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The Biochemistry of Bacterial Toxins

2. THE ENZYMIC SPECIFICITY OF *CLOSTRIDIUM WELCHII* LECITHINASE

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The general properties of a lecithinase present in the toxic culture filtrates of *Clostridium welchii* were described by Macfarlane & Knight (1941), who concluded that this enzyme was probably identical with *Cl. welchii* alpha toxin, the main lethal component of these filtrates. Since none of the *Cl. welchii* toxins examined hydrolyzed diphenyl-, monophenyl- or β-glycero-phosphate, or nucleic acid, this enzyme appeared to be a true lecithinase, as distinct from a phosphodiesterase, although the linkage attacked is of the diester type leading to the formation of phosphorylcholine and a diglyceride. In a further study of the enzyme specificity, Macfarlane (1942) reported briefly that kephalin was not decomposed by *Cl. welchii* toxin, but sphingomyelin was slowly hydrolyzed; the products of this hydrolysis, however, were not isolated.

Recently Zamecnik, Brewster & Lipmann (1947), who were unaware of this finding, examined the specificity of the toxin by an elegant adaptation of the manometric technique, and stated that it was inactive towards sphingomyelin, as well as to the other phospholipins tested. It seemed possible that the negative result of these authors with sphingomyelin was due to the short reaction time and low toxin concentration employed, but it was desirable to confirm the earlier positive results in more detail, since the specificity of the lipolytic activity of the toxin is a matter of some interest. *Cl. welchii* toxins in general appear to be free from phosphoesterases, lipase and nuclease, and the specificity of action might therefore be exploited for the differential estimation of phospholipins, for the elucidation of phospholipin structure or for the stepwise degradation of lipoprotein compounds.

The sample of sphingomyelin used in these experiments was kindly given to me by Dr O. Rosenheim, as one which, from its method of preparation as an ether-insoluble fraction, was free from lecithin; it contained, however, about 25% cerebroside. Several hydrolyses of this crude sphingomyelin with *Cl. welchii* toxin have been carried out, in which from 70 to 90% of the phosphorus present was converted into a water-soluble compound, with a satisfactory recovery of phosphorylcholine. It proved difficult, however, owing to the overlapping solubilities in the solvents tried, to separate the fatty product, which on the classical formula of sphingomyelin should be lignocerylsphingosine, from the accompanying cerebroside in sufficient yield and purity to characterize the product, though evidence of the formation of a nitrogenous product free from phosphorus and carbohydrate was obtained. It is clear also, since the starting material may be a mixture of 'sphingomyelins', that the hydrolysis product may be a mixture containing a variety of fatty acid residues,

quite apart from any cerebroside impurity in the crude lipin.

Dr Lipmann has since informed me that his early data (manometric) showed a very slow reaction between the toxin and sphingomyelin, which was attributed to contamination of the sphingomyelin with a little lecithin, but that using larger amounts of toxin and a longer reaction time he has confirmed the decomposition of sphingomyelin. It appears therefore that *Cl. welchii* toxins have a curious specificity, which is presumably due to a single enzyme, in splitting off phosphorylcholine from lecithin and sphingomyelin, though neither kephalin (phosphatidylethanolamine or phosphatidylserine) nor, according to Zamecnik *et al.* (1947), glycerophosphorylcholine nor lysolecithin are attacked.

EXPERIMENTAL

Methods. The methods used for testing the enzymic activity of the toxin were similar to those previously described (Macfarlane & Knight, 1941), the reactions being followed by estimation of the acid-soluble organic P formed. One enzyme (lecithinase) unit (e.u.) = the amount producing 0·1 mg. P from excess lecithin at pH 7·6 and 37° in 15 min. under standard conditions.

Toxin. Glycerinated or dried toxin derived from *Cl. welchii*, type A, strain S 107, was used for the study of reaction rates, and a highly purified dry preparation, 354 H, for which I am indebted to Dr van Heyningen and Miss Bidwell, for the isolation experiments.

Substrates. The crude sphingomyelin used contained 2·7 % P, 2·5 % N, and 5·0 % galactose by orcinol estimation; calculated from this, the sample contained approximately 71 % of sphingomyelin and 23 % of cerebroside.

