welchii lecithinase. Evidence of the existence of such a lecithinase has now been obtained by quantitative isolation of the products of hydrolysis, phosphorylcholine and a neutral fat. Macfarlane & Knight (1941) designated a lecithinase of this type as lecithinase C, following the designation by Belfanti, Contardi & Ercoli (1936) of the two types of lecithinase known at that time as lecithinases A and B; lecithinase A splits off lysolecithin and lecithinase B two fatty acids from lecithin. Bard & McClung (1948) have stated that the designation of the *Cl. welchii* enzyme as a lecithinase C was incorrect, as the Italian authors (Contardi & Ercoli, 1933) had previously named the enzyme liberating choline from lecithin as a lecithinase C. It has, however, been pointed out by Miles & Miles (1950) that the existence of this enzyme was merely postulated by Contardi & Ercoli and in the subsequent review by Belfanti *et al.* (1936) only the lecithinases known to exist were designated. The designation of the *Cl. welchii* enzyme as a lecithinase C is therefore not incorrect.

The lecithinase activity of different samples of *Cl. haemolyticum* toxin has not been directly correlated with their lethality, but the fact that the samples examined had an enzymic activity comparable to that of *Cl. welchii* toxins, taken in conjunction with Jasmin's findings, affords reasonable evidence that the main lethal and haemolytic agent in *Cl. haemolyticum* toxin is this lecithinase. The inhibitory action of heterologous antitoxic sera on this enzyme shows that it is immunologically distinct from the lecithinases of *Cl. welchii* and *Cl. oedematiens* type A; it is however closely related, and probably identical antigenically, with *Cl. oedematiens* type B lecithinase, as was expected from the finding of Oakley *et al.* (1947) that the L.V. factor of *Cl. haemolyticum* was apparently identical with *Cl. oedematiens* β-toxin.

SUMMARY

1. *Clostridium haemolyticum* toxin contains a lecithinase decomposing lecithin into phosphorylcholine and a neutral fat.

2. The toxin also decomposes sphingomyelin, but does not attack the kephalin types of phospholipin.

3. The enzyme is inhibited by *Cl. haemolyticum* and *Cl. oedematiens* type B antitoxic sera, but not by *Cl. welchii* or *Cl. oedematiens* type A antitoxic sera, and is apparently identical antigenically with *Cl. oedematiens* β-toxin.

I am indebted to Dr C. L. Oakley for giving me the opportunity to examine this toxin and for the gift of antitoxins.

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The Biochemistry of Bacterial Toxins

5. VARIATION IN HAEMOLYTIC ACTIVITY OF IMMUNOLOGICALLY DISTINCT LECITHINASES TOWARDS ERYTHROCYTES FROM DIFFERENT SPECIES

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As a result of related work in several laboratories, it has been established with reasonable certainty that the lecithinases present in the culture filtrates of *Clostridium welchii*, *Cl. oedematiens* type A, and *Cl. oedematiens* type B are identical respectively with the specific antigenic components designated *Cl. welchii* α-toxin, *Cl. oedematiens* β-toxin and *Cl. oedematiens* γ-toxin, while *Cl. haemolyticum* lecithinase is apparently identical antigenically with *Cl. oedematiens* β-toxin (Nagler, 1939; Macfarlane, R. G., Oakley & Anderson, 1941; Macfarlane & Knight, 1941; Oakley, Warrack & Clarke, 1947; Macfarlane, 1948, 1950). Culture filtrates of various strains of *Cl. bifermentans* also contain a lecithinase which has a similar biochemical action and is related antigenically to *Cl. welchii* lecithinase (Miles & Miles, 1947, 1950).

Cl. welchii α-toxin, the specific lecithinase, is a lethal, haemolytic and necrotic toxin, so it might be supposed *a priori* that lecithinases with the same

biochemical action—decomposition of lecithin into phosphorylcholine and a diglyceride—would have the same haemolytic and lethal capacity, per unit of enzyme, as this toxin, however variable the enzyme content of the crude toxin, in units/ml., might be. It was pointed out by Oakley *et al.* (1947) that in fact these lecithinases differ in their haemolytic capacity; thus *Cl. welchii* α-toxin readily attacks sheep erythrocytes, which are relatively insensitive to the *Cl. oedematiens* γ-toxin, but hardly affects horse erythrocytes which are very sensitive to the *Cl. oedematiens* toxin. (Dr Oakley informed me that the statement by Oakley *et al.* (1947) that *Cl. oedematiens* β-toxin was similar to the γ-toxin was an error, and that horse and sheep cells are equally sensitive to the β-toxin.) Miles & Miles (1947, 1950) have pointed out that, relatively to the lecithinase activity, samples of *Cl. bifermentans* toxin are much less toxic to mice than *Cl. welchii* toxin. The examination of the *Cl. bifermentans* toxins carried out by these authors does not exclude the possibility that these toxins contained another lipolytic enzyme in addition to a lecithinase C, since the identification of phosphorylcholine and a neutral fat as sole end products of the hydrolysis of lecithin was not unequivocal. The presence of such an enzyme, however, though it might account for some discrepancies in titres and the apparent differences in the toxicity of lecithinases from different strains of *Cl. bifermentans*, could scarcely affect the gross difference in toxicity for mice between the lecithinases of *Cl. welchii* and *Cl. bifermentans*, nor the qualitative differences in the haemolytic effect on erythrocytes of different species.

