The Lecithinase of *Bacillus cereus* and its Comparison with *Clostridium welchii* α -toxin

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SUMMARY: *Bacillus cereus* and *B. mycoides* produce lecithinases which split lecithin into phosphorylcholine and a diglyceride in the same way as the lecithinase (α -toxin) of *Clostridium welchii*. These enzymes also possess most of the biological activities associated with *Cl. welchii* α -toxin, e.g. produce the Nagler reaction and the egg-yolk reaction, lyse red blood cells, and are dermonecrotizing and lethal.

The enzymes are activated by Ca and Mg ions, but inhibited by Na, K, NH_4 ferric and Al ions. Optimal enzyme activity requires the presence of Ca ions within a narrow range of concentration of $1-4 \times 10^{-3}$ M. It is interesting that at this concentration of Ca ion lecithin flocculates most readily from its emulsion. The enzyme thus seems to have a maximal affinity for lecithin when the latter adsorbs an optimal amount of Ca ion in reaching its isoelectric point.

Like the *Cl. welchii* lecithinase, the *B. cereus* lecithinase is fairly resistant to heat. The lysis of red blood cells and the hydrolysis of free lecithin by *B. cereus* lecithinase was strongly inhibited by normal sera of all the animals tested. But when lecithin was bound in egg-yolk lipoproteins, its hydrolysis by the enzyme was unaffected by normal serum. Specific serum, on the other hand, was capable of inhibiting the hydrolysis of both free lecithin and protein-bound lecithin. The *B. cereus* and *B. mycoides* lecithinases are immunologically related, but they are not so related to *Cl. welchii* lecithinase.

In a previous communication (McGaughey & Chu, 1948) it was reported that, in the *Bacillus* group, *Bacillus cereus*, *B. mycoides* and *B. anthracis* were able to produce an egg-yolk reaction similar to that caused by *Clostridium welchii* α -toxin. In the case of *Cl. welchii* α -toxin, the reaction was found to be due to the action of a lecithinase which splits lecithin into phosphorylcholine and diglyceride (Macfarlane & Knight, 1941). A similar type of lecithinase has now been demonstrated in *B. cereus* and *B. mycoides*, and the activity also runs parallel with the yolk reactivity. In view of the close correlation between the lecithinase activity and the haemolytic, necrotic and lethal activities of *Cl. welchii* α -toxin, it was considered interesting to see whether the lecithinases obtained from these new sources and causing the same biochemical reaction on the substrate lecithin also possess the biological properties of the *Cl. welchii* lecithinase.

MATERIALS AND METHODS

Cultures. Although many strains of *B. cereus*, *B. mycoides* and *B. anthracis* were used, for most of the work the enzyme was prepared from a strain of *B. cereus* (N.C.T.C. 945).

Preparation of lecithin. Lecithin was prepared and purified by the methods of Maclean & Maclean (1927) and Welch (1945). Fresh egg yolks were extracted with several changes of ethanol. The ethanol extract was evaporated to dryness *in vacuo* and taken up in a small volume of ether. Crude phospholipids were precipitated

from the ethereal solution with excess of acetone. The precipitate was taken up again in ether and kept in the refrigerator to allow sphingomyelin and galactolipin to separate out. After centrifuging, the clear ethereal solution was again treated with acetone and the process was repeated three times. Finally, the acetone precipitate, which contained chiefly lecithin and cephalin, was taken up in absolute ethanol. After standing in the refrigerator overnight, the cephalin which precipitated was removed by centrifugation. The purification was repeated until the product gave a perfectly clear solution in ice-cold ether or ethanol.

As lecithin is very unstable in air, it was found convenient to make a thick emulsion of the product as soon as it was prepared and to distribute it in fixed quantities, usually 250 mg., in small ampoules. They were then dried from the frozen state and sealed *in vacuo*. Lecithin kept in this form remained apparently unchanged for a long time. For use, the contents of an ampoule were washed out with water and diluted to the required volume. Lecithin kept in this form emulsifies very readily. The product generally contained 1.95–2 % N, 3.75–3.9 % P, 13.8–14.2 % choline with a molecular ratio of P and choline 1:0.95–0.97.

Preparation of cephalin. Cephalin was prepared from sheep brain according to Maclean & Maclean (1927). The product contained 3.76 % P but no choline. It was not hydrolysed by *Cl. welchii* lecithinase, and was thus practically free from lecithin.

Preparation of lipovitellin and lipovitellenin. Lipovitellin and lipovitellenin, the two egg-yolk lipoproteins, were prepared according to Alderton & Fevold (1945) and Fevold & Lausten (1946). The former contains 16–18 % and the latter 35–40 % of phospholipins, of which about 80 % is lecithin.

Chemical analysis. Total P was determined photoelectro-colorimetrically by Brigg's method after ashing with H_2SO_4 and HNO_3 . N was estimated by the micro-Kjeldahl method and Ca by precipitation as oxalate and permanganate titration. For the estimation of choline, a modification of Beattie's reineckate method (Glick, 1944) was used.

Clostridium welchii α -toxin. The *Cl. welchii* α -toxin used in this investigation was a glycerinated preparation containing 2800 lecithinase units (Macfarlane & Knight, 1941) per ml., kindly supplied by Dr van Heyningen (1941*b*).

