The Lecithinase of *Clostridium bifermentans* Toxin

BY GILLIAN M. LEWIS AND MARJORIE G. MACFARLANE

Lister Institute of Preventive Medicine, London, S.W. 1

(Received 14 November 1952)

Miles & Miles (1947, 1950) showed that culture filtrates of *Clostridium bifermentans*, an organism previously shown to give the Nagler reaction (Hayward, 1943), decomposed lecithin enzymically with production of a water-soluble organic phosphoric compound. This action was inhibited not only by homologous antisera but also, though less specifically, by *Clostridium welchii* antitoxin. Miles & Miles concluded from their experimental evidence that the action of the *Cl. bifermentans* filtrates was due to a lecithinase of the same biochemical type as the *Cl. welchii* lecithinase (*Cl. welchii* α -toxin), i.e. a lecithinase C, decomposing lecithin in a single step to phosphorylcholine and a diglyceride (Macfarlane & Knight, 1941). Miles & Miles also showed that, comparing the *Cl. welchii* and *Cl. bifermentans* preparations in enzymically equipotent doses, the *Cl. bifermentans* lecithinase was relatively non-toxic to mice, and relatively non-haemolytic towards red cells of various species.

The validity of any discussion of this interesting variation in the toxicity of similar enzymes depends on the identification of the *Cl. bifermentans* enzyme as a lecithinase C, and upon this point the evidence obtained by Miles & Miles is not conclusive. The chemical evidence adduced, i.e. the formation from lecithin of a water-soluble phosphorus compound, with liberation of choline on acid hydrolysis, does not exclude the presence of glycerylphosphorylcholine, nor establish that phosphorylcholine is a primary product. The discrepancies noted by Miles & Miles (1950) and also by Macfarlane (1948) between the capacities of the *Cl. welchii* and *Cl. bifermentans* preparations to decompose lecithin and the capacity to produce turbidity in egg yolk suggested that the *Cl. bifermentans* preparations might contain a lecithinase B, i.e. one producing fatty acids and glycerophosphorylcholine from lecithin, or even a mixture of lipolytic enzymes. The *Cl. bifermentans* enzyme also differed from the *Cl. welchii* enzyme in that calcium was apparently not required for activation.

Cl. bifermentans lecithinase is inhibited by *Cl. welchii* (antilecithinase) antitoxin. This fact, though it argues some similarity in structure, is not evidence that the enzymic groups are identical, for it is not known that the enzymic groups are antigenic *per se*.

Indeed the fact that the antilecithinase to *Cl. oedematiens* β - or γ -lecithinases does not inhibit the heterologous enzyme indicates that the lecithinase group is not the only or the dominant antigenic group.

We have examined the biochemical action of the *Cl. bifermentans* preparations used by Miles & Miles in their studies, and have obtained more conclusive evidence that the lecithinase present is of the same biochemical type as *Cl. welchii* α -toxin.

MATERIALS AND METHODS

Enzyme preparations. Two freeze-dried preparations of *Cl. bifermentans* toxin, designated by us as B_1 and B_2 , were used. These were derived from the least toxic strains used by Miles & Miles; for the same lecithinase activity, the toxicity was less than one-fortieth of that of *Cl. welchii* toxin.

The *Cl. welchii* toxin used was precipitated from a culture filtrate of *Cl. welchii*, S107, with $(\text{NH}_4)_2\text{SO}_4$, dialysed and freeze-dried. A solution in 50% glycerol was used routinely.

Lecithin. This was prepared from egg yolk by the method of Macfarlane & Knight (1941).

Egg-yolk extract. Egg yolks were washed and beaten up in 0.9% NaCl to give a 10% (v/v) suspension. This was clarified by centrifugation for 30 min. at 7000 rev./min. in a Servall angle centrifuge. The preparation was stored at -10° , either alone, or buffered with 0.2 vol. of Palitsch borate buffer, pH 7.2, or 0.2 M-acetate buffer, pH 5.5.

Lecithinase activity. This was estimated in standard conditions: 1.0 ml. 2.5% (w/v) aqueous lecithin, 1.0 ml. Palitsch borate buffer, pH 7.2, containing 0.01 M-CaCl₂, and 2.0 ml. of toxin diluted in water were incubated 30 min. at 37° ; 1.0 ml. 20% (w/v) trichloroacetic acid was added, the mixture filtered after 10 min. and the acid-soluble P estimated. The amount of toxin liberating 50 μg . P in these conditions was defined as an L unit. Within the range 0.5–3.0 L units the amount of P liberated was a linear function of the enzyme concentration.