Oakley *et al.* (1947) concluded that something was involved in the haemolysis of different types of cells by different lecithinases besides the enzymic attack on lecithin. Miles & Miles (1947) suggested that differences between the lecithinases might reside either in a readiness of absorption to certain tissue structures that is independent of lecithinase activity, or in the presence in the body of activators to one but not to the other. It appeared probable to the writer (Macfarlane, 1948) that haemolysis by these toxins is dependent upon the hydrolysis of the cell phospholipin and that differences in sensitivity are due primarily to variations in the rate of the hydrolysis with the species of red cells for any one lecithinase, or with the kind of lecithinase for cells of any one species, because the mutual access of enzyme and substrate might be affected by the specific structure of the enzyme or the cell. A direct comparison of the rate of decomposition of the phospholipin of intact sheep and horse erythrocytes by amounts of *Cl. welchii* and *Cl. oedematiens* lecithinases equal in activity towards aqueous emulsions of lecithin has now been made, and shows that striking differences exist. An attempt was also made to correlate the rate of decomposition of the cell phospholipin with the rate of haemolysis; these experiments were complicated by the phenomenon of 'hot-cold' lysis, and some observations upon this are included in this paper.

EXPERIMENTAL

Materials and methods

Lecithinases. The toxins used as sources of the immunologically distinct lecithinases, which are designated throughout as the specific antigen, were: for *Cl. welchii* α-toxin, glycerinated or dry toxins derived from *Cl. welchii* type A, strain S107; for *Cl. oedematiens* β-toxin, dry *Cl. haemolyticum* or *Cl. oedematiens* type B toxin; and for *Cl. oedematiens* γ-toxin, dry *Cl. oedematiens* type A toxins. The lecithinase activity of the toxins was determined, and checked from time to time, and solutions of the required strength were made up in 0·9% NaCl immediately before use.

The *Cl. welchii* toxin used was found to be free from θ-toxin, as the haemolytic activity was not decreased in presence of θ-antitoxin. In several of the haemolysis experiments, the toxins were heated in borate buffer pH 8·0 for 15 min. at 38° to inactivate any receptor-destroying enzyme (McCrea, 1947) which might have affected the cells, and the lecithinase activity was then redetermined before use. This treatment, however, made no consistent difference to the course of haemolysis.

Erythrocytes separated from samples of defibrinated, oxalated or citrated blood were washed three times by centrifuging with 0·9% NaCl immediately before use, and the packed cells were suspended in 0·9% NaCl at the desired concentration, usually 50 or 80% (v/v).

Lecithinase activity. The enzyme activity was measured by the liberation of acid-soluble P from an aqueous emulsion of egg lecithin at pH 7·6 and 37°, one enzyme unit (e.u.) being the amount liberating 0·1 mg. P in 15 min. under standard conditions (Macfarlane & Knight, 1941).

Ether-soluble phospholipin of erythrocytes. Measured amounts of the reaction mixtures, usually 5 ml. containing 2 ml. cells, were extracted twice, for 2 hr. at room temperature with occasional stirring, with 6 vol. of a 1:2 ethanol-ether mixture. The two extracts, separated by centrifuging, were combined and evaporated *in vacuo* in a filter flask warmed up by occasional immersion in hot water; the residue was extracted with ether, and the ethereal solution, cleared by centrifuging, was measured or made up with ether to a known volume. The phospholipin content was then estimated by colorimetric determination of total P in samples after wet ashing.

It is not considered that this method of extraction is ideal, i.e. that 100% of the phospholipin is extracted by ether-ethanol in these conditions, but it is practicable. A third extraction made no substantial difference to the total, and duplicate determinations usually agreed within ±5%. The values for lipid P were in reasonable accord with others quoted in the literature; thus 2 ml. packed sheep cells generally yielded about 0·2 mg. lipid P, of which about 50% was not decomposed by *Cl. welchii* lecithinase and was therefore regarded as kephalin. Erickson, Williams, Bernstein, Avrin, Jones & Macy (1938) found, for two sheep, an average of 330 mg. phospholipin/100 g. cells, which is approximately 13 mg. P/100 ml. cells, of which 50% was kephalin.

In the present experiments, the lipid P liberated by the lecithinase would include both lecithin and sphingomyelin P, but the fraction is termed 'lecithin'.

Rate of hydrolysis of phospholipin of horse and sheep erythrocytes in situ *by different lecithinases*

In these experiments, the different lecithinases were diluted freshly in saline (0·9 % sodium chloride) to give solutions of known enzymic activity, determined by the rate of hydrolysis of egg lecithin in standard conditions. A measured amount of red cells in saline was diluted with more saline according to the final concentration of cells required, sufficient borate (Palitsch) buffer, pH 7·6 containing 0·06 M-calcium chloride, was added (1/5 of the final volume) to give 0·012 M-Ca^{++} overall, and the mixture warmed to 37°. A measured amount of the required enzyme was then added. Samples (5 ml.) were removed at once, and at intervals during incubation at 37°, into 50 ml. centrifuge pots and treated immediately with 30 ml. of the ethanol-ether mixture, which stopped the enzyme action. The extraction and estimation of lipid P was then carried out as described.

Table 1. *Hydrolysis of phospholipin of sheep and horse erythrocytes by* Clostridium welchii *α-toxin*

(The values are given for 5 ml. mixture containing 0·5 or 1·0 ml. cells and 1 e.u. lecithinase, incubated at 37°.)

	Sheep cells		Horse cells	
Initial lipid P (μg.)	Exp. *A* 100, 108	Exp. *B* 47, 46	Exp. *A* 82, 86	Exp. *B* 30, 30
Incubation (min.)	P hydrolysed (μg.)			
10	14	15	0	0
20	24	22	0	0
30	40	23	4	4
45	38	24	14	13
60	44	23	14	13

Table 2. *Hydrolysis of phospholipin of sheep and horse erythrocytes by* Clostridium oedematiens *γ-toxin*

(The values are given for 5 ml. mixture containing 1·0 ml. cells and 0·5 e.u. lecithinase at 37°.)

