THE KINETICS OF PROGRESSIVE REACTIONS IN SYSTEMS CONTAINING SAPONIN, DIGITONIN, AND SODIUM TAUCHELOCATE* 

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It has recently been pointed out (Ponder and Cox, 1952) that certain lysins, among which are saponin and digitonin, may initiate a process in the red cell which is not adequately described by the terms reversible and irreversible, but rather by the term progressive. Such reactions cannot easily be stopped once they are well under way. There is no a priori reason for thinking that the kinetics of this new type of reaction are more likely to be of one form rather than of another, and so it is necessary to determine what they are by the direct experiments to be described in this paper.

Method

The washed cells of 4 ml. of heparinized human blood are suspended in 20 ml. of NaCl-buffer at pH 7. Two calibration curves are constructed, using a model 6A Coleman junior spectrophotometer at a wave length of 6500. The first (calibration curve I) gives the optical densities corresponding to values of percentage hemolysis P of 10, 20, . . . 100 per cent in systems consisting of 1.6 ml. of saline and 0.4 ml. of the suspension. These standards are prepared by mixing various proportions of the suspension and of the suspension after hemolysis by freezing and thawing. The systems are contained in small cuvettes (6-302) held in an adapter (6-100). This calibration curve is concave to the P-axis. The second calibration curve (calibration curve II) gives the optical densities corresponding to the same values of P in a system containing 19.6 ml. of saline and 0.4 ml. of the suspension; i.e., to the same values of P in the first system diluted tenfold. A large cuvette (6-300) with no adapter is used to contain the diluted systems. The relation between P and the optical density is much more nearly linear than it is in calibration curve I, and should be plotted with the density on about four times the scale used for the first calibration curve. Matched cuvettes are used to establish and maintain the spectrophotometer zero.

A series of dilutions of the lysin, in NaCl-buffer, are required. In the case of saponin, a series containing 2.5 times the quantity which is to be contained in 2 ml. of the completed system is prepared, the useful concentrations being 200, 150, 133, 100, 80, 67, 50, 40, 35, and 30 γ per 0.8 ml. The quantity of lysin in the completed system of 2 ml. volume is denoted by c. In the case of digitonin, the useful concentrations are

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50, 25, 20, 15, 12, and 10 *γ* per 0.8 ml. The hemolytic system is prepared by mixing 0.8 ml. of one of the concentrations of lysin with 0.8 ml. of saline in a small cuvette and adding, at zero time, 0.4 ml. of the red cell suspension. The cuvette should be in place in the spectrophotometer.

When the hemolytic system is completed, the galvanometer moves away from its zero to the scale reading for zero per cent hemolysis on calibration curve I. After an interval which depends on the lysin concentration *c*, it begins to travel towards scale readings corresponding to increasing values of *P*. Just before it reaches the scale reading for a value of *P₀* previously decided upon (e.g., *P₀* = 25 per cent hemolysis), the cuvette containing the hemolytic system is emptied into 18 ml. of saline in a large cuvette, the adapter is removed and set aside, and the large cuvette is placed in the light path. All this must be done very quickly. A reading is made at once; it should correspond, very nearly, to *P₀* on calibration curve II. If the reaction is progressive, the galvanometer moves towards its zero; i.e., through scale readings corresponding to larger and larger values of *P* on calibration curve II. The movement is usually relatively rapid at first and then becomes slower. When the rate of movement has become very small, the scale reading corresponds to a value *P₁* on calibration curve II. The difference (*P₁* - *P₀*) is the extent to which the lytic reaction has progressed beyond the value *P₀* or the "gain," and will be denoted by *g*.

Experiments of this kind have two principal sources of error associated with them. The first is that the value of *P₀* on calibration curve II, after the system has been diluted, is not always the same as the value of *P₀* on calibration curve I. This is particularly liable to happen when the reaction is rapid, and the observation should be discarded if the discrepancy is more than about 5 per cent. The second is that the final value *P₁* may be reached very slowly, and this raises the practical question as to how long the observation ought to be continued before the final value is recorded. In digitonin and taurocholate systems in which *P₀* has more than a very small value, *P₁* is always substantially 100 per cent if one waits long enough, but in saponin systems the rate of increase in *P* decreases and a final value of *P₁* can be recorded after 10 minutes, 20 minutes, etc. For practical purposes, it is sufficient to record it 10 minutes after the system has been diluted, and then to set the system aside so that any further increase in *P* can be recorded after several hours.

