LII. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

V. THE LYTIC EFFECT OF MIXTURES OF TWO LYSINS.

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THE only existing paper on this subject is one by Sen and Mitra [1929], in which a study of the lytic effects of mixtures of saponin, sodium taurocholate, sodium glycocholate and sodium oleate, in various proportions, led to the conclusion that the effects of two lysins acting together are probably additive in most cases, although in some instances the effect of the mixture may be greater than the sum of the individual effects. Sen and Mitra's experiments, however, are hardly conclusive, for two reasons. (1) No account is taken of the p_H changes which occur when two lysins such as saponin and sodium taurocholate are mixed together, which may result in either an acceleration or an inhibition of the action of one or both lysins. Sen and Mitra recognise this themselves. (2) The existing data are not presented in such a way as to be susceptible of exact analysis, and the conclusion that the effects of two lysins are additive in most cases really rests on nothing more substantial than an impression.

This paper is concerned with a re-investigation of the problem, and, as will be seen, leads to conclusions somewhat more definite than those of Sen and Mitra.

1. *Methods.*

Except in the case of systems containing sodium oleate, all the lysins were dissolved in an isotonic phosphate buffer at p_H 6.77, this buffer being selected because at this p_H the time-dilution curves for sodium taurocholate and sodium glycocholate are of the usual regular form and devoid of the "zones" so characteristic of these lysins in systems of certain p_H . In the case of systems containing sodium oleate the buffer used was a phosphate buffer at p_H 8.5, for this lysin is not sufficiently active in buffers of lower p_H . The cell suspension was a "standard" suspension consisting of the thrice washed cells of human blood finally suspended in 20 ml. of 0.9 % NaCl for every ml. of blood used. It is well to let the lysins stand for some hours before they are used, for as Sen and Mitra remark, their lytic activity often decreases with time.

To illustrate the procedure, consider the case in which the combined effects of saponin and sodium taurocholate are to be investigated quantitatively. First we plot a time-dilution curve at 25° for the saponin, the systems consisting of 0.8 ml. of various dilutions of the lysin (1 in 10,000, 1 in 20,000, etc.), 0.8 ml. of buffer, and 0.4 ml. of the standard suspension. Let the quantity of lysin present in each system be c_1 and the time for complete lysis t_1 . Next we take a system consisting of 0.8 ml. of taurocholate, 1 in 2000 (say), 0.8 ml. of buffer and 0.4 ml. of suspension and find the time taken by it to show complete lysis. Suppose that it is the

same time as that taken by a dilution of 1 in 60,000 saponin (33.3 γ in the system of total volume 2 ml.). Then 1 in 2000 taurocholate is equivalent to 1 in 60,000 saponin, or 1000 γ of taurocholate to 33.3 γ of saponin, equivalence being judged by the taking of equal times to produce complete lysis. Call this equivalent quantity of saponin c_3 .

Now prepare systems containing both lysins. Each contains 0.8 ml. of the 1 in 2000 taurocholate, 0.8 ml. of various dilutions of saponin, and 0.4 ml. of cell suspension. Let the time for complete lysis be t_2 . Referring to the time-dilution curve for saponin acting alone, we find t_2 corresponding to a quantity c_2 ; then, if the effects are additive, one would expect to find

$$c_3 + c_1 = c_2,$$

c_1 being the initial quantity of saponin present in the system containing the mixed lysins which haemolyses in time t_2 , c_2 the quantity of saponin which, acting alone, would produce lysis in time t_2 , and c_3 having the meaning defined above. I say that one would expect to find this relation, because it seems to follow so obviously from the reasoning, but, as a matter of fact, I shall show that the relation would be true under special conditions only, and that these are far from obvious.

If ($c_3 + c_1$) be not equal to c_2 , the activity of the mixture appears to be depressed below, or increased above, the sum of the activities of the two lysins by the factor $c_2/(c_3 + c_1)$, and the value of this ratio can be used to measure the extent of the departure from a simple additive effect.