Measurement of the lecithinase activity. Like *Cl. welchii* lecithinase, *B. cereus* lecithinase splits lecithin into acid-soluble phosphorylcholine, and the rate of increase of acid-soluble phosphate also serves as a convenient measure of the enzyme activity. The method employed was essentially the same as that used for *Cl. welchii* lecithinase (Macfarlane & Knight, 1941). The reaction mixtures were made up with 1 ml. 0.05 M borate buffer pH 7.1; 0.1 ml. 0.1 M- $CaCl_2$ (for *Cl. welchii* lecithinase 0.2 ml. was used); aqueous solution of enzyme and distilled water to 3 ml. After this mixture had been warmed to 37°, 1 ml. of a 2.5 % lecithin emulsion at the same temperature was added from a 1 ml. blow-out pipette to ensure an immediate mixing. The reaction was stopped after 15 min. incubation at 37° by the addition of 1 ml. 20 % trichloroacetic acid, which also flocculated the undecomposed lecithin. The mixture was then filtered through a no. 50 Whatman filter-paper and the total phosphate in the filtrate estimated. The enzyme activity in the following experiments was generally expressed as mg. of acid-soluble phosphate liberated from 25 mg. of lecithin in 15 min. at 37° and one arbitrary unit of lecithinase was defined as that amount of enzyme which under the above conditions would catalyse the production of 0.1 mg. acid-soluble P.

Measurement of the egg-yolk reaction. The production of opalescence in egg-yolk extract by the action of lecithinase was measured by a modification of

van Heyningen's (1941*a*) method. The standard conditions finally adopted were as follows: 1 ml. enzyme diluted in water, 1 ml. 0.05 M borate buffer (pH 7.1) and 2 ml. distilled water were put in a test-tube or preferably in the cups of the photoelectric colorimeter, and warmed to 37°; 2 ml. 5 % egg-yolk extract at the same temperature were then added. After 15 min. at 37° the turbidity was read in a photoelectric colorimeter fitted with a grey filter.

Fig. 1. The effect of lecithinase concentration on development of turbidity in egg-yolk extract. Reaction mixture: amount of lecithinase indicated above, 1 ml.; borate buffer pH 7.1; 2 ml. yolk saline; water to 6 ml.; reaction time 15 min. at 37°.

In the photoelectric colorimeter (King, 1946) the reading ('extinction coefficient *E*' multiplied by 100) is not a linear function of the turbidity, especially when the reading is greater than 30. For this reason the turbidities were read from a standard curve prepared by plotting the colorimeter readings of a series of suspensions of BaSO₄. Using this curve the turbidity of a given egg-yolk reaction mixture was expressed in terms of its equivalent BaSO₄ concentration. The standard BaSO₄ suspensions were prepared as follows: a stock suspension of BaSO₄ was prepared by adding 1 % (v/v) conc. H₂SO₄ to 2.0 ml. 1 % (w/v) BaCl₂ with stirring to a total volume of 100 ml. The resultant suspension was calculated to contain 224 mg. of BaSO₄/100 ml. A series of suspensions containing 1–200 mg. BaSO₄/100 ml. were then prepared by diluting the stock BaSO₄ suspension with 1 % conc. H₂SO₄.

A dry enzyme preparation containing 3 lecithinase units per mg. was used as the standard for the egg-yolk turbidity estimation. Fig. 1 shows the turbidities produced in 15 min. by 0–3 units of lecithinase under the above

conditions. It can be seen that in the region of from 1 to 2 units, the turbidity is a linear function of the enzyme concentration.

It must be emphasized that under the above standard conditions the final reaction mixture (total volume of 6 ml.), is 0.3 % with respect to NaCl and contains no Ca ion except that present in the egg yolk. In the case of *Cl. welchii* sufficient Ca ion is added to a final concentration of 0.005 M. A slight change in the salt concentration has a great influence on the turbidity produced. As the absolute turbidity produced by a certain amount of enzyme is influenced by a number of conditions which can hardly be identical in two occasions, it is advisable to run a few standards with each set of estimations. When the enzyme to be estimated is present in a complicated medium the standard enzyme preparation must be diluted with the same medium so that the effect of the salts and other substances present in the medium can be eliminated.

Measurement of haemolytic activity. Haemolytic activity was measured by modifications of the methods of Herbert (1941) and van Heyningen (1941*b*). Decreasing amounts of haemolysin diluted to a total volume of 2 ml. with borate buffer saline (pH 7.1) containing 10^{-4} M- Ca^{++} were incubated with 1 ml. of a washed sheep red-cell suspension standardized to contain 20 mg. haemoglobin/ml. (equivalent to c. 6 % cell suspension). After 1 hr. at 37° the reaction was stopped by the addition of 7 ml. of normal saline containing 5 % of normal horse serum, which was found to inhibit strongly the haemolytic activity of *B. cereus*. The unhaemolysed cells were then removed by centrifugation and the percentage haemolysis was estimated from the haemoglobin content of the supernatant fluid in a photo-electric colorimeter fitted with a green filter. The colorimeter readings were transformed directly into percentage haemolysis by reference to a standard curve prepared with a series of standard haemoglobin solutions. This curve was also used for the standardization of the red-cells suspension.

