The Relation of Toxicity and Enzyme Activity in the Lecithinases of *Clostridium bifermentans* and *Clostridium welchii*

BY ELLEN M. MILES AND A. A. MILES

National Institute for Medical Research, Hampstead, London

SUMMARY: Crude preparations of the lecithinase of *Clostridium bifermentans* are lethal for mice. Their toxicity, haemolytic activity, and the power of liberating acid-soluble phosphorus from lecithin are neutralized by *Cl. welchii* α -antitoxin; in terms of lethal doses of *Cl. welchii* α -toxin and the *bifermentans* lecithinase, α -antitoxin is less than one five-hundredth as effective against the *bifermentans* lecithinase as against the α -toxin.

In terms of *in vitro* lecithinase activity in standard conditions, equipotent amounts of lecithinase from three different strains of *Cl. bifermentans* were respectively 9, 60 and 75 times less toxic than the corresponding amount of *Cl. welchii* lecithinase. The toxicity of the *Cl. bifermentans* lecithinase therefore is determined, not only by its lecithinase activity, but by unknown factors that vary from strain to strain of the bacillus. These factors are not likely to be contaminating substances with a synergic or inhibitory effect, since the differences in toxicity were maintained in several preparations from each of the three strains studied.

The analysis of the two antigenically related lecithinases from *Cl. welchii* and *Cl. bifermentans* provided no positive evidence against the hypothesis that the α -toxin of *Cl. welchii* is identical with the lecithinase.

In our last paper (Miles & Miles, 1947) we established that *Clostridium bifermentans* produced an enzyme which appeared to be a lecithinase C, and was antigenically related to the α -toxin of *Cl. welchii*. The potency of the α -toxin is proportional to its power to hydrolyse lecithovitellin with the liberation of acid-soluble phosphorus (Macfarlane & Knight, 1941). At the time, we had not enough *bifermentans* lecithinase for measuring its toxicity in terms of its phosphate-liberating power. In this paper we compare the *welchii* lecithinase (WL) with toxic preparations of the *bifermentans* lecithinase (BL).

MATERIALS AND METHODS

Preparation of WL and BL. As before, the WL (*welchii* lecithinase) preparations consisted of a crude dried toxin, 'Welchpool' (abbreviated to W'pool), and a glycerinated toxin, 290. The chief difficulty of preparing potent BL (*bifermentans* lecithinase) is the poor yield of toxic material from culture; in optimum conditions, about 100 ml. of culture yield one minimum lethal dose for 20 g. mice. We found no better medium than Macfarlane & Knight's (1941) glucose peptone muscle-extract medium; and BL was prepared from it as follows. Six litres of medium in three Winchester quart bottles filled almost to the top were warmed to 37° and seeded with c. 20 ml. of an overnight anaerobic culture of *C. bifermentans* in the same medium. After 18 hr. incubation the turbid cultures were filtered successively through a Seitz clearing disk and a Berkfeld candle. The filtrate was held at 5–7°, and concentrated c. 40-fold in

cellophane bags under a pressure of $\frac{3}{4}$ atm., and the concentrate dialysed at room temperature for 18 hr. against running tap water. The whole process took 2½–3 days. The concentrate was freeze-dried in flasks and held at -5° . Four such dried preparations were then pooled in a minimum amount of water and the pool freeze-dried. The resulting fluffy, pale yellow, readily water-soluble substance was distributed in 0.5 g. lots into ampoules, dried over P_2O_5 , sealed in N_2 and stored in the dark at -5° . The yield from 24 l. was c. 9 g. In all, over nine large batches were made. The material kept indefinitely in the cold under N_2 , but lost over 50 % of its lecithinase activity in a fortnight when exposed to air for the purposes of sampling, and held in the intervals at room temperature over P_2O_5 *in vacuo*. Glycerinated solutions were more stable.

