 of this paper
Streptolysin O (0–10°C.)	43,000	Fig. 3 of this paper
Tetanolysin (5–15°C.)	40,000	Fig. 3 of this paper

Further Observations on Lysis Induced by Cl. septicum Hemolysin

The general properties of *Cl. septicum* hemolysin have been described elsewhere (2) and in connection therewith it was pointed out that the lysin possesses some of the properties of an enzyme. Although further investigation failed to reveal the chemical nature of the substrate upon which the lysin acts, certain additional observations relating to the mechanism of lysis have been made.

In a study of the action of *Cl. septicum* hemolysin, Menk (16) discovered that the hemolytic reaction consisted of two phases: a phase of combination of lysin with erythrocytes followed by a phase of actual lysis. It was reported that combination of lysin and erythrocytes occurred in an environment of dextrose solution but that lysis did not occur unless the cells were washed and resuspended in saline solution. It was suggested that the rôle of salt in hemolysis was analogous to that of electrolyte in bacterial agglutination.

Significance of the Induction Period.—The addition of *Cl. septicum* hemolysin to erythrocytes never results in immediate hemolysis; lysis is always preceded by a latent or induction period of definite duration (16, 2). Under the condi-

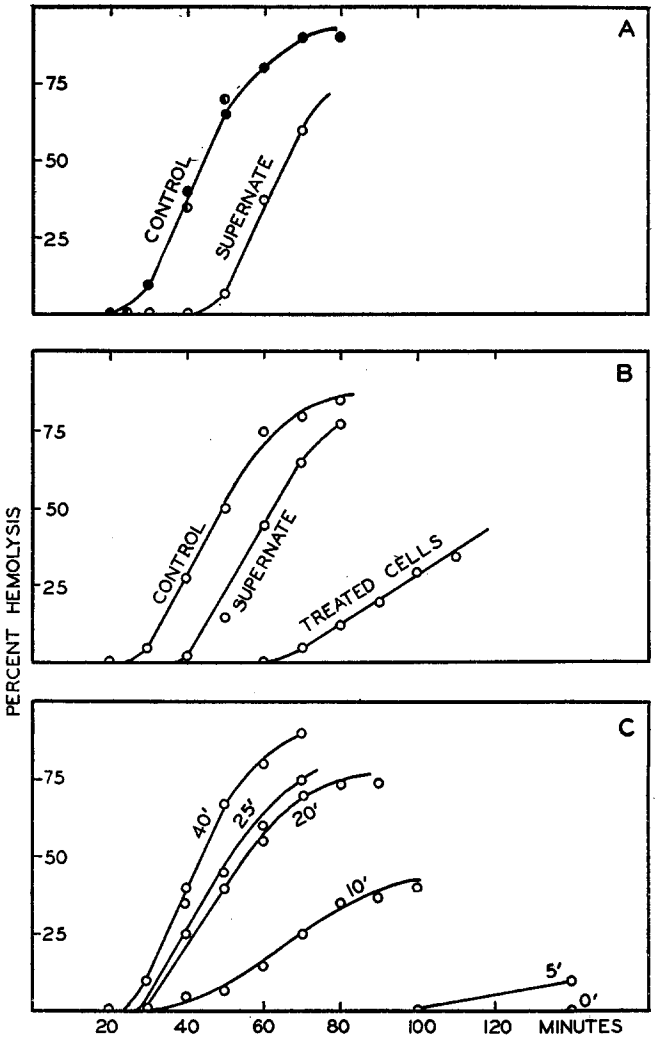


FIG. 4. Analysis of induction period (primary reaction) preceding lysis induced by *Cl. septicum* hemolysin.

A, comparison of hemolysis with lysin removed at end of primary reaction and with lysin present throughout course of both primary and secondary reactions. Red cells incubated with *Cl. septicum* hemolysin for 20 minutes at 20°C. followed by centrifugation. Half-shaded circles: lysis of treated cells plus buffered saline. Open circles: lysis of fresh cells plus supernatant fluid. Solid circles: lysis of uncentrifuged control cells. Temperature 20°C.

B, effect of reduced temperature on primary reaction between cells and lysin. Red cells incubated with lysin 20 minutes at 2°C. followed by centrifugation. Curve marked "treated cells:" lysis of treated cells plus buffered saline at 20°C. Curve marked "supernate:" lysis of fresh cells plus supernatant fluid at 20°C. Control: red cells plus lysin at 20°C. during the entire experiment.

