

THE INHIBITION OF HEMOLYSIS, AS STUDIED BY THE
TECHNIQUE USED FOR INVESTIGATING PROGRESSIVE
REACTIONS, AND BY A TECHNIQUE USING
RADIOACTIVE HEMOLYSINS*

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Our understanding of inhibition and acceleration of hemolysis is still in an unsatisfactory state. One possibility is that an inhibitor reacts with a lysin to form a relatively non-lytic compound or complex, as when cholesterol reacts with digitonin to form the almost insoluble cholesterol digitonide. Such a reaction (termed a Δ reaction) would decrease the concentration of lysin in the system, and lysis would be correspondingly slower. Another possibility is that the inhibitor reacts with the red cell surface making it more (or less) resistant to a reaction with the lysin; this possibility is particularly attractive when the system contains, not an inhibitor, but an accelerator, and there are instances in which reactions of this kind (termed R reactions) can be demonstrated directly (Ponder, 1939).

In investigating Δ reactions and R reactions it is usual to mix the lysin with the inhibitor or the accelerator, and then to add the red cells. The times for complete hemolysis in different concentrations of the lysin, without and with the addition of the inhibitor or accelerator, are measured; if a concentration of lysin c_1 to which inhibitor has been added takes the same time to produce complete hemolysis as a smaller concentration c_2 to which the inhibitor has not been added, the amount of lysin rendered inert in a Δ reaction is $\Delta = c_1 - c_2$, while the increase in resistance of the cells in an R reaction is $R = c_1/c_2$. If the values of R are less than 1.0, there is acceleration instead of inhibition; in the case of acceleration of hemolysis, however, Δ has no clear meaning.

This simple method of approaching the problem has been very useful, but it rests on at least three assumptions. The first is that the reaction between the lysin and the inhibitor is substantially irreversible, the second is that the system surrounding the red cells is spatially homogeneous, and the third is that hemolysis is the result of a simple one stage process in which the lysin reacts with components of the red cell structure. For most purposes, the first assumption has seemed to be valid, although there are effects, involving the variation in

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the amount of lysin rendered inert by serum when the number of red cells in the system is varied, which have led to attempts to introduce some degree of reversibility (Ponder and Gordon, 1934). The second and third assumptions are no longer tenable since the demonstration that hemolytic reactions may be *progressive* (Ponder and Cox, 1952, Ponder, 1953), and since the demonstration that there may be a rapid uptake of lysin at the red cell surfaces to form an "internal lysin phase" (Croes and Ruysse, 1951 *a*, 1951 *b*, Ponder and Ponder, 1954). The evidence now points to lysis in some systems containing simple hemolysins being a process involving two stages in time and two lysin phases. The first state in time is the entry of the lysin into an "internal lysin phase" situated at the cell surfaces, a process which may be partially reversible at first, and which may be referred to as "fixation" of the lysin. The second stage is the irreversible reaction of the fixed lysin in the "internal lysin phase" with the cell components, and this is the hemolytic reaction proper.

In view of these developments, it is desirable to investigate inhibitory phenomena by the same methods as have established that lytic reactions may be progressive and that there may be an internal lysin phase in the neighborhood of the red cell surfaces, particularly as such investigations show that there are inhibition-producing mechanisms in addition to those already considered. This paper will deal primarily with the inhibitory effects of human plasma, but some notes on the effects of lecithin, cholesterol, and serum albumin will be added.

Technique

The method used for showing that some hemolytic reactions are progressive consists in allowing the reaction to proceed until a certain percentage, P_0 , of complete hemolysis has occurred, and at this moment diluting the system with sufficient saline (usually 10 volumes) to render the concentration of lysin in the system less than asymptotic. If lysis continues from P_0 to $(P_0 + g)$ in spite of this dilution, the process is progressive and there is a method (Ponder, 1953) by means of which the extent of the dilution of lysin in the internal phase can be calculated; in experiment, the dilution of the system as a whole is usually 10-fold, and the calculation yields the result that the dilution of the lysin in the internal phase is F -fold. Except in some special cases, $F = <10$ in saponin systems; when the lysin in the internal lysin phase is completely unaffected by the addition of the saline, $F = 1.0$ (systems containing digitonin and the bile salts).

