98. THE BIOCHEMISTRY OF BACTERIAL TOXINS

1. THE LECITHINASE ACTIVITY OF *CL. WELCHII* TOXINS

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UNTIL recently the specific toxic substances produced by bacteria have been investigated mainly by pathological and serological methods and further knowledge of their chemical nature and modes of action is clearly desirable. In addition to the intrinsic value of such knowledge there would be an immediate practical advantage to be derived from the purification and isolation of the specific toxins from toxic culture filtrates for use in the preparation of antitoxic sera, particularly if the toxins are mixtures of several antigens, as for instance in the *Cl. welchii* group. In these cases a full complement of antibodies to all the toxic components of the invading organisms is the goal in serum therapy, but the response to immunization even with a single antigen is variable in individual animals and it is not necessarily the case that with a mixture of antigens the relative proportions of the different antibodies in the serum will reflect those of the corresponding antigens in the toxin or toxoid used for immunization.

The isolation of particular toxic components would also be an advantage in studying the mode of action of the toxins on the cell. That a toxin may have an affinity for a particular type of tissue or cell is well known, and in some cases the physiological effect of a particular toxic component in a complex toxin has been demonstrated by the use of suitable antitoxic sera to inhibit the effect of the other components, but little is known of what these effects mean in terms of cell chemistry. In searching for the biochemical mechanisms by which the bacterial toxins affect the cells of the host two main hypotheses may be considered.

First, the toxin may be a substance which blocks a metabolic reaction in the host by competing with the normal substrate for the enzyme catalysing this reaction; this hypothesis is the counterpart of the theory of Fildes [1940] concerning the mode of action of chemotherapeutic substances, according to which the chemotherapeutic agent blocks an essential metabolic reaction in the invading organisms. This conception has already received some attention. Wooldridge & Higginbottom [1938] showed that the α-toxin present in *Cl. welchii* toxins inhibited the aerobic oxidation of succinate by various minced tissues of the guinea-pig, and that this inhibition was in turn considerably decreased by the specific antitoxin; they did not, however, detect which of the various enzyme systems concerned in the oxidation was inhibited. Peters & Cunningham [1941] have examined the action of diphtheria toxin on tissue respiration, and on various dehydrogenase and phosphorylation systems, but none of the enzymes examined was affected by the toxin.

The second hypothesis is that the toxin itself may be an enzyme exerting its toxic function by attacking, according to its degree of enzyme specificity, one or more substances which are normal constituents of a cell. It consequently distorts the metabolism of the cell in one or other particular direction, either primarily by destruction of an essential structure or the inhibition of a metabolic mechanism or secondarily by the production from a normal cell constituent of a toxic substance with such powers. On this hypothesis the toxin can primarily affect only those cells which contain the appropriate substrate for its enzymic activity and then only if this substrate is accessible to it. It is of course well known that certain bacterial culture filtrates have enzymic activity, but such activity has not in general been related to their toxic effects. The hydrolysis of lecithin by snake venom with the formation of lysolecithin has been for long the only instance in which the action of a toxin on a pure substance *in vitro* affords a satisfactory basis for its effect *in vivo*, namely haemolysis; for it is known that the red blood cells contain lecithin and that lysolecithin is a highly haemolytic substance. Recently the presence in various bacterial culture filtrates and in snake and spider venoms of an enzyme, hyaluronidase [Chain & Duthie, 1940], which rapidly reduces the viscosity of certain mucoproteins, has been correlated with that of a 'diffusing factor' which has a marked effect on the permeability of skin. McClean & Hale [1941] have pointed out the possible relationship of this enzyme to the local invasiveness of the organism. There is thus already evidence that certain toxins contain enzymes which may contribute to the pathogenic effect.

The basis of both these hypotheses of the modes of action of bacterial toxins is that the initial lesion in the host cell is one of molecular dimensions and high chemical specificity. Since a distortion in the metabolism of one type of cell or group of cells may affect the metabolism of neighbouring cells or tissues a characteristic pathological picture may develop throughout the tissues of the host as a result of this primary lesion. The total pathogenic effect of a bacterial infection is no doubt a summation of all the metabolic disturbances caused by the growth of the micro-organism, including those due to serologically non-specific toxic substances, for example histamine.