All the experiments to be described below were carried out at 25°C.


Fig. 1 shows typical results obtained for a fixed value of *P₀*, in this case 50 per cent hemolysis, and for a variety of values of *c*.

If the value of *c* is sufficiently small, the reaction does not progress after the system is diluted, and *g* = 0. As *c* is increased, a value is reached at which hemolysis continues after the system is diluted, and for which *g* is greater than zero. The largest concentration of lysin which can be employed without a progressive reaction occurring after the system is diluted will be called *cₙ₀*. As *c* is increased above this value, *g* increases rapidly at first and then more slowly, until *g* = 100 - *P₀*. This corresponds to 100 per cent hemolysis, the first 50 per cent (in this case) having been produced by the lysin in the original
system of 2 ml. volume and the remaining 50 per cent having been produced, as the result of a progressive reaction, in the system diluted tenfold. The value of c corresponding to complete lysis will be called \( c_{\infty} = (100 - P_0) \).

Between \( c = 0 \) and \( c = (100 - P_0) \), \( g \) is a function of \( c \), and, more specifically, of the difference between \( c \) and \( c_{\infty} \):

\[
g = f(c - c_{\infty})
\]

Except that it is concave to the \( c \)-axis, little can be said about this function at present. One extreme possibility, however, is ruled out, viz. that the lysin is distributed in two phases, one at the red cell surface (an "internal" phase) and the other in the intercellular fluid (a "bulk" phase), and that the addition of the system to 10 times its volume of saline dilutes the bulk phase but leaves the internal phase unaffected. Were this so, \( g \) would be constant and equal to 100 \( - P_0 \), since all the concentrations of lysin above and including \( c_{\infty} \), can eventually produce complete hemolysis in the undiluted system. At the same time, the possibility remains that there is an internal phase and that it undergoes some dilution.

2. Saponin Systems. Limiting Conditions

There are two limits to the family of curves of which the curve in Fig. 1 is one. The first is the asymptotic concentration for the undiluted hemolytic system of 2 ml. volume. This is found in the usual way by finding the smallest value of \( c \) which will produce complete hemolysis in the undiluted system in a
very long time (3 to 6 hours at 25°C.), and is shown in Fig. 1 by the vertical dotted line.

The second is the locus of all the values of \( c_{\text{em}} \).

When the reaction is slow, i.e., when \( c \) is small, this can be found by repeating the procedure in section 1 with increasing concentrations of lysin, until a concentration is found at which the reaction becomes progressive and at which \( g \) is larger than zero. When the reaction is rapid, i.e., when \( c \) is large, it is simpler to keep \( c \) constant and to allow \( P_0 \) to vary. To do this, one completes the hemolytic system of 2 ml. volume, and adds it after an interval \( t \) (in seconds) to the large volume of saline; one then notes the value of \( P_0 \) in the diluted system, and observes whether or not the reaction is progressive. If it is not, the same procedure is repeated but the time interval \( t \) is made a little longer; if it is, the procedure is repeated with the time interval a little shorter. In this way, for any concentration \( c \), one arrives at a small value of \( P_0 \) at which \( g \) is certainly zero, and a slightly greater value of \( P_0 \) at which \( g \) has a small positive value; the largest value of \( P_0 \) for which \( g \) is zero in a system containing the concentration \( c \) is somewhere between the two. When this procedure is repeated for a series of values of \( c \), the locus of the concentrations \( c_{\text{em}} \) can be obtained with a fair degree of accuracy, even although the concentrations are great enough (e.g. 200 \( \gamma/\text{ml.} \)) to bring about rapid hemolysis.