An arbitrary haemolytic (H.U.) unit is defined as the amount of haemolysin causing 50 % haemolysis of 1 ml. suspension of sheep red blood cells containing 20 mg. haemoglobin after 1 hr. at 37°. Fig. 2 shows the degree of haemolysis produced by 0.5–3.5 H.U. of *B. cereus* haemolysin. The shape of the curve is similar to that of streptolysin O (Herbert, 1941) and *Cl. welchii* α -toxin (van Heyningen, 1941*b*).

RESULTS

Concentration of the enzyme

A 20 hr. nutrient broth culture of *B. cereus* was used as a source of the enzyme. To the culture filtrate, which generally contained 1–2 units of lecithinase, ammonium sulphate was added slowly with stirring to two-thirds saturation. The precipitate, which contained most of the enzyme, was then redissolved in one-twentieth of its original volume of water. To this solution half a volume of saturated ammonium sulphate solution was added and the resultant dark brown sticky precipitate was centrifuged off. The clear supernatant was dialysed against running tap water for 8 hr., and then against several changes of distilled water at 2° for 24 hr. The dialysed solution was either dried to

a solid *in vacuo* or concentrated by dialysing against 50 % aqueous glycerol. The latter preparation retained its enzymic activity unchanged for over 1½ years. The glycerinated solution generally contained 50–80 lecithinase units per ml. and the dry preparation about three lecithinase units per mg. The discarded fractions precipitated by one-third saturation, and by more than two-thirds saturation, with ammonium sulphate contained 0·02 or less lecithinase units per mg.

Fig. 2. Standard curve for titration of *B. cereus* haemolysin.

Mode of hydrolysis of lecithin by Bacillus cereus lecithinase

The mode of hydrolysis of lecithin by *B. cereus* lecithinase was compared with that of *Cl. welchii* lecithinase. Of three samples of 760 mg. of lecithin containing 30 mg. of P, one was hydrolysed by *B. cereus* lecithinase, one by *Cl. welchii* lecithinase and the third served as a control. The reaction mixtures were made up with distilled water to a total volume of 20 ml. and contained 0·005 M-CaCl₂. On incubation the enzyme mixtures rapidly became acid and were frequently neutralized with 0·1 N-NaOH during the course of the hydrolysis. After 8 hr. at 37° the enzyme mixtures became fairly clear with a layer of fat on the surface, while the control still remained milky. The mixtures were then extracted four times with ether, and the ethereal solutions were separated by centrifugation. The combined ethereal solutions were evaporated to dryness, and extracted with acetone. The residues from the ether extracts of the

enzyme mixtures were almost completely soluble in acetone; that from the control mixture was completely insoluble, showing that most of the lecithin had been hydrolysed by the enzymes yielding acetone-soluble material. The acetone-soluble materials from both mixtures had the same acid value of 2.0, and thus appeared to be neutral fats.

Table 1. *Comparison of hydrolysis of lecithin by Clostridium welchii lecithinase and Bacillus cereus lecithinase*

	<i>B. cereus</i> lecithinase	<i>Cl. welchii</i> lecithinase	Control (no enzyme)
Before hydrolysis:			
Lecithin (mg.)	760	760	760
Total P (mg.)	29.9	29.9	29.9
Total N (mg.)	15.4	15.4	15.4
After hydrolysis:			
(1) Water soluble products:			
Total P (mg.)	29.0	28.5	0.3
Total N (mg.)	14.5	14.0	0.15
Inorganic P (mg.)	0	0	0
Free choline (mg.)	0	0	0
(2) Ether soluble products:			
Acetone soluble material (neutral fat) (mg.)	529.6	510.0	0
Acetone insoluble material (unhydrolysed lecithin) (mg.)	20.0	31.0	720

Table 2. *The action of Bacillus cereus and Clostridium welchii lecithinases on free and protein-bound phospholipins*

Substrate	Time of incubation	Acid soluble P (mg.) liberated by the action of	
		1 mg. <i>B. cereus</i> lecithinase preparation	1/900 ml. <i>Cl. welchii</i> lecithinase preparation
25 mg. of free lecithin	{ 15 min. 3 hr.	0.30 0.64	0.30 0.62
25 mg. of free phospholipins extracted from lipovitellenin	{ 15 min. 3 hr.	0.31 0.65	0.30 0.61
70 mg. of lipovitellenin con- taining about 25 mg. of bound phospholipin	{ 15 min. 3 hr.	0.15 0.34	0.03 0.05

The aqueous solution from the two enzyme mixtures contained more than 95 % of the original lecithin P in an organic form and an equivalent amount of N but no free choline. In the case of *Cl. welchii* the organic P compound was crystallized as Ca salt and identified as phosphorylcholine (Macfarlane & Knight, 1941). By means of their technique 85 mg. of crystals were obtained from *B. cereus* lecithinase reaction mixture and 105 mg. from that of *Cl. welchii* lecithinase. Analysis showed that their compositions were very similar and agreed fairly well with that required for crystalline Ca compound of phosphorylcholine. (Ca, 12.0 and 12.0 %; N, 4.4 and 4.3 %; total P, 8.8 and 9.0 %;

free choline and inorganic phosphorus 0; first figures for the crystalline compound from *B. cereus* lecithinase reaction mixture and second figures for that from *Cl. welchii* lecithinase mixture.) Both crystalline organic P compounds were rapidly hydrolysed by a bone phosphatase preparation free from diesterase (kindly supplied by Dr M. G. Macfarlane). Equivalent amounts of free choline and inorganic phosphate were liberated from the two crystalline compounds after the hydrolysis, i.e. both compounds were phosphorylcholine. The changes of the three reaction mixtures after incubation are shown in Table 3. It can be seen that *B. cereus* lecithinase acts on lecithin in the same way as *Cl. welchii* lecithinase, in that neutral fat and phosphorylcholine are produced.

