THE MECHANISM OF THE INHIBITION OF HEMOLYSIS

V. INHIBITORY PROCESSES OCCurring IN THE COURSE OF SIMPLE HEMOLYTIC REACTIONS

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Most investigations on the kinetics of hemolysis have been based on the idea that the lysins enter into irreversible combination with components of the red cell membrane, and imply that the lytic reaction is accompanied by a diminution in the quantity of free lysin present in the system. Attempts at expressing the velocity of hemolytic reactions accordingly contain, in one form or another, the assumption that lysis occurs when a certain amount of a cell component is transformed into a new compound as a result of a reaction with a quantity of lysin $x$, and so are concerned with the form of an expression of the type

$$\frac{dx}{dt} = f(c - x)$$

in which $c$ is the initial concentration of lysin. The term $(c - x)$ is the amount of free lysin in the system, and this is supposed to decrease as the reaction between lysin and cell component proceeds, so that it stops, with $t = \infty$, when $x = c$. Usually lysis is complete long before this happens, but the time for complete lysis is infinite in a system with an initial concentration of lysin equal to some particular value of $x$ associated with complete hemolysis, and this concentration corresponds to the asymptote of the time-dilution curve. Now there are two points, essential to this theory, which are not satisfactorily settled. The first is whether there is an accumulation of lysin at the red cell surfaces, in which case the concentration of lysin to be considered in the kinetics of the systems would be some function of $c$ rather than $c$ itself, and the second is whether the concentration of lysin falls during the hemolytic process as the theory demands.

1. If an accumulation of lysin takes place at the red cell surfaces, it ought to be possible to demonstrate it by separating off the bulk phase immediately after the cells are added to the lysin in such concentrations as do not produce lysis in the short time necessary for the separation; the quantity of lysin present in the separated bulk phase can then be found by adding red cells and measuring the time for their complete hemolysis; $i.e.$, by a hemolytic titration. When this is done, it is found that the concentration of lysin (saponin, digitonin, and the bile salts) in the separated bulk phase is smaller than the initial concentration, and this has been interpreted as evidence for the accumulation of the...
lysins at the red cell surfaces (Ponder, 1934 a). This explanation has now to be reviewed in the light of the possibility that inhibitory substances are present in the fluid in which the cells are suspended or are liberated immediately on the addition of the lysins. When a point like this is involved, it is clearly helpful to have an independent method, in which the measurement of the velocity of hemolytic reactions is not involved, for determining the concentration of lysin in the bulk phase. The results of applying such a method, in which the concentration of lysin is measured colorimetrically, is described in section I of this paper.

2. If the concentration of free lysin becomes less during the course of a hemolytic reaction, it ought to be possible to demonstrate the progressive decrease by separating the bulk phase after the reaction has proceeded for various lengths of time, adding red cells, and finding the time t for their complete hemolysis; this will provide, by reference to a curve showing t as a function of concentration, the concentration of lysin present at the time at which the separation was made. When this is done in the case of a reaction between saponin and stromata, the concentration of lysin in the bulk phase is indeed found to fall with time (Ponder, 1935), although in a manner different from that which is predicted on the basis of the simple reaction of expression (1). The demonstration of this progressive fall in lysin concentration once seemed to be good evidence for the correctness of the underlying idea that the hemolytic process uses up lysin, but now, in the light of what is known about the reactions between lysins and inhibitors, we have to consider the possibility that the progressive decrease in the concentration of active lysin is due to the progressive addition to the system of inhibitory substances derived from the stromata. Again one would think that the situation would be clarified if we could measure the concentration of the lysin in the bulk phase by a method which does not depend on a hemolytic reaction, such as a color reaction. The results of applying such a method are described in section II of this paper, and while this approach to the problem does not simplify the situation as much as might be hoped, it leads to a clearer understanding of the processes involved.

I. The Supposed Accumulation of Lysins at Red Cell Surfaces

The question as to whether a lysin accumulates at the surfaces of red cells as soon as they are added to it, so that the effective concentration in the neighborhood of the cell surfaces is not the concentration $c_i$ added, but a function of $c_i$, can be answered unequivocally in certain cases by an experiment of the following type, in which the quantity of lysin present in the bulk phase of a hemolytic system, after the addition of red cells and their subsequent rapid removal, is determined both directly by colorimetry and indirectly by a measurement of its hemolytic activity. It is necessary for this type of experiment that the lysin shall give a color reaction which is a function of its concentration.
and which is not interfered with by substances such as cholesterol, proteins, etc., in the concentration in which they occur in the bulk phase, and that the concentrations of lysin used shall lie between certain limits: they must not be so great as to produce rapid lysis in step (a) of the procedure, nor must they be so small that they cannot produce lysis in step (b). These conditions are met by sodium taurocholate in dilutions of 1 in 1000 to 1 in 3000, so the procedure will be described as carried out with this lysin.

