HEMOLYSIS BY SAPONIN AND SODIUM TAUROCHOLATE, WITH SPECIAL REFERENCE TO THE SERIES OF RYVOSH.

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This paper is concerned with the investigation of the occurrence of the resistance series known as the series of Ryvosh, when (*a*) saponin and (*b*) sodium taurocholate are employed as hemolytic agents.

The essential literature on the subject is readily summarised. Ryvosh (1907), using non-quantitative methods, has investigated the hemolysis of the cells of certain animals by saponin and by hypotonic saline solution. The series of Ryvosh consists of these animals arranged in the order of the resistance of their cells to saponin hemolysis, which order is the reverse of that obtained when the same animals are arranged according to the resistance of their cells to hemolysis by hypotonic saline. This series has been reinvestigated and confirmed by Ponder (1927) who has employed a strictly quantitative technique. Kofler and Lázár (1927), making use of different and less exact methods, have been able to confirm the series of Ryvosh for hemolysis by saponin. Using other glucosides they have failed to obtain the series. Finally, Ponder and McLachlan (1927) have obtained values of the resistance constant, *R*, for several of the animals included in the series, using hemolytic agents of bacterial origin. They have concluded that the values so obtained are not sufficiently different to justify the arrangement of the various animals in a series. The question arises as to whether the series of Ryvosh is of general applicability or whether saponin may be considered as a special hemolytic agent in this respect. It is of special importance to extend the investigation of the series of Ryvosh to other hemolytic agents, as yet not utilised in this respect, and with this in mind the following experiments with saponin and sodium taurocholate were carried out.

I. The Series of Ryvosh and Saponin Hemolysis. Method.

The method used in the experiments of both parts of this paper and in the analysis of all the curves is essentially that developed and fully described by Ponder (2–6). These papers contain a complete explanation of the procedures, which will be briefly described here.

The dilutions of lysin are prepared in such a way that when the lysin is added to the systems the final dilution of the hemolytic agent is that desired. With the exceptions of the sheep and the ox, in which cases the dilutions used were one-tenth as great, the final dilutions used in the experiments with saponin were 1 in 10,000 to, usually, 1 in 60,000, *i.e.*, 1 part of saponin to 10,000 parts of 0.85 per cent NaCl. The test-tubes used were small white glass tubes, 4 by 0.5 inches. These were thoroughly cleaned, finally steamed with live steam and then dried in an oven. To secure a single point on the experimental curve, 0.8 cc. of the lysin dilution is placed in a test-tube and to this is added 0.8 cc. of 0.85 per cent NaCl. The tube is then placed in a glass-sided water bath having a white illuminated background and kept at a constant temperature of 25°C. In the water bath is also kept the blood suspension and the pipette used to transfer the suspension to the test-tube. Time being allowed for these to all acquire the temperature of the bath, the hemolytic system is completed by the addition of 0.4 cc. of the blood suspension, the blood corpuscles of which are kept in suspension by frequent shakings. The blood suspension used is the "standard" suspension of Ponder, consisting of the corpuscles from 1 cc. of oxalated blood, twice washed and suspended in 20 cc. of 0.85 per cent NaCl. With a stop-watch the time is taken for complete hemolysis to occur. This time plotted against the corresponding dilution gives one point on the experimental curve.

In each experiment two such time-dilution curves are secured, one for man, taken as a standard, and one for the animal whose relative resistance to the lysin is being investigated. The times of complete hemolysis corresponding to a dilution of, say, 1 in 10,000 are taken simultaneously for the cells of man and of the animal. These two times are then plotted against the corresponding dilution and the first point on each of the two experimental curves thus obtained. The procedure is then repeated in order to secure the times corresponding to the next higher dilution of the lysin, *i.e.*, 1 in 20,000, etc.

