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The Biochemistry of Bacterial Toxins

4. THE LECITHINASE ACTIVITY OF *CLOSTRIDIUM HAEMOLYTICUM* TOXIN

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Oakley, Warrack & Clark (1947) observed that culture filtrates of *Clostridium haemolyticum* contained a haemolytic factor producing opalescence in egg-yolk emulsion; this lecithovitellin (L.v.) reaction could be neutralized by *Cl. oedematiens* type B antitoxic sera containing β -antitoxin. These facts indicated that *Cl. haemolyticum* toxin contained a lecithinase similar in biochemical action to those of *Cl. welchii* and *Cl. oedematiens* toxins, and closely related antigenically to *Cl. oedematiens* type B lecithinase, the β -toxin (Oakley *et al.* 1947; Macfarlane, 1948a). The presence of such a lecithinase has been verified by isolation of the products formed from lecithin by the action of the toxin, and by the examination of the inhibitory power of appropriate antitoxic sera upon the enzymic activity. A preliminary note on these findings has been published (Macfarlane, 1948b).

EXPERIMENTAL

Methods. Lecithinase activity was determined by the rate of liberation of acid-soluble P from lecithin under standard conditions, one enzyme unit (e.u.) liberating 100 μ g. P in 15 min. at 37°. The antilecithinase activity of sera was measured by their inhibitory action on P liberation by a test dose of toxin. These and other methods were similar to

those used previously (Macfarlane & Knight, 1941; Macfarlane, 1948a).

Toxins and antitoxins. Dry toxins prepared at the Wellcome Laboratories from a strain of *Cl. haemolyticum* originally isolated by Dr Vawter were used. These toxins, and samples of *Cl. haemolyticum* and *Cl. oedematiens* antitoxic sera, were given by Dr Oakley; the titres of the sera in β - and γ -antitoxin units by the L.v. test are referred to arbitrary standards (Oakley *et al.* 1947). The *Cl. welchii* antitoxin used was the Elstree no. 1 standard, containing 95 i.u./ml.

Hydrolysis of lecithin by Clostridium haemolyticum toxin

Isolation of products. Lecithin (0.65 mmol.) prepared from egg yolk was emulsified in 20 ml. 0.01 M-calcium chloride, pH 7.4, and incubated at 37° with 10 mg. toxin, further quantities of toxin (10 mg.) being added after 3 and 24 hr. incubation, and the mixture titrated back to pH 7.4 at intervals with NaOH. In 3 hr. 0.43 mmol. and in 19 hr. 0.56 mmol. acid were produced, equivalent to 67 and 86 % hydrolysis of the lecithin if only one linkage were attacked. Production of acid continued very slowly to a total of 0.79 mmol. at 42 hr. when the experiment was stopped. The mixture was separated, by extraction with ether several times, into a water-soluble fraction which contained 95 % of the original

lipin P in an organic form, and an ether-soluble fraction. The two fractions were worked up for the isolation of phosphorylcholine and a diglyceride respectively, as in previous isolations.

Phosphorylcholine was isolated as the crystalline calcium compound; the crude crystals (164 mg.), equivalent to 80% yield on the original lipid, were analysed without recrystallization. (Found: N, 4.3; total P, 8.8; inorganic P, nil; Cl, 11.8. Calc. for $C_5H_{13}O_4NCIPCa$, $4H_2O$: N, 4.3; Cl, 10.8; P, 9.5%.) The substance was rapidly and completely hydrolysed by purified bone phosphatase free from diesterase, and was therefore a monophosphoric ester.

From the ethereal fraction, acetone-soluble material with a saponification value of 167 and an acid value of 10 was obtained in amount (366 mg.) equivalent to a 95% yield of a palmityleoyl glyceride.

Cl. haemolyticum toxin therefore decomposes lecithin substantially into phosphorylcholine and a neutral fat; but the acid value of the fat, and the slight excess of acid produced during the enzymic hydrolysis, indicate that in addition to the lecithinase the toxin may contain a trace of a lipase whose action becomes apparent if the incubation time is prolonged.

Lecithinase activity of *Clostridium haemolyticum* toxins. The two samples of toxin examined (HC 231046 and NX 605) had activities of 2.5 and 3.0 e.u./mg. respectively, similar to the activity of dry toxins from highly toxigenic strains of *Cl. welchii* type A, and much higher than that of *Cl. oedematiens* toxins (0.05–0.3 e.u./mg.) prepared in the same way by precipitation from the culture filtrate with ammonium sulphate.

The lecithinase showed more than 50% of its maximum activity over the pH range 5.0–9.0, with a broad optimum from pH 7.0 to 7.6. With these crude toxins, the enzyme was active without addition of calcium ions, though the activity was increased by such addition; it was inhibited by fluoride, but the inhibition was not immediately apparent, presumably because the precipitation of calcium fluoride was gradual. Thus, in one experiment under

standard conditions, the enzyme liberated, in two successive 15 min. periods, 126 and 97 μ g. P in presence of 0.01 M-calcium chloride, 62 and 24 μ g. P in presence of 0.08 M-sodium fluoride, and 57 μ g. P in absence of added calcium ions.

Substrate specificity of Clostridium haemolyticum lecithinase

Sphingomyelin. The action of the toxin on lecithin and sphingomyelin at different substrate concentrations was compared with that of *Cl. welchii* toxin. Table 1 shows that sphingomyelin is decomposed by *Cl. haemolyticum* toxin, and that, as with *Cl. welchii*, the rate of decomposition is considerably slower than that of lecithin.

Table 1. *Hydrolysis of lecithin and sphingomyelin by Clostridium welchii and Clostridium haemolyticum toxins*

(1 e.u. *Cl. welchii* or *Cl. haemolyticum* toxin in total vol. 6.0 ml., including 1 ml. borate buffer pH 7.6; Ca^{++} 0.01 M overall; incubation 15 min. at 37°.)