(a) Preparation of Systems.—A suspension of the thrice washed red cells of 2 ml. of human blood in 40 ml. of 1 per cent NaCl is prepared. To four tubes are added 2 ml. of sodium taurocholate in dilutions of 2.5 in 1000, 2.5 in 1500, 2.5 in 2000, and 2.5 in 3000 in phosphate buffer at pH 6.5, and 2 ml. of saline (1 per cent NaCl). The four tubes are cooled to 4°C. in ice water, and about 8 ml. of the red cell suspension is cooled at the same time. As quickly as possible, 1 ml. of the cold cell suspension is added to each of the four tubes, which are then immediately transferred to a centrifuge and spun at 5000 R.P.M. for 1 minute. The supernatant fluids, which should show no trace of either red cells or free hemoglobin, are transferred to four empty tubes without delay, the cells being discarded. The remainder of the cooled cell suspension is centrifuged at the same speed, and the supernatant fluid is removed and kept for step (d), below.

(b) Colorimetric Estimation of Lysin.—To four empty boiling tubes is added 0.5 ml. of sodium taurocholate diluted 1 in 1000, 1 in 1500, 1 in 2000, and 1 in 3000 in phosphate buffer, and to another four boiling tubes 0.5 ml. of the four supernatant fluids obtained in step (a). To each boiling tube is added 0.5 ml. of saline and 1 ml. of freshly prepared 0.9 per cent furfural in water and 6 ml. of 16 N H$_2$SO$_4$ (Reinhold and Wilson, 1932). After the contents have been mixed, the eight tubes are placed for 8 minutes in a water bath at 70°C. They are cooled in running water, and the intensity of color in each is determined with a Lumetron photometer at 6400 Å. The furfural and acid alone give a small blank which is subtracted from the readings.1

(c) Hemolytic Titration of Lysin.—Hemolytic systems containing 1.6 ml. of the supernatant fluids from the four tubes containing red cells and lysin (step (a), above) are completed by adding 0.4 ml. of red cell suspension, and the times for complete hemolysis at 37°C. are determined. A standard time-dilution curve for systems containing 0.8 ml. of various dilutions of taurocholate in phosphate buffer, 0.8 ml. of saline, and 0.4 ml. of suspension is plotted at 37°C., and reference to this, together with a division by 5/4 to allow for the additional dilution of the lysin in the systems prepared in step (a), gives the quantities of lysin present in the supernatant fluids.

1 The standards and the unknowns should be heated simultaneously, and in every experiment the values for the standards should be found and used as applying to that experiment only. The supernatant fluid from the red cell suspension gives a color value slightly in excess of that of the furfural-H$_2$SO$_4$ blank; this is due to small amounts of chromogenic substances (not the bile salts) in the supernatant fluid, and their contribution to the total color has to be allowed for (see footnotes 2 and 3). The method is sensitive enough to detect a difference between $c_1$ and $c_2$ amounting to about ± 2 per cent of $c_1$. 

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(d) Hemolytic Titration of Inhibitory Material.—To 0.8 ml. of various dilutions of taurocholate in phosphate buffer is added 0.8 ml. of saline and 0.4 ml. of the supernatant fluid obtained from the red cell suspension in step (a). After standing at 37°C. for 60 minutes to allow the inhibitory material and the lysin to react, the hemolytic systems are completed by adding 0.4 ml. of red cell suspension, and the times for complete lysis at 37°C. observed. Comparison with a standard time-dilution curve obtained at 37°C. for systems containing 0.8 ml. of various dilutions of lysin in phosphate buffer, 1.2 ml. of saline, and 0.4 ml. of cell suspension enables the quantities of lysin rendered inactive by the inhibitory material to be calculated.

<table>
<thead>
<tr>
<th>Initial lysin in bulk phase</th>
<th>Lysin in bulk phase after contact with cells</th>
<th>Effect of inhibitory material</th>
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<tbody>
<tr>
<td>( \delta_1 )</td>
<td>( \delta_2 )</td>
<td>( \gamma_2 )</td>
</tr>
<tr>
<td>1000</td>
<td>2000</td>
<td>1950</td>
</tr>
<tr>
<td>1500</td>
<td>1333</td>
<td>1330</td>
</tr>
<tr>
<td>2000</td>
<td>1000</td>
<td>1020</td>
</tr>
<tr>
<td>3000</td>
<td>667</td>
<td>670</td>
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Values of \( \gamma \) and of \( \Delta \) are in \( \gamma \) throughout.

