XVIII. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

III. TIME-DILUTION CURVES AND ZONES OF ACTION.

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THIS paper, which is intended to amplify one already published [Ponder, 1926, 2] is concerned with the expression

$$\kappa t = \frac{1}{S - \psi S} \log \frac{c}{c - \psi x - x} \dots\dots\dots(1),$$

which, when applied to a population of cells whose resistances are distributed according to a normal frequency curve, describes in a very satisfactory manner the action of the simple haemolysins¹. In the expression, c is the concentration of lysin acting on the cells, x the quantity used up in the reaction with the cells, κ a velocity constant, and t the time taken for the lysin to bring about any specified degree of lysis; S is a term relating to the surface of the suspension, while ψ denotes a probability which can vary between 0 and 1. The derivation of the expression is discussed in the paper quoted.

This paper falls into two parts. In the first part we shall examine the extent to which the expression (1) describes the time-dilution curves of various lysins, and in the second part we shall find values for ψ , and thence values for the size of the hypothetical "zone of action" surrounding the cells of the particular haemolytic systems considered, within which the reaction between the cells and the lysin may be imagined to proceed.

1. THE TIME-DILUTION CURVES.

The time-dilution curve for any haemolysin is obtained by plotting the dilution of lysin against the time which it takes to bring about complete haemolysis of a certain number of cells. The technique is fully given in a previous paper [Ponder, 1926, 1] and papers quoted therein. In all the cases given below the suspension used was prepared by suspending the thrice washed cells from 1 cc. of blood in 20 cc. of 0.85 % NaCl. Of this suspension, 0.4 cc. was added to 1.6 cc. of a solution of lysin of such a strength as to give a known dilution of lysin after the mixture. In this way the dilution of lysin

¹ In the paper quoted, the final x in the denominator has been dropped by a printer's error

upon which the calculations are based is that present in the fluid in which the cells are suspended at the beginning of the haemolytic reaction. The lysins were all dissolved in 0.85 % NaCl.

We are here concerned with the extent to which equation (1) describes these time-dilution curves, and shall therefore tabulate experimental and calculated results. In these tables, the dilution of lysin will be replaced by a concentration—the number of milligrams of lysin present in the tube containing 2 cc. of fluid. This is for convenience in calculation only.

In using equation (1) for the calculation of the time-dilution curves, we may simplify it by putting S equal to unity, and ψ equal to zero. The expression then reduces to the well-known expression for a monomolecular reaction

$$\kappa t = \log \frac{c}{c-x}.$$

It cannot be too strongly emphasised, however, that this simplification is permissible only because of the special use to which the expression is to be put: in general the simplification cannot be made.

Concentration mg.	t experi- mental mins.	t calcu- lated mins.	Concen- tration mg.	t experi- mental mins.	t calcu- lated mins.
1. Ammonia and human erythrocytes at 25°			2. Sodium stearate and human erythrocytes at 25°		
		$x=15, \kappa=0.25$			$x=0.32, \kappa=0.20$
64	0.9	1.0	2.0	0.9	0.85
34	2.1	2.2	1.0	2.0	1.9
23	4.1	4.25	0.5	4.6	5.1
17	9.0	8.5	0.4	8.0	8.0
			0.3	16.0	15.0
3. Sodium palmitate and human erythrocytes at 25°			4. Sodium oleate and human erythrocytes at 25°		
		$x=0.25, \kappa=0.25$			$x=0.048, \kappa=0.166$
2.0	0.5	0.56	0.2	1.5	1.56
1.0	1.0	1.0	0.1	3.6	3.8
0.5	2.6	2.8	0.06	8.0	7.8
0.4	4.2	4.0	0.05	20.0	19.2
0.3	7.7	7.2			
0.285	9.0	8.4			
5. Sodium taurocholate and human erythrocytes at 25°			6. Saponin and human erythrocytes at 25°		
		$x=0.33, \kappa=0.178$			$x=0.028, \kappa=0.303$
2.0	0.8	0.9	0.2	0.4	0.49
1.0	1.9	2.1	0.1	1.0	1.1
0.5	5.8	6.0	0.05	2.7	2.7
0.4	9.7	9.7	0.04	4.0	4.0
			0.03	6.2	6.1
			0.0285	13.0	12.8

