KINETICS OF LYSIS BY CLOSTRIDIUM SEPTICUM HEMOLYSIN*

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Culture filtrates of *Clostridium septicum* not only are lytic for erythrocytes of various species of mammals (1), but also are lethal for small laboratory animals (2). In studying the relation of the hemolysin to the lethal toxin (3), parallelism in the hemolytic and lethal activity was invariably observed. Attempts to separate a hemolytic from a lethal component were unsuccessful, and it was concluded that the hemolytic and lethal properties were functions either of a single substance or of two substances having closely similar physical and chemical properties. In view of the possible identity of hemolysin and lethal toxin, it seemed worth while to study in some detail the hemolytic reaction. The kinetics of this reaction forms the subject of the present communication.

Although rigorous proof is lacking, existing information indicates that the hemolysin of *Cl. septicum* is protein. It is non-dialyzable, markedly thermolabile, can be precipitated with ammonium sulfate, and is antigenic (3). These properties, moreover, are shared by the oxygen-labile hemolysin of group A hemolytic streptococcus, whose protein nature is suggested by the chemical analyses of Smythe and Harris (4) and Herbert and Todd (5).

Methods

Preparation of Hemolysin.—*Cl. septicum*, strain 44, obtained from the National Institute of Health, was grown in cultures of 500 ml. each in the protein-free medium described (6). After incubating 16 to 20 hours, the cultures were centrifuged. The clear supernatant fluids were kept in the refrigerator with or without a few drops of toluene. These preparations, which are referred to as "hemolysin," or as culture supernate, were used without further purification.

Red Cell Suspension. —The red cell suspension consisted of the erythrocytes from 10 ml. of human blood twice washed in phosphate-buffered saline (M/12.9 NaCl and M/15 ((mono- and dibasic)) sodium phosphate, pH 7.0), and then made to a final volume of 750 ml. with the same phosphate-buffered saline. The sensitivity of the red cell suspension to the action of *Cl. septicum* hemolysin was found to remain unaltered for at least a week, provided the cell suspension was kept at 0 – 4°C. Unless otherwise stated, the hemolysin was always diluted in saline, buffered with phosphate at pH 7.

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Measurement of Rate of Hemolysis.—The rate of hemolysis was determined by thoroughly mixing 30 ml. of an appropriate dilution of hemolysin with 30 ml. of the red cell suspension, following a 10 minute period of temperature equilibration. 5 ml. samples, withdrawn after suitable intervals of time, were immediately centrifuged, the supernatant fluid removed from the unlysed red cells, and the hemoglobin in the supernatant fluid estimated colorimetrically using a Coleman spectrophotometer at a wave length of 5790 Ångström units. The degree of hemolysis is expressed in per cent, 100 per cent being complete hemolysis of the cell suspension. Per cent hemolysis is plotted against time, as illustrated by Fig. 1, and the rate of hemolysis determined by measuring the slope of the linear portion of the curve.