The locus is shown by the curved dotted line in Fig. 2. It will be noticed that the value of \( c_{\text{em}} \) becomes less as \( P_0 \) becomes greater. It cannot become less indefinitely, however, because it can never be less than \( c_{\text{em}} \), the asymptotic concentration for the system. It seems likely, indeed, that when \( P_0 \) is almost 100, \( c_{\text{em}} \) is almost as small as \( c_{\text{em}} \), and the locus of \( c_{\text{em}} \) is represented in Fig. 2 as passing towards this limit although the experimental range does not allow observations in this region to be made.
3. Saponin Systems. $P_0$ and $c$ Both Varying

To determine what happens when $P_0$ and $c$ are both varied, it is necessary to find the course of curves such as the one shown in Fig. 1, but for a number of values of $P_0$. Fig. 2 shows as extensive results as can be obtained in a single experiment, in which the relation between $c$ and $g$ has been found for $P_0 = 80, 60, 30, 20$, and $10$ per cent hemolysis. The position of the asymptotes and the locus of $c_{p=0}$ are shown by the dotted lines.

4. Digitonin and Taurocholate Systems

The relations between $g$ and $c$ are more difficult to explore in systems containing digitonin than they are in systems containing saponin. The experiments show, however, that the diluted systems containing digitonin hemolyze completely, at varying rates, if left long enough, provided that $P_0$ has more than a very small value and provided that the lysin concentration is a little more than asymptotic. In experiments similar to those described by Ponder and Cox (1952, Fig. 2), the dilution of the hemolytic system is followed by progressive hemolysis as in the case of saponin systems. This hemolytic reaction slows down, but does not come to a steady state as rapidly as it does in saponin systems; it continues slowly at a rate which is less as the concentration of lysin is less. Investigation of the rates of hemolysis in undiluted and in diluted systems shows that this slow progressive lysis is largely due to the form of the timedilution and percentage hemolysis curves for digitonin, and that it is not a phenomenon of a new kind.

Systems containing sodium taurocholate behave similarly. Even if $P_0$ is very small, hemolysis in the diluted system proceeds to completion, although at varying rates. One can add 0.4 ml. of the cell suspension to 1.6 ml. of saline containing 150 $\gamma$ of lysin (a little more than the asymptotic concentration of 133 $\gamma$/ml.), dilute the system tenfold within 10 seconds, and yet obtain complete hemolysis if the system is left for an hour or so. By comparison, a system in which 0.4 ml. of red cell suspension is added to 19.6 ml. of saline containing 150 $\gamma$ of lysin shows no lysis at all.

5. Analysis and Discussion

Looked at from the standpoint of existing theory, every value of $P_0$ corresponds to a value of $x$, the quantity of lysin combined with the reactive cell components, the special relation between $P_0$ and $x$ being determined by the form of the frequency distribution of red cell resistances (Ponder, 1948). Those cells whose resistance, measured in units of $x$, is less than the $x$ for the value of $P_0$ under consideration are hemolyzed, while the remainder, which have a resistance greater than the $x$ for $P_0$, remain intact. This divides the cells of the population into two categories. The foregoing experiments show that the unhemolyzed cells can be again divided into two categories, those which hemolyze progressively in the diluted hemolytic system, and those which do not.
There are two altogether different situations which would account for this. The first is that in which the combination of \( x \) molecules of lysin with certain cell surface components has an ultimate effect on neighboring components which depend on the former for their stability; this is the kind of situation suggested by evidence (principally derived from electron microscope studies) that agglutination and lytic phenomena start locally and then spread (Ponder, Bessis, Bricka, and Gorius, 1952). The second alternative is that the system contains an internal phase in which the lysin is less affected by dilution than it is in the bulk phase, and in which the concentration may also be different from that in the bulk phase. It should be noticed that these two hypothetical situations are not mutually exclusive. The first alternative cannot be developed along quantitative lines because it involves an undefined property of the red cell surface, the stabilizing effect of molecules on their neighbors. The second alternative, on the other hand, is relatively easy to put into a quantitative form.