To illustrate the method used in the analysis, the curves for rabbit and man may be taken as an example. The two curves are plotted together upon the same piece of graph paper. A single point on each curve corresponding to a certain time is noted. Twice the reciprocal of the dilution thus secured in the case of the human cells is taken as equal to c_1 and twice the reciprocal of the dilution for the rabbit cells taken as c_2. In the same way the values of c_1 and c_2 are obtained for several other pairs of points on the two curves. When the c_1 values are plotted against the c_2 values an approximately straight line should be secured. Ponder (4) has discussed this c_2/c_1 ratio and has pointed out that the curves ob-

tained by plotting c_1 against c_2 in this type of experiment is not a straight line but that for all practical purposes it may be taken as such. Having thus determined the straight line, the best value of R, the resistance constant, is obtained from the ratio c_2/c_1 and R is taken to be constant for all corresponding points on

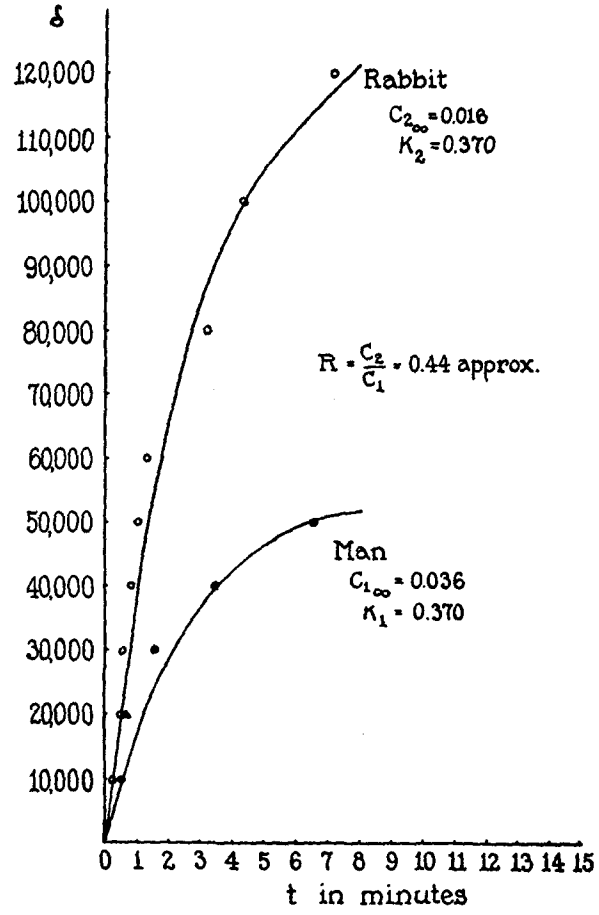

FIG. 1.

the two curves. The theoretical curves are then fitted to the experimental points.

The equation of the time-dilution curves is the well known first order expression

$$t = \frac{1}{\kappa} \log \frac{c}{c - x},$$

where c is the initial concentration of the lysin, x is the amount of lysin used up by the system at any time, t, and κ is a composite constant. The value of c for any dilution, δ, is obtained by taking twice the reciprocal of the dilution and is in milligrams. When $t = \infty, x = c_\infty$, *i.e.*, the total amount of lysin that the system uses up. In practice the value of x is determined by doubling the reciprocal of the dilution corresponding to the asymptote of the curve. The value of log $c/(c - x)$ is then calculated for each observed time and the value of κ derived from that theoretical curve which best fits the experimental points. Table I gives the values of c, the number of milligrams of lysin in the system, the observed values of t and the calculated values of t for the rabbit and for man. R for this pair of curves is approximately equal to 0.44.

TABLE I.

c	Man		Rabbit	
	t obtained	t calculated	t obtained	t calculated
	min.	*min.*	*min.*	*min.*
0.2	0.5	0.54	0.3	0.23
0.1	0.6	1.20	0.5	0.47
0.06'	1.5	2.13	0.5	0.75
0.05	3.5	3.44	0.8	1.04
0.04	6.5	6.2	1.0	1.38
0.03'			1.3	1.76
0.028				
0.025			3.2	2.76
0.022				
0.020			4.3	4.35
0.018				
0.017			7.2	7.65

In Fig. 1 the theoretical curves are represented by lines and the experimentally determined points as small circles (rabbit) and large dots (man). The second point in the curve of man, represented by a triangle, obviously results from an error. In both curves the experimental points corresponding to the higher concentrations fall inside the calculated curves. That this is a general occurrence has been pointed out by Ponder (4). With the exception of the two points mentioned, both experimental curves are found to fit the calculated ones sufficiently satisfactorily for the purposes of this paper.