Recent advances in our knowledge of the biochemistry of micro-organisms permit a rational approach to these problems on several interrelated lines. The separation of the individual toxic components should be easier if the culture medium contains the minimum amount of substances similar to these toxic factors in chemical nature, and even more so if the proportion of a particular toxic component can be increased or decreased at will. The results of recent work on bacterial metabolism point to the possibility that the amount and nature of the toxic substances excreted by the bacteria are, like their content of endogenous enzymes, largely determined by the conditions in which the bacterial growth is taking place. Modern work on bacterial nutrition has demonstrated the effect of the composition of the culture medium on bacterial growth and made possible the use of relatively simple culture media of known chemical composition. It is therefore now easier to ensure that the toxin production shall take place under controlled conditions which can be varied at will. The value of this approach to the problem has been strikingly exploited by Mueller, Pappenheimer and co-workers [see Mueller, 1940; Mueller & Miller, 1941; Pappenheimer, 1937] in the case of diphtheria toxin. Its application to antigenically complex toxins would however be greatly facilitated by a knowledge of the biochemical action of the separate toxic components, if such knowledge made possible the substitution of a rapid specific chemical reaction for the complicated

animal tests necessary in the serological method. Moreover, the biochemical action of the toxin might give some clue to a variation in the culture medium likely to enhance the yield of a toxin, as in the analogous case of adaptive enzymes.

Because of the importance of *Cl. welchii* in war-wound infections it was decided to begin this investigation by examining the enzymic activity of the toxic culture filtrates of this organism. The present paper gives an account of a method used for the routine production of *Cl. welchii* (Type A) toxin[1] and of a lecithinase in the toxin which is probably identical with the lethal factor, α-toxin. The detection of this lecithinase was a direct consequence of the work of Dr R. G. Macfarlane and his colleagues on the action of the α-toxin of *Cl. welchii* on egg-yolk emulsions, following on the observations by Nagler [1939] that this toxin had a specific action on human serum.

Cl. welchii *toxins*

Wilsdon [1931; 1932–33] examined the causative organisms of several diseases and showed that they could be classified into 4 types of *Cl. welchii*, A, B, C and D, differing from each other serologically. The toxins produced by these four types are different though they have one or more toxic factors in common; the nomenclature and distinguishing effects of these components are set out in Table 1.[2] The differentiation of these toxic factors has been made

Table 1. *Antigenic analysis of* Cl. welchii *toxins*

Type	Disease	Toxins α	β	δ	ε	θ
A	Gas gangrene	+ + +	−	−	−	+
B	Lamb dysentery	+	+ + +	±	+ +	−
C	'Struck' in sheep	+	+ + +	+	−	−
D	'Pulpy kidney' in sheep	+ +	−	−	+ +	−
Characteristics of toxins:		Lethal Haemolytic Dermo-necrotic	Lethal Dermo-necrotic	Haemolytic ? Lethal	Lethal Necrotic	Haemolytic Lethal ? Dermo-necrotic

almost entirely by serological methods, chiefly by the work of Glenny and his colleagues [Glenny *et al.* 1933], although Prigge [1937] obtained from Type A filtrates, by fractional salting-out with Na_2SO_4 and $(NH_4)_2SO_4$, fractions with different proportions of the α- and θ-toxins, and thus demonstrated that at least two different substances were involved. Recently van Heyningen has achieved a considerable purification of the α-toxin from Type A filtrates and obtained it free from θ-toxin; this will be described in a paper to be published in a later issue of this Journal.

Nagler [1939] observed that when *Cl. welchii* was grown in human serum an opalescence developed and eventually a layer of fat rose to the surface of the liquid medium. This phenomenon took place with the exogenous toxin from all

[1] The word toxin is used in this paper to cover the unfractionated toxic culture filtrates, which may contain also immunologically non-specific substances. The various antigenically distinct factors will be specifically designated; thus α-toxin, and the corresponding antibody as α-antitoxin or anti-α serum.