The material consisted of all the soluble large-molecular products of *Cl. bifermentans*, including various enzymes (Dr E. S. Duthie, for example, found powerful proteinase activity in our preparations). Our attempts to fractionate with ammonium sulphate or acetone, at room temperature and in the cold, and at various pH values, were unsuccessful, since a great deal of lecithinase was destroyed in the process. We therefore used the crude material for our experiments, relying on the specific neutralization of BL activity by *Cl. welchii* antitoxin to distinguish effects due to BL itself from those due to impurities in the preparation. The validity of this antitoxin control depends on the assumption that *Cl. welchii* and *Cl. bifermentans* are serologically related only in respect of their lecithinases.

With subculture, strains partly lost their power to produce BL in liquid media. Three strains of *Cl. bifermentans*, namely, M58e used in our previous work, and two other wound strains M10g and A250c, were plated on human-serum agar. Colonies producing a large Nagler reaction were grown for 18 hr. in cooked-meat medium, and the centrifuged deposit of growth distributed into ampoules and preserved by Stamp's (1947) gelatin-ascorbic acid method, to serve as stable, uniform starting material for each batch.

In spite of its containing so many bacterial products, the final material was remarkably non-toxic. Only BL from M58e was toxic, and its toxicity was entirely neutralized by *Cl. welchii* antitoxin. Material from the other two strains could be given intravenously to 20 g. mice in doses as large as 50 mg. without producing more than half an hour's discomfort and staring coat, 30 min. after injection.

Measurement of enzyme activity. The lecithinase activity of WL and BL solutions was measured (a) by titrating serial dilutions against yolk extract, expressing the result as arbitrary BL or WL units/ml. (Miles & Miles, 1947), and (b) by estimating the acid-soluble P liberated from fine suspensions of crude hen-egg lecithin. Crude lecithin was prepared by the method of Macfarlane & Knight (1941), stored under N_2 at 2° and used within 7–10 days. For enzyme titrations it was suspended in water, 20 mg./ml. The maximum acid-soluble P liberable from 1 mg. of successive preparations varied between 0.028 and 0.084 mg. Solutions of the crude WL and BL had a pH between 6.8 and 7.8, and were readily buffered by M/15–20 buffers. Accordingly, the reaction mixture usually consisted of 1 vol. each of lecithin suspension, lecithinase solution, and either

2 vol. $\text{m}/5$ Palitzsch's borate buffer (Palitzsch, 1915) pH 7.5, or 1 vol. buffer and 1 vol. anti-enzymic serum. Although BL has an optimum activity in the region of pH 5, the higher pH was used as a routine, partly because at pH 5 the lecithin suspensions were slightly unstable and filtration of trichloroacetic acid precipitates was difficult; but mainly because for our purpose it was more important to measure enzyme activity at pH values similar to those of animal tissues. For titrations of WL, CaCl_2 was added to make a 0.0188 M solution.

The hydrolyses were carried out in test-tubes immersed in a 37° bath so that half the fluid column was above the water-level, to promote continuous mixing by convection. All solutions were warmed to 37° before mixing. Acid-soluble P was estimated by the method of King (1947) with slight modifications. Four ml. of reaction mixture were treated with 2 ml. of trichloroacetic acid for 5–10 min. and filtered. The filtrate was rather resistant to oxidation; 0.5 ml. were evaporated to dryness with one drop of a saturated solution of Na_2CO_3 in a 15 ml. volumetric flask, and digested with 60 % perchloric acid. Digestion was usually complete 5 min. after the disappearance of colour. The molybdate colour was estimated against phosphate standards in an EEL (Evans Electro-selenium Ltd., Harlow, Essex) photoelectric colorimeter. With an EEL 'tricolour red' screen the ammeter readings were strictly proportional to concentrations of P.

We depended on the added trichloroacetic acid to stop enzyme activity at the moment of sampling. Blanks were made by adding the trichloroacetic acid to the mixture of lecithin and buffer, and immediately afterwards adding the lecithinase solution. In definitive tests, samples were taken from the reaction mixture in triplicate, and estimated separately.