Instead of adding saline to dilute the system when P_0 has reached a certain value, an inhibitor can be added, and F can be calculated as before. If the inhibitor is without effect on the lysin in the internal phase, $F = 1.0$, whereas if it reacts with the lysin in the internal phase to produce inhibition there, F may attain considerable values.¹ Except that an inhibitor, instead of a large volume of saline, is added to the

¹ The values reached, however, are never as large as the values of R obtained when plasma is added to the lysin, the cells being added afterwards. There is a cer-

system when P_0 has a certain value (*e.g.*, $P_0 = 20$, $P_0 = 40$, etc.), the technique is similar to that already described (Ponder, 1953). Specifically, the systems consist of 1 ml. of lysin in various concentrations, 0.5 ml. of a standard red cell suspension (1.25×10^8 cells), and inhibitor contained in 0.5 ml. of saline. The quantity of inhibitor is denoted by I , the undiluted material, *e.g.* plasma, being one for which $I = 1.0$; thus, when the inhibitor is plasma, $I = 0.1$ means that the 0.5 ml. of inhibitor added to the system consists of plasma diluted 1 in 10, $I = 0.02$ means that the 0.5 ml. of added inhibitor consists of plasma diluted 1 in 50, and so on. In the calculations, F is always

$$F = \frac{\text{Initial concentration of lysin } c_1}{\text{Concentration of lysin which gives } (P_0 + g) \text{ per cent lysis}}$$

In the case of saponin, g was measured after 10 minutes, and the denominator of the expression for F was taken from a percentage hemolysis curve similar to that in Ponder, 1953, Fig. 3 a, but in which the percentage hemolysis in various lysin dilutions was measured after 10 minutes. In the case of digitonin, a curve showing the percentage hemolysis after 2 hours was used instead, and g was measured at the end of 2 hours. All the experiments were carried out at 25°C.

Since the technique is complicated and since there are so many combinations of conditions which could be considered, the description of the experimental results will be confined to those which throw new light on what is known about inhibitory phenomena.

1. Saponin and Plasma²

(a) P_0 Constant, c_1 constant (two values), I Varying.—Table I, upper part, shows values of g and of F obtained when $P_0 = 20$, when $c_1 = 100 \gamma$, and when I varies from zero to 0.10. The progressive nature of the reaction, measured by g , becomes less as I increases; this means that if lysis is allowed to proceed to 20 per cent hemolysis before the inhibitor is added, the further progress of the reaction is inhibited more and more as the quantity of added inhibitor is increased. Fig. 1 results from plotting F against I . When $I = 0$, $F = 1.0$, *i.e.* there is no inhibition, and as I increases F increases, at first sharply and then more slowly, towards a limiting value. This value is reached when $P_0 = 20$, tain similarity between F and R , as can be seen from their definitions. F is the extent to which a system containing a concentration c_1 of lysin must be diluted to give a concentration c_x which provides just enough lysin for $(P_0 + g)$ per cent hemolysis; R (measured at the asymptotes) is the extent to which a system containing a concentration c_1 of lysin must be reduced in concentration to give the concentration c_{100} which provides just enough lysin for 100 per cent hemolysis.

² Neither lecithin (500 γ) nor cholesterol (100 γ) prevents the lytic reaction from progressing, even if these inhibitors are added to saponin-cell systems in which P_0 is as small as 20. In lysin-inhibitor-cell systems, on the other hand, 500 γ of lecithin gives an R value of about 1.5, while as little as 10 γ of cholesterol gives an R value of 2.0.

$g = 0$; $(P_0 + g) = 20$, γ for $P = 20 = 55 \gamma$, and $100/55 = 1.8$, the value of F for complete inhibition. A rough extrapolation shows that this limit is reached when I is about 1.5. A similar relation between F and I is found when $c_1 = 67 \gamma$ (dotted line in Fig. 1). Here the limiting value of F is 1.22.

(b) c_1 and I Constant, P_0 Varying.—The results shown in the lower part of Table I were obtained with systems in which $c_1 = 100 \gamma$ and $I = 0.1$, P_0 being varied from 5 to 55. The value of g increases as P_0 increases; *i.e.*, as the lysin becomes more and more firmly attached to the cell surfaces. The values of F vary from 2.0 when $P_0 = 5$ to 1.2 when $P_0 = 55$, and the relation between P_0 , c_1 , and g is quite similar to that found when lysin-cell systems are diluted

TABLE I

$c_1 = 100, P_0 = 20$				
I	g	$(P_0 + g)$	γ for $(P_0 + g)$	F
0.0	80	100	100	1.0
0.01	65	85	77	1.3
0.02	25	45	66	1.5
0.05	15	35	62	1.6
0.10	8	28	59	1.7
$c_1 = 100, I = 0.1$				
P_0	g	$(P_0 + g)$	γ for $(P_0 + g)$	F
5	5	10	50	2.0
15	13	28	59	1.7
25	20	45	66	1.5
35	30	65	70	1.4
55	40	95	85	1.2

with large volumes of saline (Ponder, 1953, Fig. 2). It should be noticed that the relation is quite different from that found with digitonin-cell-plasma systems (Section 2, below).