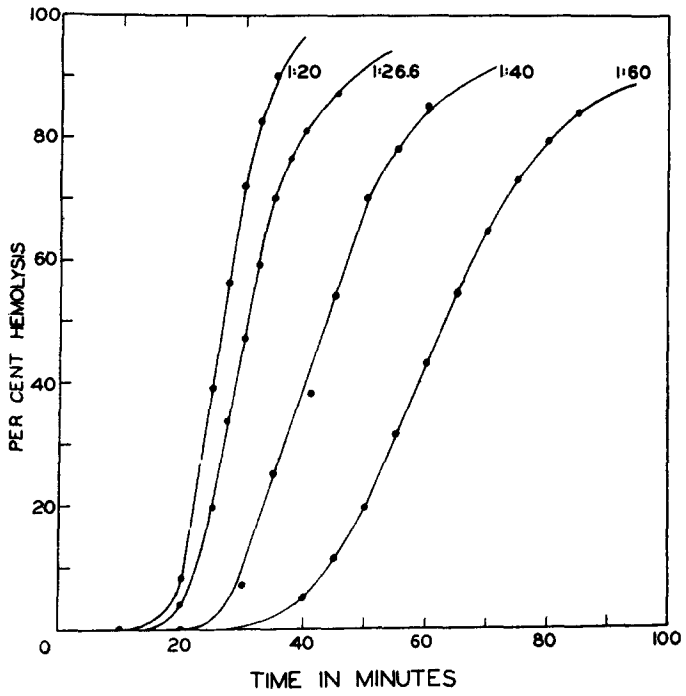

FIG. 1. Course of hemolysis for four different dilutions of the same culture supernate. The four curves, from left to right, represent dilutions of 1:20, 1:26.6, 1:40, and 1:60.

EXPERIMENTAL

Rate of Hemolysis as a Function of Concentration of Hemolysin—The purpose of this experiment was to establish the relation between rate of hemolysis and the concentration of hemolysin. 4 dilutions of hemolysin — 1:20, 1:26.6, 1:40, and 1:60— were mixed with red cell suspension, and the rate of hemolysis at 20°C., at pH 7.0, was measured in the manner described. The results are shown in Fig. 1. In Fig. 2, the slopes of the linear portions of these curves and of curves similar to them, but based upon measurements made at 37°C. upon another lot of hemolysin, are plotted against concentration of hemolysin.

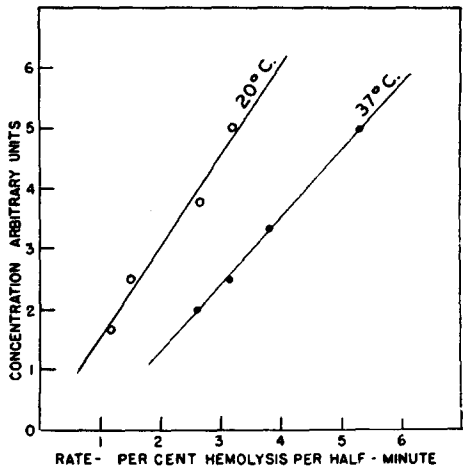

FIG. 2. Relation between rate of hemolysis and concentration of hemolysin. ○, rates at 20°C. obtained by measuring slopes of linear portions of curves of Fig. 1. ●, rates at 37°C. from similar curves in which another preparation of hemolysin was used.

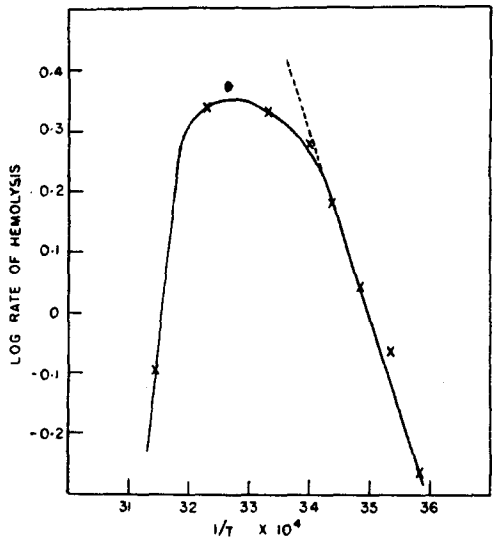

FIG. 3. An Arrhenius plot of the effect of temperature on rate of hemolysis. Below approximately 20°C., μ = 12,700 calories.

From Fig. 2, it can be seen that the rate of lysis is directly proportional to the concentration of hemolysin.

Rate of Hemolysis as a Function of Temperature.—In this experiment, the hemolysin concentration was constant, and the temperature was varied.

