X. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

II. THE SERIES OF RYVOSH.

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THE series of Ryvosh is concerned with the order in which the erythrocytes of the mammalia may be placed with respect to haemolysis by saponin and hypotonic saline respectively. Ryvosh [1907] places the types of cell in the following order, the most resistant species occurring first.

Saponin. Sheep, goat, ox, cat, grey mouse, pig, grey rat, dog, white rat, rabbit, guinea-pig.

Hypotonic saline. Guinea-pig, white rat, dog, grey rat, rabbit, pig, grey mouse, cat, ox, goat, sheep.

It will be observed that the order of resistance to saponin is the reverse of that to hypotonic saline, with the rabbit forming an exception.

This antagonism between the resistance to saponin and that to hypotonic saline has often been remarked upon, and various reasons have been put forward as to why it should exist. In particular, Orahovats [1926] has recently referred to the series in connection with his researches on the resistance of red cells in splenic blood, and as a result of these researches the subject assumes considerable importance. The results of Ryvosh having been obtained by methods which are open to considerable objection, we propose to investigate the matter afresh by a more satisfactory experimental procedure.

The objections to the original methods are principally the three following.

(1) Ryvosh added defibrinated blood to his solutions of NaCl and of saponin. The haemolytic systems accordingly contained serum, the presence of which renders the measurements of resistance to saponin unreliable, for serum itself inhibits saponin haemolysis [Ponder, 1923].

(2) The amount of blood thus added was in all cases such as to produce a 1 % suspension of cells. Now for experiments such as these, we can prepare suspensions in one of three ways. (*a*) We may have, in the case of the suspension from each animal, 1 cc. containing the cells from a constant volume of blood. This is the manner in which the suspensions used by Ryvosh were prepared. (*b*) We may arrange the suspensions so that each contains, in 1 cc., a constant number of cells. (*c*) We can make the suspensions so that 1 cc. of each contains a number of cells which present constant surface. To

decide which of these three alternative methods is the best is a difficult matter. The first is the most arbitrary, and in the case of saponin haemolysis the third probably the best, since the kinetics of saponin haemolysis are described by expressions which contain terms relating to the surface of the cells [Ponder, 1926, 1]. In this paper we shall, however, consider the resistances of suspensions prepared according to methods (*a*), (*b*) and (*c*), in preference to selecting one of these methods to the exclusion of the others.

In the case of haemolysis by hypotonic saline this difficulty does not arise, since the mechanism of the haemolysis is entirely different from that of saponin lysis, and since in the case of haemolysis by hypotonic saline we are not dealing with a process in which lysin is used up. We can accordingly compare, as Ryvosh does, suspensions containing the cells from a constant volume of blood.

(3) In the series of Ryvosh we have the members arranged merely according to order, and the order of resistance to saponin the reverse of that to hypotonic saline. When we compare the series for saponin with that for hypotonic saline and arrive at the conclusion that the one is the reverse of the other, we imply that if the members of the series are correlated according to rank, we shall find a coefficient of rank correlation of -1. It is well known, however, that the significance of such a value of a coefficient of rank correlation may be very much less than it appears to be, for the coefficient of rank correlation may differ very considerably from the coefficient of real correlation. We therefore seek to replace the arrangement by order by an arrangement according to some absolute measurement, so that we may be able to find the coefficient of real correlation, the value of which is the only reliable guide.

Methods.

Saponin haemolysis. The method used for the measurement of the relative resistance of various types of cell to saponin haemolysis has been fully described in previous papers [Ponder, 1926, 1, 2, 3]. In brief, it consists of plotting the time-dilution curves for the action of saponin on some type of cell selected as a standard—in these experiments, the cells of man—and on the type of cell whose relative resistance is required. The asymptotes of the two curves are found, and the concentrations of lysin corresponding to these asymptotes written down; the division of the one figure by the other supplies a resistance constant R, the magnitude of which gives the resistance of the one type of cell in terms of that of the standard type. The method is somewhat involved, but exceedingly accurate, and has the advantage that it expresses the resistance as the ratio of two absolute quantities. Moreover, R is constant not only for the concentrations corresponding to the asymptotes, but for all other concentrations of lysin, and thus the disadvantage of employing an arbitrarily selected concentration of lysin, or of estimating haemolysis after an arbitrarily selected time, is overcome.