Turbidity development in egg yolk. This was measured in an EEL photoelectric colorimeter. The toxin solution (1.0 ml.) was mixed with 1.0 ml. buffered egg-yolk extract and incubated at 37° . The turbidities were read against a blank containing egg yolk and water, and expressed as the scale reading. For quantities of toxin between 0.5 and 1.2 L units the turbidity was proportional to the concentration of enzyme, and within this range the turbidity was proportional to the time of incubation for at least 60 min.

Estimations of P were made colorimetrically (Martland & Robison, 1926) after wet-ashing.

RESULTS

Products of the hydrolysis of lecithin by Clostridium bifermentans toxin

Course of hydrolysis. An emulsion of lecithin (5 ml., 94 μ moles, 2.9 mg. P) in 0.002M-CaCl₂, pH 7.2, was mixed with 60 mg. *Cl. bifermentans* toxin B₂ (30 L units) in 2 ml. water. Samples were removed for the initial analysis, and the remainder (87 μ moles) incubated at 37°. The hydrolysis was followed by titration of the acid formed with 0.05N-NaOH; this was taken only to pH 7.2 (phenol red) to minimize saponification of glycerides. After 5.5 hr., 66 μ moles acid had been formed, and 77 μ moles acid-soluble P (89% hydrolysis). The mixture was then separated into water-soluble and ether-soluble fractions by three extractions with 10 ml. ether.

Fig. 1. Production of acid from lecithin by *Cl. welchii* and *Cl. bifermentans* toxins, followed manometrically. ○—○, 2 L units *Cl. bifermentans* B₂ toxin; ●—●, 2 L units *Cl. welchii* toxin.

Characterization of products. The water-soluble fraction after heating to 100° for 5 min. and filtration contained 1.9 mg. P, all in organic form (70% recovery of original phospholipin P). On treatment of a sample with a purified bone phosphomonoesterase at pH 8.0 in presence of 0.005M-MgCl₂, all the P present was converted to orthophosphate in 1 hr. at 38°; a parallel test with potassium diphenyl phosphate showed that the bone phosphatase was free from diesterase. The hydrolysis product was therefore a monophosphoric ester.

The ether-soluble fraction gave 38.0 mg. of acetone-soluble material, acid value 2.9; calculated as oleylpalmityl-diglyceride, this represents 73% recovery on the original lecithin, and 83% on the actual hydrolysis. The fatty acids formed on saponification were not isolated.

The above hydrolysis was carried out on a small scale because of the low activity of the *Cl. bifer-*

mentans enzyme and the limited amount available. As a check on the findings, a parallel experiment was run simultaneously with 30 L units of *Cl. welchii* toxin instead of *Cl. bifermentans* enzyme; the analytical figures and the recovery were practically identical. The production of acid from lecithin by equal quantities (L units) of the two lecithinases was also followed manometrically using the technique of Zamecnik, Brewster & Lipmann (1947) and found to correspond closely (Fig. 1). There appears little doubt that the *Cl. bifermentans* lecithinase is a lecithinase C liberating a monophosphoric ester, a neutral fat and one equivalent of acid from lecithin.

Tests for presence of other enzymes in Clostridium bifermentans toxin

Phosphodiesterase. The test mixture contained 2.0 ml. 0.1M-citrate buffer, pH 6.5, 1.0 ml. 0.02M-potassium diphenylphosphate, and 1.0 ml. toxin B₂ (10 mg.), with appropriate controls. After 30 min. at 37° the free phenol present was estimated using the Folin & Ciocalteu reagent. No liberation was observed, indicating that the toxin had no diesterase activity and that the phosphorylcholine formed during the hydrolysis of lecithin had not arisen by a secondary reaction.

Lipases. The action on triacetin, tributyrin and Tween 20 (a polyoxyalkylene derivative of sorbitan-monolaurate) was examined by the manometric technique of Singer & Hofstee (1948). With 50 mg. toxin B₂, in a total vol. of 3.3 ml., no increase in carbon dioxide evolution above that of the substrate without toxin took place in 1 hr. at 37°. The action of the toxin on triolein was examined by the method of Willstätter, Waldschmidt-Leitz & Memmen (1923), using 100 mg. toxin, with 1 hr. incubation at 37°, and again no lipase activity was detected.