The saponin curves have been analysed and the resistance constant, R, determined for each animal of the series. These values have been correlated with the values of R obtained by Ponder (5) according to the expression

$$r = \frac{S\,(xy)}{\sqrt{S\,(x^2)} \cdot \sqrt{S\,(y^2)}},$$

where r is the coefficient of correlation, x and y deviations from the mean of the values of R in the two series and $S(x^2)$ and $S(y^2)$ the sums of the squares of the deviations.

Results.

The results of these experiments may be briefly expressed as in Table II, which shows the values of R given by Ponder (5) and the values of R derived from the present experiments.

The coefficient of correlation between these two sets of values has been found to be 0.89. Considering that the animal described as "goat" was *Capra hircus* in Ponder's series and *Hemitragus jemlaicus* in this series, the value of r is a very good one. These two series

TABLE II.

Animal	Ponder	Author
Rabbit	0.4	0.44
Rat	0.7	0.7
Pig	1.3	1.0
Man	1.0	1.0
Guinea pig	1.1	1.1
Dog	1.2	1.65
Cat	2.1	4.2
"Goat"	2.5	5.0
Sheep	7.0	6.0
Ox	6.6	7.3

were obtained, one by Ponder in Edinburgh and the other by the author in New York. It is of interest that similar results have been given by two so widely separated groups of animals.

It may be concluded that the series of Ryvosh is obtained when saponin is used as the hemolysing agent.

II. The Series of Ryvosh and Sodium Taurocholate Hemolysis.

Method.

The method used in the experiments with sodium taurocholate is essentially that described in the first part of this paper, such modifications as occur being necessary because of the fact that sodium taurocholate very rapidly changes its hemolytic properties in solution. These modifications are as follows: (1) while dilutions of saponin will keep on ice for several days, a stock dilution of sodium taurocholate must be prepared at the latest possible moment before use. The

stock solution is such that, if itself used as the hemolytic agent, would give a final dilution of 1 in 100. (2) Unlike the saponin system, the sodium taurocholate system necessitates rapid working; for each pair of points corresponding to the curves of man and of the animal the dilution must be made individually from the stock solution and the two points determined simultaneously. With saponin, however, the time interval between the preparation and the use of the dilutions need not be at a minimum and so a number of tubes may be allowed to hemolyse together. (3) It is even more important with sodium taurocholate than with saponin that the glassware used be thoroughly cleaned and dried, especially the test-tubes which are to contain the hemolytic systems.

The analysis of the curves is carried out in the same manner as in the case of the saponin curves, with the following exceptions. Plotting c_1 against c_2 does not result in a straight line, since the two curves compared are found to approach their respective asymptotes with different values of κ. The resistance constant, R, has therefore been determined from the ratio

$$\frac{c_{2\infty}}{c_{1\infty}} = R_\infty.$$

R_∞ furnishes no information concerning the velocity constants, κ_2 and κ_1, of the curves of the animal and man respectively. Unlike saponin hemolysis, sodium taurocholate hemolysis yields curves having different values of the velocity constant. The values of κ may be quite different for two comparable curves. Further, κ_1 is found to have different values in different experiments. There are several reasons for attributing the latter variation to changes in the hemolytic properties of the sodium taurocholate while in solution. In the first place, it is well known that the bile salts are very unstable in solution, undergoing changes which affect their ability to hemolyse red cells. Secondly, it has been found difficult to make the time interval between the preparation of the stock solution of taurocholate and its use the same for each of the experiments. Thirdly, the blood of man used was always taken in the same manner from the same individual and the standard suspension was made up in the same way in each experiment, thus greatly reducing the probability that changes may have occurred in the cell component of the system. It is therefore desirable to have an expression of the velocity constant of the curves of the series which will enable the values of κ_2 to be more readily compared. This may be done by taking the ratio

$$\frac{\kappa_2}{\kappa_1} = A.$$

Better relative values of κ_2 are offered by A than by κ_2 itself.