These limiting values, corresponding to complete inhibition of the progressive reaction, are much smaller than the values of R which would be obtained if the lysin were to be mixed with the inhibitor and the cells added afterwards. This means that the fixation of lysin to the cell surfaces during the time required for 20 per cent hemolysis results in some of the lysin being no longer subject to the inhibitory action of the inhibitor, presumably because it is already fixed with relation to the cell components. Because it is fixed, the reaction progresses; there might, however, be a quantity of lysin at the cell surfaces, not fixed in the same sense as the foregoing but which would become fixed at a later stage of the reaction, and it is possible that this quantity might be removed from the cell surfaces as a result of a combination with the inhibitor, but without the progressive reaction being affected (see Section 4,

which gives an answer to the question: In a system in which some of the lysin has formed an internal lysin phase at the cell surfaces, does the addition of an inhibitor result in some of the lysin being displaced into the bulk phase?).

2. Digitonin and Plasma

When tested by the method which dilutes them, systems containing digitonin are progressive even if diluted when $P_0 < 5$; $F = 1.0$, and the extent to which the lytic reaction progresses seems to be virtually unaffected by the dilution, so firmly and so quickly is the lysin attached to the cell surfaces (Ponder, 1953).

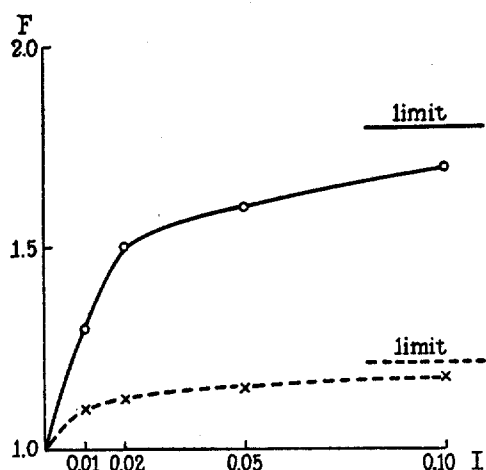


FIG. 1. Ordinate, inhibitory effect of plasma in the internal lysin phase, measured by F ; abscissa, quantity of plasma added, measured by I . Full curve, 100 γ of saponin; dotted curve, 67 γ of saponin. For further explanation, see text.

When plasma is added to systems containing digitonin, the results again point to the lysin being firmly and rapidly attached to the cell surfaces. Using enough digitonin to produce complete hemolysis and plasma diluted 1 in 10 ($I = 0.1$) we find that when $P_0 = 5$, $g = 95$, when $P_0 = 20$, $g = 80$, and so on, ($P_0 + g$) being always approximately 100 and F being always nearly 1.0. It is true that the added plasma slows the hemolytic process, and so it may be inferred that it affects the reaction in some way as yet undefined³, but it is

³ Why both dilution and the addition of an inhibitor reduces the velocity of lysis in digitonin systems but leaves the final effect unaffected is a point which is still obscure. Perhaps there is a continual uptake of lysin from the bulk phase as the reactions in the internal lysin phase proceed, and the inhibitor may render the lysin in the bulk phase relatively inactive, thus slowing the hemolytic reaction while still permitting it to go to completion. Perhaps the inhibitor itself enters the internal

almost without effect on the final result; digitonin, once allowed to come into contact with the cell surfaces rapidly forms an internal lysin phase (Ponder and Ponder, 1954), and the lysin in this phase is so firmly attached that its final effect is almost unaffected either by dilution or by the addition of plasma.⁴

3. Sodium Lauryl Sulfate and Plasma

This lysin was selected for the study of inhibitory effects because it can be prepared in a radioactive form (Croes and Ruysen, 1951 *a*, 1951 *b*). It produces complete hemolysis of 0.5 ml. of a standard human red cell suspension in a concentration of about 50 γ /ml., while the concentration which produces only a trace of lysis is about 25 γ /ml. In the latter concentration, the reaction is not progressive, dilution with 10 volumes of saline not being followed by any