Action of Bacillus cereus lecithinase on egg-yolk extract

When egg-yolk extract was incubated with the enzyme preparation, there was a gradual increase of turbidity followed by formation of scum. Analysis of the reaction mixture showed a great increase of acid-soluble P as a result of the action of the lecithinase on the egg-yolk phospholipins. The close correlation of the lecithinase activity with the development of opalescence in egg-yolk extract was shown by the following experiment.

To a mixture of 10 ml. of diluted enzyme, 10 ml. of borate buffer (pH 7.1) and 20 ml. of distilled water at 37°, 20 ml. of yolk saline at the same temperature were added. Immediately and at intervals after mixing, the turbidities of 6 ml. samples were determined. As soon as each turbidity was read, 1 ml. 20 % trichloroacetic acid was added to stop the reaction and the total acid-soluble phosphate estimated. The rate of liberation of acid-soluble P and the development of turbidity were almost parallel. There is little doubt that the production of turbidity in egg-yolk extract in this case is due primarily to the lecithinase activity, but the quantitative development of the egg-yolk reaction is determined also by a number of conditions which must be well controlled when the egg-yolk reaction is used for the titration of lecithinase.

*The action of Bacillus cereus and Clostridium welchii
lecithinases on egg-yolk lipoproteins*

Under the above experimental conditions *B. cereus* lecithinase preparation was more active in producing turbidity in egg-yolk extract than *Cl. welchii* lecithinase. About 3 units of the latter is required to develop in egg-yolk extract a turbidity comparable to that produced by 1 unit of the former. As the egg-yolk reaction, also known as the lecitho-vitellin (L.V.) reaction, is supposed to be due to the action of lecithinase on egg-yolk lipoproteins, it was considered interesting to see if these two lecithinases also vary in their activities against purified egg-yolk lipoproteins. Lipovitellenin, the egg-yolk lipoprotein containing more phospholipins and with less phosphorus in its protein part, was chosen for this purpose. The preparation used contained 1.5 % P, 9.9 % N, and 4.99 % choline. It could be dissociated with ethanol and repeatedly extracted with ether and ethanol to give 63 % vitellenin (N, 15.4 %; P, 0.23 %; choline, nil) and 36 % phospholipin (N, 1.8 %; P, 3.54 %; choline, 11.1 %;

choline/P=0.79/1). From the analysis it can be seen that about four-fifths of the phospholipin was choline-containing and that any cephalin present could not be more than one-fifth of the total phospholipin. As shown in Table 2, similar amounts of *B. cereus* and *Cl. welchii* lecithinases, based on their rate of hydrolysis of free lecithin, also had about the same activity on the free phospholipin extracted from lipovitellenin, but varied very much in their activities against phospholipin bound in lipovitellenin. *Cl. welchii* lecithinase appeared to be much less active against vitellenin-bound lecithin as compared with *B. cereus* lecithinase. This might also account partially for its weaker

Fig. 3. Effect of Ca concentration on hydrolysis of free lecithin by *B. cereus* and *Cl. welchii* lecithinases. Reaction mixture: 1 mg. *B. cereus* lecithinase preparation or 0.001 ml. *Cl. welchii* α -toxin; 1 ml. borate buffer pH 7.1; 1 ml. 2.5% lecithin; 0.08 ml. 0.1 M-CaCl₂; water to 4 ml.; reaction time 15 min. at 37°.

activity on egg-yolk extract. As *B. cereus* lecithinase preparation also acts on cephalin, its relatively higher activity on lipovitellenin, as measured by the amount of acid-soluble P liberated, might be due to the additional effect of cephalinase. But estimation of the choline content of the hydrolysis products showed that over 90% of the acid-soluble P was, in fact, liberated from lecithin.

Characteristics of Bacillus cereus lecithinase

The effect of Ca and Na ions on the lecithinase activity. Like *Cl. welchii* lecithinase, the activity of *B. cereus* lecithinase is greatly affected by Ca ion. Fig. 3 compares the effects of Ca ion on *B. cereus* and *Cl. welchii* lecithinases. The latter has a wide range of optimal Ca concentration, whereas the activity of the former is maximal in the narrow range of $1-4 \times 10^{-3}$ M-Ca. As lecithin is readily precipitated from an emulsion by Ca salts, it was first thought that the inhibitory effect of Ca ion at higher concentrations might be simply due

to precipitation of lecithin. In order to elucidate this, the effect of Ca ion on the lecithin emulsion itself was investigated. It was expected that lecithin emulsion would be stable at the concentration of Ca ion optimal for the lecithinase activity and precipitation might take place at a higher concentration. Contrary to these expectations, lecithin emulsion was found to be most unstable in the presence of $1-4 \times 10^{-3}$ M-Ca, which is the optimal concentration for the activity of *B. cereus* lecithinase. This correlation is shown in Table 3.