The results obtained in a typical experiment with sodium taurocholate as the lysin are shown in Table I. The first two columns show the initial dilution \( \delta_1 \) and corresponding concentration \( \delta_1 \) in \( \gamma/2 \) ml. The third and fourth columns show the quantity of lysin present in the bulk phase after rapid separation from the cells, and as measured by a colorimetric estimation (\( \gamma_2 \)) and by a hemolytic titration (\( \gamma_1 \)). The decrease in the free lysin in the bulk phase can be expressed either as \( R = \gamma_1/\gamma_2 \), or as \( \Delta_1 = \gamma_1 - \gamma_2 \). The last two columns show first the concentration of active lysin, \( \gamma_1 \), in the systems containing inhibitory material derived from the cells (step (d), above), and then the quantity of lysin rendered inert by the inhibitory material, \( \Delta_4 = \gamma_1 - \gamma_4 \).

Table II shows the results of an experiment carried out in exactly the same way, but with sodium glycocholate as the lysin.

The following conclusions can be drawn from the results in Tables I and II. (1) There is no evidence of an accumulation of the lysin at the red cell surfaces, since colorimetric determinations show that \( \gamma_2 = \gamma_1 \), substantially. (2) The hemolytic activity of the lysin in the bulk phase, after contact with the cells, is reduced so that the value of \( \gamma_1/\gamma_2 \) is about 1.56. This is the same result as was obtained in earlier experiments (Ponder, 1934 a), when it was interpreted as showing that the lysin accumulates at the cell surfaces, so that \( \gamma_2 = c_0(1 - \gamma) \), where \( 1 - \gamma = 1/R \). (3) Part of the reduction in the hemolytic activity of the bulk phase is due to the presence of inhibitory material derived...
from the cells and present in the fluid in which they are suspended. In the experiment in Table I about one quarter, and in the experiment in Table II a larger fraction, of the reduction of lytic activity in the bulk phase can be accounted for in this way. (4) The remainder of the reduction in the lytic activity in the bulk phase must be due to some reaction over and above that between the lysin and the inhibitory material present in the fluid in which the cells are suspended. The possible nature of this reaction will be discussed below.

The same type of experiment can be carried out in systems containing larger numbers of cells. A suspension of washed red cells with a volume concentration as high as 0.3 is prepared, and to 2 ml. of this is added 2 ml. of lysin (taurocholate or glycocholate) in various concentrations, at 4°C. The cells are centrifuged off at

<table>
<thead>
<tr>
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<th>Effect of inhibitory material</th>
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<tbody>
<tr>
<td></td>
<td>$c_1$</td>
<td>$c_2$</td>
</tr>
<tr>
<td>1000</td>
<td>2000</td>
<td>2020</td>
</tr>
<tr>
<td>1500</td>
<td>1333</td>
<td>1350</td>
</tr>
<tr>
<td>2000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>3000</td>
<td>667</td>
<td>660</td>
</tr>
</tbody>
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Values of $c$ and of $A$ are in $\gamma$ throughout.

once, and the concentration of bile salt in the supernatant fluids is found both colorimetrically and by a hemolytic titration. As before, $c_4$ will be found to be substantially the same as $c_1$. Because of the larger number of cells in the systems, the inhibitory effects are very pronounced, and it is usually impossible to measure $c_4$, the concentration of active lysin in the supernatant fluids, because lysis takes so long to complete, but $c_2$ is certainly much less than $c_1$, and at the same time considerably greater than $c_4$, the concentration of active lysin present in systems containing the supernatant fluid from the cell suspension.

The only value attached to experiments in which the volume concentration is large is that such experiments show that the bile salts do not accumulate measurably at the red cell surface even when it is very extensive; they also provide the incidental information that the bile salts do not enter unhemolyzed cells to any measurable extent.

The same type of experiment as that referred to in Tables I and II can be carried out with saponin as the lysin, but in this case the color in step (b) of the procedure is developed with the Mecke reagent (Kobert, 1887; Kofler, 1927).
This reagent is made by dissolving 1 gm. of selenious acid in 200 ml. of concentrated H₂SO₄, and is used by adding 6 ml. of the reagent to 3 ml. of saponin in saline in dilutions from about 1 in 1000 to 1 in 10,000. A brown color develops almost immediately. The intensity of color is measured with a Lumetron photometer at 5300 Å, and this must be done within 2 minutes; in practice, the measurement is made as soon as the bubbles which result from the addition of the reagent to the saponin solution have disappeared. An opalescence, followed by a cloudiness and finally by the formation of a reddish-brown precipitate, appears if one waits longer and if the saponin is sufficiently concentrated. Beer’s law is followed only over a small range and in dilute solutions, as is shown by the following relation between dilution and log I: 1 in 6,400, 9; 1 in 3,200, 18; 1 in 1,600, 32; 1 in 1,200, 46; in more concentrated solutions the opalescence and precipitate appear. The reagent is not specific for the saponins, although different saponins develop different colors with it.