2. Results.

1. *Saponin and digitonin.* These lysins are both glucosides and have haemolytic properties which are similar in almost every respect. When mixed, however, their combined effect is much less than their individual effects added together, as can be seen from the values for the ratio $c_2/(c_3 + c_1)$ which are given in Table I.

Table I.

Saponin γ	Digitonin γ		
	40	25	18
100	0.89	0.53	0.59
50	0.90	0.52	0.55
40	0.92	0.56	0.59
25	0.87	0.60	0.54
20	0.83	0.57	0.51

In the case of these two lysins, the inhibition is greater when the lysins are present in the smaller concentrations, but, as will be seen below, this is not an invariable rule. I may remark that the second decimal of the value given for the ratio is probably not very reliable.

2. *Saponin and sodium taurocholate.* These two lysins differ quite considerably in their haemolytic properties, but as in the case of saponin and digitonin, there is mutual inhibition.

Table II.

Saponin γ	Taurocholate γ		
	2000	1000	667
100	0.73	0.98	0.98
67	0.71	1.00	0.97
40	0.72	0.89	0.93
25	0.84	0.85	0.86
20	0.89	0.88	0.80

As in Table I there is a tendency for the inhibition to increase as the quantity of saponin becomes less, but it does not change in a similar way as the quantity of taurocholate is varied. The greatest inhibition of all, indeed, is met with when the bile salt is present in the greatest concentration.

3. *Saponin and sodium oleate*. These two lysins are quite unlike in many respects, and the activity of a mixture of the two is far greater than that which would result from summing the individual activities.

Table III.

Saponin γ	Oleate γ		
	200	100	67
100	1.74	1.63	1.41
67	1.52	1.67	1.40
50	1.39	1.60	1.37
40	1.34	1.50	1.26
33	1.16	1.37	1.05
25	1.09	1.11	1.03

The acceleration in this case is greatest when the lysin concentration is greatest and falls off as either the quantity of saponin or of oleate present in the system is reduced.

4. *Sodium taurocholate and sodium glycocholate*. In the case of these two similar haemolysins, we again find more than an additive effect, and again the acceleration is greatest when the lysins are present in greatest quantity.

Table IV.

Taurocholate γ	Glycocholate γ		
	1000	800	670
1330	2.14	1.44	1.27
1000	1.81	1.36	1.20
800	1.39	1.33	1.16
667	1.33	1.29	1.11
500	1.20	1.18	1.07
400	1.12	1.09	1.04

5. *Sodium taurocholate and sodium oleate*. This combination of lysins gives the most curious results of all:

Table V.

Taurocholate γ	Oleate γ		
	200	100	67
2000	0.62	0.78	0.76
1000	1.02	0.70	0.79
500	1.54	1.07	0.89
400	1.70	1.20	0.93

Here we find more than an additive effect, or less than an additive effect, depending on the concentrations of the haemolysins present in the system.

DISCUSSION.

At first sight these results appear to dispose of Sen and Mitra's contention that the total effect of mixtures of lysins is "in most cases probably additive," for the ratio $c_2/(c_3 + c_1)$ is generally greater or less than unity. Before drawing such a

conclusion, however, we must carefully scrutinise the principles on which the investigation is based.

To simplify matters, let us first assume that both lysins, which we shall refer to as A and B , act on the same cell component. Existing theory states that lysis is complete when $x\gamma$ of lysin are used up in combining with a proportional quantity of cell component. Suppose that this occurs when lysin A transforms the component, and when $x=25$. Further, suppose that complete lysis takes time t_1 when A is used. When B is used, let the time be t_1 also: then we would say that the quantity c_2 of B has the same activity as the quantity c_1 of A , for both give complete lysis in the same time t . If the two lysins were acting together in these same concentrations, we should at first sight expect the time taken for complete lysis to be the same as that taken by (c_1+c_1) of A , if A were acting alone.