Haemolysin tests. Haemolysis was measured on red cells thrice washed in 0.85 % saline, and suspended in saline, 5 % (v/v) of packed centrifuged deposit. Falling dilutions of BL and WL were titrated, and approximate 50 % end-points (HD 50) determined by comparing haemoglobin liberated after 1 hr. at 37° with that in comparison tubes containing the same red-cell suspension lysed with saponin, and diluted to contain 90, 70, 50, 30 and 10 % of the original haemoglobin.

Toxicity tests. WL and BL preparations were injected intravenously in 18–22 g. mice; 0.2 ml. of preparation with 0.2 ml. of either saline or antiserum.

RESULTS

The hydrolysis of crude lecithin by BL

The comparison of hydrolytic activity. The curves for the liberation of acid-soluble P from lecithin by a given concentration of BL after varying periods of time ('time-P' curves; Fig. 1) and by varying concentrations of BL after a fixed time ('dose-P' curves; Fig. 2) are similar. In both, the lower portion of each curve is approximately linear, and both tend to become horizontal at the level of the maximum amount of liberable P for the preparation of lecithin used. The method of varying the enzyme concentrations was preferred, because it corresponds to the method of Macfarlane & Knight, and was technically less

laborious when comparisons of enzyme potency were made. Moreover, the dose-P curves are more regular than the time-P curves, with a longer and more definite part of the curve that is approximately straight. Owing to the relatively feeble potency of BL preparations, 30 min. was chosen as the

Fig. 1. The rate of liberation of acid-soluble P at 37°, pH 7.5, from suspensions of crude lecithin containing 0.140–0.160 mg. liberable P, by the following preparation of BL: (a) 5 mg. of M10g, BL18; (b) 5 mg. of A250c, BL18 and (c) 2.5 mg. of M58e, BL19.

standard time, instead of the 15 min. of Macfarlane & Knight, so as to have measurable amounts of P and at the same time economize in the use of BL preparations. There was some variation in repeated tests with different batches of lecithin; all comparative estimates of lecithinase potency were made therefore on one batch. Three concentrations were tested, in a range chosen to fall on the straight part of the curve. The linearity, however, of any substantial length of the P-liberation curve was difficult to establish, and for definitive estimates of potency a regression line was fitted to the three points plotted as log concentrations of the enzyme, against P liberated. In Fig. 8, the results for the two WL preparations and BL preparation, two from strain M58e, and one each from M10g and A250c are summarized. Each point is the mean of estimates

made on three samples taken from the same reaction mixture. The line was fitted and an analysis of variance made by the method of Fisher (1948). The variance of these means is very small, as the following typical figures for BL M10g show:

Dose (mg.)	Acid-soluble P liberated (mg.)		
5.00	0.087,	0.068,	0.069
2.50	0.039,	0.041,	0.039
1.25	0.018,	0.019,	0.017

Fig. 2. The liberation of acid-soluble P after 30 min. at 37°, pH 7.5, from suspensions of crude lecithin containing 0.140–0.170 mg. liberable P, by graded amounts of various lecithinase preparations (a) WL W'pool; (b) and (c) A250c, BL18, on two batches of lecithin.

The weights of the means are very large, and hence all the departures from linearity, obvious in Fig. 8, are significant. It is therefore impossible from these figures to decide whether the most convenient linear relation is in fact given by plotting log concentration of enzyme against weight of P liberated, either for WL or BL; though it is certainly more convenient than that of concentration of enzyme against weight of P. We estimated the standard dose of enzyme by calculating from the fitted line the weight of lecithinase preparation liberating 50 μg. acid-soluble P in 30 min. at 37°. This we have symbolized as

DL50P, the dose liberating 50 μg . P (the symbol is awkward, but was so devised to distinguish it from analogues of the established LD50 notation of Trevan (1927), with which it is not strictly analogous). The DL50P of the six preparations are listed in Table 1, with the slopes of the regression lines. By regression analysis, the four slopes for the BL preparations proved to be