**TABLE III**

<table>
<thead>
<tr>
<th>Initial lysin in bulk phase</th>
<th>Lysin in bulk phase after contact with cells</th>
<th>Effect of inhibitory material</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>c₁ Colorimetric estimation c₂ Hemolytic titration R ΔΔₐ c₄</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>1000</td>
<td>970 910 1.10 90</td>
</tr>
<tr>
<td>4000</td>
<td>500</td>
<td>505 455 1.14 45</td>
</tr>
<tr>
<td>6000</td>
<td>333</td>
<td>330 300 1.11 33</td>
</tr>
<tr>
<td>8000</td>
<td>250</td>
<td>255 220 1.14 30</td>
</tr>
</tbody>
</table>

Values of c and of Δ are in γ throughout.

and small color values are obtained when it is added to dilute plasma or the supernatant fluids of cell or stroma suspensions; this means that suitable blanks must be set up along with the standards and unknowns.²

Table III shows the result of a typical experiment carried out in the same way as the experiments on which Tables I and II are based, except that sap-

²Because Beer’s law is followed only over short ranges of dilution, it is doubtful whether the color values for these blanks can be subtracted from the color values of the unknowns in the usual way to give the final values, unless the blank values are quite small. A similar difficulty is encountered in connection with the furfural determinations. For example, in Table IV a 1 in 3000 taurocholate gives a value for log I of 19, a 1 in 10 serum a value of 12, and the mixture of the two a value of 30; the mixture would thus seem to contain (30 - 12)/19, or 95 per cent of the lysin actually present. The general effect of subtracting the serum blanks is to make the lysin apparently present less than that really present, but this causes trouble only when the blanks have a color value of more than 10 per cent of the color value of the unknown. Even when these sources of uncertainty are taken into account, however, the colorimetric determinations in Tables I, II, III, V, and VI with furfural and the Mecke reagent are sufficiently good to make it certain that c₂ is substantially the same as c₁, and that c₃ is much greater than c₂.
onin is used as the lysin instead of the bile salts. The contents of Table III are arranged in the same way as those of Tables I and II.

The results of experiments of this type, with saponin as the lysin, are substantially the same as those of the experiments in which the lysin is sodium taurocholate or sodium glycocholate, except that the $R$-values tend to be smaller. The conclusions of this section accordingly are: (1) The suspension medium of a thrice washed red cell suspension contains inhibitory substances which render inert a small quantity of lysin, $\Delta_4$, so far as its hemolytic effect is concerned. (2) On the addition of the lysin to the cell suspension, a further quantity of lysin is rendered non-hemolytic within the short time necessary for the separation of the cells from the bulk phase of the system, and before any lysis takes place. This quantity ($\Delta_4$) is several times greater than $\Delta_4$. (3) The colorimetric measurements show that the quantity of chromogenic material in the bulk phase, after contact with the cells, is substantially the same as that present initially, and that no appreciable quantity of the lysin

The observation that the concentration of taurocholate or glycocholate $c_3$ found colorimetrically in the bulk phase, after contact with the cells, is substantially the same as the initial lysin concentration $c_4$ although a considerable quantity of the lysin in the bulk phase is non-hemolytic, leads to the conclusion that lysin combined with the inhibitory material of the system is as chromogenic in the furfural reaction as is free lysin. This important conclusion can be arrived at in another way by showing that such inhibitory substances as cholesterol and the serum proteins do not interfere with the color reaction. This is done in Table IV, which shows the quantity of taurocholate, as a percentage of the quantity added, which is found by the furfural color reaction in the presence of cholesterol, serum albumin, and serum itself. The quantity of cholesterol added is equimolar with the quantity of taurocholate, the amount of serum added such as to give dilutions of 1 in 20, 1 in 100, and 1 in 200 in the serum-taurocholate mixture, and the amount of serum albumin added such as to give a concentration of 0.25 per cent in the albumin-taurocholate mixture.

<table>
<thead>
<tr>
<th>TABLE IV</th>
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<tr>
<td>Taurocholate 1 in Serum, 1/10 Serum, 1/50 Serum, 1/100 Cholesterol, equimolar Serum albumin, 0.25 per cent</td>
</tr>
<tr>
<td>1000</td>
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The only important interference with the development of color occurs when serum, diluted 1 in 10, is present, and here the interference becomes greater as the concentration of taurocholate becomes less. The interference with color production is
initially present accumulates in increased concentration at the red cell surfaces.  