	Lipid P (μg.)			
	Sheep cells		Horse cells	
Time (min.)	Exp. *A*	Exp. *B*	Exp. *A*	Exp. *B*
0	104	110	105	87
	105	110	104	87
10	108	110	103	85
20	108	108	91	79
30	106	104	86	68
45	106	104	79	60
60	110	104	73	53

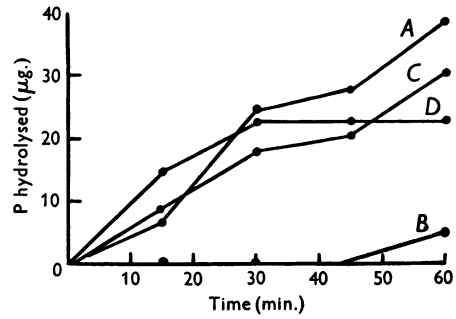

Fig. 1. Rate of hydrolysis of phospholipin of sheep and horse erythrocytes by *Cl. oedematiens* β- and γ-toxins. Curve *A*, γ-toxin, horse cells; *B*, γ-toxin, sheep cells; *C*, β-toxin, horse cells; *D*, β-toxin, sheep cells. 0·5 e.u. enzyme, 1 ml. cells per 5 ml. mixture.

Experiments were carried out with the concentration of cells varied from 0·5 to 2·0 ml. packed cells per 5 ml. mixture, and with the concentrations of enzyme varied from 0·5 to 2 e.u. per 5 ml. mixture. As it was technically awkward to obtain hydrolysis curves with short time intervals simultaneously for the six combinations tested (three lecithinases, two kinds of cell) the experiments were varied so that on one day different enzymes were used with one sample of cells, and on another day the same enzyme solution was tested simultaneously on horse and sheep cells and so forth. The absolute results, in μg. P hydrolysed in a given time, and particularly in the total amount

Table 3. *Comparative hydrolysis of phospholipin of sheep and horse erythrocytes by* Clostridium welchii *α-toxin and* Clostridium oedematiens *γ-toxin*

(The values are given for 5 ml. mixture containing 1·0 ml. cells and 0·5 e.u. of the specific lecithinase, incubated at 37°.)

Cells ...	Sheep				Horse			
Toxin ...	*Cl. welchii*		*Cl. oedematiens*		*Cl. welchii*		*Cl. oedematiens*	
Time (min.)	Lipid P (μg.)	Decrease (μg.)	Lipid P (μg.)	Decrease (μg.)	Lipid P (μg.)	Decrease (μg.)	Lipid P (μg.)	Decrease (μg.)
0	95, 96	—	95, 96	—	92, 100	—	92, 100	—
15	99	—	99	—	99	—	Lost	
30	86	10	100	—	92	4	74	22
45	78	18	110	—	85	11	71	25
60	72	24	94	—	75	21	60	26

hydrolysed in experiments continued until no further hydrolysis occurred, varied in experiments which were nominally identical except that the sample of blood had been drawn from a different individual or perhaps stored at 4° for a day or two. The most probable explanation of this variability is that, as will be shown later, the hydrolysis of lecithin precedes the haemolysis (i.e. precedes the actual liberation of haemoglobin into the medium); sooner or later, however, haemolysis occurred in all these mixtures, but the time of onset of haemolysis varied somewhat with different samples of blood in nominally identical systems. The lecithinases are affected by laked cells (for instance, under the standard conditions of testing, the activity of 1 e.u. of *Cl. welchii* or *Cl. haemolyticum* lecithinase was reduced by 75% by the presence of laked sheep cells at a final concentration of 1/100), so that in nominally identical experimental mixtures the lecithinase would begin to be affected at a time determined by the 'fragility' of the particular cells used. It is therefore the differences in the initial rates of hydrolysis of the cell phospholipin that most truly reflect the differences of affinity in the combinations studied.

Some representative experiments are shown in Tables 1–3 and Fig. 1. It may be emphasized that in these experiments the actions of equal amounts in standard enzyme units of the different lecithinases were being compared. Table 1 shows that *Cl. welchii* α-toxin hydrolyses the phospholipin in sheep cells more rapidly than that in horse cells and that the hydrolysis, on occasion, ceased before the whole of the hydrolysable P had been liberated. Table 2 shows that *Cl. oedematiens* γ-toxin attacks the horse cell more rapidly than the sheep; this table shows the actual amounts of P estimated. Table 3 shows an experiment in which the actions of equal amounts of these two toxins on the same samples of horse and sheep cells were examined almost simultaneously, and again shows the marked differences in the initial rates of hydrolysis. Fig. 1 illustrates the results of a similar experiment comparing the action of *Cl. oedematiens* β-toxin on horse and sheep cells; this toxin attacked both kinds of cell at about the same rate. The curves for *Cl. oedematiens* γ-toxin from the values in Table 2 are included in Fig. 1 to show the differences between the two toxins.

Summarizing these results, it appears that amounts of the different lecithinases which are equal in biochemical activity, judged by the rate of hydrolysis of egg lecithin in aqueous emulsion, attack the phospholipin of intact erythrocytes at different rates, and that the rate can vary with the kind of erythrocyte as well as with the kind of lecithinase. The actual differences found were consistent with the finding of Oakley *et al.* (1947), based on estimations of the minimum haemolytic dose of the toxins in standard conditions, that sheep cells are more sensitive than horse cells to *Cl. welchii* α-toxin, but less sensitive to *Cl. oedematiens* γ-toxin, and that sheep and horse cells are about equally sensitive to *Cl. oedematiens* β-toxin.

Comparative rates of hydrolysis of the extracted phospholipin of sheep cells by immunologically distinct lecithinases

It seemed possible that the differences in the rate of decomposition of the phospholipin in intact cells might be due to differences in the relative proportions of lecithin and sphingomyelin in cells from different species, or perhaps to differences in the proportions of α- and β-lecithin present; that is to say, the differences might be due mainly to differences in the nature of the substrate available, the different lecithinases having different affinities for different substrates. The phospholipin was therefore extracted from sheep cells and the rates of hydrolysis of the cell lipin and of egg lecithin by *Cl. welchii* and *Cl. oedematiens* γ-toxin were compared.