C, effect on the primary reaction of *Cl septicum* antihemolysin added at 0, 5, 10, 20, 25, and 40 minutes after mixing cells and lysin. Temperature at 20°C.

tions employed in the present study the duration of the induction period was approximately 25 minutes. It was considered of interest to inquire further into the nature and significance of the induction period.

The technique of following lysis was the same as that employed in the earlier experiments. Unless otherwise stated, experiments were carried out at 20°C. To two tubes containing 15 ml. cell suspension were added 15 ml. hemolysin diluted 1:40. One of the two tubes served as a control. After 20 minutes the other tube was quickly centrifuged, the sedimented cells resuspended in buffered saline solution, and the colorless supernatant fluid added to fresh cells. Lysis was then followed in all three tubes. The results (Fig. 4A) show that cells exposed to lysin for only 20 minutes (during which period no lysis occurred) subsequently hemolyzed at approximately the same rate as cells which were allowed to remain in the presence of lysin during the entire experiment. It is evident that during the induction period the cells became altered in such a manner that lysis followed automatically. Little of the lysin was used up in altering the cells since the hemolytic activity of the supernatant fluid was almost as great as that of the control (Fig. 4A).

Experiments similar to the one just described showed clearly that at least two reactions are involved in the hemolytic system: a *primary reaction* between cells and lysin, occurring during the induction period and involving alteration of the cells, and a *secondary reaction* consisting of actual lysis and characterized by the liberation of hemoglobin. It was considered desirable to observe the effect of reduced temperature on the primary reaction. For this purpose the experiment just described was repeated except that one tube was kept at 2°C. instead of at 20°C. for the first 20 minutes. After that time the tube was centrifuged, and the course of hemolysis of the treated cells, the supernatant fluid plus fresh cells, and the control cells, followed at 20°C. It can be seen in Fig. 4B that the cells treated at 2°C. for 20 minutes lysed at a considerably slower rate than the control cells. The critical thermal increment of the primary reaction rate, calculated from the rates of lysis obtained in this experiment, was found to be 13,300, a value which does not differ significantly from that (12,700) found (2) for the system as a whole.

Effect of Antihemolysin.—If lysis occurs as a distinct step following a primary alteration in the cells during the induction period it follows that antibody to the hemolysin should inhibit the primary reaction but not the secondary, lytic reaction. This concept was tested by adding to the system 0.01 ml. monovalent *Cl. septicum* horse antitoxin containing 14 units, at 0, 5, 10, 20, 25, and 40 minutes after mixing lysin 1:40 with cells. The curves of hemolysis are shown in Fig. 4C. The results show that lysis was completely inhibited when antibody was added at the same time as the lysin was mixed with the cell suspension. When antibody was added at 5 and 10 minutes lysis occurred slowly; when added at 20 or 25 or 40 minutes the rate of lysis was only slightly less than that

of tubes containing no antibody (cf. Fig. 4C with controls of Fig. 4A and 4B). The findings indicate that the primary reaction occurring during the induction period is inhibited by antibody while the secondary reaction (actual lysis) is affected little or not at all.

Effect of Sucrose on Hemolysis.—Since the action of some hemolytic agents is known to be inhibited by sugars it was of interest to observe the effect of

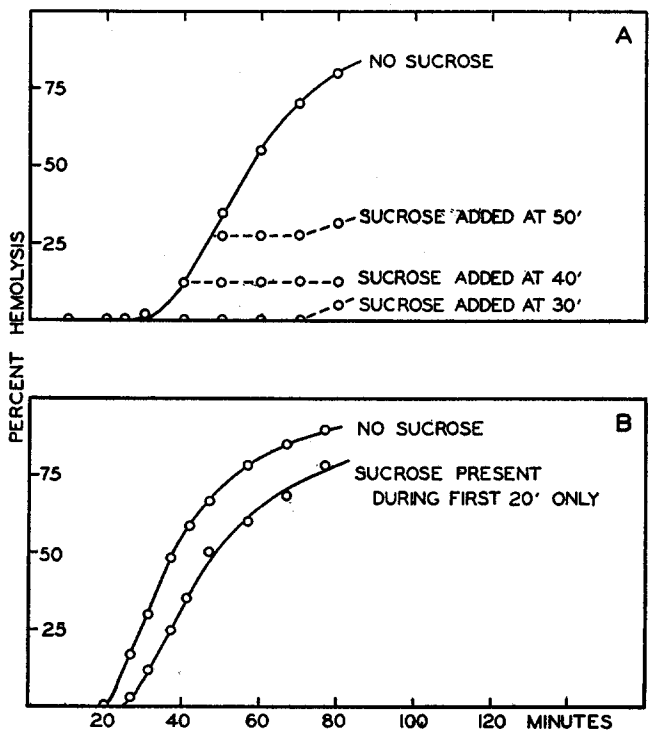


FIG. 5. Effect of 0.15 M sucrose on course of hemolysis induced by *Cl. septicum* hemolysin.