Fig. 3. A comparative estimate of phosphate liberation from one batch of lecithin by BL and WL preparations, the amount of P liberated being plotted against log dose enzyme, and regression lines fitted (see Table 5): (a) WL290; (b) WL W'pool; (c) and (d) BL20; (e) BL18; (f) BL17.

homogeneous and had a common slope of 0.0718; thus providing evidence that the four enzyme preparations behaved in the same way. The two slopes for WL differ significantly from the BL slopes; and from one another, though to a lesser extent. It follows that though the ratios of the DL50P indicate the relative potencies for a wide range of concentrations of the BL preparations, ratios of WL to BL potencies, or of WL potencies to one another, are strictly valid only for the DL50P itself.

The DL50P of successive batches of BL from one strain was of the same order of size, varying much as the two batches of M58e recorded in Table 1, i.e. within about 20 % of each other. Preparations from strains M10g and A250c were always weaker in lecithinase than those from M58e.

The nature of the hydrolysis. Additional evidence that the acid-soluble P compound liberated from lecithin by BL is phosphorylcholine was obtained by a chromatographic analysis of the breakdown products. BL and WL were added

in amounts calculated to liberate most of the acid-soluble P present in the lecithin; each was added to 40 mg. crude lecithin in 8 ml. water, and held at 37° for 30 min. The mixtures were then treated with trichloroacetic acid and filtered. Our colleague, Dr T. S. Work, devised and carried out the analysis.

Table 1. *The doses of BL and WL preparations liberating 50 µg. acid-soluble P (DL50 P) from crude lecithin at 37° under standard conditions; and the slopes of the log dose/phosphate-liberation regression line*

Bacterium	Batch	DL50 P	Slope of regression line
<i>Cl. bifermentans</i> : A250c M10g M58c	BL17	4.63 mg.	0.0664
	BL18	3.13 mg.	0.0830
	BL19	1.80 mg.	0.0747
	BL20	2.24 mg.	0.0653
<i>Cl. welchii</i>	WL W'pool	0.203 mg.	0.0997
	WL 290	0.00143 ml.	0.1583

The filtrates were extracted with an equal volume of CHCl_3 . The extracted aqueous solutions were filtered and evaporated to dryness *in vacuo*. The residues were dissolved in 0.2 ml. water; 0.1 ml. was placed directly on no. 4 Whatman paper, the remaining 0.1 ml. after hydrolysis with 5N-HCl for 16 hr. at 110°, to split the phosphoryl radical from any phosphorylcholine present. The four samples, two of unhydrolysed and two of acid-hydrolysed material, were run in phenol against authentic samples of choline. After drying the paper was sprayed with Kraut's reagent (von Oettingen & Bowman, 1933), which showed spots as follows:

Lecithin split by	Acid-soluble material hydrolysed by	Choline
WL W'pool	{ Nil { HCl	Trace Large quantity
BL 17	{ Nil { HCl	Nil Large quantity

It is clear that BL, like WL, liberates an acid-soluble compound, which in turn liberates choline on acid hydrolysis; in conjunction with the fact that BL liberates acid-soluble P, this strongly suggests that the compound is phosphorylcholine, and confirms our original classification of the *bifermentans* enzyme as a lecithinase C.

Nomenclature of the enzyme. In so classifying the enzyme we followed Macfarlane & Knight's (1941) procedure with the α -toxin of *Cl. welchii*. Bard & McClung (1948), however, object to this designation for enzymes splitting phosphorylcholine from lecithin, on the grounds that Contardi & Ercoli (1933*a*) had already used the label C for a hypothetical enzyme yielding choline from lecithin, and suggest that the new enzyme should have been named 'Lecithinase D'.

It is true that in two papers (1933*a, b*) Contardi & Ercoli mentioned four theoretically possible enzymes attacking different linkages in the lecithin molecule, of which lecithinases A (giving lysolecithin) and B (giving glycerophosphorylcholine and two fatty acids) were already known to exist, C was

postulated as yielding choline and D as yielding phosphorylcholine; but at that time (1933*b*) these authors also pointed out that there was no evidence that types C and D existed. In a subsequent review, Belfanti, Contardi & Ercoli (1936) designated only lecithinases A and B, and no other possible lecithinases, such as C and D, were mentioned.