Sheep cells (400 ml.) were washed with saline, haemolysed in 20 vol. of cold water and the stroma collected after acidification to pH 5·8. The stroma were extracted twice with 2 vol. ethanol-ether (1:2) mixture, the extract was evaporated to small bulk and treated with acetone. The precipitated phospholipin was washed with acetone, and emulsified in water (15 ml.). The emulsion contained 0·89 mg. P/ml. The action of *Cl. oedematiens* γ-toxin and of *Cl. welchii* α-toxin on this cell-lipid preparation and on an emulsion of egg lecithin containing 0·91 mg. P/ml. were compared simultaneously. As only 50% of the phospholipin of the cell is hydrolysed by these lecithinases, the amounts of sheep lipid and lecithin added were adjusted to give nominally the same concentration of hydrolysable P.

The results (Table 4) show that both the toxins hydrolysed the extracted phospholipin more slowly than lecithin; probably a large proportion of the cell phospholipin is sphingomyelin, which is more slowly hydrolysed than lecithin. The relative rates of hydrolysis of the two substrates by the two toxins, however, were practically the same. There is no indication in this experiment that the different toxins

Table 4. *Hydrolysis of extracted phospholipin of sheep cells by* Clostridium oedematiens *γ-toxin and* Clostridium welchii *α-toxin*

(The mixture contained 1 ml. (approx. 0·5 e.u.) toxin; 1·8 mg. sheep lipid P or 0·9 mg. egg lipid P; 1 ml. Caborate buffer; and water to 6·0 ml. Incubated at 37°.)

Toxin	Phospho-lipin	Hydrolysis (μg. P) 15 min.	30 min.	Ratio of rates Sheep/egg
Cl. oedematiens	Sheep	25	34	0·36
	Egg	70	133	
Cl. welchii	Sheep	16	28	0·33
	Egg	49	79	

have a different rate of attack upon the extracted phospholipin of the sheep cell which would account for the difference in the rate of attack in the intact cell. But it may be noted that the hydrolysis of the extracted phospholipin did not go to completion (approximately 50 %); this may be due to differences in the dispersion of the extracted substrates, but more extensive investigation on the nature of the phospholipins and their relative rates of hydrolysis are necessary before it could be concluded that there is no difference at all in the substrate affinities of the different toxins.

The extent of 'hot-cold' lysis with erythrocytes of different species

Haemolysis, in the sense of liberation of haemoglobin from the cell into the medium, is clearly secondary to some alteration of the cell membrane by the lytic agent. As it was intended to look for a correlation between the degree of decomposition of the phospholipin of the cell and the degree of haemolysis it was necessary to know whether the secondary effect, the haemolysis, had the same course in different kinds of cell, for the phenomenon is complicated by the fact that *Cl. welchii* α-toxin is a so-called 'hot-cold' lysin (cf. Glenny & Stevens, 1935). It was shown by van Heyningen (1941) that there might be no visible haemolysis in a mixture of sheep cells and *Cl. welchii* α-toxin after incubation at 37°, but that if the mixture were cooled a rapid and extensive haemolysis took place; the lysis during the 'cold' period was apparently independent of the action by the toxin, as it still took place when antitoxin was added after incubation and before cooling. The extent of 'hot-cold' lysis with erythrocytes from different species was therefore examined.

The general method adopted was to incubate a mixture of 10 ml. cells (50% v/v), 5·0 ml. borate buffer pH 7·6 containing 0·06 M-$CaCl_2$ and 10 ml. of diluted *Cl. welchii* toxin in 0·9% NaCl at 37°; 1 ml. samples were pipetted out at the desired times into each of a number of centrifuge tubes containing 5 ml. *Cl. welchii* antitoxin diluted in similar buffer-saline and already warmed in the same thermostat. The amount of antitoxin (50 i.u.) was fifty times the amount required for neutralization of the toxin, to ensure that the action was stopped at once. The contents of the tubes were mixed by inversion and the tubes cooled for 10 min. in beakers of water at different temperatures. The water was stirred by hand with a thermometer about every 2 min. during the period of cooling and kept at the desired temperature by the cautious addition of ice water. The tubes were then centrifuged simultaneously for 4 min.; the haemoglobin in the supernatant was estimated by colorimetric comparison with a standard prepared by haemolysing 1 ml. of the reaction mixture in 5 ml. water. The amount (e.u.) of toxin added, or the time of incubation, was adjusted as necessary to give from 1 to 30% haemolysis at 37°. Control experiments showed that in the absence of toxin no haemolysis occurred during the incubation or on cooling. Cooling the tubes for 30 min. instead of 10 min. slightly increased the 'cold' lysis at temperatures below 10°, presumably because of slower diffusion at the lower temperatures, but as the trend of the results was not affected the shorter period of cooling was used.

Extent of 'cold' lysis in sheep cells. A mixture of sheep cells and *Cl. welchii* toxin was incubated as described above, and at intervals two samples were removed into warm antitoxin-saline, one being held at 37° and the other cooled at 1°. Table 5 shows that after 5 min. incubation a trace (0·3 %) of haemolysis in the tube at 37° was increased to 10 % on cooling; and after 10 min. incubation, though the 'hot' lysis increased only from 0·3 to 0·6 %, the 'cold' lysis increased to 75 %.

Table 5. *'Hot-cold' lysis of sheep cells with* Clostridium welchii *toxin*

(The reaction mixture (60 ml.) contained 12 e.u. lecithinase and 12 ml. 50% (v/v) cells. The haemolysis was measured after addition of antitoxin to the sample.)