A, sucrose added at 30, 40, and 50 minutes.

B, sucrose present only during first 20 minutes.

sucrose on the primary and secondary phases of the *septicum* system. As noted earlier, Menk (16) reported that lysis did not occur if the cells were exposed to hemolysin dissolved in glucose solution instead of saline solution.

One and one-half ml. of 3 M sucrose solution were added to the system 30, 40, and 50 minutes after mixing 15 ml. lysin 1:40 with 15 ml. cell suspension. The resulting hemolysis curves and a control curve (no sucrose) are presented in Fig. 5A. It can be seen that lysis was promptly interrupted when the sugar was added at 40 and 50 minutes, and that initiation of lysis was delayed when sucrose was added at 30 minutes. The inhibitory effect of sucrose was tempo-

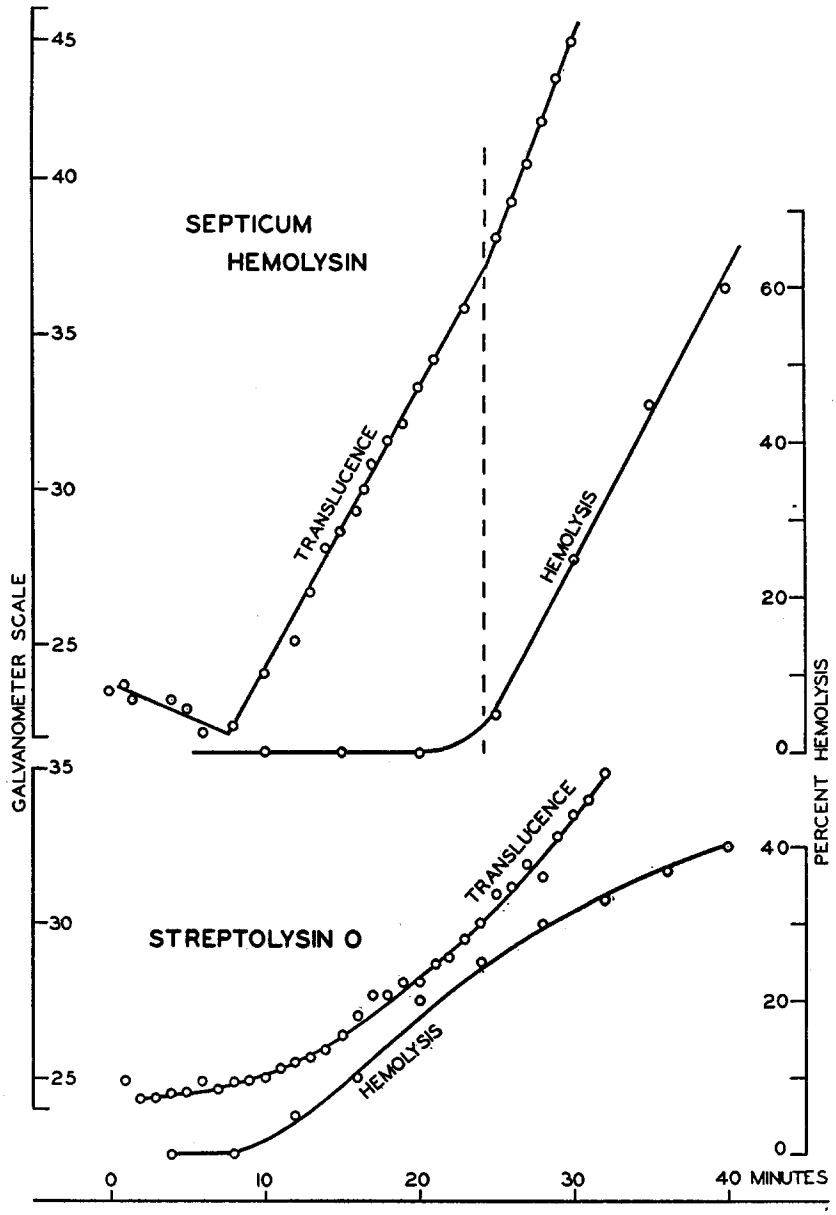


FIG. 6. Change of light transmission prior to and during hemolysis of cells exposed to *Cl. septicum* hemolysin (upper part of figure) and to streptolysin O (lower part of figure). Broken line indicates time of beginning lysis in the *septicum* system.

rary since lysis began again after the period of inhibition. In another experiment 0.15 M sucrose was allowed to be present in the system only during the

first 20 minutes, after which time the cells were centrifuged and resuspended in buffered saline. Under these conditions the initiation of lysis was slightly delayed although the rate of lysis was almost as great as that of the control (Fig. 5B). The results of these experiments show that sucrose has little or no effect on the primary action of the lysin and that the effect of sucrose is an interference with the secondary reaction (actual lysis) or a reaction closely associated therewith.