Correlation between turbidity production and acid-soluble P liberated in egg-yolk extract

It appeared from the above experiments that the *Cl. bifermentans* toxin examined contained a lecithinase C, but no other lipolytic enzymes which could account for the discrepancies previously noted between the lecithinase activity and the rate of development of turbidity in egg yolk compared with *Cl. welchii* toxin. Nor does it appear that the discrepancy was due to proteolytic action (Miles & Miles, 1950). In the earlier experiments, however, the turbidity produced in egg yolk was referred to the number of L units used, i.e. to the activity of the two toxins acting on purified lecithin at pH 7.2. In the following experiments the turbidity produced was directly compared with the amount of acid-soluble P liberated in egg-yolk extract, as well as with the rate of hydrolysis of lecithin.

Dilutions of the *Cl. welchii* toxin and the *Cl. bifermentans* toxins B_1 and B_2 were made up so that 1 ml. samples produced turbidity at approximately the same rate when mixed with 1.0 ml. egg-yolk extract at pH 7.2 on incubation at 37°. The turbidity was measured at 20, 40 and 60 min. to check that it was proportional to the time of reaction, and at each time the acid-soluble P was estimated in 1.0 ml. samples: simultaneously, samples of the same toxin solutions were added to standard lecithinase test mixtures and the L units present determined in the usual way.

The results (Table 1) show that the ratio of turbidity to acid-soluble P production in egg yolk was practically the same for all three toxins, but the ratio of turbidity in egg yolk to acid-soluble P production from lecithin was considerably lower for

Table 1. Correlation between turbidity production and hydrolysis of egg-yolk phospholipin by *Cl. bifermentans* and *Cl. welchii* lecithinases

Reaction	<i>Cl. welchii</i>	<i>Cl. bifermentans</i>	
		B_1	B_2
(a) Turbidity in egg-yolk units/40 min.	46	46	54
(b) P liberation from egg yolk ($\mu\text{g.}/40$ min.)	69	76	90
(c) P liberated from lecithin ($\mu\text{g.}/30$ min.)	150	50	63
Ratio a/b	0.66	0.58	0.60
Ratio a/c	0.31	0.92	0.86

the *Cl. welchii* toxin than for the two *Cl. bifermentans* toxins, i.e. in the egg-yolk test the phospholipin was hydrolysed more slowly than in the standard lecithin test by *Cl. welchii* toxin, and more rapidly by the *Cl. bifermentans* toxins. Another experiment was made in which the rate of hydrolysis of egg-yolk phospholipin by the same number of L units of *Cl. welchii* and *Cl. bifermentans* toxin was compared. This experiment (Fig. 2) illustrates the difference in velocity and also shows that in these conditions both the toxins were apparently stable for 48 hr.

Effect of pH. Miles & Miles (1947) found the pH optimum for *Cl. bifermentans* lecithinase to lie between pH 5 and 6, whereas Macfarlane & Knight (1941) found that of the *Cl. welchii* was 7–7.6. The pH-activity curves for the samples of toxin used in the present experiments are shown in Fig. 3. The two curves intersect at pH 7.2 which was the initial pH used in the tests. It was found, however, that in the egg-yolk tests the pH fell rather rapidly to 6.0, and if a greater concentration of buffer were used this in itself inhibited the *Cl. welchii* enzyme (which was considerably purer than the *Cl. bifermentans* samples). Miles & Miles (1950) used a pH of 5.1 for their standard egg-yolk tests. It appears that the

discrepancies between the lecithinase and the egg-yolk tests are largely due to the pH of the egg-yolk test being below the optimum for *Cl. welchii*

Fig. 2. Liberation of acid-soluble P from egg-yolk extract. ○—○, 2 L units *Cl. bifermentans* toxin; ●—●, 2 L units *Cl. welchii* toxin.

Fig. 3. pH-Activity curve of *Cl. welchii* and *Cl. bifermentans* lecithinases. ○—○, *Cl. bifermentans*; ●—●, *Cl. welchii*.

lecithinase, and to the difficulties of adjusting the salt concentrations so that both enzymes are equally affected. When the turbidity is referred to the hydrolysis of phospholipin in the egg-yolk

extract, rather than to the hydrolysis of lecithin in a separate test, there is no discrepancy, i.e. no evidence suggesting the existence of a second turbidity-producing factor.

