CIV. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

V. A FURTHER INVESTIGATION OF THE INHIBITION PRODUCED BY PLASMA AND SERUM.

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If a quantity of plasma or serum is added to a haemolytic system containing a quantity c_1 of a lysin such as saponin or one of the bile salts, the time t_2 required for complete lysis is longer than the time t_1 which would be required if the quantity c_1 were to act in the absence of the added plasma or serum. It seems, indeed, that a quantity of lysin Δ is rendered inactive by the plasma, the remainder of the lysin, c_2 , producing lysis in the time $t_2 > t_1$. The problem is to find a simple and satisfactory relation between the variables.

One of us attempted to do this in 1923 [Ponder, 1923], and influenced by the idea that plasma-proteins might "adsorb" some of the lysin, sought a relation between Δ , the amount "adsorbed," and c_2 , the amount left free. These two quantities were found to be related by an expression not unlike the adsorption isotherm,

$$\Delta = \alpha c_2^{1/\mu} \quad \dots\dots(1),$$

where α and μ are constant, provided that any one amount of plasma M is used as the inhibitor. Plotting $\log \Delta$ against $\log c_2$ thus resulted in a straight line with a slope of $1/\mu$, but when the value of M was varied, μ was found to vary from about 3.5 when M was 0.04 ml. to about 0.035 when M was 0.00125 ml. The straight lines corresponding to various values of M accordingly cut one another: "an impossible state of affairs" [Ponder, 1925].

This difficulty was in part overcome in 1925 by the observation that the curves obtained by plotting Δ against c_2 do not pass through the origin, but cut the c_2 axis at different levels. To allow for this, expression (1) was re-written

$$\Delta = \alpha (c_2 - K)^{1/\mu} \quad \dots\dots(2),$$

where K is the intercept on the c_2 -axis just referred to. The plotting of $\log \Delta$ against $\log (c_2 - K)$ now resulted in a series of parallel straight lines with $\mu = 1.75$: this is a much simpler state of affairs, offset, however, by the fact that no good explanation was forthcoming either for the existence of K or for the fact that K was found to vary with M in a quite inexplicable way [Ponder, 1925].

In view of this new difficulty, and in view of the possibility that the premises underlying the analysis might be altogether wrong, a very rigorous test was applied [Ponder, 1932]. If the effect of the added inhibitor is to render lysin inert, and not to interfere with the haemolytic reaction in any other way, we must have the following relations fulfilled,

$$\left. \begin{array}{l} \Delta_1/\Delta_2 = 1 \\ \Delta_1 = c_1 - 1.2c_2 \\ \Delta_2 = c_1 - 1.2c_3 \end{array} \right\} \quad \dots\dots(3),$$

and

where c_1 is the initial amount of lysin in the system containing the plasma, c_2 the quantity apparently left free after some of the lysin Δ_2 has been rendered inert as judged by the time required for complete haemolysis, and c_2 the quantity left free after some of the lysin Δ_1 has been rendered inert, as judged by the time required to produce 50 % (or any other percentage) haemolysis. It was found that this condition was very exactly fulfilled, and so the hypothesis that the plasma inactivated lysin without interfering with the other properties of the system was supported by evidence not easy to set aside.

The present situation is therefore very unsatisfactory. The assumption that the plasma reacts with the lysin so as to render some of it inert is apparently sound, but the relation between Δ and c_2 cannot be left as it stands, for either we have to explain the variation of μ with M , or we have to introduce a third constant K , itself a function of M , and devoid of physical meaning. The following investigations will therefore be divided into three parts.

1. Remembering that all previous analyses have been based on data obtained by plotting "short" time-dilution curves, we shall show that essentially the same relations result from analysing data based on the more accurate "long" time-dilution curves [see Ponder and Yeager, 1930].

2. We shall show that the order of the fundamental reaction between cell and lysin is not changed as a result of the presence of the inhibitor, *i.e.* we shall confirm by independent means the result of the test contained in expression (3).

3. Abandoning the idea of an "adsorption," we shall seek a relation between Δ and c_1 . This will be found to involve the constants α , μ and K , but the last mentioned will be assigned an obvious physical meaning, and it will be shown that μ is independent of the amount of inhibitor used. In this way the problem will be solved in terms of a single expression, which, although empirical, has a real physical significance. It will further be shown that we are now able to predict, in a qualitative sense at least, the changes which are observed in the amount of inhibition when the number of cells in the systems is varied.