Incubation with toxin at 37° (min.)	Haemolysis (%)	
	At 37°	At 1°
5	0·3	10
10	0·6	75
20	4·8	92
30	10·4	100

Variation in 'cold' lysis with temperature of cooling. A mixture of sheep cells and *Cl. welchii* toxin was incubated for 15 min. at 37°, and samples after dilution with the warm antitoxin were cooled at different temperatures in the range 37–1°. Fig. 2, in which the percentage haemolysis is plotted against the temper-

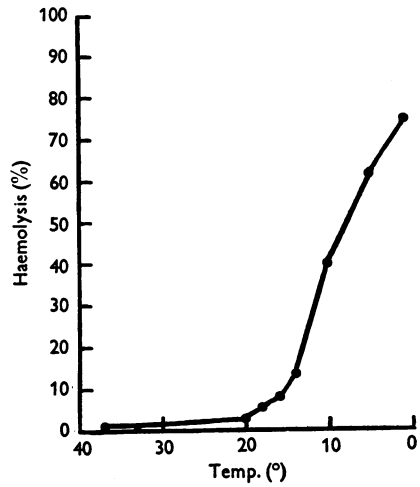

Fig. 2. Variation in 'cold' lysis with temperature of cooling. The mixture of sheep cells and *Cl. welchii* toxin (1 e.u./5 ml.) was incubated 15 min. at 37°; samples were then mixed with antitoxin and cooled for 10 min.

ature of cooling, shows that, with a 'hot' lysis at 37° of 1 %, cooling from 37 to 20° caused little 'cold' lysis, but about 18–14° there is a marked inflexion in the curve, and at 10 and 1° the 'cold' lysis was 40 and 75 % respectively.

Variation in 'cold' lysis with erythrocytes from different species. Similar experiments were carried out with cells from horse, rabbit and human blood. The amount of *Cl. welchii* toxin added had to be varied owing to the different sensitivities of the cells, and was arranged by trial to give between 1 and 20 % haemolysis at 37° in 15 min. The results show that the extent of 'cold' lysis is very different in different species (Table 6). With horse cells there is an inflexion in the temperature curve similar to that with sheep cells, but the increase in haemolysis is much less marked, e.g. with an initial 'hot' lysis at 37° of 1 %, the total haemolysis on cooling to 1° was 75 % with sheep cells, but only 14 % with horse cells. With rabbit and human cells, cooling had practically no effect on the total haemolysis; sometimes the values were slightly lower after cooling, presumably owing to slower diffusion. Similar results were obtained with *Cl. haemolyticum* toxin.

Table 6. *Extent of 'cold' lysis in erythrocytes of different species*

(Mixtures of the cells with *Cl. welchii* toxin were first incubated at 37° for 15 min.; samples were mixed with antitoxin and cooled 10 min. The 'hot' lysis (37°) was varied in different experiments by varying the amount of toxin.)

Species of cell	Haemolysis (%) after cooling at							
	37°	20°	18°	16°	14°	10°	5°	1°
Sheep	1	3	5	9	14	41	63	75
Horse (*a*)	1	1	2	2	2	3	8	14
Horse (*b*)	18	17	15	17	16	20	24	38
Rabbit	19	14	—	14	—	14	14	14
Human	12	13	—	13	—	13	14	15

It appears from the experiments that the phenomenon of 'hot-cold' lysis is dependent not only on the action of a particular kind of lysin (for not all haemolysins give this effect), but also on the ability of the cell to undergo 'cold' lysis, which may vary with cells from different species. It is interesting that the curve relating temperature to the degree of 'cold' lysis resembles those relating temperature to the surface area of films of fatty substances (Adam, 1922); possibly the effect is directly connected with the fact that the lytic agent is a lecithinase.

Correlation between the onset of hydrolysis of the cell phospholipin and the onset of haemolysis

It was clear from the foregoing experiments that the degree of haemolysis after cooling was not a fair measure of the action of the haemolysin. A comparison was therefore made of the rate of hydrolysis of the phospholipin in a mixture of cells and toxin with the rate of haemolysis, measuring the haemolysis which had actually occurred in the mixture at 37° at the time the sample was taken for phospholipin estimation.

The experiments were carried out with different combinations of the specific toxins and sheep or horse cells, in a similar manner to those already described (p. 272), the cells being incubated with the toxin in buffer-saline containing $CaCl_2$ in a thermostat at 37°; the amount of toxin (e.u.) was, however, varied so as to get suitable rates for measurement over experimental periods of 2–3 hr. Samples for haemoglobin determination were removed into tubes already warmed in the thermostat, rapidly centrifuged and the supernatant removed, approx. 5 min. after taking the sample, for estimation by colorimetric comparison with a laked standard. The sample for haemoglobin was therefore in contact with the active toxin slightly longer than its pair for phospholipin determination; but the specific antitoxins were not all available in sufficient amount to enable the use of the alternative method of neutralizing the toxin and then taking both samples.

Representative experiments with *Cl. welchii* α-toxin and *Cl. oedematiens* γ-toxin acting on sheep and horse erythrocytes are shown in Fig. 3, the percentage hydrolysis of phospholipin and the percentage hydrolysis being plotted against time. In all the systems, the hydrolysis of phospholipin preceded the haemolysis in the early stages of the reaction. The distinct lag in haemolysis is consistent with the idea that haemolysis is dependent upon the primary action of the lecithinase.