Effect of fluoride and citrate on Clostridium bifermentans lecithinase

Miles & Miles (1947) found that *Cl. bifermentans* lecithinase differed from *Cl. welchii* lecithinase in that it was independent of activation by Ca^{2+} , i.e. the rate of hydrolysis was the same with and without Ca^{2+} and on addition of 0.01 M-oxalate. It has been found previously in this laboratory that when tests are made with toxins of low activity or with high concentrations of substrate it may be difficult to demonstrate activation by Ca^{2+} , as sufficient calcium may already be present in the toxin or substrate: in experiments with inhibitors such as fluoride and oxalate care must be taken that the pH and the time of contact are suitable for the effective precipitation of the insoluble calcium salt, e.g. at pH 5.1 the precipitation of calcium fluoride is slow and incomplete.

Effect of fluoride on egg-yolk reaction. Mixtures containing *Cl. bifermentans* toxin and egg yolk, pH 7.2, with or without sodium fluoride were used, and the tests arranged so that the sodium fluoride was in contact either with the egg yolk or the toxin for 15 min. before addition of the second compound, to compare with tests in which the sodium fluoride was added immediately after the two other components were mixed. Table 2 shows that inhibition by sodium fluoride in this system is demonstrable but it is by no means immediate.

Table 2. *Effect of fluoride on hydrolysis of egg-yolk phospholipin by Cl. bifermentans lecithinase*

(1.0 ml. *Cl. bifermentans* toxin, 1.0 ml. egg-yolk extract $\pm$ 0.2 ml. 0.5 M-NaF in a total vol. 4.0 ml., incubated at 37°.)

System	P hydrolysed ($\mu\text{g.}$)		
	1 hr.	3 hr.	19 hr.
No fluoride	23	45	74
NaF added to toxin-yolk mixture	17	20	23
NaF added to yolk before incubation	17	17	21
NaF added to toxin before incubation	21	26	28

Effect of citrate on lecithinase reaction. The activity of *Cl. bifermentans* and *Cl. welchii* toxins in the standard lecithinase test at pH 7.2 in presence and absence of added Ca^{2+} , and in presence of citrate, was compared. The citrate was added to the lecithin 20 min. before the addition of the toxin. Table 3 shows that with both toxins the activity was increased in the presence of added Ca^{2+} , and considerably decreased by the prior addition of citrate.

Table 3. *Effect of citrate and Ca^{2+} on hydrolysis of lecithin by Cl. bifermentans and Cl. welchii toxins*

(1.0 ml. lecithin, 1.0 ml. borate buffer, pH 7.2, 1.0 ml. toxin, water or CaCl_2 to total vol. 4.0 ml., 30 min. at 37°.)

System	Acid-soluble P liberated ($\mu\text{g.}$)	
	<i>Cl. welchii</i> toxin	<i>Cl. bifermentans</i> toxin
No added Ca^{2+}	75	143
0.01 M- CaCl_2	115	180
0.05 M-Citrate	20	30

DISCUSSION

The experiments described here confirm the conclusion reached by Miles & Miles (1947, 1950) that the lecithinase present in *Cl. bifermentans* culture filtrate is of the same biochemical type as that in *Cl. welchii* toxin (i.e. one splitting lecithin into phosphorylcholine and a diglyceride), but has a different pH optimum. It has been found that the action of this enzyme, like that of the *Cl. welchii* enzyme, is dependent on Ca^{2+} ions, and the lecithinase is apparently the only lipolytic enzyme present in the samples examined. It is desirable to establish this fact unequivocally, in view of the extraordinarily interesting differences in toxicity of the two enzymes shown by Miles & Miles (1950) and their possible use in comparative studies of enzyme-antienzyme systems.

Miles & Miles (1947) suggested that the difference in toxicity between the *Cl. bifermentans* and *Cl. welchii* enzymes 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. There is, however, no *in vitro* evidence that any activation apart from the presence of Ca^{2+} ions is necessary. Later, Miles & Miles (1950) observed differences in the toxicity of lecithinase from different strains of *Cl. bifermentans*. Differences in the rate of attack of the lecithinases of *Cl. welchii* and *Cl. oedematiens* on the phospholipin of erythrocytes of various species are known. Macfarlane (1950) suggested that the differences in structure of the enzymes, which is clearly evidenced by their immunological specificity, conditioned the accessibility of the enzyme to its substrate when the substrate was itself part of a specific structure, and might also determine the toxicity of the enzymes and therefore the virulence of the microbes producing them.

SUMMARY

1. The lecithinase present in culture filtrates of *Clostridium bifermentans* is of the same biochemical type as *Clostridium welchii* lecithinase.