Table I.

	δ_1	c_1	t	δ_2	c_2	Δ
Saponin <i>plus</i> 0.02 ml. serum	30,000	66.7	4.0	62,000	32.4	34.3
	35,000	57.1	10.2	75,000	26.6	30.4
	40,000	50.0	30.0	87,000	23.0	27.0
	45,000	44.5	90.0	99,000	20.2	24.3
Saponin <i>plus</i> 0.16 ml. serum	30,000	66.7	2.5	54,000	36.7	30.0
	40,000	50.0	11.0	78,000	25.6	24.4
	45,000	44.5	26.0	86,000	23.2	21.3
	50,000	40.0	120.0	101,000	19.8	20.2
Saponin <i>plus</i> 0.01 ml. serum	35,000	57.1	3.5	56,000	35.7	21.4
	40,000	50.0	5.1	66,000	30.3	19.7
	45,000	44.5	9.0	76,000	26.3	18.2
	50,000	40.0	16.0	84,000	23.8	16.2
	55,000	36.3	37.0	89,000	22.4	13.9
Saponin <i>plus</i> 0.006 ml. serum	40,000	50.0	2.6	55,000	36.3	13.7
	50,000	40.0	6.1	72,000	27.8	12.2
	55,000	36.3	11.0	79,000	25.3	10.7
	60,000	33.3	26.0	86,000	23.2	10.1
	65,000	30.3	48.0	91,000	22.0	8.0
Saponin <i>plus</i> 0.004 ml. serum	45,000	44.5	3.5	56,000	35.7	8.8
	50,000	40.0	4.5	63,000	31.8	8.2
	55,000	36.3	7.0	70,000	28.6	7.7
	60,000	33.3	10.5	77,000	26.0	7.3
	65,000	30.3	40.4	89,500	23.7	6.6

Data for "long" time-dilution curves.

These are presented in Table I, and arranged in exactly the same way as in the Table on p. 486 of the paper which contains the results obtained from "short" time-dilution curves [Ponder, 1925]. All the figures refer to time-dilution curves for saponin and a standard suspension of human red cells at 30°. Values of c_1 , c_2 and Δ are in γ .

The effect of added plasma on the order of the fundamental reaction.

The time-dilution curves for systems containing various quantities of plasma (or serum) can be individually subjected to analysis for the values of the constants in the equation which describes them,

$$kt = \frac{p}{p-1} \cdot \left\{ c \frac{p-1}{p} - (c-x) \frac{p-1}{p} \right\} \quad \dots\dots(4),$$

and, using the data given *in extenso* above together with those for some intermediate curves, we get the values shown in Table II.

Table II.

Serum, ml.	$n (=1/p)$	k	x	$R (=x_1/x_2)$
0.0	2.6	0.00213	19.0	1.00
0.02	2.6	0.000557	40.0	2.11
0.016	2.6	0.000813	36.4	1.92
0.010	2.6	0.00104	30.8	1.62
0.008	2.6	0.00108	28.6	1.51
0.006	2.6	0.00147	26.7	1.41
0.004	2.6	0.00152	24.1	1.27
0.002	2.6-2.7	0.00174	23.0	1.21

Thus n is constant, irrespective of the quantity of inhibitor added, as required if the test in expression (3) is to apply. When the analysis of the curves is carried out in this way, k decreases as M increases, but this is only because the analysis in terms of expression (4) takes no account of the inhibitor rendering lysin inert. The decreasing value of k is really only another way of expressing the relation of Δ to c_1 , as in expression (5).

The accuracy with which the various time-dilution curves are described by expression (4), with the above values of the constants inserted, is really quite surprising.

Results of plotting Δ against c_1 .