The rate of hemolysis effected by 30 ml. of a 1:25 dilution of culture supernate was determined at pH 7.0, and at temperatures of 6°, 10°, 14°, 17°, 21°, 27°, 37°, and 45°C. In Fig. 3, the logarithm of the rate of hemolysis is plotted against the reciprocal of the absolute temperature. From 6°C. to approximately 20°C., the curve is linear, while above approximately 20°C., there is departure from the straight line, the degree of departure increasing with increasing temperature. The energy of activation, between 6°C. and 20°C., calculated from the equation of Arrhenius (7) is 12,700 calories per mole. Q_{10} equals 2.0 between 10° and 20°C. The maximum rate of hemolysis occurs at or near 37°C.

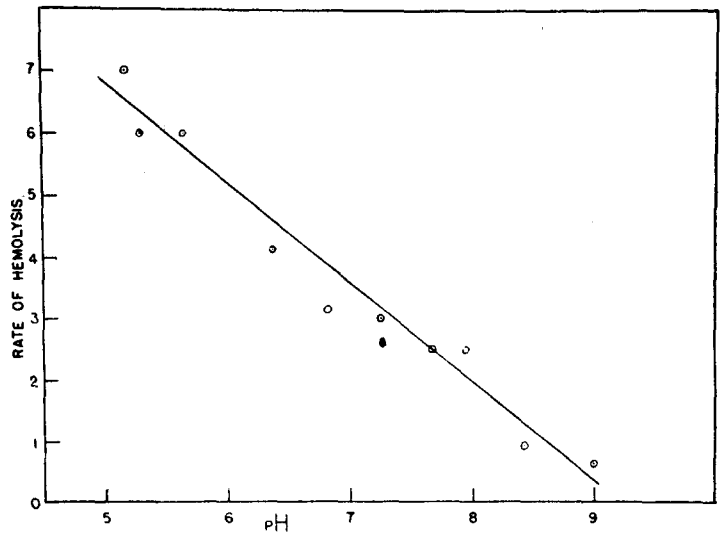

FIG. 4. Rate of hemolysis as a function of pH.

Rate of Hemolysis as a Function of Hydrogen Ion Concentration.—In order to minimize variation in total ionic strength at different hydrogen ion concentrations, the diluent for the hemolytic system was M/6.45 in NaCl and M/60 in phosphate, the latter having been added as H_3PO_4, NaH_2PO_4, Na_2HPO_4, or Na_3PO_4 in amounts necessary to give the desired pH, but always keeping the total phosphate concentration M/60. The hemolysin employed was a 1:60 dilution of culture supernate, and the tests were carried out at 20°C. The rate of hemolysis at pH 5.2 to pH 9.0 is shown in Fig. 4. The red cells in control tubes containing no hemolysin remained unhemolyzed over this pH range. Below pH 5, hemolysis was observed to occur in the absence of *Cl. septicum* hemolysin. Fig. 4 shows that the rate of hemolysis is very slow at pH 9, but increases with decreasing pH to about pH 5, below which the cells will lyse in the absence of hemolysin.

DISCUSSION

The foregoing experiments throw a certain amount of light on the nature of the reaction underlying hemolysis. The rate of hemolysis is shown in Fig. 2 to be directly proportional to the hemolysin concentration over the range studied. In these experiments relatively low concentrations of hemolysin were used; lower concentrations are likely not to effect complete hemolysis of the red cell suspension employed. The linear relationship between rate of reaction and concentration of catalyst is a property of most, if not of all enzyme-catalyzed reactions. The finding that the rate of hemolysis is a linear function of the concentration of hemolysin suggests that the lytic reaction may be enzymic.

The linear relationship between rate of hemolysis and concentration of hemolysin does not seem to apply to lytic agents such as saponin, judging, at least, from the results of Ponder's extensive investigation (8) of the hemolytic properties of saponin. Reference to the upper portion of Ponder's Fig. 24, in which quantity of hemolysis is plotted against time for 6 different concentrations of saponin, shows that the decrease in rate of hemolysis with decrease in concentration of saponin, is very much greater than it would be were there a linear relationship between rate of hemolysis and concentration of saponin. It is possible that lower concentrations of saponin would give different results. In the absence of further information on this point, one is led to conclude, in contrast to the conclusion of Ponder and McLachlan (9), that the kinetics of lysis by bacterial hemolysins may be fundamentally different from that of lysis by saponin.