Table 3. *The effect of calcium on the flocculation of lecithin and the activity of Bacillus cereus lecithinase*

	Concentration of Ca (10^{-3} M)							
	25	10	5	2.5	1.25	0.5	0.25	0
Rate of flocculation* of lecithin	—	—	++	+++	++++	+	—	—
Lecithinase activity† (mg. P liberated)	0.030	0.230	0.300	0.312	0.310	0.075	0.024	0.022

* The rate of flocculation of lecithin was roughly estimated by the relative time required for flocculation of lecithin after adding various amounts of Ca^{++} into lecithin emulsions. + + + +, + + +, + +, + = relative rates of flocculation; — = no flocculation after 3 hr.

† The lecithinase activity was expressed as mg. of acid-soluble phosphate liberated from 25 mg. of lecithin in 15 min. by the action of 1 mg. *B. cereus* lecithinase.

The fact that *B. cereus* lecithinase activity is greatest at the Ca ion concentration at which lecithin flocculates most readily from its emulsion suggests that the enzyme might have a maximal affinity for lecithin when the latter has absorbed an optimal amount of Ca ion in reaching its isoelectric point.

Although the hydrolysis of free phospholipins by *B. cereus* lecithinase requires the presence of free Ca ion, the egg-yolk reaction and the hydrolysis of phospholipins bound in lipovitellenin were found to proceed with maximal velocity even in the presence of oxalate. The addition of Ca ion, in concentration as low as 10^{-3} M, greatly decreased these activities (see Fig. 4). The independence of *B. cereus* lecithinase of free Ca ion for the hydrolysis of phospholipin present in lipovitellenin might be due to a small amount of Ca (0.08 %) bound in the lipovitellenin which is not readily precipitated by oxalate and is sufficient to activate the enzyme. It is also possible that the combination of phospholipin with protein has so changed their configurations as to render Ca ion unnecessary for the action of the enzyme on the phospholipin.

Later, it was found that Na ion has a strong inhibitory action on the enzyme activity, the activity of the enzyme in physiological saline being only 5 % of that in water. Fig. 5 shows the amount of acid-soluble P liberated from lecithin by a given amount of *B. cereus* lecithinase at various concentrations of Na ion. A similar, but smaller effect was observed with *Cl. welchii* lecithinase.

The effect of pH. Since the enzyme activity is greatly affected by a small change in the Na concentration, and since sodium salt buffers of different pH generally vary in their Na content, it is necessary to equalize the final Na concentration of reaction mixtures containing buffers of different pH, otherwise the enzyme activity might be influenced not only by the difference

Fig. 4. The effect of Ca on hydrolysis of free and bound phospholipins by *B. cereus* lecithinase. x-x-x, hydrolysis of free lecithin; o-o-o, hydrolysis of phospholipins bound in lipovitellenin; ●-●-●, production of turbidity in egg-yolk extract. Percentage activity is calculated by assuming the maximal activity as 100 %. (No Ca was added to these reaction mixtures; the small amount of Ca present came from the enzyme and the substrate.)

Fig. 5. The effect of Na concentration on lecithinase activity of *B. cereus*. Reaction mixture: 1 mg. *B. cereus* lecithinase preparation; 1 ml. borate buffer pH 7.1; 0.1 ml. 0.1 M-CaCl₂; 1 ml. 2.5 % lecithin; 0.0-0.6 ml. 6 % (w/v) NaCl; water to 4 ml.; reaction time 15 min. at 37°.

of pH, but also by the variation of the salt concentration. Fig. 6 compares the enzyme activities at various pH's with and without adjustment of the final Na concentration. Borate buffers were used for pH 6.7–9.0 and acetate buffer for pH 5.7. The final strength of the buffer in the reaction mixture is 0.012 M. Although the optimal pH in both cases was around neutrality, the effect was greatly exaggerated in curve I, where the final salt concentrations were left unadjusted. This additional effect was doubtless due to the lower Na ion concentrations at pH's near neutrality.

Fig. 6. The effect of pH on the activity of *B. cereus* lecithinase. Reaction mixture: 1 mg. *B. cereus* lecithinase preparation, 0.1 ml. 0.1 M-CaCl₂; 1 ml. 2.5 % lecithin; 1 ml. 0.05 M borate buffer pH 6.7–9.0, or acetate buffer pH 5.7; water to 4 ml.; reaction time 15 min. at 37°. Figures on the curves indicate the calculated [Na⁺] of the reaction mixtures (× 10⁻³ M). The variation of [Na⁺] at various pH's as shown in curve I is due to the different Na content of the buffers. In curve II, the [Na⁺] of the reaction mixture at various pH's were all adjusted to that at pH 9, i.e. 20 × 10⁻³ M by the addition of NaCl solution.