A typical example of the time-dilution curves obtained from the sodium taurocholate experiments is that seen in Fig. 2 for the cells

of the rabbit and of man. In Table III are shown the concentrations of the lysin and the corresponding observed and calculated times for each of the curves. Fig. 2 shows the experimental and calculated curves. It is to be noted that the two curves cross as they approach

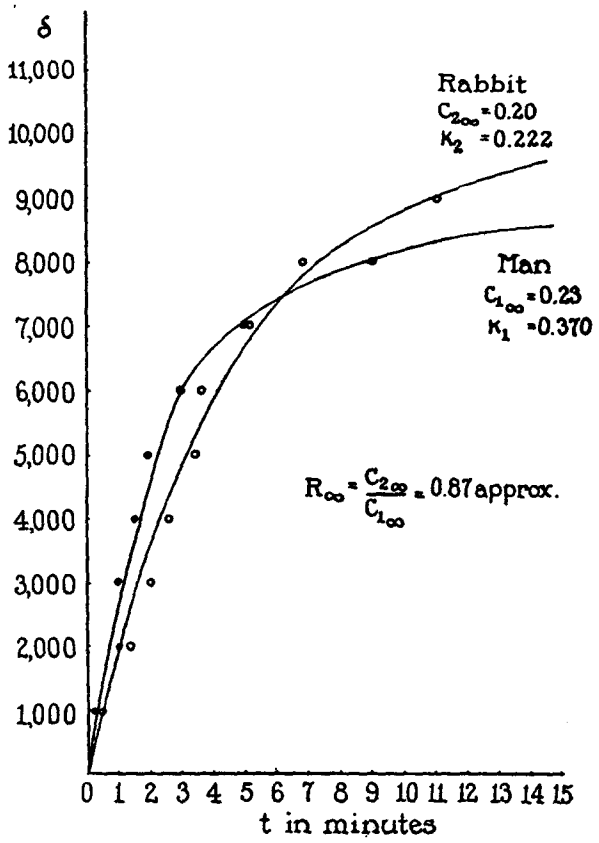

FIG. 2.

their respective asymptotes; crossing has not been found to occur in every case but the curves compared always show different values of κ.

Results.

The results of the experiments with sodium taurocholate may be briefly expressed. Table IV gives the values of R derived from the

saponin experiments, the values of R_∞ obtained from the taurocholate experiments and the values of A. It should be remarked that the values of R_∞ do not vary greatly from unity. In determining A, κ_1 is taken equal to 1, but the experimental values of κ_1 have been found to vary from 0.37 to 0.43 with a mean of 0.40 ± 0.32.

TABLE III.

c	Man		Rabbit	
	t obtained	t calculated	t obtained	t calculated
	min.	*min.*	*min.*	*min.*
2.0	0.2	0.33	0.4	0.47
1.0	1.0	0.7	1.4	1.0
0.6'	1.0	1.13	2.0	1.58
0.5	1.5	1.7	2.6	2.3
0.4	1.9	2.3	3.4	3.12
0.3'	2.9	2.9	3.6	4.1
0.28	4.9	4.7	5.0	5.6
0.25	9.0	8.7	6.8	7.2
0.22			10.5	10.8

TABLE IV.

Animal	$R = \frac{c_2}{c_1}$ (Saponin)	$R_\infty = \frac{c_{2\infty}}{c_{1\infty}}$ (Na taurocholate)	$A = \frac{\kappa_2}{\kappa_1}$
Rabbit	0.44	0.87	0.60
Rat	0.7	1.00	0.62
Pig	1.0	1.09	0.36
Man	1.0	1.00	1.00
Guinea pig	1.1	0.64	0.28
Dog	1.65	0.85	0.36
Cat	4.2	1.05	1.52
"Goat"	5.0	0.78	0.34
Sheep	6.0	0.76	0.25
Ox	7.3	1.00	0.24

The coefficient of correlation for the values of R and R_∞ has been calculated as before and r found to be approximately -0.21, a correlation value of no significance in this connection.

It must be concluded that the series of Ryvosh is not obtained when

sodium taurocholate is used as the hemolytic agent and when R_∞ is taken to represent the resistance constant, R. This increases the probability that saponin may be a special hemolytic agent in so far as it can produce the series of Ryvosh and adds sodium taurocholate to the group of lysins, exemplified by bacterial lysins and a number of glucosides other than saponin, which give different resistance series (7, 9).

SUMMARY.

1. The series of Ryvosh is obtained when hemolysis of the red cells of the animals concerned occurs with saponin as the lytic agent.

2. The series of Ryvosh is not obtained when R_∞ is taken as the resistance constant and sodium taurocholate is used to hemolyse the cells of the same animals.

3. The hemolysin sodium taurocholate has been found to differ from saponin in that the time-dilution curves are found to approach their respective asymptotes with different values of κ.

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