But a little consideration will show that this is only so if the value of n in the expression for the fundamental reaction for A and B is the same, *i.e.* the kinetics of the reaction between the cell component and A and between the cell component and B must both be described by the expression

$$kt = \frac{p}{p-1} \left\{ c^{\frac{p-1}{p}} - (c-x)^{\frac{p-1}{p}} \right\},$$

with the same value of $p=1/n$. Or, in words, if two lysins produce complete haemolysis by transforming the same amount of cell component in the same time, they will transform different amounts of cell component in shorter times, unless the value of n is the same for the time-dilution curves of both lysins. This is a very important point, and is related to another point which has been discussed elsewhere [Ponder, 1932], *viz.* that if we have a quantity of lysin c_1 which, as the result of the presence of some inhibitor, appears to have been reduced to the smaller quantity c_2 , then the quantity of lysin rendered inert by the inhibitor is (c_1-c_2) only if the value of n is the same in the presence and in the absence of the inhibitor.

Let us take a concrete example, in which 100γ of lysin A (*e.g.* saponin) act on cells to produce complete lysis when $x=25$. Suppose that $n=1.0$ and $1/k=10$. The relation between t and the quantity of component transformed is then shown in Fig. 1, curve A, and the time for complete lysis is 1.23 minutes. Now let 100γ of B act, and suppose that $n=2.0$ and $1/k=0.59 \times 10^{-3}$. Curve B of the same figure shows the relation between t and x , and again complete lysis occurs in 1.23 minutes. In these concentrations the two lysins are accordingly equal to each other, at least when the time for complete lysis is used as a criterion. But if the transformation of 12.5γ is taken as a criterion, they are unequal, for A transforms this quantity in 0.57 minute and B in 0.51 minute. And similarly for the transformation of any quantity less than 25γ .

Now suppose that we are ignorant of this effect of the different values of n , and mix 100γ of A with 100γ of B . Then we again get complete lysis when 25γ of lysin are transformed, and referring to the A-curve (arbitrarily used as a standard) we expect this to occur in 0.57 minute, for then if n were 1.0 for both A and B , we should get a transformation of 12.5γ of A and 12.5γ of B . In fact, we get 25γ used up in combining with the cell component in 0.54 minute as the result of 12γ of A being used up, and 13γ of B . This looks like an acceleration, and referring to the time-dilution curve for A , we find $c=200\gamma$ giving complete lysis in 0.57 minute and $c=210\gamma$ giving complete lysis in 0.54 minute. In such a case we should find the ratio $c_2/(c_3+c_1)$ to be 1.05, although in fact the effects of the two lysins were strictly additive.

This apparent acceleration is not very spectacular, but it becomes far greater if we use a smaller quantity of lysin, *e.g.* 35γ of *A* and 42.5γ of *B*¹. These both produce lysis in 5.44 minutes if the values of *n* and of $1/k$ are the same as the above. If *n* were the same for *A* and *B*, and the values of $1/k$ so adjusted as to give $t=5.44$ for $x=25$, then we should have 25γ transformed in 1.9 minutes in a system containing both *A* and *B* in the above concentrations. In fact, when $n=2.0$ for *B* and 1.0 for *A*, we get complete lysis in 1.25 minutes, for at this value

Fig. 1. Two curves showing the relation between time and the quantity of lysin transformed, *A*, when $n=1.0$, *B*, when $n=2.0$. For details, see text.

of t we have 8.25γ of *A* transformed and 16.75γ of *B*, the total being 25γ . Referring to the time-dilution curve for *A*, we get $t=1.25$ when $c=99\gamma$ and $t=1.9$ when $c=70\gamma$. So the ratio in this case turns out to be 1.4, which represents quite a considerable acceleration, although the activities of the two lysins are still strictly additive.

By the same reasoning, if we select as the standard curve that for the lysin with the higher value of n , instead of that for the lysin with the lower value of n , as above, the apparent accelerations become replaced by apparent inhibitions of the same magnitude.