Correlation between percentage hydrolysis and percentage haemolysis

It was particularly noticeable in the experiments with *Cl. welchii* toxin and horse cells that after an appreciable hydrolysis of phospholipin had occurred, with little accompanying haemolysis, there was often a sudden increase in haemolysis so that the latter reached 100 % before the maximum hydrolysis of phospholipin (about 50 %) took place. Presumably the rate at which haemoglobin leaks from the cell at a particular time is a function, not directly of the rate of action of the lecithinase, but of the damage incurred by the cell at that time, i.e. the rate of leakage is constantly increasing, while the rate of enzymic action is tending to decrease as the substrate disappears. Upon this function may be superim-

posed the normal variation in fragility amongst a heterogenous population of cells. In a particular experiment with one sample of blood, therefore, the

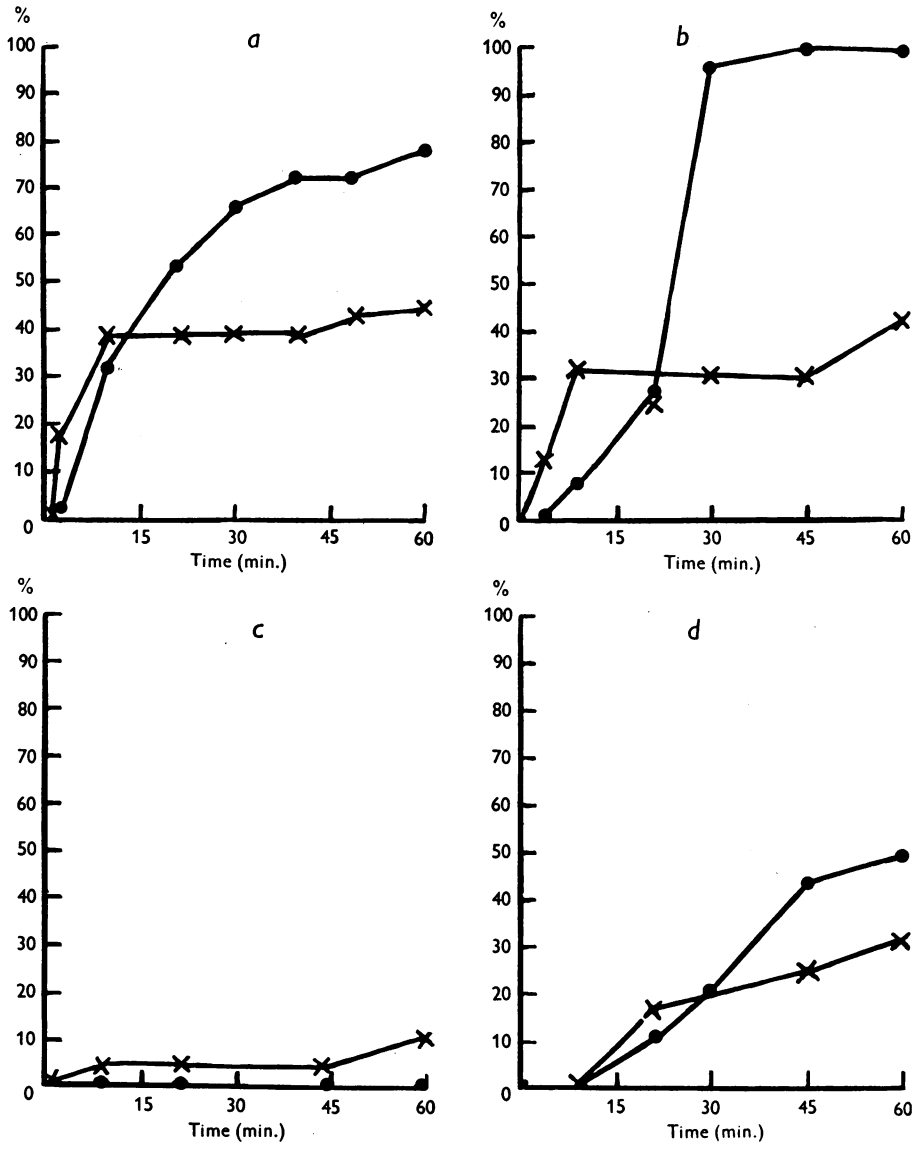

Fig. 3. Correlation between rate of hydrolysis of phospholipin and rate of haemolysis of erythrocytes by *Clostridium* toxins. (*a*), *Cl. welchii* α-toxin (15 e.u.), sheep cells; (*b*), *Cl. welchii* α-toxin (7·5 e.u.), horse cells; (*c*), *Cl. oedematiens* γ-toxin (1 e.u.), sheep cells; (*d*), *Cl. oedematiens* γ-toxin (1·5 e.u.), horse cells.

The mixtures contained 2 ml. packed cells, 1 ml. Ca-borate buffer and the stated amount of toxin per 5 ml. Percentage hydrolysis of phospholipin, × — × ; percentage haemolysis, ● — ●.

extent of haemolysis at a particular time depends on: (1) the rate of enzymic action, which may vary with the kind of lecithinase or the species of cell; (2) the damage, in the sense of liability to leak, which is caused to the cell by the decomposition of a particular amount of phospholipin; this is not necessarily the same in cells from different species, as the architecture of the membrane may be different; and (3) the variation in the mean fragility of the particular sample of cells from the normal mean.

In Fig. 4 the percentage hydrolysis of the 'lecithin' fraction (= 50 % of the total phospholipin) has been plotted against the percentage haemolysis for all the

samples from three experiments with *Cl. welchii* toxin with different lots of sheep cells (Fig. 4*a*), and from three experiments with this toxin on horse cells (Fig. 4*b*). The figures indicate that the critical level of damage is different in the two kinds of cells, the horse cells being apt to lyse completely at any level over 50% hydrolysis of the 'lecithin' fraction, whereas the sheep cells were more resistant. It appears also that while at the lower values there is some correlation between hydrolysis and haemolysis, the values of phospholipin hydrolysis corresponding to 50% haemolysis are very variable and lie in the critical region. The activity of haemolysins is sometimes assessed by the time taken to reach 50% haemolysis, or by the dose necessary to reach this value in an arbitrary time. It seems possible that a lower value for the end point, e.g. 25–30% haemolysis, would give more reproducible results with different samples of blood, at least with this particular type of haemolysis.