II. The Utilization of Lysin by Stromata

The problem as to whether lysin is used up during the hemolytic process is seriously complicated by the fact that the lysis of red cells results in the liberation of inhibitory material (hemoglobin and intracellular substances). These substances are present in such large amounts in completely hemolyzed hemolytic systems that they interfere with the colorimetric determinations of both saponin and the bile salts, while if a hemolytic titration is used to determine the quantity of lysin remaining, there is difficulty in deciding whether the smaller amount of active lysin found is the result of its having been used up in the lytic reaction or to its having been rendered inert by liberated inhibitors.

To simplify the situation, the reaction between lysins and red cell ghosts (stromata) can be studied instead of that between lysins and the red cells themselves, and in this way some of the difficulties associated with the liberation of hemoglobin are avoided. In saponin-stroma systems, indeed, the effects of inhibitory material are very small, and so these systems are ideal in so far as those aspects of the problem which can be elucidated by hemolytic titrations are concerned. Unfortunately, colorimetric measurements of lysin concentrations are not satisfactory in saponin-stroma systems. Colorimetric measurements are excellent, on the other hand, in bile salt-stroma systems, although here the interpretation of the results of hemolytic titrations are rendered negligible, however, when serum in dilutions greater than 1 in 50, equimolar cholesterol, or 0.25 per cent serum albumin are present in the systems containing the lysin in concentrations from 1000 γ/ml. to 250 γ/ml. This means that taurocholate, largely combined with cholesterol or serum proteins in such a way as to be non-lytic, is as chromogenic in the furfural reaction as is hemolytically active taurocholate. Similar results are obtained with sodium glycocholate.

Differences in the inhibitory effect of serum are observed when either the number or the kind of red cell used in a hemolytic system is varied, or when the pH is changed, and I have tried to account for this in terms of a competition for lysin between the inhibitor and the cell surfaces (Ponder, 1945a, b). This competition is supposed to result from the lysin-inhibitor combination being partially reversible, and to lysin accumulating at the surfaces of different numbers and kinds of red cells to different extents. The greater the tendency (measured by γ) for the lysin to accumulate at the cell surfaces, the smaller the amount bound in the lysin-inhibitor combination; i.e., the less the inhibition produced by a given amount of inhibitor. It now seems clear that the proper explanation for the effects of changing the number or kind of red cell, or of changing the pH of the system, does not depend upon differences in the tendency of the lysin to accumulate at the red cell surfaces. Some other explanation is required.
somewhat equivocal by the relatively large effects of inhibitory substances present in the fluid in which the stromata are suspended. Taken together, however, the observations which can be made in saponin-stroma systems and those which can be made in bile salt-stroma systems enable us to form a fairly clear picture of what happens to the lysin.

The experimental procedure is very similar to that described in section I. A suspension of the stromata of thrice washed human red cells is made by hemolyzing the cells with water, bringing the pH of the hemolysate to about 5.5 by bubbling CO₂ through it, and throwing down the ghosts by gentle centrifuging. The ghosts are washed, with very slow centrifuging, with saline saturated with CO₂, and a suspension of stromata, equivalent to a standard red cell suspension, is prepared as already described (Ponder, 1934 a).

To 2 ml. of various dilutions of lysin are added 2 ml. of saline, and, when the mixture has been brought to the temperature at which the experiment is to be conducted, 1 ml. of the stroma suspension, at the same temperature, is added. The stromata are either thrown down by centrifuging at high speeds immediately, and are thus separated from the bulk phase, or are allowed to react with the lysin for various times before being separated by centrifuging. In either case, the supernatant bulk phase is removed from the layer of stromata, and the concentration of lysin in it is found by a color reaction (as in step (b) of section I, furfural being used to develop the color in the case of the bile salts, and the Mecke reagent in the case of saponin), and by a hemolytic titration similar to that of step (c) of section I. Supernatant fluid from the stroma suspension is obtained at the same time as the lysin-stroma systems are centrifuged, and the inhibition produced by the inhibitory material contained in it is found by a procedure similar to that described in section I, step (d).

Results obtained in a typical experiment in which stromata react with sodium taurocholate are shown in Table V. Here \( c_1 \) (the initial concentration of lysin in \( \gamma/2 \) ml.), \( c_2 \) (the concentration of lysin found in the supernatant fluid by the color reaction), \( \Delta_4 \) (the quantity of lysin rendered inert as found by the hemolytic titration), and \( \Delta_4 \) (the quantity of lysin rendered inert by the inhibitory material in the suspension medium of the stroma suspension) have the same meanings as in Tables I, II, and III. Table VI shows similar results for an experiment in which the lysin is saponin; unfortunately, it is possible in this case to measure \( c_2 \) only when \( c_1 \) is 200\( \gamma \), for if \( c_1 \) is smaller the color developed with the Mecke reagent is too small to allow of a satisfactory determination being made in view of the dubiety which exists as regards the blanks (see footnote 2).