All measurements are carried out at 25°; the saponin used is Merck's pure white saponin. The cell suspensions are prepared in the manner described in previous papers, and suitably diluted so as to contain either (*a*) the cells from a constant volume of the animal's blood, (*b*) a constant number of cells, or (*c*) a number of cells which present a constant surface. The dilution necessary is to be found from the figure for the red cell count per mm.^{3} of blood, and the figure for the surface of the cell as calculated from photographic measurements.

Hypotonic saline. In order to ascertain the resistance of different types of cell to hypotonic saline, a special series of solutions of varying tonicity is prepared. These are adjusted so as to give convenient tonicities when 0·2 cc. of suspension—consisting of cells suspended in 0·8 % NaCl—is added to 1·8 cc. of each solution. The following series is suitable.

cc. of 0·8 % NaCl	Resulting tonicity g. NaCl %	cc. of 0·8 % NaCl	Resulting tonicity g. NaCl %
0	0·08	44·4	0·40
5·5	0·12	50·0	0·44
11·1	0·16	55·5	0·48
17·5	0·20	61·1	0·52
22·2	0·24	66·6	0·56
27·5	0·28	72·0	0·60
33·3	0·32	77·7	0·64
39·0	0·36	83·3	0·68

To the quantity of 0·8 % NaCl, as shown in this table, there is added distilled water to 100 cc.; the result is a solution which gives, when to 1·8 cc. of it is added 0·2 cc. of the suspension in 0·8 % NaCl, a tonicity as shown by the corresponding figure in the table. The use of these solutions is much more convenient than is the drop method of Hamburger. If tonicities intermediate to those given in the table are required, they are easily obtained by mixing two of the solutions in the proper quantities.

The method used is to add to 1·8 cc. of each of the solutions 0·2 cc. of a suspension prepared by suspending the thrice washed cells from 1 cc. of blood in 20 cc. of 0·8 % NaCl, the entire experiment being carried out at 25°. After 60 minutes the tubes are examined, and the greatest tonicity which brings about complete haemolysis is noted. Solutions which give tonicities near this figure are now prepared, and with them the experiment is repeated, so as to determine the greatest tonicity which gives complete haemolysis to 0·01 % of NaCl.

Certain observations on this technique have to be made. (1) Brinkmann [1922] has pointed out that, for tonicity experiments in general, it is better to use solutions containing NaCl, KCl, $CaCl_2$, and $NaHCO_3$, than to use solutions of pure NaCl. The tonicity is varied by altering the NaCl content, the other components being kept the same. In this series of experiments we do not follow Brinkmann's suggestion for the following reason. It is well known that the most suitable balance of NaCl, KCl and $CaCl_2$ varies greatly for the erythrocytes of the different mammals; a solution such as Brinkmann recommends, although excellent for the study of the resistance of one particular

type of cell, will accordingly be unsuitable when cells of different species are to be compared, for the particular NaCl, KCl and $CaCl_2$ balance which is suitable for one animal may be very unsuitable for another, and thus we should obtain effects not directly connected with that of hypotonicity. We accordingly use pure NaCl. (2) We measure the resistance by the greatest tonicity which will complete haemolysis in 60 minutes in order to proceed in the same general way to that by which we measure the resistance to saponin. In the latter case we determine the asymptote of a time-dilution curve; here too, we determine what corresponds to an asymptote, for we imagine, although we do not actually do it, that we are plotting tonicity against the time taken to produce complete lysis, and we select that tonicity which produces complete lysis in 60 minutes—a time which, in these experiments, is as near infinity as we require. The justification of this step is that a tonicity a little greater than that selected would never complete haemolysis at all. (3) It is very important to remember that in these experiments, as in those determining the resistance to saponin, we are determining the resistance of the most resistant cells of the sample, and not of the cells of average resistance. The determination of the resistance of the average cell of the sample would be an exceedingly difficult matter. The importance of this point is that when we find a series in which the various cells may be placed with respect to their resistance, this series only holds for the most resistant cells; unless the standard deviation is the same for every suspension, the series may not hold for the average cells of the various suspensions.