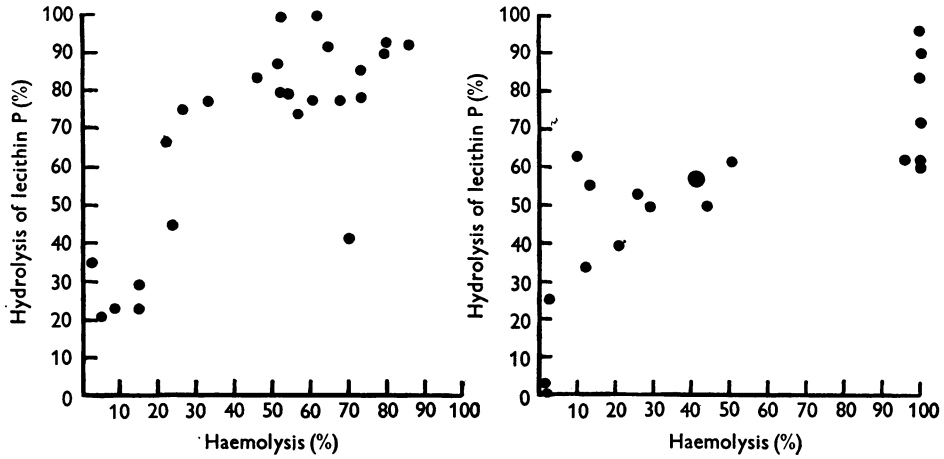

Fig. 4. Correlation between percentage hydrolysis of lecithin P and percentage haemolysis. *Cl. welchii* toxin on (*a*) sheep cells; (*b*) horse cells.

Adsorption of lecithinases by erythrocytes

It seemed possible that there might be differences in the rate of adsorption of the different lecithinases by different kinds of cells which might be the basis of the different rates of attack on the phospholipin. A considerable number of experiments with different conditions were carried out to test this possibility. A measured amount of lecithinase (from 0·5 to 5 e.u.) was added to a mixture containing washed cells (1–3 ml.) in a total volume of 5 ml., and after a time the cells were centrifuged down and the residual activity of the lecithinase determined in the supernatant. Tests were carried out at 0°, room temperature and 37°, in the presence and absence of calcium ions, with different cells and with different lecithinases. The experiments had to be arranged so that the supernatants were removed before haemolysis took place, as the activity of the lecithinase is decreased in the presence of laked cells.

In none of the experiments was there any significant decrease in the enzymic activity of the supernatants. In comparative experiments, for instance, keeping one sample of a mixture at 0° until another sample at 37° had haemolysed, there was still no measurable adsorption at 0°, although any adsorption would presumably have been about as rapid at 0° as at 37° and there had clearly been sufficient contact between the cell and the enzyme at 37° for the reaction to take place. In many of the experiments (e.g. with 0·5 e.u. of enzyme) the potential rate of haemolysis was limited by the concentration of the enzyme, so that a substantial, or at least a significant, decrease in the enzyme was expected if adsorption (that is to say, fixation) took place. It may be noted here also that no agglutination of the cells occurred on mixing with the toxin.

DISCUSSION

It has been generally accepted, though never formally proved, that the haemolytic, lethal and lecithinase activities of *Cl. welchii* type A toxins free from the θ-haemolysin are all properties of a single agent, the *Cl. welchii* α-toxin. Direct evidence that haemolysis is dependent upon the decomposition of the phospholipin of the red cell, however, has hitherto been lacking. The experiments which have now been carried out with the three immunologically distinct lecithinases of *Cl. welchii* and *Cl. oedematiens*

toxins show that haemolysis was always preceded by decomposition of some of the phospholipin of the cells. Moreover, on comparing the rates of action of equal amounts (in enzyme units) of these three lecithinases on sheep or horse erythrocytes, striking differences were observed which appear to be sufficient to account for the differences in sensitivity to the haemolysins noted previously by other workers. Thus, for example, *Cl. oedematiens* γ-toxin hydrolysed the phospholipin in sheep cells more slowly, *Cl. welchii* α-toxin more quickly, than that in horse cells; these findings are in agreement with the observations of Oakley *et al.* (1947) in haemolytic tests that sheep cells are less sensitive than horse cells to *Cl. oedematiens* γ-toxin, but more sensitive to *Cl. welchii* α-toxin. There seems little doubt that haemolysis by these toxins is due primarily to the action of the lecithinase present, and that differences in haemolytic sensitivity are due basically to differences in the rate of hydrolysis of the phospholipin of the cell, though other differences in the structure of the cell may have an effect. It seems probable that the relative inactivity of *Cl. bifermentans* lecithinase as a haemolysin (Miles & Miles, 1950) is due to failure to attack the phospholipin in the cell.

It appears that the differences in rate are not due to differences in the affinity of the different enzymes for the substrate as such, for no difference was detected in the action on phospholipin extracted from sheep cells. The differences must then be regarded as reflecting the 'goodness of fit' between the individual enzyme and the cell surface in which the substrate is embedded. It is clear from immunological data that differences in configuration amongst the lecithinases on the one hand, and amongst the species-specific red cells on the other, in fact exist and it seems probable that a difference in configuration which can determine immunological specificity would be sufficient to condition the approach of the enzyme to the cell surface. There is, of course, no evidence in the present work that the particular groups which determine the antigenic specificity of the enzyme or the erythrocyte would also determine the 'goodness of fit' between the enzyme and the cell, for non-antigenic groups might intervene, though it is convenient for argument to assume that the immunologically specific groups are those which also determine access.