Effect of substrate concentration. Fig. 7 shows the effect of substrate concentration on the initial velocity of hydrolysis with a given concentration of enzyme. Since lecithin is present as a colloidal solution, it is difficult to calculate the Michaelis constant (*K_m*) for the enzyme. Following the method of calculation, as used by Zamecnik, Brewster & Lipmann (1947) for *Cl. welchii* lecithinase, and making similar assumptions, the *K_m* of *B. cereus* lecithinase is calculated to be 4.8–5.4 × 10⁻³ M, which is near to that of *Cl. welchii* lecithinase, viz. 5 × 10⁻³ M.

Effect of enzyme concentration. Fig. 8 shows that the initial velocity of hydrolysis, as measured by determining the amount of acid-soluble P formed in 15 min., is directly proportional to the enzyme concentration over at least an eightfold range.

The stability of the enzyme. Like *Cl. welchii* lecithinase, the *B. cereus* enzyme

Fig. 7. The effect of substrate concentration. Enzyme concentration 0.25 mg./ml.; pH 7.1; total volume of reaction mixture 4 ml.; reaction time 15 min. at 37°.

Fig. 8. The effect of enzyme concentration. Reaction mixture: 25 mg. lecithin; 1 ml. borate buffer pH 7.1; 0.1 ml. 0.1 M -CaCl₂; enzyme preparation 0–1.0 mg.; water to 4 ml.; reaction time 15 min. at 37°.

is fairly heat stable. Heating the enzyme in pH 7.1 borate buffer for 30 min. at 60° in a sealed tube destroyed only 20 % of its activity, and 30 % of its activity still remained after standing in boiling water for 10 min. The enzyme is also resistant to oxidation; treatment with a 1:100 dilution of 30 vol. H_2O_2 for 1 hr. at room temperature had no effect on its activity. It is rather easily inactivated by surface denaturation; bubbling N_2 through a dilute enzyme solution for 60 min. destroyed 40 % of its activity. It can also be inactivated by incubating with dilute formalin. When kept in 50 % aqueous glycerol at 2°, the enzyme activity remained unchanged for over 2 years. Dilute solutions of the enzyme in water or saline are rather unstable at room temperature.

The haemolytic activity of Bacillus cereus lecithinase preparations

The close association of lecithinase activity and haemolytic activity of *B. cereus* and *B. mycoides* has already been discussed (McGaughey & Chu, 1948). One lecithinase unit is generally associated with ten haemolytic units. Further studies showed that this haemolysin has many characteristics in common with *Cl. welchii* lecithinase, which is also haemolytic.

Fig. 9. The effect of Ca on haemolytic and lecithinase activities of *B. cereus*. * No Ca was added to these reaction mixtures, the small amount of Ca present as shown in the figure came from the enzyme and the substrate.

Effect of Ca ions. The lecithinase activity of *B. cereus* is optimal at Ca concentration around $1-4 \times 10^{-3}$ M. The haemolytic activity is maximal at a lower concentration of Ca, ranging between $0.05-0.2 \times 10^{-3}$ M. The activity can be decreased either by very slight increase of Ca concentration or by the addition of citrate or oxalate as shown in Fig. 9.

Susceptibility of erythrocytes of different species. Oakley & Warrack (1941) showed that α -toxin of *Cl. welchii* was comparatively inactive against goat or horse red blood cells; *B. cereus* haemolysin behaves in the same way. Red cells of sheep, pig, rabbit, guinea-pig and man are almost equally susceptible to its action; horse cells less susceptible; and goat cells very resistant. About twenty times more haemolysin is required to haemolyse goat cells than sheep cells.

Table 4. *The inhibition of lecithinase activity and haemolytic activity of Bacillus cereus by normal horse serum*

Lecithinase activity: 0.0-2 ml. normal horse serum diluted with saline to 0.2 ml. were allowed to act with 1 ml. of diluted enzyme in water at 37° for 15 min., one ml. pH 7.1 borate buffer, 0.1 ml. 0.1 M-CaCl₂, 0.7 ml. H₂O and 1 ml. 2.5 % lecithin were then added and the amount of hydrolysis estimated in the usual way after another 15 min. incubation.

Haemolytic activity. Decreasing amounts of normal horse serum were incubated at 37° for 15 min. with 0.1 mg. of lecithinase preparation containing 3 H.U. in a total volume of 2 ml. made up with borax buffer saline. 1 ml. standard suspension of red cells were then added and the degree of haemolysis determined after 1 hr. incubation.

Amount of normal horse serum (ml.)	Lecithinase activity of 1 mg. lecithinase preparation		Haemolytic activity of 0.1 mg. lecithinase preparation	
	P liberated (mg.)	Inhibition (%)	Haemolysis (%)	Inhibition (%)
1/5	0.000	100.0	0	100
1/10	0.001	99.6	0	100
1/20	0.004	98.5	0	100
1/40	0.028	90.0	0	100
1/80	0.050	82.5	0	100
1/160	0.110	62.3	0	100
1/320	0.156	45.0	0	100
1/640	0.222	25.4	0	100
1/1280	0.256	9.9	3.0	97
1/2560	—	—	50.0	50
1/5120	—	—	97.0	3
0	0.284	—	100.0	—