The values for \( g \) shown in Figs. 1 and 2 are those found 10 minutes after the hemolytic system has been diluted with ten times its volume of saline; if one waits for 30 minutes, 1 hour, or longer, the values of \( g \) are a little greater. By experiment it is easy to find a relation between the concentration of lysin \( c \) and the percentage of complete hemolysis at the end of 10 minutes in the undiluted system. This relation is an asymmetric sigmoid curve (Fig. 3 \( a \)), and, if the times were much longer than 10 minutes, existing theory would identify \( c \) with \( x \), the amount of lysin which had entered into combination with the cell components. For this reason, and to distinguish them from the con-
centrations in Figs. 1 and 2, the concentrations on the abscissa of the sigmoid curve will be referred to as \( c \) in what follows. The sigmoid relation shows that to produce hemolysis of all the cells, 33 \( \gamma \) of lysin must be present, to produce 90 per cent hemolysis, 22 \( \gamma \), to produce 80 per cent hemolysis, 17 \( \gamma \), and so on.

Suppose that each cell in the hemolytic system is initially surrounded by a region (an internal phase) in which the concentration of lysin \( c' \) is equal to the initial concentration \( c \) in the entire system. (This simplifies the situation somewhat, for \( c' \) need not become equal to \( c \) until after some time has elapsed.) Further suppose that, when the system is diluted at a moment when the percentage hemolysis is \( P_0 \), \( c \) is diluted tenfold whereas \( c' \) is diluted \( F \)-fold, where \( F \) can have a variety of values about which nothing is known as yet. To get 100 per cent hemolysis in the system, 33 \( \gamma \) of lysin is required, and this will be supplied by a concentration \( c/F = 33 \). Similarly, to get 90 per cent hemolysis, 22 \( \gamma \) is required, and this will be supplied by another concentration \( c/F = 22 \), perhaps with a different value of \( F \), and so on; in fact, the total amount of lysis \((P_0 + g)\) which will occur in any concentration \( c \), after dilution at a moment at which the percentage hemolysis is \( P_0 \), can be found provided we know the amount of lysin \( c_0 \) which corresponds to a given degree of hemolysis \((P_0 + g)\), together with the value of \( F \). For example, if the concentration of lysin in the system is 120 \( \gamma \), if the system is diluted when \( P_0 = 30 \), and if we assume that \( F \) is 8, 120/8 = 15 \( \gamma \), which is not enough to produce 100 per cent hemolysis (this would require 33 \( \gamma \)), but is enough to produce 73 per cent hemolysis (from Fig. 3 a). By referring to Fig. 2, the experimental value is found to be 68 per cent, a reasonably good agreement which depends, of course, entirely on the assumed value of \( F \). If, however, we calculate a series of values of \( F \), by taking a large variety of values of \( P_0, c \), and \((P_0 + g)\), and if we find that \( F \) is constant for each value of \( P_0 \), it will point quite clearly to the internal phase of the system undergoing an \( F \)-fold dilution and to \( F \) being determined by \( P_0 \); i.e., by the extent to which the lysin has reacted with the cells up to the moment of dilution.

The results of the computation are shown in Table I. This table is prepared in the following way. A value of \( P_0 \) is selected, e.g., \( P_0 = 10 \). The concentrations \( c \) which give \((P_0 + g)\) per cent hemolysis are read off from the curve corresponding to \( P_0 = 10 \) in Fig. 2, entries being made for \( g = 0, g = 10, g = 20 \), and so on. The values of \( c \) are divided by the value of \( c_0 \) for \((P_0 + g)\) per cent hemolysis, taken from the sigmoid curve, Fig. 3 a; the values are also given on the bottom line of Table I. The result is \( F \), the extent to which the concentration \( c \) must be diluted in order to give the concentration \( c_0 \) which provides just enough lysin for \((P_0 + g)\) per cent hemolysis. This calculation is repeated for the other curves in Fig. 2, corresponding to \( P_0 = 20, P_0 = 30 \), and so on.