A suspension of stromata which is prepared so as to be equivalent to a standard red cell suspension as regards its effectiveness in reacting with lysin in one dilution is not exactly equivalent when reacting with lysin in other dilutions.
By taking the results contained in Tables V and VI together, the conclusions of this section are: (1) The suspension medium of a stroma suspension contains inhibitory substances which render a quantity of lysin, $\Delta_4$, inert so far as its hemolytic effect is concerned, and this quantity is considerably greater when the bile salts are used as lysins than when the lysin is saponin. (2) When lysin is allowed to react with a stroma suspension, a much greater quantity of lysin $\Delta_4$ is rendered non-hemolytic, the quantity depending on the length of time during which the reaction takes place (see Ponder, 1935, for extensive time-concentration relations for systems containing saponin and stromata). (3) Colorimetric measurements show that the concentration of chromogenic material in the bulk phase of the lysin-stroma systems at the end of several hours of reaction is substantially the same as it was initially, before the reaction began.

### DISCUSSION

The first observation to be accounted for is that the concentration of lysin in the bulk phase of a lysin-stroma system, as determined colorimetrically, is substantially the same as the initial concentration, even though a reaction between the lysin and the stromata has continued for hours, and though a large fraction of the lysin in the bulk phase can be shown to be inert as regards its hemolytic properties. This would be so if the lysin molecules were as chromogenic after they had reacted with the cell components as before the
reaction, and if the reaction were not to involve the lysin molecules being bound to the portion of the cell or of the ghost which remains and can be separated after hemolysis, or stromatolysis, has occurred. Such a picture of what takes place during the "fundamental reaction" of hemolysis, involving the interaction of the lysin molecules of the bulk phase with components of the cell membrane (and perhaps with more deeply placed components as well) to form lysin-stroma compounds or complexes, and the subsequent diffusion of these compounds or complexes outward into the bulk phase in which they appear as chromogenic but non-lytic, has many similarities to Schulman and Rideal's picture of what happens in the process of film penetration (Schulman and Rideal, 1937 a, b). Other possibilities are that the amount of lysin utilized is so small (less than 2 per cent of \( c_t \)) that its disappearance cannot be measured by the color reactions employed, or even that there is no utilization at all. These alternative explanations, which would call for a completely new theoretical treatment, and which lead to great difficulties in accounting for the relation between the velocity of hemolysis and the concentration of lysin, will be referred to again below.

The question which next arises is whether the disappearance of the quantity \((\Delta_a - \Delta_t)\), occurring within a minute of the addition of the lysin to the cells (Tables I, II, and III) can be accounted for as being a utilization of lysin. An attempt can be made at answering this question by comparing the values of \((\Delta_a - \Delta_t)\) for different values of \( c_t \) with the values of \( x \), calculated for \( t = 1 \) minute from the expression which describes the experimental data best on the assumption that a reaction between lysin and cell components is really involved. The expression

\[
K_t = \frac{p}{p - 1} \left\{ c \frac{p-1}{n} - \left( c - x \right) \frac{p-1}{n} \right\}
\]

derived from

\[
\frac{dx}{dt} = K(c - z)^n
\]

with \( p = 1/n \), is one which describes the experimental values for time-dilution and percentage hemolysis curves excellently, even if empirically, and so, as a specific instance, we can calculate \( x \) for various values of \( c_t \) and for \( t = 1.0 \),

\(^8\) There seems to be no reason in the meantime why the processes of hemolysis and stromatolysis should not be thought of side by side with the process of penetration and breakdown of mixed protein-lipoid film, each kind of process throwing light on what happens in the other. Questions of mechanism are more likely to be settled by studying film penetration, and questions of kinetics by studying hemolytic and stromatolytic processes. While the kinetics of penetration have not been fully worked out, it is probable that they will require the same kind of equations as have been developed in connection with hemolytic systems, and that the same kind of difficulties, both mathematical and physical, will make their appearance once more.
using \( n = 1.5 \) and \( K = 0.0012 \), these being the values which give the best fit to the time-dilution curve for sodium glycocholate at 4°C., with its asymptote at \( c_\infty = 500 \). The resulting values of \( x \) can then be compared, in Table VII, with the values of \( (\Delta_4 - \Delta_1) \) derived from Table II.