Swelling of Erythrocytes during the Induction Period.—Macroscopic examination of a mixture of *Cl. septicum* lysin and cells revealed that a decrease in opacity of the suspension occurred during the induction period. In order to study in a more quantitative manner the change in opacity, the light transmission of the system was measured in a Coleman spectrophotometer at a wave length of 6500 A.u. From a duplicate tube samples were removed, centrifuged, and the hemoglobin in the supernatant fluid measured in order to follow hemolysis. The results are shown in the upper portion of Fig. 6. A slight decrease in translucence during the first 7 minutes was followed by a marked increase in translucence prior to, as well as during the period in which lysis occurred. A small but definite change in the slope of the curve of translucence of Fig. 6 can be observed at $t = 24$ minutes, the approximate time at which lysis commenced. For comparison a similar experiment was carried out employing streptolysin O. The results shown in the lower portion of Fig.6 reveal that the marked increase in translucence which occurred during the induction period of the *septicum* system was absent from the streptolysin O system.

The foregoing results suggest that the cells increase in volume prior to lysis. That an increase in volume does occur was shown by hematocrit measurements carried out during the induction period before lysis set in. Owing to the very small volume occupied by the packed cells it was necessary to construct special hematocrit tubes, the lower portion of which had a bore of 0.88 mm. Employing conditions similar to those of the previously described experiments the volume occupied by the cells in 5 ml. samples of the reaction mixture was measured after 5 minutes' centrifugation at 2000 r. p. m. The average volume occupied by the cells which had been exposed to the hemolysin for 13 minutes prior to centrifugation was found to be 24.8 c. mm. while that found for cells which had not been exposed to the hemolysin was 22.1 c. mm. The difference was found to be reproducible. No consistent difference in volume was found when cells exposed to heat-inactivated hemolysin were compared to untreated cells. The difference in volume revealed by the hematocrit measurements (12 per cent) is consistent with the increased translucence observed photometrically.

DISCUSSION

In Ponder and McLachlan's study (17) of the comparative kinetics of bacterial hemolysis, it was found that lysis induced by hemolysins of bacterial

origin was described by equations similar to those which have been found to describe saponin hemolysis. The kinetics of the fundamental reation, that is, the reaction between lysin and erythrocytes, was considered by them to be identical regardless of whether the hemolysin was saponin or a lysin of bacterial origin. The opinion was expressed, however, that the observed similarity in the mode of action of the two kinds of lysins might be largely superficial. The latter opinion is supported by some of the observations made in the present study.

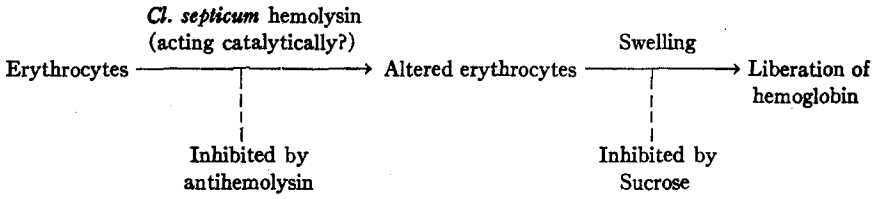
The present study of hemolysis differs from most others in several respects not the least important of which is the avoidance of assumptions concerning the nature of the primary interaction between lysin and erythrocytes. The almost universal practice of employing as a measure of rate of hemolysis the elapsed time between the mixing of reagents and the attainment of 50 per cent, 100 per cent, or some other definite degree of hemolysis has also been avoided. Instead, the course of hemolysis was carefully followed, and the rate of maximum hemoglobin liberation was calculated from curves expressing the rate of appearance of extracellular hemoglobin.