[2] It was agreed at a representative colloquium held at Cambridge in April 1941, under the chairmanship of Prof. T. Dalling, that Glenny's original description of the lethal, haemolytic and dermonecrotic factor in Type A filtrates as α-toxin should be adhered to by English workers; and that the second haemolytic toxin in these filtrates should be called θ-toxin [cf. Smith, 1941; Stephenson & Dalling, 1941].

types of *Cl. welchii* but not with other bacterial filtrates, and was specifically inhibited by *Cl. welchii* antitoxin. He found that the activity in this respect ran parallel with the lethal action of type A toxins and that it could be used in an end-point method for the titration of the appropriate antitoxin. Later he showed [Nagler, 1941] that the reaction could be used to estimate the combining power (toxin + toxoid) of a toxin, or of toxoid alone, with antitoxin. Macfarlane *et al.* [1941] found that *Cl. welchii* toxin acts on a clear emulsion of egg-yolk in saline to give a similar but more marked opalescence and finally a cream due to the coalescence of fat globules. They showed that this reaction was due entirely to the α-toxin, which is the lethal factor predominant in type A filtrates and that β-, δ-, ϵ- and θ-toxins had no effect. They found also that this egg-yolk reaction and the haemolysis caused by α-toxin are activated by Ca^{++} ions and remarked that 'the strict parallelism between haemolysis by α-toxin and the production of free lipoid from lecitho-vitellin led us to suppose that both these activities might be manifestations of the same enzyme reaction'.

These authors considered that the biochemical action of the toxin was to liberate fat from combination in a lipoprotein. We have found however that after the action of the toxin the whole of the original lipin P is present as a water-soluble compound, while the protein and neutral fat are almost unchanged chemically. The toxin also decomposes an aqueous emulsion of lecithin with the production of phosphocholine and a diglyceride. It seems probable therefore that the creaming of the yolk-emulsion is due to the decomposition of the lecithin present which acts as a stabilizing agent in the emulsion of protein and fat. The available evidence points to the fact that the lecithinase responsible for this action is identical with the enzyme responsible for the reaction on egg-yolk and human serum and therefore, from the work of Macfarlane *et al.* [1941], with the α-toxin.

Preparation of the toxins

Some details of the medium and the method of cultivation of the organisms may be recorded here as they have proved useful to other workers to whom they have been already communicated. The technique has been evolved in the Serum Department during a number of years, mainly by Dr D. W. Henderson. The medium described has given good yields of toxin (100–200 M.L.D./ml.), with the strain used, in a suitable condition for biochemical investigation. Variations of peptone and glucose concentration may be necessary for maximum yields of toxin with other strains.

The organisms were grown in a medium the final composition of which was essentially as follows:

(*a*) 3·3% Evans[1] peptone ... ... 800 ml.
(*b*) Na_2SO_4 muscle-extract (see below) 100 ml.
(*c*) 0·5% glucose (added as a 4·5% solution in (*b*)).

The Na_2SO_4 muscle-extract (*b*) is the only ingredient of the medium which calls for special comment. This was prepared from horse or beef muscle by the method of Deutsch *et al.* [1938], which yields a protein-free extract containing all the water-soluble constituents of the muscle including the more heat-labile materials. The constituents of this extract which appear to enhance toxin formation on this medium are being investigated.

Deutsch *et al.* used a small fruit-press warmed to 32° for expressing the juice from the warm mass of tissue and sulphate. For 10 kg. quantities of meat mince we have found it convenient to use 8–10 in. Büchner funnels attached to filter flasks and to apply a vacuum, the whole apparatus standing in a hot-room. A rubber sheet tied over the funnels by narrow rubber tubing is a con-

[1] Manufactured by Evans Sons, Lescher and Webb, Ltd., Runcorn, Cheshire.

siderable aid in expressing the juice. The layer of mince-sulphate sludge on a filter should be not more than 2 in. thick before sucking. The further stages of the preparation follow Deutsch *et al.* The extract usually has a *p*H of about 5; alkali is added, with stirring to avoid local excess, bringing the *p*H to 6·5. A more alkaline *p*H causes a precipitate and loss of potency for toxin production. We have been content with one extraction of the minced muscle, 10 kg. of mince yielding on an average 1500 ml. of extract.