A comparison of the calculated values of \( x \) and of the experimental values of \( (\Delta_4 - \Delta_1) \) shows that some occurrence, not recognized in the simple theory on which expressions (2) and (3) are based, takes place in the hemolytic system within a short time (1 minute, and perhaps much less) of the addition of the lysin to the red cells. The nature of this occurrence is still an open question, for it might be either (a) a rapid utilization of lysin, or (b) a rapid giving-off of inhibitory material from the cell surfaces into the bulk phase of the system. It should be emphasized, however, that it is not simply the fixation of lysin molecules which precedes film penetration, for \( c_\alpha \), measured colorimetrically, is substantially the same as \( c_\beta \); this means that no appreciable amount of lysin is fixed to the material of the cell in such a way as to be carried down when the cells are separated by centrifuging.

If these two possibilities exist as regards the values of \( (\Delta_4 - \Delta_1) \) in Tables I, II, and III, the same two possibilities exist as regards the values of \( (\Delta_4 - \Delta_1) \) in Tables V and VI. Here the large amount of lysin which disappears after a 4 hour reaction between lysin and stromata, in excess of the amounts which can clearly be accounted for by the inhibitory effects of material in the stroma suspension medium, can also be the result of (a) utilization of lysin with a diffusion of the non-lytic but chromogenic lysin-stroma compounds back into the bulk phase, or (b) the giving-off of inhibitory material from the stroma into the bulk phase and the formation of non-lytic but chromogenic compounds with the free lysin there. It would be helpful if we could make a calculation, similar to that upon which the data of Table VII are based, show-

It is possible that the red cell as a whole represents a system which is in equilibrium with its environment, and that the addition of lysin results in a sudden transfer of material from the cell phase to the bulk phase so as to satisfy some new equilibrium conditions. One instance in which something of this sort occurs is when red cells in saline are placed between closely applied glass surfaces; the "antisphering substance," a protein, then leaves the cells to be adsorbed on the glass, and the cells become spheres (Furchgott, 1940; Furchgott and Ponder, 1940).
ing the quantity of lysin which would be expected to be utilized at the end of a 4 hour reaction period between lysin and stromata, and if we could show that the values of \((A_0 - A_4)\) in the last two columns of Tables V and VI are greater than the calculated values of \(x\); the excess would then represent the result of an inhibitory process. It has been shown, however, that although the use of expressions (2) and (3) results in a good, if empirical, description of the velocity of hemolysis, the same expressions cannot be used to describe the subsequent process of stromatolysis (Ponder, 1935). As a result, we have no means of knowing to what extent a process of utilization, or alternatively a process of inhibition, is responsible for the value of \((A_0 - A_4)\) in the last two columns of Tables V and VI. The explanation of the differences \((A_0 - A_4)\) in the third and fourth columns of these tables presents the same problem as the explanation of the differences \((A_s - A_4)\) in Tables I, II, and III; here again we have no way of deciding whether the differences are the result of a process of utilization or of a process of inhibition.

The essence of the difficulty as regards the kinetics of hemolysis thus seems to be that we have to write

\[
\frac{dx}{dt} = f(e - x - \Delta)
\]

instead of expression (1), without being able to decide on the relative values of \(x\) and of \(\Delta\). In one extreme case the inhibitory effects would be negligible or absent, and we would have an expression such as expression (1), which, in one form or another (e.g. expressions (2) and (3), and the expressions which result from putting \(A = Bx\), where \(B\) is a constant\(^8\)) has been the basis of my treatment of the kinetics of hemolysis up to now (Ponder, 1934 b). In the other extreme case, \(x\) would be zero, and there would be no reaction involving a utilization of lysin. If this were the true state of affairs, colorimetric methods would certainly not reveal any disappearance of lysin, for there would be none, and we would have to account for the fact that hemolysis takes infinite time in a concentration \(c_n\) by saying that all the lysin in this concentration is rendered inert by inhibitory material. We would then have to develop some completely new theory, in which a reaction between lysin and cell components was not involved, in order to account for the experimental observation that the velocity of hemolysis is a function of lysin concentration. It may remain a possibility that no utilization of lysin occurs during the hemolytic process.

\(^8\) The inhibition term \(\Delta = Bx\) or \(Bx\) is introduced into the equation for the fundamental reaction of hemolysis in order to take account of the inhibitory effect of the hemoglobin liberated as the cells hemolyze. In a complete theory, it would be required in addition to the inhibitory terms discussed in this paper, which deals only with the events which occur on the addition of lysin and before lysis begins, and the events which occur in virtually hemoglobin-free lysin-stroma systems.
but, if so, there is no indication in the meantime as to the form which a new theory might take.  