With these prerequisites it has been found that the lysins studied can be grouped into several classes according to the manner in which rate of lysis is affected by lysin concentration and by temperature. The main findings are summarized in Tables I and II. In Table I it can be seen that rate of lysis is directly proportional to concentration in the case of streptolysin S', *Cl. septicum* hemolysin, pneumolysin, theta toxin, and tetanolysin. It is notable that all of these lysins are derived from bacteria. All of the remaining lysins, two of which are of bacterial origin, exhibited a non-linear relationship between rate and lysin concentration. In the case of saponin, sodium taurocholate, and cetyl pyridinium chloride, the relationship is exponential, and the same may be true also of tyrocidine, duponol C, and *atrox* venom-lecithin mixture during the rapid phase of lysis. These differences are interpreted as reflecting differences in the mode of action of the lytic agents. In view of the fact that all of the lytic agents showing a direct proportionality between rate and concentration appear to be proteins, it seems not improbable that some or all of the lysins of this class are enzymes. The findings concerning the effect of temperature on the rate of lysis as shown in Table II are consistent with this view. None of the evidence at hand, however, proves that any of the lysins studied actually are enzymes.

Cl. septicum hemolysin and streptolysin S' are the only lysins which exhibited a long induction period preceding the initiation of lysis. It is of interest that the values of the critical thermal increment of rate of lysis induced by these two lysins lie very close to each other and that both values are much lower than those found for the other lysins.

The lysis induced by *Cl. septicum* hemolysin has been studied in greater detail than that induced by the other hemolysins, particularly in regard to the

significance of the induction period. The findings indicate that the process by which hemolysis occurs can be represented by the following scheme:



This formulation of the events leading to lysis, without doubt an oversimplification, serves to delineate two problems requiring further investigation, namely: (1) the precise nature of the alteration of the cells induced by the lysin, and (2) the process by which the altered cells swell. Until the nature of the chemical action of the lysin becomes known, little can be said about the first problem. Concerning the second, it may be noted that swelling has been observed to precede hemolysis in a number of instances including lysis induced by such diverse lytic agents as X-radiation (18), lysolecithin (?) (*cf.* 19 and 20), lipid solvents, bee venom, and others (21). A commonly accepted explanation of the swelling is one proposed by Davson (22) and Wilbrandt (21) according to which an increased permeability to cations alters the conditions of Donnan equilibrium in such a way that salt and water penetrate the cell causing it to swell until it bursts. Hemolysis which depends upon increase in cation permeability has been designated by Wilbrandt (21) as "colloid osmotic hemolysis." The present findings indicate that lysis induced by *Cl. septicum* hemolysin may be of the colloid osmotic type. If this is correct the primary action of the hemolysin would be an alteration of the cell membrane leading to increased cation permeability. Swelling and lysis would then follow automatically. It is also possible, however, that the swelling observed in connection with the action of *Cl. septicum* hemolysin could result from an increase in osmotically active substances within the erythrocyte and occur independently of changes in permeability. On the basis of the evidence now available it cannot be decided whether the observed swelling is to be accounted for by the theory of Wilbrandt and Davson or by another mechanism.

SUMMARY

A study has been made of the kinetics of lysis induced by various hemolytic agents. The course of hemolysis was followed by mixing lysin with washed human erythrocytes, removing samples from the mixture, and estimating colorimetrically the hemoglobin in the supernatant fluid of the centrifuged samples. Most of the curves (but not all of them, *e.g.* tyrocidine) obtained by plotting degree of hemolysis against time, were S-shaped. The initiation of lysis by streptolysin S′ was delayed, and in this property, streptolysin S′ was similar to *Cl. septicum* hemolysin. None of the other lysins studied exhibited a long latent period preceding lysis.

The maximum rate of hemoglobin liberation was found, in the range of lysin concentrations studied, to be a linear function of concentration when theta toxin of *Cl. welchii*, pneumolysin, tetanolysin, or streptolysin S′ was the lytic agent. With comparable concentrations of saponin, sodium taurocholate, cetyl pyridinium chloride, tyrocidine, duponol C, lecithin-*atrox* venom mixture, or streptolysin O, the relation between rate and concentration was non-linear.

The critical thermal increment associated with hemolysis was determined for systems containing pneumolysin, theta toxin, streptolysin S′, streptolysin O, tetanolysin, and saponin.

The findings concerning the effect of concentration and temperature on the rate of hemolysis provide a basis for classifying hemolytic agents (Tables I and II).

Hemolysis induced by *Cl. septicum* hemolysin was found to be preceded by two phases: a phase of alteration of the erythrocytes and a phase involving swelling. Antihemolytic serum inhibited the first but not the second phase while sucrose inhibited the second but not the first phase.

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