The locus of \( c_{w=0} \) is provided by the concentrations which, when divided by \( c_0 \), give \( P_0 \) itself, \( g \) being zero; thus \( c_{w=0} = Fc_0 \). For example, \( P_0 = 80 \), so \( F = 2 \) (from Table I). \((P_0 + g) = 80 \) when \( g = 0 \); \( c_0 \) for \((P_0 + g) = 80 \) is 17. \( Fc_0 = 34 \) (actually 33).
The values of $F$ in Table I are remarkably constant for any value of $P_0$, considering the size of the experimental errors which can affect them. This gives considerable support to the idea that there is an internal phase which is diluted to an extent determined by $P_0$. It should be noticed that the lysin in this internal phase is active lysin, but that the tenacity with which it is held at the red cell surface is nevertheless determined by $P_0$; i.e., by the extent to which the lytic reaction has proceeded at the moment of the dilution of the system. The relation is shown in Fig. 3 b, and the simplest explanation is that the internal phase becomes more stable as time goes on and as greater numbers of its molecules become attached, irreversibly, to the cell components. It should also be noticed that the values of $F$ corresponding to $P_0 = 10$ and $P_0 = 20$ are greater than 10, although the system is diluted only tenfold. This suggests that the internal phase takes some time to reach the concentration $c' = c$, and that it appears to be excessively diluted if the system is diluted before the concentration of the internal phase has reached that of the bulk phase.1

1 If hemolysis is thought of as the result of a process similar to film penetration, molecules of active lysin can be pictured as becoming more or less reversibly attached to surface components in such a way as to form an internal phase near the red cell surface, and as becoming reoriented at a later stage of the process, when they enter the surface ultrastructure and destroy it. A diagrammatic representation of this has already been given (Ponder, 1948, Fig. 18). At first the active lysin will be anchored more or less reversibly, and the firmness of the anchoring may vary from system to system. Later the attachment will become irreversible. Looked at in this way, the transition from active lysin to combined lysin (as the terms are used in existing theory) need not involve anything more than a change in the spatial arrangement of the lysin molecules with respect to the molecules of the surface ultrastructure.
When the data for systems containing digitonin or sodium taurocholate are treated in the same way, the internal phase appears to be almost unaffected by the dilution of the system, i.e. it is very firmly held at the red cell surface, so that if $c$ is only slightly greater than the asymptotic concentration, $(P_0 + g) = 100$. The value of $F$ is substantially 1.0 throughout. This result, when contrasted with the results obtained for saponin, supports the idea (based on a different kind of evidence) that the attachment of digitonin and sodium taurocholate to the red cell surfaces is firmer than the attachment of saponin (Ponder and Cox, 1952). The “firmness of attachment,” however, refers to the state of the active lysin held in the internal phase rather than to the combination of lysin and cell component which results in lysis, although the two seem to be related. While the latter combination is probably irreversible, the formation of the internal phase appears to be a process which can (in the case of saponin), or cannot (in the case of digitonin), be partially reversed by diluting the system.

These results, although favorable to the existence of an internal phase as the explanation for hemolytic reactions being progressive, do not allow us to set aside completely the alternative explanation that the reaction is progressive because combination between lysin molecules and certain molecules of the cell ultrastructure ultimately renders neighboring molecules unstable. Work in progress with tagged lysins points to the possibility of making direct measurements of the variations in the concentration of a lysin in an internal phase. It is altogether possible that such direct measurements might show that an internal phase actually suffers dilution, but not to the extent which would give the numerical values of $F$ in Table I. In such a situation, both of the alternative explanations for the progressive nature of the reaction might have to be considered simultaneously.

SUMMARY

The relations between lysin concentration, percentage hemolysis at the moment at which the lysin concentration is reduced by dilution, and the amount of hemolysis which follows the dilution as a result of the reaction being “progressive” point to there being an “internal” phase at the red cell surfaces, in which the lysin is less affected by the dilution than in the system as a whole. A second possibility, i.e. that the combination of lysin molecules with certain components of the cell surface has an ultimate effect on neighboring components which depend on the former for their stability cannot, however, be ruled out.

In systems containing digitonin or sodium taurocholate, this internal phase, once formed, seems to be almost unaffected by the dilution of the system; i.e., these lysins are very firmly held at the cell surfaces.
saponin the lysin is less firmly attached, so that dilution of the system affects its concentration appreciably.

REFERENCES