Thermostability. The effect of heating on *B. cereus* haemolysin is interesting. The haemolytic activity was completely destroyed after heating at 60° for 10 min., while boiling for 10 min. only destroyed 80 % of its activity. This peculiar heat resistance has also been observed with staphylococcus α -haemolysin (Arrhenius, 1907) and *Cl. welchii* α -toxin. (Guillaumie, Kreguer & Fabre, 1946). Heating affects the haemolytic activity of *B. cereus* filtrate more than it affects the lecithinase activity. Heating at 60° for 10 min. has very little effect on lecithinase activity but completely abolishes the associated haemolytic activity. However, this does not necessarily mean that the haemolysin is unrelated to the lecithinase, as it is quite possible that heating at 60° only upsets the access of lecithinase to the substrate on the red cells but does not affect the approach of the enzyme to free lecithin. The haemolytic activity of *Cl. welchii* α -toxin is also more sensitive to heat than its associated lecithinase activity.

Hot-cold effect. Unlike *Cl. welchii* α -toxin (van Heyningen, 1941b), *B. cereus* haemolysin is not a hot-cold lysin.

Resistance to oxidation. Like *Cl. welchii* α -toxin, the haemolytic activity of *B. cereus* is not affected by oxidation.

Inhibition by normal serum. Lysis of red cells as well as hydrolysis of lecithin by *B. cereus* are both strongly inhibited by normal sera of all the animals tested, including horse, sheep, rabbit, ox, guinea-pig, mouse and man (see below).

Effect of peptone. The activity of *B. cereus* haemolysin is greatly enhanced in the presence of a small amount of peptone. About 50 % more haemolysin is required to lyse sheep red cells in the absence of peptone than in its presence. Similar observation has been made on *Cl. welchii* toxin by Oakley & Warrack (1941).

Inhibition of lecithinase activity by normal serum

The hydrolysis of free lecithin and the lysis of red blood cells by *B. cereus* filtrate were strongly inhibited by normal sera of all the animals tested, including horse, ox, sheep, rabbit and man. The inhibitor was found to be associated with both serum globulin and serum albumin. But it was not

Table 5. *The effect of normal serum proteins on the hydrolysis of free phospholipins and protein-bound phospholipins by Bacillus cereus phospholipinase*

Reaction mixture: 1 mg. *B. cereus* lecithinase; 1 ml. borate buffer; 0.1 ml. 0.1M-CaCl₂ (only for the hydrolysis of free phospholipins); 1 ml. of 2.5 % free phospholipins or 7 % lipovitellenin; serum proteins as indicated above; water to 4 ml. Reaction time: 15 min. at 37°.

	Hydrolysis of free phospholipin		Hydrolysis of bound phospholipins	
	'P' liberated from 25 mg. free phospholipins extracted from 70 mg. lipovitellenin	Inhibition (%)	'P' liberated from 70 mg. of lipovitellenin containing about 25 mg. bound phospholipin	Inhibition (%)
Control. No serum protein	0.307	—	0.140	—
Horse serum globulin 0.2 mg.	0.090	71.0	0.141	0
Horse serum globulin 1.0 mg.	0.061	80.0	0.142	0
Horse serum albumin 0.2 mg.	0.060	80.5	0.141	0
Horse serum albumin 1.0 mg.	0.045	85.5	0.143	0

destroyed by heating to 70° for 10 min., which had apparently denatured the serum proteins. The extent of the inhibition is shown in Table 4. In the experiments cited the inhibition was measured by comparing the rate of hydrolysis during the first 15 min. of incubation. The inhibitory effect has also been demonstrated in experiments where the reaction was allowed to proceed for several hours. The inhibition is thus not just a temporary lag effect.

It is interesting that normal serum proteins, although inhibiting the hydrolysis of free lecithin in high dilution, have very little effect on the yolk reaction. This is partially explained by the finding that normal serum proteins also fail to inhibit the hydrolysis of phospholipins bound in lipovitellenin and lipovitellin, the two egg-yolk lipoproteins (Table 5). The fact that *B. cereus*

lecithinase preparation also acts on cephalin and that the cephalinase activity is not inhibited by normal serum, suggested that the acid-soluble P liberated from lipovitellenin in the presence of normal serum proteins might largely come from cephalin. This possibility was ruled out, however, by the finding that the choline contents of the acid-soluble P compounds liberated from lipovitellenin in the presence of normal serum proteins or in its absence were practically the same. The reason why the combination of lecithin with proteins like vitellenin or vitellin should counteract the inhibition of the lecithinase by serum protein is not clear.

Production of specific anti-lecithinase in rabbits and its differentiation from normal serum inhibitor

Specific *B. cereus* anti-lecithinase has been produced by immunizing rabbits with the enzyme preparation. Unlike the inhibitor in normal serum the specific anti-lecithinase is capable of inhibiting not only the hydrolysis of free lecithin but also the yolk reaction and the hydrolysis of protein-bound lecithin. Possibly, the specific anti-lecithinase has a more direct effect on the enzyme than the inhibitor present in normal serum. Table 6 compares the effect of normal rabbit serum and immune rabbit serum on various activities on *B. cereus* lecithinase preparation.