Nature and concn. (mm) of substrate	Hydrolysis by toxin (μ g. P)	
	<i>Cl. welchii</i>	<i>Cl. haemolyticum</i>
Lecithin		
6.9	112	114
1.4	79	103
0.7	58	70
0.4	40	47
Sphingomyelin		
1.6	33	35
0.6	23	30

Kephalin. The action towards kephalin was determined by comparison of the maximal production of acid-soluble P from the ether-soluble, acetone-insoluble phospholipins prepared from egg yolk and from brain, which contain very different proportions of lecithin and kephalin. Parallel determinations of the maximal hydrolysis of these phospholipin preparations by *Cl. welchii* toxin, which has been shown to have no action on phosphatidylethanolamine or

Table 2. *Hydrolysis of phospholipins of egg yolk and brain by Clostridium welchii and Clostridium haemolyticum toxins*

(Tubes containing 5 e.u. lecithinase, 0.5 ml. 2% phospholipin emulsion, Ca^{++} 0.01 M, and 1.0 ml. borate buffer pH 7.6 in total vol. 6.0 ml. were incubated at 37° for various times, and the acid-soluble P estimated.)

Incubation time (min.)	Hydrolysis (% of lipid P)			
	Egg-yolk lipid (0.41 mg. P)		Brain lipid (0.48 mg. P)	
	<i>Cl. haemolyticum</i>	<i>Cl. welchii</i>	<i>Cl. haemolyticum</i>	<i>Cl. welchii</i>
15	84	82	37	36
30	94	92	42	41
45	95	92	39	42
60	95	100	41	44

phosphatidylserine (Macfarlane, 1948c), were also made. Table 2 shows that the *Cl. haemolyticum* toxin hydrolysed 95 % of the egg-yolk phospholipin and 47 % of the brain phospholipin, the corresponding figures for *Cl. welchii* toxin being 100 and 44 %. The *Cl. haemolyticum* lecithinase therefore appears, like the *Cl. welchii* enzyme, to be specific for lecithin and sphingomyelin, and to have no action on the cephalin type of phospholipin.

Inhibition of Clostridium haemolyticum lecithinase by homologous and heterologous antitoxic sera

The inhibitory action of antitoxic sera was determined by allowing measured volumes of a suitable dilution of serum to combine with the test dose of the toxin (2 e.u.) at room temperature for

The cross-inhibition by the two latter antitoxins was examined more closely to determine whether the *Cl. haemolyticum* and *Cl. oedematiens* type B enzymes were immunologically identical. The two sera used were found to be of similar strength, though this was fortuitous, and were therefore used at the same dilution, 1/500. Table 4 shows that the same volume of either antitoxin inhibited the test doses of both toxins to nearly the same degree. Considering that the two toxins may differ in the total content of antigen (toxin + toxoid) contained in the test dose, which was selected to give equal enzymic activity, the similarity of the neutralizing power of the antitoxins towards the homologous and heterologous toxins strongly suggests that the *Cl. oedematiens* β -lecithinase and the *Cl. haemolyticum* lecithinase are immunologically identical.

Table 3. *Inhibition of lecithinases by heterologous antitoxic sera*

Specific antitoxin		Inhibition of test dose (2 e.u.) of lecithinase (%)		
		<i>Cl. haemolyticum</i> toxin	<i>Cl. oedematiens</i> toxin	
			Type B	Type A
Kind	Units			
<i>Cl. welchii</i> α -antitoxin	2	Nil	Nil	Nil
<i>Cl. oedematiens</i> γ -antitoxin	3	—	—	27
	14	—	6	69
	70	3	7	100
	140	6	7	100
<i>Cl. oedematiens</i> β -antitoxin	3	50	57	—
	6	85	89	—
	15	100	100	4
	75	100	100	17

Table 4. *Cross-inhibition of Clostridium haemolyticum and Clostridium oedematiens Type B lecithinases by the specific antitoxins*

(The test dose of *Cl. haemolyticum* toxin contained 1.56 e.u. and of *Cl. oedematiens* toxin 1.50 e.u.)

Antitoxin (ml.)	Inhibition of enzyme (%)			
	<i>Cl. oedematiens</i> β -antitoxin Todd 3, 1/500		<i>Cl. haemolyticum</i> antitoxin EX 1250, 1/500	
	<i>Cl. haemolyticum</i> toxin	<i>Cl. oedematiens</i> toxin	<i>Cl. haemolyticum</i> toxin	<i>Cl. oedematiens</i> toxin
0.2	37	35	37	20
0.4	65	60	62	36
0.6	79	66	75	53
0.8	82	81	83	70

10 min., and then estimating the residual lecithinase activity in the standard manner (Macfarlane, 1948a). Table 3 shows that *Cl. haemolyticum* lecithinase was not appreciably inhibited by an amount of *Cl. welchii* antitoxin which was known to be fifty times greater than that required to inhibit 2 e.u. *Cl. welchii* lecithinase, nor by amounts of *Cl. oedematiens* γ -antitoxin more than sufficient to neutralize the γ -lecithinase; it was, however, readily inhibited by *Cl. haemolyticum* antitoxin and by *Cl. oedematiens* β -antitoxin.

DISCUSSION

While this work was in progress Jasmin (1947) reported that *Cl. haemolyticum* toxin gave reactions with human serum and lecithovitellin similar to those described by Nagler (1939) and Macfarlane, Oakley & Anderson (1941). He showed, further, that a parallelism existed between the intensity of these reactions and the haemolytic and lethal activity of the toxin, and suggested that all these properties were due to the activity of a lecithinase similar to *Cl.*

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 cephalin 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