We think it is proper therefore to use Macfarlane & Knight's designation 'lecithinase C' for an enzyme splitting phosphorylcholine from lecithin, on the following grounds:

(1) The designation uses the next available letter for a properly characterized enzyme. Earlier use of a letter for a hypothetical enzyme, to which the evidence did not point unequivocally, in a scheme of classification that was neither generally accepted, nor maintained by its proposers, cannot be held as a valid pre-emption of that letter.

(2) The designation 'lecithinase C' for the *Cl. welchii* enzyme and other enzymes of this type has been used in the literature since its introduction in 1941, and to change it would confuse the situation unnecessarily.

Toxicity tests

Table 2 summarizes a number of toxicity tests, typical of many more. Exps. 1-6 show that in doses of 40-50 mg., various batches of BL from M10g were slightly toxic for mice, and that the toxicity was neutralized by *welchii*

Table 2. *The toxicity of BL and WL preparations, given intravenously in 18-22 g. mice*

Exp. no.	Strain and batch of lecithinase	Dose (mg.)	No. of mice dying/group	
			Without α -antitoxin	With α -antitoxin
1-3	M10g	BL9 40	0/3, 1/3, 1/3	—
4, 5		BL13 40	0/3, 0/3	—
6		BL18 50	2/8	0/8
7, 8	A250c	BL10 50	0/3, 0/3	—
9		BL12 50	0/5	—
10		BL17 50	1/9	2/9
11	M58c	BL19 40	5/5	1/5
		20	4/5	0/5
		10	3/5	0/5
		5	1/5	0/5
	M58c	BL19 20	(20/20)*	
		10	7/20 (LD 50 15.01 mg.	
		5	3/20	± 0.232)
		0.25	(20/20)*	
	WL W'pool	0.20	19/20 (LD 50 0.15 mg.	
		0.15	10/20	± 0.0021)
	WL290	0.00132†	15/20	
		0.00110†	3/20 (LD 50 0.00122 ml.	
		0.00090†	2/20	± 0.000016)

* Omitted in calculation of LD 50.

† ml.

antitoxin. BL from A250c was less specifically toxic, though there was some toxicity not neutralized by an antitoxin (Exps. 7–10). The toxicity of BL19, from M58e, was considerable and was neutralized by antitoxin (Exp. 11). The toxicity of BL19 and of the two WL preparations was determined in a single experiment (12), and the LD50 calculated for each by the method of Gaddum (1938), with the results shown in the table.

Non-specific deaths usually occurred within $\frac{1}{2}$ hr. of injection. Specific BL deaths took place between 2 and 24 hr. and resembled those due to WL; the more rapidly dying animals discharged blood-stained watery fluid from nose and urethra, and died with slowed respiration and staring coat. Post-mortem, the bladder was filled with blood-stained fluid and the muscles were stained a pale yellow-brown, presumably due to haemolysis. The degree of intravascular haemolysis however, was not large during life, for in mice given an intravenous dose of BL sufficient to kill in 2–4 hr., only from 2–5 % of the total haemoglobin in the blood had been liberated from the red cells in 1 hr. It is, of course, possible that BL modified the remaining red cells so that they became more susceptible to destruction in the tissues. No intravenous haemolysis was demonstrable in mice given maximum sublethal doses of BL.

Cl. welchii antitoxin, which was allowed to act on BL at 37° for 30 min. before injection, though saving the life of BL-intoxicated mice, did not immunize completely. The protected animals, like the unprotected, became ill within 1–2 hr. of injection; but they recovered at the third hour, whereas the unprotected animals grew progressively worse until death. It is possible that this early illness was due to substances other than the lecithinase.