Coefficients of correlation. These are calculated in the usual way. The coefficient of real correlation, r, is given by

$$\frac{S(xy) - Nd_1d_2}{\sqrt{\{S(x^2) - Nd_1^2\}}\sqrt{\{S(y^2) - Nd_2^2\}}},$$

where x and y are measured from points distant d_1 and d_2 from their respective means, and the coefficient of rank correlation, ρ, by

$$1 - \frac{S(g^2)}{\frac{1}{6}N(N^2-1)},$$

where $S(g)$ denotes the sum of the gains in rank of the second series over the first.

Results.

1. *Suspensions containing cells from constant volume of blood.*

Each suspension was prepared so that the thrice washed cells from 1 cc. of the blood of the animal were finally suspended in 20 cc. of saline. The resistances to saponin and to hypotonic saline were determined by the above methods; in the table which shows the results the resistance to saponin is indicated by the figure for the asymptote of the time-dilution curve, the dilution of lysin being converted, for convenience in calculation, into the corresponding concentration of lysin in mg. The value of R is also given, the

cells of man being taken as the arbitrary standard. The resistance to hypotonic saline is given as the percentage of NaCl which gives the greatest tonicity capable of producing complete lysis in 60 minutes.

Table I.

	Resistances			Rank.	
	Saponin				
Animal	Asymptote	*R*	Saline	Saponin	Saline
Man	0·030	1·0	0·35	3	1
Guinea-pig	0·033	1·1	0·40	4	2
Rat	0·021	0·7	0·40	2	3
Rabbit	0·012	0·4	0·42	1	4
Dog	0·036	1·2	0·44	5	5
Pig	0·039	1·3	0·45	6	6
Cat	0·063	2·1	0·48	7	7
Ox	0·198	6·6	0·49	9	8
Goat	0·075	2·5	0·52	8	9
Sheep	0·210	7·0	0·56	10	10

An inspection of this table will show that the different types of cell fall, as regards their resistance to hypotonic saline, in Ryvosh's series. As regards their resistance to saponin, they fall in a series which is nearly the reverse of the first, exceptions being provided by the goat, rabbit, and rat. Ryvosh gives the rabbit as the only animal which falls out of place in the series, for he finds the resistance of the cells of the goat greater than that of ox cells to saponin; in general, however, the order in which Ryvosh places the different types of cell is confirmed.

We now look at the correlation coefficients. Taking the series as given by Ryvosh, and correlating according to rank, we obtain a value of ρ of $-0{\cdot}94$. This value is very high, as might be expected from the fact that the order for resistance to saponin is the reverse of that for resistance to hypotonic saline, with the one exception of the rabbit. Taking next the series as found in Table I, and correlating according to rank, we get a value of ρ of 0·88, to which a negative sign must be prefixed, as the figure giving the resistance to saline becomes greater as the resistance becomes less. Next taking the correlation of the resistances as measured in absolute quantities, we obtain a much lower figure for r, the coefficient of real correlation, for it works out as 0·75, to which, again, a negative sign is to be attached.

2. *Suspensions containing a constant number of cells.*

In this series of experiments, each suspension was so prepared as to contain $2{\cdot}5 \times 10^8$ cells in 1 cc. In the table showing the results, the same arrangement as in Table I is adopted, and there is added a column to show the number of cells present in 1 mm.^{3} of the blood of each animal examined. This last figure was obtained by a red cell count made in the usual way.

Table II.

Animal	Cells per mm.^{3} $\times 10^{-6}$	Resistances: Saponin: Asymptote	Resistances: Saponin: R	Resistances: Saline	Rank: Saponin	Rank: Saline
Man	5·0	0·030	1·0	0·35	3	1
Guinea-pig	5·0	0·033	1·1	0·40	5	2
Rat	7·0	0·019	0·6	0·40	2	3
Rabbit	6·0	0·011	0·4	0·42	1	4
Dog	7·0	0·032	1·1	0·44	4	5
Pig	5·0	0·039	1·3	0·45	6	6
Cat	6·0	0·057	1·9	0·48	8	7
Ox	6·0	0·180	6·0	0·49	10	8
Goat	15·0	0·045	1·5	0·52	7	9
Sheep	10·0	0·140	4·6	0·56	9	10

The change in the method of preparing the suspension, it will be observed, causes a very considerable difference in the order of resistance to saponin. The coefficient of rank correlation now works out at $-0{\cdot}80$, and the coefficient of real correlation at no greater figure than $-0{\cdot}68$.