'Lecithin' was prepared from egg yolk, by repeated precipitations of the portion soluble in ether at 0° with acetone. The product usually contained 80–90 % lecithin.

A mixed preparation of 'lecithin-kephalin' was obtained from acetone-dried sheep brain by extraction with ether and repeated precipitation with acetone of the ether-soluble portion.

A purer preparation of 'kephalin' was obtained by repeated precipitation of the ethereal extract with 4 vol. ethanol.

Hydrolysis of sphingomyelin

Rate of hydrolysis. A representative protocol showing the comparative rate of hydrolysis of sphingomyelin and lecithin is shown in Table 1. At the same concentration, the hydrolysis of sphingomyelin is considerably slower than that of lecithin, but proceeded to the extent of 70 % in the conditions used. The hydrolysis, like that of lecithin, is activated by Ca ions and inhibited by NaF.

Isolation of products: Procedure (*a*). Crude lipin (0·69 g.) was emulsified in 25 ml. 0·02 M-$CaCl_2$, the pH adjusted to 8·4 with NaOH, and 10 mg. (600 e.u.) *Cl. welchii* toxin 354 H added in three portions at intervals of 3 hr. The mixture was incubated at 37°, being titrated back to pH 7·4 at intervals with 0·05 N-NaOH and finally to pH 8·4. The hydrolysis was allowed to proceed for 22 hr. in all, at which point 86 % of the P was water-soluble.

The aqueous portion was separated by filtration, evaporated to a small bulk on a water bath, refiltered, evaporated to dryness and re-extracted into 2 ml. water from which the crystalline Ca compound of phosphorylcholine was obtained on addition of ethanol. (Found: P, 9·1; N, 3·7; Cl, 10·1 %. Calc. for $C_5H_{13}O_4NPClCa.4H_2O$: P, 9·46; N, 4·25; Cl, 10·75 %.) The identity was further confirmed by determination of the hydrolysis constant in N-HCl at 100°, and by the rapid and complete hydrolysis of this phosphoric ester by bone phosphatase (phosphomonoesterase) to orthophosphate with liberation of choline.

The water-insoluble portion was extracted with cold acetone, dried and re-extracted with acetone. The total acetone-soluble material amounted only to 40 mg.; assuming that this material was a diglyceride derived from lecithin, the maximum amount of lecithin possibly present as an impurity in the crude sphingomyelin corresponded to less than 10 % of the organic P present. The acetone-insoluble material was fractionated by deposition from ethanolic solutions at different temperatures, the course of fractionation being followed by the P, N and carbohydrate content, as shown in Table 2.

Isolation of products: Procedure (*b*). Another hydrolysis, using 0·5 g. crude sphingomyelin, was carried out in a similar way, but the water- and acetone-insoluble material was extracted with cold chloroform. The fractions soluble and insoluble in chloroform, redeposited from ethanolic

Table 1. *Comparative hydrolysis of lecithin and sphingomyelin at 37°*

(Reaction mixtures contained 1·0 ml. aqueous emulsion of phospholipin (100 μg. P), 1 ml. borate buffer (Palitzsch), pH 7·6, and the additions below in a total volume of 6·0 ml.)

Cl. welchii toxin (Enzyme units)	Phospholipin	Additions	Time of incubation (min.)	P hydrolyzed (μg.)
1·1	Sphingomyelin	Nil	30	15
1·1	Sphingomyelin	$CaCl_2$	30	20
1·1	Sphingomyelin	$CaCl_2$	180	37
1·1	Sphingomyelin	NaF	180	12
1·1	Lecithin	Nil	30	30
1·1	Lecithin	$CaCl_2$	30	51
1·1	Lecithin	$CaCl_2$	180	76
1·1	Lecithin	NaF	180	21
3·3	Sphingomyelin	$CaCl_2$	180	71
3·3	Sphingomyelin	NaF	180	19

Table 2. *Balance sheet of the hydrolysis of crude sphingomyelin by* Cl. welchii *toxin*