Table 6. *The effect of normal rabbit serum and immune rabbit serum on various activities of Bacillus cereus lecithinase preparation*

This table compares the effects of 0.1 ml. normal serum and 0.1 ml. immune serum on the various activities produced by 1 mg. of *B. cereus* lecithinase preparation.

	Lecithinase activity					
	Hydrolysis of free lecithin			Hydrolysis of phospholipins bound in lipovitellenin		
	P liberated		Inhibition (%)	P liberated		Inhibition (%)
	(mg.)			(mg.)		
Control (no serum)	0.310	—		0.140	—	
Normal serum 0.1 ml.	0.005	98		0.140	0	
Immune serum 0.1 ml.	0.005	98		0.055	61	
	Haemolytic activity		Yolk reaction		Toxicity	
	Haemolysis (%)	Inhibition (%)	Increase of turbidity	Inhibition (%)	mice dying mice tested	Protection (%)
Control (no serum)	100	—	168*	—	12/12	—
Normal serum 0.1 ml.	0	100	167	0	12/12	0
Immune serum 0.1 ml.	0	100	8	95	3/12	75

* Turbidity expressed as mg. BaSO₄/100 ml.

Antisera against *B. cereus* lecithinase have no effect on *Cl. welchii* lecithinase, and vice versa; the enzymes therefore are antigenically different. On the other hand, lecithinase produced by *B. mycoides* can be neutralized by antisera against *B. cereus* lecithinase. This provides further evidence of their close relationship.

The toxicity of Bacillus cereus lecithinase

The fact that the hydrolysis of free lecithin and lysis of red blood cells by *B. cereus* lecithinase are inhibited by normal serum suggests that the enzyme might be innocuous *in vivo*. On the other hand the inability of normal serum to inhibit the hydrolysis of lecithin bound in egg-yolk lipoprotein suggests that normal serum might fail to inhibit the action of the enzyme on other lipoproteins occurring in animal tissue; the enzyme might therefore not be so innocuous *in vivo* as first expected. In fact, the enzyme preparation was found to be toxic for experimental animals, although the toxicity might be due to factors other than lecithinase.

Intravenous inoculation of a dose of the enzyme preparation containing 2-4 lecithinase units is lethal to mice. The M.L.D. of *Cl. welchii* α -toxin for mice, on the other hand, generally contains 0.5 lecithinase unit. Intradermal injection in rabbits and guinea-pigs produces marked inflammatory lesions with central necrosis. The toxicity can be neutralized by immune rabbit sera containing specific anti-lecithinase. As the enzyme preparation is very crude, it is impossible to say whether its toxicity is due to lecithinase activity exclusively or to some other factors as well. The enzyme preparation has already been found to possess cephalinase activity which is likely to account for part of its toxicity.

The action of Bacillus cereus lecithinase preparation on cephalin

Unlike the *Cl. welchii* lecithinase (Macfarlane, 1942) the preparation from *B. cereus* was capable of hydrolysing not only lecithin but also cephalin. Whether the hydrolysis of cephalin is due to the lecithinase or to another enzyme remains to be seen. A few preliminary experiments have shown that acid-soluble organic P but not inorganic P is split from cephalin by the enzyme. An enzyme preparation containing three lecithinase units was able to catalyse the production of 0.12 mg. acid-soluble P from 25 mg. of cephalin in 15 min. under the same conditions as used for lecithinase estimation. The cephalinase activity also requires the presence of Ca ion, but is not inhibited by normal serum, which has a very strong inhibitory effect on the lecithinase activity.

DISCUSSION

The data showing the close similarity of *B. cereus* and *Cl. welchii* lecithinases in their enzymic properties and biological activities are summarized in Table 7. *B. cereus* lecithinase possesses most of the biological activities found to be associated with *Cl. welchii* α -toxin. Although these two lecithinases are very similar in most respects, differences have been noted between them. Some of these differences may have a bearing on their activities *in vivo*; the different reactions to normal serum is an interesting example. Thus, although these two enzymes exhibit similar chemical activities *in vitro*, they might differ very much in their activities *in vivo*.

The inhibitory action of normal serum protein on the hydrolysis of free

lecithin by *B. cereus* lecithinase, in contrast to its failure to suppress the hydrolysis of lecithin bound in egg-yolk lipoproteins is another illustration of the complexity of the biological system.

Table 7. *Comparison of Bacillus cereus lecithinase and Clostridium welchii lecithinase*

Lecithinase activity	Ca requirement	<i>B. cereus</i> <i>Cl. welchii</i> lecithinase lecithinase	
		+	+
	Optimal pH	6.8-7.2	7.0-7.6
	Thermostability (%):		
	activity after { 60° 10 min.	82	87
	{ boiling 10 min.	32	45
	Resistance to oxidation	+	+
	Inactivation by formalin	+	+
	Precipitation by 2/3 sat'd. (NH ₄) ₂ SO ₄	+	+
	Activity on egg-yolk lipoproteins	++	+
	Inhibition by normal serum proteins	+	—
Biological activity	Egg-yolk reaction and Nagler reaction	+	+
	Haemolytic activity	+	+
	Inhibition of haemolysis by normal serum proteins	+	—
	Dermonecrotic effect in guinea-pigs	+	+
	Toxicity to mice	+	++

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