3. *Suspensions presenting constant area.*

In order to prepare these suspensions, one has to take account of the number of cells per mm.^{3} of blood, and the surface area of each cell. The first figure can be found by a count in the usual way; the second must be determined by calculation from the figures for the diameter and thickness of the cells. These can be obtained from photographic measurements of the erythrocytes suspended in plasma; the surface area is then calculated from the expression

$$\text{Area} = 2\pi A^2 + 2\pi AB\,\frac{\sinh^{-1} e}{e}.$$

A and B are the semi-axes major and minor of the cell, the biconcavities being imagined to be turned inside out, so that the cell assumes the form of a spheroid. The eccentricity about the minor axis is e, and it may be pointed out that the part of the expression, $\sinh^{-1} e/e$, is remarkably constant for all the cells of the mammalia, being equal to 0·6.

Multiplying the number of cells per mm.^{3} of blood by the figure for the area of each cell in μ^2 gives a figure which is proportional to the surface area presented by a suspension containing the cells from 1 cc. of the animal's blood. This figure may be compared with a similar figure for a suspension of human cells, which it is convenient to take as an arbitrary standard; in this way a series of suspensions from the blood of different animals can be prepared, each presenting the same surface to the lysin. In this series of experiments, the surface presented by the cells of 1 cc. of any of the suspensions used was $30 \times 10^9\ \mu^2$.

In Table III the value of a constant S, denoting the ratio of the surface presented by the cells in 1 cc. of the animal's blood to the surface presented by the cells in 1 cc. of human blood, is given in order that the strength of the various suspensions may be readily compared.

Table III.

Animal	S	Resistances: Saponin: Asymptote	Resistances: Saponin: R	Resistances: Saline	Rank: Saponin	Rank: Saline
Man	1·0	0·030	1·0	0·35	3	1
Guinea-pig	0·8	0·039	1·3	0·40	5	2
Rat	1·0	0·021	0·7	0·40	2	3
Rabbit	0·95	0·012	0·4	0·42	1	4
Dog	1·0	0·036	1·2	0·44	4	5
Pig	0·53	0·050	1·7	0·45	6	6
Cat	0·62	0·085	2·8	0·48	7	7
Ox	0·66	0·230	7·7	0·49	9	8
Goat	0·72	0·090	3·0	0·52	8	9
Sheep	0·7	0·250	8·3	0·56	10	10

The order of resistance to saponin under the conditions of this experiment is very nearly the same as that shown in Table I, for there is a tendency for the greater number of cells, as found in the sheep and goat, to cancel out with a small figure for the surface area.

The coefficients of correlation work out as follows: $\rho = -0{\cdot}85$, $\blacktriangleright = -0{\cdot}76$. These are very close to the values obtained from Table I, and considerably higher than those obtained from Table II.

Discussion.

Regarding the coefficients of rank correlation, we may set these aside at once. The high value obtained from Ryvosh's original series, and the not much lower value obtained from Table I, are quite misleading, and no deductions can be drawn from them except that the order in which the various types of cell fall with respect to resistance to saponin and to hypotonic saline is extremely unlikely to be brought about by mere chance. The odds against this order occurring by chance alone, are, in fact, about 6000 to 1.

We are also inclined to set aside the results of Table II, with a value of r of $-0{\cdot}68$. There appears no good reason for comparing suspensions containing a constant number of cells, when we have a comparison between suspensions presenting constant surface to the lysin. In any case, the value of r obtained by this method of comparison is the lowest of all; if the comparison of suspensions containing a constant number of cells should be insisted upon, the remarks about to follow would apply *a fortiori*.

Tables I and II each yield a value of r of about $-0{\cdot}75$. This is a high value for a coefficient of correlation, but not so high as to justify us in saying that there is only one factor determining the resistance—a factor which gives a high resistance to saponin and a low one to hypotonic saline. It appears permissible, however, to say that one important factor must exist, coupled with perhaps one, or more, subsidiary factors, the operation of which may affect, in one direction or the other, the operation of the primary factor.

The nature of this primary factor is a matter for interesting speculation. Port [1922] has suggested that the phosphoric acid content of the cell is the

essential factor in determining the resistance, for he observes that the order of resistance to saponin for the cells of different animals is the same as the order of phosphoric acid content as given by Abderhalden [1895], while the order of resistance to hypotonic saline is the reverse of the order of phosphoric acid content. In applying this suggestion to his own researches Orahovats apparently takes the term "phosphoric acid" to include inorganic phosphates; with this interpretation, he finds little in support of Port's suggestion.