Haemolysis by BL

The haemolytic tests reported in our last paper (Miles & Miles, 1947) were extended to stronger preparations of BL. Table 3 records the HD50 for red cells of various animal species, and the value of the lecithinase 'units' of various

Table 3. Comparison of the 50 % haemolytic dose (HD50) and lecithinase units, of various BL and WL preparations

		Lecithinase preparation				
		M10g BL18 (mg.)	A250c BL17 (mg.)	M58e BL19 (mg.)	WL W'pool (mg.)	WL 290 (ml.)
Red cells tested	Human	> 20*	> 20*	> 20†	—	—
	Horse	> 20	> 20	> 20	—	—
	Sheep	> 20	> 20	> 20	—	—
	Rabbit	> 20†	> 20†	> 20‡	—	—
	Guinea-pig	> 20	> 20	> 20	—	—
	Mouse	3.75	8.0	0.625	0.625	0.00312
1 lecithinase unit (yolk extract)		0.10	0.04	0.05	0.3125	0.00156

— No test.

* Trace haemolysis at 20 mg./ml.

† 10 % haemolysis at 20 mg./ml.

‡ 10 % haemolysis at 5 mg./ml.

All recorded haemolysis neutralized by *Cl. welchii* α -antitoxin.

preparations of BL and WL. Our previous findings with 64 units BL/ml. are largely confirmed, but the use of preparations containing up to 500 units BL/ml. reveals minor activity against rabbit and human red cells. All the haemolytic activity of the BL preparations was neutralized by *welchii* α -antitoxin. Of the common laboratory animals, the mouse is the most convenient source of susceptible cells for haemolysin tests. The relative haemolytic inactivity of BL preparations in terms of 'lecithinase units', as compared with WL, is noticeable.

The efficacy of Cl. welchii α -antitoxin against BL

With the BL from strain M58e, it was possible to measure the serological relation between *Cl. bifermentans* lecithinase and the α -toxin of *Cl. welchii*, by titrating α -antitoxin, in mice, against approximately one LD₅₀ of WL and BL. The mixtures of toxin and antitoxin were held for 30 min. at 37° before intravenous injection (Table 4). Though 13.5 mg. BL was about LD₅₀, the dose

Table 4. *Protective power of Cl. welchii α -antitoxin in mice receiving approximately one LD₅₀ of BL (M58e; BL20) and of WL (Welchpool)*

Antitoxin given (i.u.)	No. of mice dying/group given		Minimum protective dose of α -antitoxin (i.u.)
	13.5 mg. BL	0.15 mg. WL	
26.4	0/10	—	—
18.2	0/10	—	18.2
6.6	5/10	—	—
3.3	3/10	—	—
1.6	5/10	—	—
0.100	—	0/10	—
0.050	—	0/10	—
0.025	—	0/10	0.025
0.0125	—	3/10	—
0.000	4/10	9/10	—

given of WL W'pool proved to be about LD₉₀; the protective dose of antitoxin will therefore be larger than that required for an LD₅₀. The ratio of the minimal protective doses, measured on a twofold dilution scale is 528:1 (18.2:0.025). That is, compared with homologous toxin more than 500 times the amount of α -antitoxin is required for neutralization of the heterologous toxin.

Comparison of the lecithinase, haemolytic and toxic activities of BL and WL

Table 5 summarizes the results; the actual amounts in (a) are converted in (b) to percentages of the phosphate-liberating activity. The striking differences between BL and WL are the relatively high activity of BL in the 18 hr. yolk-extract titration, and the relatively low toxicity of BL. The high yolk-extract titres confirm our suggestion that BL is more stable than WL in mixtures with yolk extract. When yolk-extract titres are read at 1 hr. instead of 18 hr. the titre of BL is comparatively much lower. Of greater importance is the ratio of the LD₅₀'s in the two lecithinases. Averaging the results with WL290 and W'pool, the ratio is 670:72, or 9.3:1. That is, in terms of *in vitro* lecithinase activity at pH 7.5, BL is about one-ninth as toxic as WL.