These results are sufficient to show that the degree of exactness with which the times for complete haemolysis can be calculated from equation (1) is considerable. The nature of the lysins, too, is sufficiently varied to show that the expression is one of general applicability. This list might be greatly extended by including the action of each of these lysins on the cells of various

animals; it has been thought unnecessary to do this, for we have shown in the first paper of this series that the relation of the action of saponin on human cells to the action on the cells of any of the mammalia is such that the resistance of the two systems can be expressed by a constant R . Reference to another publication [Ponder, 1926, 2] will show that the appearance of this constant indicates that, if the action of saponin can be described by an expression such as (1), the action on the cells of the sheep, dog, cat, or whatever animal is selected, must be described by an expression of the same type.

We might extend the list by including other lysins. Those given above are the common ones, and the action of the rarer lysins must be left for future investigation.

2. THE ZONE OF ACTION.

In a haemolytic system such as one containing red cells and saponin, it is plain that the reaction which results in lysis cannot occur generally throughout the system, but only in the neighbourhood of the cell surfaces. The reaction is therefore discontinuous, and in order to take the idea of discontinuity into account, we use the idea that each cell is surrounded by a zone within which the reaction can be localised, and to which the term "zone of action" is applied.

It is shown in the paper quoted above [Ponder, 1926, 2] that the size of the zone of action may be approximately obtained in a very simple way. We take the most dilute suspension of red cells with which it is possible to work, plot its time-dilution curve, and find $c_{1\infty}$, the concentration of lysin which corresponds to the asymptote. A suspension of a known cell content, some four times greater than that of the very dilute suspension, is then prepared, its time-dilution curve plotted, and $c_{2\infty}$, the concentration of lysin corresponding to the asymptote found.

Then we have $\frac{c_{2\infty}}{c_{1\infty}} - 1 = \psi$ (2).

But in the nomenclature of another paper [Ponder, 1926, 1], the ratio $c_{2\infty}/c_{1\infty}$ is equal to R , the resistance constant of the second suspension as compared with the first, and this constant has the same value for all values of t . In order to avoid confusion in what follows, we shall call the resistance constant, when it is obtained from two suspensions such as the above for the purpose of working out the dimensions of the zone of action, not R , but R_z . So we have

$$R_z - 1 = \psi,$$

or $R_z - 1 = v/V$ (3),

where v is the volume of all the zones of action added together, and V the volume of the whole haemolytic system.

Call v' the volume of a single zone of action, and we get

$$\frac{V(R_z - 1)}{N} = v' \text{(4),}$$

where N is the number of cells in the haemolytic system which contains the greater number of cells.

If ρ is the radius of the zone of action measured from the surface of the cell outwards (see Fig. 1),

$$\rho = \sqrt[3]{\frac{3V(R_z-1)}{4\pi N}} - r \quad \dots\dots\dots(5),$$

where r is the radius of the cell in the spherical or Goughian form.

The determination of the dimensions of the hypothetical zone of action is therefore an easy matter, for all that has to be done is to determine R_z for a suspension of known cell content and of arbitrary strength as compared with the most dilute suspension with which it is possible to work.

Fig. 1.

Diagram of the zone of action. The cell of radius r is surrounded by the zone, extending outwards for a distance ρ . The figure rotated about the vertical line gives a solid of volume v' . The volume of the sphere representing the cell is v'' , and the volume of the spherical zone surrounding it is Z .

Suitable strengths of the suspensions are as follows: (a) a suspension containing in 20 cc. of 0.85 % NaCl the thrice washed cells from 1 cc. of blood; (b) a suspension containing one-fourth of the cells in suspension (a). With a few exceptions, it is impossible to obtain good end-points with suspensions less concentrated than (b); in any case, the zones of action appear in such suspensions to be completely separated, which is the essential point.