	Quantities (mg.)			Composition (%)				
	Wt.	P	N	P	N	Carbohydrate (as galactose)	C	H
Original material	579	15·6	14·5	2·7	2·5	5·0	—	—
Calculated sphingomyelin (from P)	410	15·6	14·1	3·79	3·42	Nil	—	—
Calculated phrenosin (from galactose)	133	—	2·2	Nil	1·70	21·7	69·6	11·2
Calculated yield lignoceryl sphingosine	319	—	7·0	Nil	2·21	Nil	77·4	12·7
Found								
(1) P equivalent of acid formed	—	12·8	—	—	—	—	—	—
(2) Water-soluble portion	—	13·5	6·5	—	—	—	—	—
Cryst. phosphoryl choline	110	10·6	4·1	—	—	—	—	—
(3) Water-insoluble portion:								
Fraction from ethanol at 20°	86	1·65	1·46	1·92	1·7	9·2	—	—
Fraction from 95% ethanol at 20°	138	0	2·88	0	2·1	1·7	—	—
Fraction from ethanol at 0°	129	0·21	2·45	0·16	1·9	12·0	—	—
Mother liquors	83	0	—	0	—	4·0	—	—
Semi-crystalline material from pooled carbohydrate-low fractions from four hydrolyses	250	—	—	0	1·9	3·0	74·9	11·9

Table 3. *Hydrolysis of monoaminomonophosphatides by* Cl. welchii *toxin*

(Reaction mixture (6·0 ml.): 0·48 mg. lipin P; 3·0 e.u. toxin; 0·002M-$CaCl_2$; pH 7·6, at 37°.)

Phospholipin	Amino N / Total N (%)	α-Amino N / Total N (%)	Calc. % of P as lecithin	% hydrolysis of P 1 hr.	2 hr.	3 hr.	4 hr.
(1) Ether-soluble acetone-insoluble (egg yolk)	22	Nil	78	70	74	78	78
(2) Ether-soluble acetone-insoluble (sheep brain)	83	23	17	17	19	22	22
(3) Ether-soluble ethanol-insoluble (sheep brain)	95	25	5	0	<5	<5	—

solution at 0°, contained 5·5 and 14·5% carbohydrate respectively, but repetition of this step on the carbohydrate-low fraction did not effect a further separation. This material was then fractionated by deposition from hot ethanol; the main fraction, which separated between 22° and 0°, contained P, 0·07; N, 1·9; carbohydrate, 4·5%.

Four hydrolyses of 0·5–0·6 g. crude sphingomyelin were carried out in all, in which from 67 to 91% of the lipin P was converted to a water-soluble form, with a satisfactory recovery of phosphorylcholine, while approx. 30–40% of the calculated sphingosine N of the phospholipin was recovered in a P-free carbohydrate-low fraction containing 1·9–2·1% N. This pooled material was insoluble in cold ethanol, acetone or ether, dissolving readily on warming in ethanol or acetone and redepositing on cooling. After several fractionations, the deposit from 95% aqueous ethanol was a semi-crystalline mass of irregular plates but still gave a reaction on heating with orcinol and H_2SO_4 which indicated the presence of 2–3% carbohydrate (Table 2). In view of the possibility that the starting material contained a mixture of 'sphingomyelins', as well as the cerebroside impurity, the further identification by degradation of this small amount of material was not attempted.

Action of Cl. welchii *toxin on kephalin*

The action of *Cl. welchii* toxin on individual monoaminomonophosphatides was determined by comparison of the maximal production of water-soluble P from ether-soluble phospholipins prepared from egg yolk and brain with the amino N and α-amino N of the sample. Table 3 shows that the maximum percentage hydrolysis of P corresponded with the percentage content of non-amino (lecithin) N, while practically no hydrolysis was observed in a 'kephalin' fraction from sheep brain containing 25% of the total N as α-amino N. The conclusion drawn from this result, that neither phosphatidylethanolamine nor phosphatidylserine was hydrolyzed by *Cl. welchii* lecithinase, has been confirmed by Zamecnik *et al.* (1947) using pure samples of these phospholipins.

SUMMARY

1. The action of *Cl. welchii* toxin on a sample of crude sphingomyelin has been examined. Approximately 90% of the lipin P was converted into a water-soluble form; the hydrolysis is slower than that of lecithin P in similar conditions, but like that of lecithin is activated by Ca ions and inhibited by NaF. Phosphorylcholine was recovered quantitatively from the hydrolysis products, together with a P-free product approximating to the composition of lignocerylsphingosine, which was not obtained pure.