2. This enzyme, like the *Cl. welchii* lecithinase, is activated by Ca^{2+} ions.

We are greatly indebted to Mrs E. M. Miles for the gift of *Cl. bifermentans* toxins.

REFERENCES

- Hayward, N. J. (1943). *J. Path. Bact.* **55**, 285.
Macfarlane, M. G. (1948). *Biochem. J.* **42**, 590.
Macfarlane, M. G. (1950). *Biochem. J.* **47**, 270.
Macfarlane, M. G. & Knight, B. C. J. G. (1941). *Biochem. J.* **35**, 884.
Martland, M. & Robison, R. (1926). *Biochem. J.* **20**, 847.
Miles, E. M. & Miles, A. A. (1947). *J. gen. Microbiol.* **1**, 385.
Miles, E. M. & Miles, A. A. (1950). *J. gen. Microbiol.* **4**, 22.
Singer, T. P. & Hofstee, B. H. J. (1948). *Arch. Biochem.* **18**, 229.
Willstätter, R., Waldschmidt-Leitz, E. & Memmen, F. (1923). *Hoppe-Seyl. Z.* **125**, 93.
Zamecnik, P. C., Brewster, L. E. & Lipmann, F. (1947). *J. exp. Med.* **85**, 395.

Biochemical Studies of Toxic Agents

4. A STUDY OF ETHEREAL SULPHATE FORMATION *IN VIVO* USING RADIOACTIVE SULPHUR

BY J. C. LAIDLAW* AND L. YOUNG†
Department of Biochemistry, University of Toronto

(Received 8 November 1952)

The present work was done to throw light on the question of whether administered inorganic sulphate can participate in the formation in the animal body of the so-called 'etheral sulphates'. This matter has long been the subject of controversy. The problem is one which lends itself to investigation by means of tracer isotope techniques, and the present paper is an account of experiments in which etheral sulphate formation *in vivo* was studied with the aid of radioactive sulphur (^{35}S).

In earlier papers of the present series descriptions have been given of the isolation of 2-amino-1-naphthylsulphuric acid from the urine of rats dosed with 2-naphthylamine (Manson & Young, 1950), and the isolation of 1-naphthylsulphuric acid and 2-naphthylsulphuric acid from the urine of rats dosed with 1- and 2-naphthol, respectively (Berenbom & Young, 1951). The formation of 2-amino-1-naphthylsulphuric acid from 2-naphthylamine is an example of etheral sulphate production from a compound which, at least at the time of its administration, does not contain a phenolic group. On the other hand, the conversion of a naphthol to the corresponding naphthylsulphuric acid is typical of a reaction which is known to occur when many phenolic compounds are administered to animals. It appeared, therefore, that 2-naphthylamine and 1- or 2-naphthol would serve as suitable compounds in a study of the participation of administered inorganic sulphate in the biosynthesis of etheral sulphates.

* Present address: Peter Bent Brigham Hospital, Boston 15, Mass.

† Present address: Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E. 1.

Three groups of experiments were carried out. The first group consisted of determinations of the distribution of ^{35}S in the inorganic and total sulphate fractions of rat urine (and hence the etheral sulphate fraction) following the administration of radioactive sodium sulphate alone, or simultaneously with the administration of 2-naphthylamine by another route. The results obtained indicated that the administered inorganic sulphate had participated in etheral sulphate formation. This was confirmed in the second group of experiments. In one of these experiments radioactive 2-amino-1-naphthylsulphuric acid was isolated from the urine of rats dosed with 2-naphthylamine and radioactive sodium sulphate by different routes. In a similar experiment radioactive 2-naphthylsulphuric acid was isolated from the urine after the administration of 2-naphthol and radioactive sodium sulphate. In the third group of experiments it was shown that radioactive inorganic sulphate does not exchange in urine with the sulphate of the etheral sulphate fraction.

EXPERIMENTAL AND RESULTS

Animals and dosing. The animals used were male white rats weighing between 170 and 210 g. During the experimental period they were housed in cages which permitted the collection of urine separate from the faeces. The rats were fed on a diet which consisted of Master Fox Breeding Ration (Toronto Elevators Ltd.) supplemented with fresh milk and whole wheat bread. In quantitative experiments the animals were allowed to feed twice daily for periods of 30 min. each from a metal cup designed to prevent scattering of food. In qualitative experiments the rats were fed in a separate cage twice daily for periods of 30 min. each and any