When two suspensions such as these are used, R_z will always be found, irrespective of the type of cell dealt with, to be less than 2. This is, of course, essential, for if R_z exceeds 2, the zones of action completely overlap, and the limits within which the experimental procedure is possible are overstepped.

We shall illustrate the method by an example.

Lysis of sheep cells by saponin. The strengths of the two suspensions were as in (a) and (b) above.

For suspension (a) the asymptote of the time-dilution curve at 25° was found to correspond to a concentration of lysin of 0.210 mg.

For suspension (*b*) the asymptote corresponded to a lysin concentration of 0.130 mg.

Dividing the one figure by the other we get $R_z = 1.63$, and so

$$(R_z - 1) = 0.63.$$

This figure is less than 1, as it ought to be. The number of cells in the system was $2 \cdot (10^8)$, the volume of the system 2 cc., and the radius of the sheep cell, in the spherical form, 2μ .

Applying equation (5) with these values inserted, we find that the value of ρ works out as 9.5μ . The zone of action accordingly extends, in the case of this haemolytic system, from the cell surface outwards for a distance of 9.5μ .

We now proceed to apply this method to the cells of various animals, with a view to determining whether the radius of the zone of action is constant, or whether it varies with the type of cell to be haemolysed.

All the experiments were carried out at 25° . The results are shown in the following table, the types of cell being arranged according to increasing size of the zone of action:

Animal	R_z	$N \times (10^{-8})$	r, μ	ρ, μ
Rabbit	1.07	1.0	2.5	4.5
Man	1.14	1.0	3.0	5.8
Dog	1.18	1.4	2.5	6.0
Cat	1.23	1.2	2.3	7.4
Ox	1.59	1.6	2.3	9.0
Sheep	1.63	2.0	2.0	9.5

A glance at the table will show the remarkable nature of the result, for in the table we have the cells arranged in Ryvosh's series [Ryvosh, 1907; Ponder, 1927]. The order of the cells arranged according to the size of the zone of action is thus the same as the order of the cells arranged according to resistance to saponin. The most resistant type of cell—that of the sheep—has the largest zone of action, the least resistant type—that of the rabbit—the smallest zone of action.

Turning to the idea of the zone of action as a zone within which there exists a concentration gradient of lysin, and regarding its boundary as a point where the slope of this concentration gradient becomes negligible, we see at once the meaning of this important result. In the case of a cell which is very resistant to saponin, such as that of the sheep or ox, a large amount of saponin requires to be used up in order to bring about lysis. As a result, the zone of action is large. If, on the other hand, we have a cell such as that of man or of the rabbit, little saponin needs to be used up to bring about lysis, and the zone of action will be comparatively small. That the size of the zone of action must be related to the resistance of the particular type of cell to the lysin of the system is therefore plain from first principles, and no result other than that of the above table could be expected.

We may, however, go further, and try to find the exact relation between the size of this zone of action and the resistance presented by the particular type of cell. As has been seen in previous papers, the resistance of a particular haemolytic system, compared with that of another haemolytic system

chosen as a standard, may be expressed as the value of a constant R . This constant is obtained by dividing the concentration of lysin which corresponds to the asymptote of the time-dilution curve at 25° (or any other convenient temperature), for a haemolytic system containing saponin and the particular type of cell whose resistance is to be determined, by the concentration of lysin corresponding to the asymptote of the time-dilution curve at 25° for a haemolytic system containing the cells to be used as a standard, together with saponin. Usually it is convenient to use human cells as the standard type. For the detail of this method a previous paper [Ponder, 1926, 1] should be consulted.

In using this method of comparing resistance of cells, it is to be remembered that R refers to an entire haemolytic system. If we wish to use values of R to show the resistance of the individual cell to the lysin—or, what is the same thing, to show the amount of lysin combining with the individual cell—we must be very careful that the haemolytic systems from which the values of R are obtained are in every respect comparable. They must be comparable as regards the state of the zones of action in the system; it is no use comparing two systems in one of which the zones overlap and in the other of which they are separate. Further, if R is to indicate the amount of lysin used up by the single cell, the systems must have the same cell content. We must therefore determine values of R for systems in which the zones of action are all separate, and in which the chance of a cell entering the zone of action of another cell is so small as to approach zero. Having done this, the systems have to be reduced to the same red cell content. This point deserves the most careful consideration. A value of R determined in this way may be properly compared with figures for the dimensions of the zone of action.