Plotting Δ against c_2 results in exactly the same type of curve as in previous investigations, the curves corresponding to each value of M cutting the c_2 -axis at various levels, and being described with a fair degree of accuracy by expression (2), which contains the arbitrary constant K , a function of M . Plotting Δ against c_1 , on the other hand, results in a series of curves which cut the c_1 -axis at a single point, so that $\Delta = f(c_1 - K)$, K now being a constant for all values of M . (This is clearly shown in Fig. 1, in which the two methods of plotting are compared.) To find a meaning for K , consider the steps of the analysis in detail. The time-dilution curve in the absence of inhibitor has an asymptote at $c_{1\infty}$, for which concentration $t = \infty$. Imagine a time-dilution curve for a system containing an indefinitely small amount of plasma: this curve will differ insensibly from the "standard" curve, and will have an asymptote $c_{2\infty}$, differing insensibly from $c_{1\infty}$. Thus when Δ tends to zero, c_2 will tend to c_1 , and the curve obtained by plotting Δ against c_1 will cut the c_1 -axis at a point $c_1 = c_{1\infty}$, $\Delta = 0$, and this will be the intercept K , equal to $c_{1\infty}$. The constant K is therefore equal to the asymptotic

concentration for the "standard" curve with which the time-dilution curve for the system containing plasma is compared, and expression (2) becomes

$$\Delta = \alpha (c_1 - c_{1\infty})^{1/\mu} \quad \dots\dots(5).$$

Plotting $\log \Delta$ against $\log (c_1 - c_{1\infty})$ now gives a series of parallel straight lines for which $\mu = 2.0$ (in this case) and for which α has the following values:

Serum, ml.

0.02
0.016
0.010
0.006
0.004

α

4.67
4.27
3.47
2.57
1.74

Fig. 1. To show the result of plotting Δ against c_1 and c_2 respectively. The five different curves correspond to five different quantities of serum.

The value of μ is therefore constant for all values of M , while the value of α decreases as M decreases. As has previously been remarked, it does not decrease linearly with M , and small amounts of inhibitor are relatively more effective than larger amounts.

Expression (5) accordingly has a comparatively simple physical meaning, and the only curious thing about it is that the quantity of lysin rendered inactive by the inhibitor varies as a power of the lysin initially present (or, for that matter, of the lysin left free). On the other hand, it is scarcely to be expected that a reaction between two such colloids as saponin and the serum-proteins would be expressible by anything better than an empirical formula.

The effect of varying the concentration of the cell suspension.

All previous investigations have been carried out with reference to suspensions of "standard" strength, but we shall now show that the phenomenon of inhibition by plasma and serum can be completely understood only if we consider what happens when the concentration of the cell suspension is varied.

Consider first what ought to happen if the foregoing account of the inhibition is adequate. Each one of a family of Δ_1, c_1 curves for different values of M , when obtained by using a suspension of a particular strength s_1 , will be described by

$$\Delta = \alpha (c_1 - c_{1\infty s_1})^{1/\mu},$$

where $c_{1\infty s_1}$ is the asymptote of the standard curve. If a suspension of strength s_2 is used, and if the foregoing analysis takes account of all the facts, the members of the new family of Δ_1, c_1 curves should be described by

$$\Delta = \alpha (c_1 - c_{1\infty s_2})^{1/\mu},$$

where $c_{1\infty s_2}$ is the asymptote of the standard curve for the s_2 suspension. Thus each member of the second family ought to be the same as the corresponding member of the first family, except that the former is translated upwards or downwards by a distance equal to $(c_{1\infty s_1} - c_{1\infty s_2})$.

Experiment shows, however, that other differences are introduced by varying the strength of the suspension. For example, using a $2x$ standard and a $0.5x$ standard suspension, we get the following figures:

	δ	c_1	$c_1 - c_{1\infty}$	Δ
Saponin <i>plus</i> 0.16 ml. serum, 2 $\times$ S suspension	20,000	100.0	72.0	31.3
	25,000	80.0	52.0	28.0
	30,000	66.7	38.7	24.8
	38,000	52.6	24.6	21.1
Saponin <i>plus</i> 0.16 ml. serum, 0.5 $\times$ S suspension	25,000	80.0	55.6	33.1
	30,000	66.7	42.3	30.0
	35,000	57.1	32.7	26.9
	37,000	54.1	29.7	26.3