The three preparations from different strains, though resembling one another more than they resemble WL, nevertheless differ significantly. The BL of A250c and M10g has less than half the toxicity for mice exhibited by BL M58e, and a fourth to a sixth of its haemolytic activity. Assuming that 50 mg. is the LD25 and LD0 of M10g and A250c respectively (Table 2), and that the slope of the dose-mortality curves for all three preparations is the same, we may estimate the LD50 of the two BL's very approximately as 170 and 200 mg. On

Table 5. *Comparison of the lecithinase, haemolytic and toxic potencies of preparations of BL and WL; (a) by weight or volume, and (b) in percentages of the amount liberating 50 µg. acid-soluble P in standard conditions*

Strain	Preparation	DL50 P (crude lecithin)	WL or BL unit (yolk ext.)	HD 50 (mouse cells)	LD 50 (mice)
(a) A250c	BL17 (mg.)	4.63	0.04	8.00	> 50 (c. 200)
M10g	BL18 (mg.)	3.13	0.10	3.75	> 50 (c. 170)
M58e	BL20 (mg.)	2.24	0.05	0.625	15.01
Welchpool	WL (mg.)	0.203	0.3125	0.625	0.15
290	WL (ml.)	0.00143	0.00156	0.00312	0.00122
(b) A250c	BL17	100	0.9	173	> 1080 (c. 4300)
M10g	BL18	100	3.2	120	> 1562 (c. 5400)
M58e	BL20	100	2.2	28	670
Welchpool	WL	100	153.9	315	59
290	WL	100	109.1	218	85

this basis A250c and M10g are between one-sixth and one-seventh as toxic as M58e; i.e. toxicity roughly parallels haemolytic activity. From the corresponding values for toxicity in part (b) of the table, BL17 and 18 were 60 and 75 times less toxic than WL, in terms of *in vitro* lecithinase activity. BL from M10g and A250c was prohibitively weak for further investigation of toxicity, but not for haemolysin tests. The varying ratio between DL50 P and the haemolytic-toxic activity in the three preparations suggests that toxin and lecithinase are not the same. Accordingly, the amounts of a strong α -antitoxin neutralizing one HD100 of BL17, 18 and 20 were measured, and on one occasion proved to be respectively 30.5, 20.6 and 2.6 i.u. and on another, 29.0, 24.0 and 2.9 i.u. These figures, though not in the strict ratio of the haemolytic activities in terms of DL50 P (173, 120, 28; Table 5a) are sufficiently close, within the limits of error of the tests, to suggest that the α -antitoxin which neutralizes lecithinase activity (Miles & Miles, 1947) also neutralizes the haemolysin in constant proportion. This result was not due to chance parallelism between α -antitoxin and a distinct antihaemolysin in the serum used; for when a number of α -antitoxic sera varying in α -antitoxin content between 3800 and 70 units/ml. were titrated against the haemolysin of BL20 (M58e), the neutralizing values were 2.6, 4.2, 3.8, 5.0, 9.0, 5.5, 4.9 and 3.5 units. With one exception (9.0) the values are sufficiently close to show that the α -antitoxin is the antihaemolysin. Another serum, containing 0.2 unit of α -antitoxin/ml. and large amounts of antibody to the β and θ lysins of *Cl. welchii*, had no antihaemolytic activity.

DISCUSSION

In our previous paper we suggested that the properties of the *bifermantans* lecithinase threw some doubt on the assumption that lecithinase activity *per se* was sufficient to account for the lethal and necrotic powers of the α -toxin of *Cl. welchii*, particularly because active BL was non-toxic compared with WL; and we concluded that the speculation could be tested fully only when we had a BL whose phosphate-liberating power was equivalent to that of at least 10 M.L.D. of *Cl. welchii* α -toxin. This we have achieved with BL20; up to 50 mg. could be given to mice, which died only because of the *bifermantans* lecithinase effect, specifically neutralized by α -antitoxin. Fifty mg. contained the phosphate-liberating equivalent (Table 5) of $50/2.24=22.3$ DL50 P of W'pool *welchii* toxin, and one DL50 P of this toxin (0.208 mg., Table 5) was c. 1 M.L.D. (i.e. 1 LD100, Table 2).