TABLE II

<i>I</i>	<i>g</i>	<i>F</i>
0.0	60	1.0
0.01	30	1.15
0.02	25	1.20
0.10	20	1.25

$P_0 = 40$ in all cases.

additional hemolysis (Ponder and Ponder, 1954). When plasma is added to higher concentrations, *e.g.* 30 γ /ml., the reaction is not progressive provided that P_0 is small enough (< 20) or that *I* is large enough (0.1), but there is some

lysin phase, reducing velocity constants of the reactions which take place there, and so slowing the reaction. The answer to such speculations will no doubt be forthcoming when radioactive saponin and digitonin are available. Meantime the observation that the progress of reactions in systems containing saponin or digitonin is scarcely affected, even with respect to velocity, when a suspension of ghosts ($\rho = 0.02$) is added when $P_0 = 10, 20$, etc., suggests that the latter possibility is more important than the former. The inhibitory components of plasma would be expected to enter the internal lysin phase more readily than would large objects such as ghosts.

⁴The addition of 0.2 ml. of a cholesterol sol (100 γ of cholesterol, Lee and Tsai, 1942) appears to be a little more inhibitory than plasma with the same cholesterol content, values of *F* between 1.2 and 1.4 resulting. As in all digitonin systems, however, the value of *g* depends on how long one waits to measure it. By contrast, 15 γ of the same cholesterol sol, if allowed to remain in contact with 10 γ of digitonin for 24 hours, gives an *R* value of 1.8; *i.e.*, cholesterol allowed to stand together with digitonin is about 20 times more effective as an inhibitor than it is when it is added after the lysin has been in contact with the cells. Lecithin (500 γ) has virtually no inhibitory effect on digitonin hemolysis if added to the lysin-cell system, even when P_0 is as small as 10.

progression if the quantity of added plasma is insufficient or if P_0 is large. In still higher concentrations, the reaction is definitely progressive, g depending on P_0 and on I ; e.g., when the system contains 40 γ /ml. of lysin, we have the values in Table II.

If the lysin is mixed with the inhibitor before the addition of the cells, the inhibition observed is considerable; e.g., when 50 γ /ml. of lysin is mixed with 0.1 ml. of plasma contained in 0.5 ml. of saline, $R = 4.0$, and when the same amount of lysin is mixed with 0.02 ml. of plasma, $R = 1.8$.

These systems are difficult to work with, partly because lysis is so rapid in systems which produce complete lysis, and partly because of special features, involving a tendency for lysis to stop before it is complete (Love, 1950, but see also a criticism by Hutchinson, 1955). When all allowances have been made for these difficulties and for the uncertainties which result from them, the way in which sodium lauryl sulfate is inhibited by plasma is similar, on the one hand, to what occurs in systems containing saponin, red cells, and added plasma, *i.e.*, the hemolytic reaction is progressive if c_1 or P_0 is large enough, or if I , the quantity of added inhibitor, is small enough; on the other hand, the systems resemble systems containing digitonin because of the small values of F obtained.

4. Sodium Lauryl Sulfate- S^{35} and Plasma

It has already been shown that the radioactive hemolysins, sodium lauryl sulfate- S^{35} , sodium cetyl sulfate- S^{35} , and sodium lauryl sulfonate- S^{35} are taken up at the surfaces of washed red cells so as to form an internal lysin phase which may contain as much as 40 per cent of the lysin present before the cells were added (Croes and Ruyssen, 1951 *a*, 1951 *b*; Ponder and Ponder, 1954). These radioactive lysins can be used to answer two questions: (*a*) If an inhibitor is added to the lysin, is the uptake of the lysin by *subsequently* added red cells interfered with?; (*b*) If an inhibitor is added to a cell-lysin mixture in which the lysin has already formed an internal lysin phase at the cell surfaces, is the distribution of lysin between the bulk phase and the internal lysin phase modified?

The greatest concentration of sodium lauryl sulfate- S^{35} to which a washed human red cell suspension of volume concentration 0.3 can be added without lysis occurring was first determined; the dilution of lysin was about 1 in 50,000. Systems were then prepared as follows, the lysin being diluted 1 in 50,000, and the plasma being diluted 1 in 2:

- A. 2 ml. lysin + 0.7 ml. saline
- B. 2 " " + 0.3 " cell suspension, $\rho = 0.3$
- C. 2 " " + 0.5 " plasma + 1 ml. cell suspension added 3 minutes later
- D. 2 " " + 1 " cell suspension + 0.5 ml. plasma added 3 minutes later

On completion, each system was centrifuged and the supernatant fluid was removed. Only a trace of lysis occurred. Samples, 0.2 ml. in volume of each supernatant fluid, were placed on planchettes and dried. The radioactivity of each sample was found by Geiger-Müller counting with a thin walled GM tube, or with a windowless flow counter operating in the GM region. A correction was applied for the greater volume of systems C and D as compared with systems A and B. Table III shows typical results.