The intermediate case of expression (4), in which neither \( x \) nor \( \Delta \) are negligible, still seems to be the one which describes the greatest number of the phenomena observed in hemolytic systems, although it must be admitted that no specific function of \( (c - x - \Delta) \) which has been proposed so far has proved capable of describing the phenomena in their entirety. Part of the reason for this is evidently that earlier attempts at formulation did not recognize the extent to which the course of hemolytic reactions is influenced by inhibitory processes. In addition to this, it is probably an oversimplification to look upon hemolysis as the escape of fluid cell contents through a thin membrane the integrity of which is broken down by the lysin as a result of a process of film penetration, or in some other way. This picture of what happens may have to be replaced by a more complex one as our information regarding red cell structure becomes more complete.  

The mass of the "fixed framework" of the cells in a standard hemolytic system is about 100 to 200 \( \mu \). It is not known how much of the fixed framework corresponds to the structures which break down in the process of hemolysis, but lysis by quantities of lysin negligible in comparison to the asymptotic concentrations would have to be the result of changes initially occurring in quite a small fraction of the framework. Once initiated, the effects might of course spread to adjacent parts of the structures involved. More specifically, assuming lysis to be the result of a breakdown of a cell membrane, 1 \( \gamma \) of lysin or less would have to be able to destroy the integrity of membranes with a minimum mass of 30 \( \gamma \). This is certainly a possibility, for the cell surfaces may break down in spots (Ponder, 1941), or each \( \gamma \) of lysin may be able to produce disorganization of a mass of material much greater than itself, whether this material is present as a membrane, or as an intracellular structure to which hemoglobin is attached.  

When the rat red cell changes from its normal form to its paracrystalline form (Ponder, 1945 c), the amount of saponin or of digitonin required to hemolyze it increases several fold, probably because more than the breaking down of a thin membrane is required for the liberation of the hemoglobin. The degree of complexity of the expressions required to describe the process will depend, of course, on the complexity of the red cell structure which has to be postulated; as soon as we pass from the simple model of a bag containing fluid enclosed in a paucimolecular membrane towards a model in which the hemoglobin is bound to an intracellular material and surrounded by a membrane varying in structure from point to point, we need to use equations developed on the basis of a three-dimensional geometry instead of simple expressions, such as (1) and (4), which apply to spatially homogeneous systems. This spatial element in the treatment of the kinetics may turn out to be necessary; for example, if disorganization of a hemoglobin-stromatin complex in the cell interior were a necessary part of the hemolytic process, a lysin would act more effectively when present in sufficient concentration to enable it to enter the cell from all over the surface than when it could enter the cell only through holes resulting from
This paper is concerned with hemolytic systems containing sodium taurocholate, sodium glycocholate, or saponin, and either human red cells or the ghosts (stromata) of human red cells. The lysins are allowed to react with the cells for a short time (1 minute or less), and with the ghosts for a long time (4 hours), and the quantity of lysin remaining in the bulk phase, after the removal of the cells or of the ghosts, is found by (a) colorimetric methods, and (b) methods which measure its hemolytic activity.

In the experiments in which the lysins react with the cells for a time so short that none of them is hemolyzed, it is found: (1) that the suspension medium of a cell suspension contains inhibitory substances which render a small amount of the lysin non-lytic, (2) that on the addition of the lysin to the cell suspension, a further and much larger amount of lysin is rendered non-lytic, and (3) that the quantity of chromogenic material in the bulk phase, after the lysin has been in contact with the cells and the latter have been removed, is substantially the same as that initially present. No appreciable quantity of lysin, accordingly, accumulates in increased concentration at the cell surfaces. The results of the colorimetric determinations show that the apparent disappearance of lysin from the bulk phase, once thought to be due to an accumulation of lysin at the cell surfaces, is the result either of an inhibitory process or of a sudden utilization of lysin unrecognized by existing theory.

In the experiments in which the lysins react with stromata for 4 hours, it is found: (1) that the suspension medium of a stroma suspension contains inhibitory substances which render some of the lysin non-lytic, (2) that when the lysin reacts with the stromata over a period of time, a much greater quantity of lysin is rendered non-lytic, and (3) that the concentration of chromogenic material in the bulk phase of the lysin-stroma system, after 4 hours of reaction, is substantially the same as it was initially. The observations can be accounted for by supposing that the lysin molecules are as chromogenic after reacting with the cell components as before, and by their not being bound to the cell or to the ghost, but diffusing back, combined with the components with which they have reacted, into the bulk phase. Such a process would have similarities to the process of penetration and breakdown of mixed protein-lipoid films. Because it is not possible at present to decide how much of the lysin is rendered inert because of utilization in a reaction with cell components, and how much because of the effect of inhibitory substances, difficulties and uncertainties arise in connection with the expressions which have been used to describe the kinetics of hemolysis.

The breakdown of the surface in spots. In this case, the difference in effectiveness would probably be reflected in a decrease in a velocity constant with decreasing lysin concentration.
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