The experimental results have an interest beyond that of the actual haemolytic systems investigated, firstly in relation to the problem of virulence, and also in the wider problem of biological differentiation. It is well known that pathogenic organisms may show greater virulence in one species of animal than another, but the host-parasite relationship *in vivo* is a complex of many factors. In the haemolytic systems studied, the relationship is simplified to the rate of reaction *in vitro* between an enzyme of specific structure and its substrate held in a particular kind of cell, and it appears that differences in these rates are sufficient to account for the differences in 'virulence' where virulence is judged by the sensitivity to haemolysis. It may be thought that the 'goodness of fit', in dimensions of the order which determine immunological or enzymic specificity, between the potentially toxic factor and the tissue, whether this is exercised at the portal of entry, or in further invasion or in actual necrosis, is the basic factor determining the virulence of a parasite in a particular host, or in determining the susceptibility of different tissues in a host to a particular parasite.

This is, so far as is known, the first time that differences in the rate of action of biochemically similar but immunologically distinct enzymes upon a biological structure have been demonstrated, and the chemical basis of the differences is unknown. Two examples of known differences in chemical constitution determining the biological effect in different species may be of interest. Dr Trevan informed me (cf. Collier, Paris & Woolf, 1948) that in its paralysing action on the diaphragm, *dextro*-tubocurarine is more effective in the mouse, but considerably less effective in the rat, than its dimethyl ether. The phenolic or methoxyl groups involved in this differentiation are not in the immediate vicinity of the quaternary nitrogen group which is the curarizing group; in the lecithinases the effect of other groups in the enzyme molecule upon the access of a substrate to the enzymic groups may be analogous to that of substituent groups in the drugs.

Bender & Krebs (1950), from an investigation of the rate of oxidation of α-amino-acids by amino-acid oxidases from different sources, concluded that the specific properties of the enzyme are no less important than the structure of the amino-acid in determining oxidizability; for example, of eight straight-chain aliphatic monoaminomonocarboxylic acids, the L-amino-acid oxidase of cobra venom did not attack the C_3, C_4, C_{11}, C_{12} and C_{18} acids, while the L-amino-acid oxidase of *Neurospora* did not attack the C_{11}, C_{12} and C_{18} acids; the relative rates of attack on the remaining acids by the enzymes were also different. Considering this selectivity of action as being generally true for immunologically distinct enzymes, it is easy to imagine the bias in metabolism inherent in a species, either by absolute failure to utilize a particular substrate, or by differences in the rate of utilization, even though the enzymic groups concerned are identical and present in similar concentrations in different species. Moreover, if in the development of an individual an enzyme molecule moves from its point of formation to some other site, its activity may be modified by the configuration of the site at which it becomes fixed, and such a modification may be perhaps a major factor in the differentiation of a tissue, the whole structure regulating

the partial functions. This conception could readily be elaborated, but whether such a modification in activity could be demonstrated is another matter.

SUMMARY

1. Haemolysis of horse and sheep erythrocytes by *Clostridium welchii* and *Cl. oedematiens* toxins was always preceded by decomposition of some of the phospholipin of the cell, indicating that haemolysis is primarily dependent upon the action of the lecithinases present.

2. The rates of hydrolysis of the phospholipin of intact erythrocytes of horse and sheep by the three immunologically distinct lecithinases—*Cl. welchii* α-toxin and *Cl. oedematiens* β- and γ-toxins—are different.

3. These differences appear sufficient to account for the known differences in sensitivity of horse and sheep cells to haemolysis by the immunologically specific lecithinases.

4. After exposure to *Cl. welchii* α-toxin at 37°, sheep erythrocytes undergo extensive haemolysis (hot-cold lysis) on cooling below a critical temperature. This effect is less marked with horse cells and does not occur with human or rabbit erythrocytes.

5. The significance of the selective action of the lecithinases on erythrocytes of different species is discussed with relation to the problem of virulence and biological differentiation.

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Studies in Detoxication

33. THE METABOLISM OF HALOGENOBENZENES. A COMPARISON OF THE GLUCURONIC ACID, ETHEREAL SULPHATE AND MERCAPTURIC ACID CONJUGATIONS OF CHLORO-, BROMO- AND IODO-BENZENES AND OF THE *o*-, *m*- AND *p*-CHLOROPHENOLS. BIOSYNTHESIS OF *o*-, *m*- AND *p*-CHLOROPHENYLGLUCURONIDES

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It is believed that the halogenobenzenes are transformed in the body into phenols and into mercapturic acids. Because cysteine is involved in mercapturic acid formation, the sulphur conjugation of the halogenobenzenes has received considerable attention. There seems, however, to be little information as to the relative amounts of the halogenobenzenes excreted as sulphur conjugates (mercapturic acids) and as oxygen conjugates (glucuronides and ethereal sulphates) (Witter, 1945). Earlier work (e.g. Binkley, 1949; Coombs & Hele, 1926; Rhode, 1923; Stekol, 1936; Shiple, Muldoon & Sherwin, 1924) attempted to correlate mercapturic acid formation with sulphate conjugation, but this gave an incomplete picture because the glucuronic acid conjugation was left out of account. The object of the present work was to find out how the halogenobenzenes are distributed over the three conjugations, sulphate, glucuronic and mercapturic acid. The view that sulphur conjugation of aromatic compounds may be the precursor of their oxygen conjugations (Quick, 1937) was kept in mind. A study of the conjugation of the chlorophenols (cf. Baumann & Preusse, 1879; Shiple *et al.* 1924; Coombs & Hele, 1926; Külz, 1883) was included for comparison, and at the same time the *o*-, *m*- and *p*-chlorophenylglucuronides were prepared biosynthetically as reference compounds and for further information on the anomalous optical rotations of acetylated *o*-substituted phenylglucuronides (Smith, 1949).