The Na_2SO_4 muscle-extract must not be heated; it is sterilized by filtration and stored in the cold, after the required amount of glucose has been added to it.

Method of cultivation. The routine adopted was as follows:

1. 3·3% Evans peptone and 0·5% NaCl were dissolved in tap-water, adjusted to *p*H 7·6 with drops of 40% NaOH, warmed to flocculate the phosphate precipitate and filtered through paper (Chardin or Whatman No. 1). This peptone-water was dispensed in 800 ml. lots into 1000 ml. conical flasks, plugged, and autoclaved for 15 min. at 115°.

2. On the morning of inoculation the flasks of peptone-water were steamed for 30 min. and cooled rapidly to about 45°.

3. To each 800 ml. of the peptone-water were at once added 100 ml. of the cold sterile Na_2SO_4 muscle-extract containing 4·5% glucose. This was run in with aseptic precautions from a bulb pipette without unduly agitating the medium, to minimize re-oxygenation. Sometimes thiolacetic acid (10 ml. of 2 *M* thiolacetic acid in *N* HCl followed by an equivalent amount of 2 *N* NaOH) was added to facilitate the maintenance of anaerobic conditions suitable for the initiation of growth. This was however in general unnecessary under the conditions used and can be omitted.

4. The inoculum was from an 18 hr. culture in Robertson's meat medium of *Cl. welchii* (type A) strain 107 (N.C.T.C. No. 6125). About 5 ml. of the actively growing culture were carefully injected from a Pasteur pipette into the bottom layers of the medium. With this technique rapid initiation of growth was secured and there was no need for anaerobic incubation. The flasks were incubated at 34–35°. Gas evolution was vigorous and the frothing sometimes blew out the plugs. It was therefore convenient to place the flasks on trays which could afterwards be sterilized.

5. The cultures were incubated for 5–6 hr. only. The toxin concentration reached a peak between 5 and 6 hr. incubation, which represents approximately the end of the log phase of growth, under the conditions described here. After this time there was a considerable diminution in the amount of toxin present. A slackening in the rate of gas evolution was a rough index of the time at which to stop incubation.

6. The cultures were filtered at once through paper-pulp[1] 'puddings' formed on the outside of Berkefeld candles and held in place by wrappings of gauze tied with string.

Dried toxin preparations. Several preparations of dried toxin were made by 2/3 or full saturation of the filtrate with $(NH_4)_2SO_4$. A scum of crude toxic material rose to the surface and was rapidly skimmed off and placed on the top of piles of filter paper. Some fluid was removed by changing the underlying sheets. The top papers carrying the crude toxic material were then placed in a vacuum desiccator containing P_2O_5 and NaOH, and rapidly evacuated by a Hyvac pump. The P_2O_5 was changed once after about an hour and the desiccator re-evacuated. The product was a friable light solid easily powdered to a fine grey powder. The average yield was about 1 g. per l. of medium; more could have been recovered by filtration or more complete skimming, but yield was sacrificed for speed of manipulation at this stage.

Glycerinated toxic culture filtrates. Sterile filtrates were filled into cellophane tubing and dialysed against glycerol at 3°. The volume of culture filtrate within the tube decreased considerably as water diffused out into the glycerol, thus effecting a concentration, while glycerol more slowly diffused in. This yielded convenient stable preparations, both the M.L.D. and L+ values remaining constant over long periods when stored at 0°. One preparation has remained constant for over 9 months and shows no evidence of deterioration. This method [Vanicek, 1930] has proved to be useful for the preparation of stable test-toxins from toxic culture filtrates or autolysates of various organisms during the past ten years in the Serum Department of this Institute.

Biological potency. The minimum lethal dose of a toxin preparation was determined by the intravenous injection of the dose, in a total volume of 0·5 ml. in normal saline, into mice weighing

[1] T. B. Ford's 'plain filter pulp, cotton mixture without asbestos'. (Agents: Alfred K. Wright, 110 Saffron Hill, London, E.C. 1.)