With this preparation of BL it is clear that, compared with WL, BL has about one-ninth the mouse-toxicity of WL, when lecithinase activity is defined in terms of acid-soluble P liberated under standard conditions. It is, of course, impossible to decide how far these conditions approximate to those in animal tissues that contain a substrate for the enzymes. All we have been able to do is to establish comparable temperature, pH, and a not too divergent tonicity. Our DL50 P, like Macfarlane & Knight's lecithinase 'unit', which it closely resembles, is a highly artificial measure; and in view of the heterogeneity of the dose-response slopes in the titration of WL and BL by this means, comparisons of DL50 P must be suspect unless in any one pair of comparisons the slopes are proved to be homogeneous. Nevertheless, a ninefold difference and, in the case of BL from two other strains, M10g and A250c, a much greater than ninefold difference in toxicity are probably significant. The differences, which were constant for several batches from each strain, between toxicities of BL from M58c on the one hand, and the substantially non-toxic BL's from A250c and M10g on the other, also destroy any simple relation between lecithinase activity and toxicity for any one 'species' of lecithinase.

We suggested (1947) that differences between BL and WL 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 now appears that similar notions apply to BL from different strains of *Cl. bifermantans*. In view of the impurity of our BL preparations, we cannot exclude synergic or inhibitory substances as the cause of the difference. Nevertheless, the constancy of this difference, exhibited by several batches of BL from each of the three strains, suggests that it is a property of the lecithinase itself. That is, there appear to be interstrain variations in some quality of the lecithinase, besides the power to hydrolyse lecithin, that determines toxicity. (This conclusion depends, of course, on the validity of our assumption that in BL preparations there is only one antigen—the lecithinase—capable of reacting with antibodies in *Cl. welchii* α -antitoxins.) It is, moreover, noteworthy that the varying susceptibility of the mouse to the lethal effect of enzymically equipotent doses of BL from the three strains studied, is paralleled

by the *in vitro* susceptibility of mouse red cells to haemolysis. Though haemolysis may not be the determining factor in toxicity, the red cell at least reflects the susceptibility of those other mouse tissues, whose poisoning determines the death of the animal.

The analysis of BL preparation yields no evidence that the serologically related *welchii* lecithinase is distinct from the α -toxin. On the other hand, though large amounts of BL are specifically toxic, it is clear that lecithinase activity *in vitro* and toxicity are not consistently related. The reasons for the comparative non-toxicity of BL are obscure, but it provides an explanation, additional to the fact that very little BL is formed by *Cl. bifermentans* in vigorously growing culture, for the lack of pathogenic power in the average strain of *Cl. bifermentans*. Chu (1949) records that the lecithinase of *Bacillus cereus* has one-quarter to one-eighth the toxicity of WL. His *cereus* lecithinases, though immunologically distinct from WL, were in other respects more closely related to WL than BL appears to be.

The importance of the antigenic relationship between BL and WL must not, however, be overrated. We have now established the reciprocity of the relation, in that WL antilecithinase neutralizes the mouse-toxicity of BL, and vice versa; and that it requires 500 times more α -antitoxin to neutralize one LD 50 of BL than one LD 50 of WL. But the chief value of the relationship has been in providing a reagent, *Cl. welchii* α -antitoxin, which, it was justifiable to assume, was monospecific with regard to BL. The significance of antigenic similarity of the two enzymes must await the establishment of more facts.

Finally, it is perhaps worth noting that our negative evidence for the identity of toxic and lecithinase activity in *Cl. welchii* α -toxin, and the positive evidence of Chu and ourselves that other lecithinases are toxic, does not in our opinion add much to the speculation that all bacterial exotoxins will prove to be enzymes when the relevant substrates are found. So far we have the unique fact that one naturally and obviously effective bacterial toxin appears to be an enzyme; and the other unrelated fact that certain enzymes, including some isolated from bacterial cultures, have proved to be toxic in concentrated preparations.

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