The conclusions which can be drawn from these results are the following. (a) There is an uptake of lysin when red cells are added to 1 in 50,000 sodium lauryl sulfate-S⁸⁵, the lysin in the bulk phase being about one-sixth of the lysin present in the system at the time the cells were added. (b) If plasma (either undiluted, diluted 1 in 2, or diluted 1 in 10) is present *before* the addition of the cells to the lysin, the concentration of the lysin in the internal lysin phase is greatly reduced. (c) If plasma (in the same dilutions) is added *after* the cells have been added to the lysin, and presumably after an internal lysin phase has

TABLE III

System	Corrected counts, bulk phase
A	121
B	17
C	73
D	84

been established, the distribution of lysin between the internal phase and the bulk phase is greatly modified, the concentration of lysin in the internal phase again being greatly reduced. In system C (conclusion (b) above) the presence of inhibitor tends to prevent the lysin, with which it can readily combine, from becoming fixed at the red cell surfaces. In system D (conclusion (c), above) there seems to be a competition for lysin between the cell surfaces and the added inhibitor, the result being that little excess of lysin remains in the internal phase. Similar systems in which the plasma was diluted 1 in 10 show similar results, but the more dilute plasma is a little less effective in preventing the lysin from forming an internal phase, and a little less effective in competing with the cell surfaces for lysin.^{5, 6}

⁵ If 2 per cent human serum albumin is used as an inhibitor instead of plasma diluted 1 in 2, the results obtained are very similar to those shown in Table III. The prevention of lysin uptake at the red cell surfaces and the redistribution of lysin which occurs when the albumin is added after the internal phase has been formed are accordingly brought about by serum albumin in much the same way as they are brought about by plasma itself.

⁶ Virtually the same results have been obtained by using sodium cetyl sulfate-S⁸⁵, plasma, and albumin.

It should be noticed that $P_0 = 0$ in these experiments, *i.e.* lysin has formed an internal lysin phase (*cf.* systems A and B), but the essential condition for the reaction being progressive, the fixation of lysin to the cell components, may not have occurred; we know, at least, that the reaction is not progressive when P_0 is so small. Investigations into what would happen were the same experiments to be repeated with $P_0 = 20, 40$, etc., present insuperable technical conditions at present; *viz.*, the difficulty of separating intact red cells and ghosts from supernatant fluid. All rapid methods of separation in systems in which there is some hemolysis result in the separation of red cells plus ghosts from a supernatant fluid containing ghosts with radioactive lysin attached to them, and there seems at present to be no way in which the results obtained by such imperfect separations can be analyzed.

SUMMARY

Inhibition of hemolysis by plasma has been studied in systems containing saponin, digitonin, and sodium lauryl sulfate, using the methods developed for the study of the kinetics of progressive reactions. The results are that the progressive nature of the hemolytic reaction in saponin systems becomes less when the inhibitor is added, that the addition of inhibitor to digitonin systems has no effect on the final result although the velocity of the progressive reaction is reduced, and that the effect of plasma in lauryl sulfate systems is intermediate between the effects in saponin systems and digitonin systems. A simple explanation is that the lysin is very strongly fixed, to form an internal phase, to the cell surfaces in digitonin systems, less strongly in laurate systems, and still less strongly in saponin systems.

To answer the question as to whether, in a system in which some of the lysin forms an internal phase, the addition of an inhibitor results in a redistribution of the lysin between the internal phase and the bulk phase, sodium lauryl sulfate- S^{35} and sodium cetyl sulfate- S^{35} were prepared, and their distribution between the internal phase and the bulk phase was measured before and after the addition of plasma, the lysins being added to the cells either before or after the addition of the inhibitor. The results show that there is a large uptake of these lysins at the red cell surfaces when they are added first, and that the subsequent addition of plasma greatly reduces the quantity of lysin held in the internal phase. Further, if the inhibitor is added first and the lysin subsequently, the internal lysin phase is very incompletely formed.

Serum albumin, used in place of plasma, gives essentially similar results.

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