Between 6°C. and approximately 20°C., the *Cl. septicum* hemolytic system obeys the equation of Arrhenius, departing widely from the theoretical curve, however, above approximately 20°C. The deviation from the theoretical expectation above 20°C. in all probability is a consequence of hemolysin destruction, for it has been found that the hemolysin undergoes appreciable irreversible inactivation at 37°C. Although we are ignorant of the nature of the chemical reaction underlying lysis, we may consider that the energy of activation of the "fundamental reaction" is 12,700 calories per mole. If, however, a chain of reactions is involved in hemolysis, then this value is the energy of activation of the slowest reaction (10).

The addition of hemolysin to red cells does not result in immediate lysis. There is always a preliminary period, or "induction period," which may be from 5 to 60 minutes or more in duration, before detectable hemolysis occurs. The existence of this induction period has been noted by Menk (11). The induction period is short if a high concentration of hemolysin is employed; long if a low hemolysin concentration is used. The induction period is also a function of temperature and hydrogen ion concentration, and when plotted against the reciprocal of temperature in degrees Centigrade, gives a curve which

is almost linear. Further study would seem necessary in order to clarify the significance of the induction period.

The failure to find an optimum hydrogen ion concentration for the hemolytic system under study is somewhat unexpected, particularly because Menk (11) and Walbum (12) detected inhibition of hemolysis in pH regions on the acid side of neutrality. In the present system, however, the existence of an optimum hydrogen ion concentration below pH 5 is not precluded. The marked decrease in rate of hemolysis with decreasing hydrogen ion concentration is very probably an effect on the hemolytic system itself, rather than an effect upon either of the reactants, as may be shown in the following way: if red cells and a suitable dilution of hemolysin are mixed at pH 9, hemolysis occurs at a very slow rate; if the pH is now adjusted to 7, hemolysis proceeds rapidly. The oxygen-labile hemolysin of group A hemolytic streptococci has been shown by Herbert and Todd (5) (who employed rabbit erythrocytes) to have a sharp optimum at pH 6.5. We have found (unpublished experiments, using human erythrocytes) that hemolysis by the oxygen-labile hemolysin of group A hemolytic streptococci is inhibited at pH 5. If the pH of the system is adjusted to 6.5 to 7.0, then the cells hemolyze rapidly. It is clear that the hemolysins of these two bacterial species behave very differently with regard to the effect of hydrogen ion concentration on the respective hemolytic systems. A further difference is brought out by the observation that the hemolysin of *Cl. septicum* is not neutralized by rabbit antistreptolysin.

The hemolysin of *Cl. septicum* is probably protein; it is non-dialyzable, thermolabile, may be precipitated with ammonium sulfate, and is antigenic. It possesses some of the properties of an enzyme. Its hemolytic activity is directly proportional to its concentration, and the temperature coefficient of the hemolytic reaction is the same as that of a number of enzyme-catalyzed reactions. It is known, moreover, that at least one other bacterial hemolysin, the alpha toxin of *Cl. welchii*, which is both lethal and hemolytic, is an enzyme capable of specifically hydrolyzing lecithin (13). Mention may be made also of the enzymic action of snake venoms upon lecithin. The identity of the substrate upon which the hemolysin of *Cl. septicum* acts, offers an attractive problem for future investigation.

SUMMARY

The kinetics of the hemolytic reaction effected by the hemolysin of *Clostridium septicum*, strain 44, has been studied with regard to the effect of concentration, temperature, and hydrogen ion concentration on the rate of the hemolytic reaction. The kinetics of hemolysis was found to resemble in several respects that of enzyme-catalyzed reactions, but differed in the absence of a clearly defined pH optimum. Attention is drawn to differences between the hemolytic system studied and certain other hemolytic systems.

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