Examining Abderhalden's table, we find four sets of figures which bear on this point; figures are given for inorganic phosphoric acid, total phosphoric acid, phosphoric acid as nuclein, and lecithin. Although only seven animals appear in common to both Abderhalden's table and Table I of this paper, a close examination of these figures throws considerable light on the suggestion of Port.

First we correlate, according to rank, the total phosphoric acid content and the resistance to saponin. This gives a coefficient of − 0·84, which is very high. This total phosphoric acid, it is now to be observed, may be broken up into the inorganic and the organic phosphoric acid, for both of which figures may be obtained from Abderhalden's table. Further, the principal sources of the organic phosphoric acid are nuclein and lecithin, for both of which Abderhalden gives figures. Correlating according to rank with the resistance to saponin, we find the following coefficients:

Total phosphoric acid and resistance	− 0·83
Inorganic phosphoric acid and resistance	− 0·52
Organic phosphoric acid and resistance	− 0·53
Lecithin and resistance	− 0·21
Phosphoric acid as nuclein and resistance	− 0·89

These being merely rank correlations, it is difficult to attach a significance to the figures, but one thing stands out clearly—the correlations are all negative, the greater content of any one of these substances being associated with the lower resistance to saponin.

This fact is very important, for it has been established with a considerable degree of certainty that saponin enters into a combination with some component of the cell, from which we should expect to find the amount of that component increasing with the amount of saponin required to be used up to bring about lysis, and the amount of that component increasing with the resistance. This would give, of necessity, a positive correlation, whether by rank or by absolute value, and not a negative one.

We have already suggested [Ponder, 1926, 1] that the component with which the saponin interacts is a protein component of the cell wall, and so we may correlate the resistance to saponin with the protein contents of the various cells, as given by Abderhalden, excluding, of course, the content of haemoglobin. This procedure at once gives the high positive coefficient of 0·79. The positive sign, moreover, gives the coefficient a real significance, for we can say that the more protein in the cell, the more saponin requires to be

used up to bring about lysis, and the more resistant is the type of cell accordingly. Or, reversing the argument, the more protein in the cell, the more readily is the cell haemolysed with hypotonic saline.

We suggest that it is the protein content which is the primary factor in the determination of the resistance to saponin and to saline, and that the observation of Port is due to the fact that there exists a coefficient of rank correlation between protein content and phosphoric acid of $-0{\cdot}75$. Why this latter figure should appear we do not know, nor is its appearance material to the question at issue. The suggestion that the protein content is the essential factor at least brings Ryvosh's series into line with the results of previous researches, all of which indicate that the action of saponin is on the protein component of the cell. The phosphoric acid content happens to be a guide to the resistance merely because the amount of this substance varies inversely, roughly speaking, with the protein content.

To express the conclusion concisely, the evidence points to the following state of affairs. In a cell such as that of the sheep, rich in protein, much saponin requires to be transformed by the formation of a compound with this protein before lysis is brought about. The cell is therefore very resistant to saponin. The same cell is readily haemolysed by hypotonic saline, whether because of its high protein content, or for some other reason which we cannot indicate. A cell poor in protein, such as that of the rat, requires little saponin for the transformation of sufficient of the cell wall to bring about haemolysis, and so appears relatively unresistant to saponin. At the same time, it requires a relatively low tonicity to bring about lysis, and the cell is thus relatively resistant to hypotonic saline.

There is no difficulty in appreciating why a large protein content should give a high resistance to saponin. The reason why a high protein content should accompany a low resistance to hypotonic saline is more difficult to discover, and must be sought in the mode of operation of hypotonic saline on the cell. It may be that, just as the resistance to saponin is determined by the protein content but indicated by the phosphoric acid content, so the resistance to hypotonic saline is indicated by the protein content, but determined by some factor which is negatively correlated to the amount of protein in the cell.

Summary.

1. The series of Ryvosh is investigated afresh by quantitative methods. Ryvosh's results are in the main confirmed, and his conclusions amplified by the calculation of the correlation coefficients applicable to the series.

2. It is suggested that the resistance of cells to saponin is principally determined by the protein content of the cell, exclusive of haemoglobin, and the evidence for this suggestion is discussed.

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