It would be possible, at the expense of a rather unwarrantable amount of trouble, to investigate each one of the ratios shown in the preceding tables and to find out if the observed departures of the $c_2/(c_3+c_1)$ ratios from unity are such as could be accounted for by one of these "pseudo-accelerations" or "pseudo-inhibitions," but a conclusion may be arrived at in a quicker way, for under all circumstances the pseudo-acceleration or pseudo-inhibition is more obvious in the systems containing the more dilute lysins. We can accordingly draw up a

¹ If two concentrations of two lysins produce complete lysis in the same time, the same fractions of these concentrations produce lysis in different times, unless n is the same for both. This is so obvious that it requires no discussion.

Table showing what we should expect to find if the departures of the ratio from unity were the result of such pseudo-accelerations and inhibitions, and also what we find in fact.

Table VI.

Standard lysin	<i>n</i>	Second lysin	<i>n</i>	Result predicted	Result found	Increase or decrease in dilute system
Saponin	1.8	Digitonin	2.5	Accel.	Inhib.	Increase
Saponin	1.8	Taurocholate	1.6	Inhib.	Inhib.	Increase
Saponin	1.8	Oleate	1.3	Inhib.	Accel.	Decrease
Taurocholate	1.6	Glyccholate	1.2	Inhib.	Accel.	Decrease
Taurocholate	1.6	Oleate	1.3	Inhib.	Both accel. & inhib.	

It is therefore quite clear that even if all allowance is made for the occurrence of these pseudo-accelerations and inhibitions, we have no evidence that the activities of these haemolysins are in fact additive. Indeed, if we were to go through the labour of making the necessary corrections, the departure of the ratio from unity would be increased in as many cases as it would be decreased.

We are thus forced to one of two conclusions: either (*a*) that the effects of two lysins are not additive, or (*b*) that the additive effect is obscured by the lysins reacting with one another in such a way as mutually to inhibit or enhance each other's activities. This they might do by reacting with each other in solution, as two colloids very frequently do, or by interfering with each other in the region of the red cell surface. The two possibilities (*a*) and (*b*) are not mutually exclusive, but, as I shall show, the second is far the more likely.

Let us see what the hypothesis that the activities of two lysins are not additive amounts to, the term additive being used in the sense of this paper, and all inhibitory and acceleratory effects of the lysins on each other being either excluded or allowed for. Under such circumstances, the effects of the lysins must be additive if they affect the same cell component. If they affect two different cell components, their effects will not be additive unless we imagine that the result of partial destruction of two different and spatially separated components is equal, in an additive sense, to the much greater destruction of a single component. There is no precedent for such an idea, which is highly improbable in itself: in fact, if the two lysins attack different cell components, their effects will almost certainly not be additive. The hypothesis that the activities are not additive is therefore tantamount to a hypothesis that the lysins react with different components and demands that they shall not react with the same component. All the available evidence, however, indicates that different lysins react with the same cell component, probably the protein "stromatin" [see Ponder *et al.*, 1930], and they certainly all react with that component which is responsible for the discoidal form [Ponder, 1934]. We have accordingly no collateral evidence supporting possibility (*a*), and, while we cannot altogether disprove it, we can conclude that it is not a very likely one.

We have therefore to fall back upon the second explanation for the results obtained in the above tables, *viz.* that the additive effects of two lysins are usually obscured by various inhibitory and acceleratory phenomena which result from an interaction of the lysins with each other.

SUMMARY.

When two simple lysins (saponin, digitonin, sodium taurocholate, sodium glyccholate or sodium oleate) act together in a system, the total activity is rarely the same as the sum of the two individual haemolytic activities. The most

likely explanation of the result is that the lysins interact with each other, thereby mutually enhancing or inhibiting each other's lytic activity.

Some curious difficulties arise in the analysis of the combined effects of two lysins, and these are discussed at some length.

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