Analysis of curves such as these shows (a) that the Δc_1 curves for the $0.5x$ standard suspension make an intercept on the c_1 -axis which is less than that made by the curves for the systems containing the $2x$ standard by the amount required by theory, but (b) that the value of α is not the same for the two families, although the value of μ is the same. This means that the quantity of lysin apparently inhibited by a particular quantity of serum or plasma depends on the number of cells present in the suspension used for obtaining the time-dilution curves, and that for the same values of c_1 more lysin appears to be inhibited when the strength of the suspension is small than when it is greater. At first this observation puzzled us a great deal, but it can be explained quite easily if we take into account certain other known properties of the systems under consideration. The explanation involves giving up the idea that there is complete irreversibility of the compound formed by the lysin and the plasma-proteins.

We know that when lysin is added to cells in concentration c_1 , a quantity of lysin $= \zeta c_1$ is concentrated at the cell surfaces, thus leaving free a quantity $c_1(1 - \zeta)$. So far as we know from rather limited observations, ζ is a constant. As lysis goes on, the quantity of "free" lysin decreases, but always in such a way that it is nearly a linear function of c_1 (see Ponder [1932] the section dealing with the "second addition effect"). In any system containing cells, lysin and inhibitor, we have therefore to distinguish between three quantities: the quantity of lysin "absorbed" on the cells, the quantity left free and the quantity inhibited as a result of its interaction with the serum or plasma-proteins. The relation of these quantities will be

	Absorbed	Free	Inhibited
Before cells added	0	c_2	c_3
After cells added	c_4	c_5	c_6

Remembering that $c_1 = c_2 + c_3 = c_4 + c_5 + c_6$, and that $c_4 = \zeta c_5$, and putting, for simplicity's sake, $c_3 = \alpha c_2$, we get

	Absorbed	Free	Inhibited
Before cells added	0	$c_1/1 + \alpha$	$\alpha c_1/1 + \alpha$
After cells added	$\zeta c_1/1 + \zeta + \alpha$	$c_1/1 + \zeta + \alpha$	$\alpha c_1/1 + \zeta + \alpha$

The addition of the cells should therefore result in the reduction of the amount of lysin inhibited from $\alpha c_1/1 + \alpha$ to $\alpha c_1/1 + \zeta + \alpha$. Clearly the greater ζ the greater the reduction; hence the greater the strength of the suspension used, the smaller will the quantity of lysin inhibited appear, for ζ increases as the number of cells increases. If $c_3 = \alpha c_2^{1/\mu}$, no simple solution is possible, but it will be obvious that the effect will be in the same direction, and that the value of μ should be independent of the strength of the suspension. This is just what is found.

This theory appears entirely adequate, but it demands one rather curious condition in the haemolytic system in order that expression (3) be satisfied. When the cells are added to the system containing the plasma or serum, a redistribution of the quantities of lysin absorbed, free and bound to the inhibitor occurs in the way shown above. The course of the ensuing lysis, however, is exactly the same as that in a system containing no inhibitor, but having the lysin present in smaller initial concentration (see expression (3)). In fact, the redistribution having once been effected by the addition of the cells, the lysin bound to the inhibitor behaves as if it were permanently removed from the system. As haemolysis proceeds, the concentration of free lysin may fall, and probably does, but it is not continually replenished by the inhibitor giving up some of its bound lysin, as in a simple reversible system. We do not pretend fully to understand this, but it undoubtedly has a relation to a series of observations which will shortly be published elsewhere, and which shows that the kinetics of haemolysis require to be considered in relation to a sort of "internal" system rather than in relation to the system considered as a homogeneous whole, and that the properties of this "internal" system depend largely on the way in which the lysin is concentrated at the cell surfaces. In such systems, characterised by extreme heterogeneity, the laws for reactions in homogeneous systems certainly do not apply, and we require to seek information regarding their properties by experiment rather than by applying existing theory.

SUMMARY.

This paper is concerned with a re-investigation of the problem of the inhibition of a simple lysin by plasma or serum, using better methods of experiment and analysis than have been used hitherto. The previous conclusion that it is permissible to treat the inhibition as a reaction between lysin and plasma-protein, by means of which the lysin is removed from the system, is confirmed. It is also shown that the quantity of lysin so removed depends on the number of cells present in the system, and this observation is shown to have a simple explanation.

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