With these values of R/N we shall tabulate values of the volume of the zone of action. Call this volume Z ; it is obtained from $(v' - v'')$, where v' is given by equation (4), and where v'' is the volume of the cell supposed to occupy the centre of the zone:

Animal	$R/N \times (10^8)$	v', μ^3	v'', μ^3	Z, μ^3
Rabbit	0.46	1400	65	1335
Man	0.88	2800	110	2690
Dog	0.71	2540	65	2485
Cat	1.4	3830	51	3779
Ox	2.5	7275	51	7224
Sheep	2.15	6300	33	6267

If the values of R/N be plotted against the corresponding values of Z , a fairly good straight line will be obtained. This line is shown in Fig. 2. The points lie along the line in a very satisfactory manner considering the amount of calculation necessary in their derivation.

The equation of this line is

$$Z = \frac{3 \cdot (10^{11}) \cdot R}{N} \dots\dots\dots(6),$$

in which R refers to suspensions in which the zones of action are completely separated, and in which Z is given in μ^3 .

We may compare the experimental results with those given by this expression, and summarise the results in the following table:

Animal	Z , exper.	Z , calc.
Rabbit	1335	1380
Man	2690	2640
Dog	2485	2130
Cat	3779	4200
Ox	7224	7500
Sheep	6267	6750

Fig. 2.

Bearing in mind that Z is in μ^3 , the correspondence is excellent.

The volume of the zone of action is thus proportional to the amount of lysin used up by the cell in the haemolytic reaction. This result, it will be observed, is by hypothesis the only possible one, for we have defined the zone of action as containing the lysin which reacts with the cell. While, however, the result could have been obtained from the original conditions as a deduction, we have here obtained it in a somewhat different way, by the consideration of Ryvosh's series, and have thus linked up this series with the fundamental equation (equation (1)), with the conception of the zone of action, and with the resistance constants for the different types of cell. The fact that so many different methods of attack yield the same result is the strongest evidence of the correctness of all the underlying assumptions, and of the applicability of the fundamental equation to the kinetics of a simple haemolytic system in which saponin is the lysin.

It will be appreciated that there is much room for the extension of these methods to other haemolytic systems. These extensions we propose to deal with later.

Finally, it is instructive to represent the conception of the zone of action, regarded as a concentration gradient, in a graphical form. This is done in Fig. 3. The cell, of radius r , is represented by a thick line. At its surface the concentration of lysin is taken to be zero, and in the concentration gradient of radius ρ this concentration is supposed to rise to a value given by R/N as measured in equation (6). Between the values of R/N and zero the concentration falls according to some curve which we cannot at present determine, so we represent it merely by a straight line. In fact, it is probably a curve of exponential character. These lines are shown in the diagrams, one of which refers to the cell of the rabbit, while the other refers to the cell of the sheep.

The fall in the case of the sheep cell is very marked, and the concentration gradient very steep, as we should expect from the great resistance of this type of cell to saponin; in the case of the rabbit cell, the concentration gradient is much less steep, corresponding to the relatively low resistance of this type of cell.

Fig. 3.

Although these figures are drawn to scale, it will be understood that they represent only in a very approximate way the conditions present, and that they are to serve no purpose except to give a general idea of the state of affairs at the cell surfaces.

SUMMARY.

1. A series of tables comparing experimental and calculated results for different haemolytic systems is given, together with the expression used for obtaining the calculated results.
2. A method for determining the dimensions of the zone of action round the cells of a haemolytic system is described. The radii of the zones are determined for the cells of different animals, and it is shown that the cells, arranged in order of increasing size of zone of action, fall into Ryvosh's series.
3. It is shown that the volume of the zone of action is proportional to the resistance of the cell to the lysin, a relation which follows from theoretical considerations.

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