2. The maximum hydrolysis of lipin P in mixed lecithin-kephalin preparations corresponded with the percentage content of non-amino (lecithin) N.

3. It is concluded, from the manner of hydrolysis, that *Cl. welchii* lecithinase hydrolyzes sphingomyelin, as well as lecithin, with production of phosphorylcholine, but does not attack phosphatidylethanolamine nor phosphatidylserine.

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The Biochemistry of Bacterial Toxins

3. THE IDENTIFICATION AND IMMUNOLOGICAL RELATIONS OF LECITHINASES PRESENT IN *CLOSTRIDIUM OEDEMATIENS* AND *CLOSTRIDIUM SORDELLII* TOXINS

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Nagler (1939) observed that when *Clostridium welchii* type A was grown in human serum an opalescence developed and eventually a layer of fat rose to the surface. Macfarlane, Oakley & Anderson (1941) found that *Cl. welchii* toxin acts on a clear emulsion of egg yolk to give a similar but more marked opalescence, and that this reaction is due entirely to the α-toxin, the main lethal component of *Cl. welchii* type A culture filtrates; this is termed the lecithovitellin (L.V.) test. Subsequently, *Cl. welchii* toxin was found to contain a lecithinase (Macfarlane & Knight, 1941) which is probably identical with the agent responsible for the Nagler and L.V. reaction, and therefore with the α-toxin.

Hayward (1941, 1943) and Crook (1942) found that other species of *Clostridium*, particularly *Cl. oedematiens*, and also some aerobic spore bearers, may give positive Nagler or L.V. tests; this fact, as pointed out by Hayward (1943), does not militate against the specificity of the test for the detection of *Cl. welchii*, as the essence of the test is not merely a positive reaction but the inhibition of such a reaction by the specific antitoxin. There is, however, a tendency to regard any bacterial agent giving a positive L.V. test as a 'lecithinase', which is clearly unwarrantable without chemical evidence and may lead to confusion on the nature of the bacterial agents.

Macfarlane (1942) briefly reported the identification of a lecithinase in *Cl. oedematiens* toxins, which was immunologically distinct from the main lethal component, and also from *Cl. welchii* lecithinase. Oakley, Warrack & Clarke (1947), in a comprehensive study of the antigenic components of *Cl. oedematiens* toxins, found that the L.V. factor (γ-toxin) in *Cl. oedematiens* type A filtrates was immunologically distinct from that (β-toxin) present in type B filtrates. Through the courtesy of Dr Oakley, I have been able to show that the immunological behaviour of the lecithinases present in each of these types was similar to that of the L.V. factor, thus indicating that *Cl. oedematiens* β- and γ-toxins are lecithinases. The identification of these lecithinases, and of a lecithinase present in culture filtrates of certain strains of *Cl. sordellii* or *Cl. bifermentans*, is described below.

EXPERIMENTAL

The general methods of procedure and analysis were similar to those used by Macfarlane & Knight (1941). Lecithinase activity was determined by estimation of acid-soluble P liberated from lecithin, and the L.V. factor by the development of turbidity in a clear emulsion of egg yolk.

Toxins and antitoxins

Cl. oedematiens. For the initial experiments in 1942, dry or glycerinated toxins derived from *Cl. oedematiens* 'Albiston' were used. This type B strain toxin was in routine use in the Serum Department of the Lister Institute at Elstree for the hyperimmunization of horses; the various antitoxic sera from Elstree examined for antilecithinase activity were B type (anti-β-toxin). *Cl. oedematiens* type B toxin, O.C. 91044, with corresponding antisera Todd 3 and Todd 61, and type A toxins, A.E. 327, and O.C. 19345 with antisera 202 and 645 were given by Dr Oakley.

Cl. sordellii (or *bifermentans*). Culture filtrates were prepared in this laboratory from five strains (referred to for convenience as *Cl. sordellii*) obtained from Miss Hayward, described as: A 127 A (*sordellii* or *bifermentans*) from necrotic wound; N.C.T.C. 2919, *sordellii*; A 90 *b*, *sordellii*, from a case of gas gangrene; A 112 (*sordellii* or *bifermentans*); 7851 *a* (*sordellii* or *bifermentans*) from a gangrenous wound.