17–20 g. A direct correlation between the M.L.D. and the content of a particular toxic component is not to be expected except in the absence of other toxic substances. The L + /5 dose of toxin is the least dose of toxin which when mixed with 1/5 unit of antitoxin and injected intravenously into 17–20 g. mice, killed some but not all of the group within 48 hr.

The M.L.D. of the toxin preparations used in the present work was as follows:

Dried toxin W.E. 6	0·008 mg.
Glycerinated toxin K 178–180	0·0025 ml.
Glycerinated toxin K 188	0·0022 ml.

The L + /5 dose of toxin K 178–180 was 0·025 ml.

Antitoxins. The ultimate reference antitoxin was the British Standard Gas Gangrene Antitoxin (*Perfringens*) containing 20 units/ml. This is an α-antitoxin standard. The working reference antitoxin was the Elstree Standard No. 1 containing 95 units/ml.

Biochemical action of *Cl. welchii* toxin

General methods. Total P was determined by preliminary ashing with perhydrol and H_2SO_4 and colorimetric estimation of the inorganic phosphate by a modification of Briggs' method [Martland & Robison, 1926]; the colour comparison was made with a Bausch and Lomb colorimeter against suitable standards ranging from 0·005 to 0·1 mg. P, with an accuracy of approximately ± 0·0005 mg. P. Microestimations of N were made by the Kjeldahl method and of Ca by precipitation as oxalate and permanganate titration. The action of the toxins on egg-yolk was followed by the method devised by van Heyningen. The diluted toxin (approximately 1–3 M.L.D.) is allowed to act on the egg-yolk under standard conditions, the reaction stopped by addition of excess antitoxin and the turbidity measured in a colorimeter against that developed simultaneously by a known amount of toxin in the same conditions. We have used as a standard the glycerinated toxin K 178–180 and arbitrarily assigned to it a value of 200 egg-yolk units per ml.

Action of Cl. welchii *toxin on egg-yolk*

Decomposition of phospholipin. A sterile emulsion of one egg-yolk was prepared in 250 ml. of 0·9% NaCl. A sample of this emulsion was extracted three times with an equal volume of ether at room temperature, and the ethereal and aqueous solutions analysed. A second sample was treated with 2 mg. dry *Cl. welchii* toxin (W.E. 6) in presence of 0·01 *M* $CaCl_2$ for 4 hr. at 37° and analysed similarly. Table 2 shows that by the action of the toxin the original

Table 2. *Analysis of egg-yolk emulsion (a) before and (b) after the action of* Cl. welchii *toxin*

Values in mg./100 ml.

		Ether-soluble fraction			Protein		Acid-soluble fraction	
Total N	Total P	Wt.	N	P	N	P	N	P
(*a*) 43	12·0	850	4·0	5·3	34	5·5	5·0	1·2
(*b*) 43	12·0	750	0·2	0·2	31	0·5	9·0	11·5

lipin P was converted into an acid-soluble form; most of the P originally present in the protein component also became acid-soluble, although the protein N was only slightly decreased.

Comparison of rate of formation of acid-soluble P and development of turbidity. A number of tests were set up in duplicate in two series A and B as follows: 3·0 ml. diluted toxin (1/300) in 0·002 *M* $CaCl_2$; 1 ml. borate or acetate buffer;

1 ml. yolk emulsion, containing 0·12 mg. total P and 0·05 mg. lipin P per ml. After incubation at 37°, 5 ml. of a saline dilution of *Cl. welchii* antitoxic serum (10 units antitoxin) were added to the tube in series A to stop the reaction, and the relative turbidity estimated in the colorimeter against an arbitrary standard. 2 ml. 10 % trichloroacetic acid were added to the tubes in series B, and the amount of total P in the filtrate estimated; the amount of P liberated was calculated by subtraction from this figure of the original acid-soluble P present. Table 3, in which the relative degrees of hydrolysis and of turbidity are referred

Table 3. *Comparison of the rate of hydrolysis of P with development of turbidity in egg-yolk emulsion by* Cl. welchii *toxin*

		20 min.			35 min.		
			Relative degree of			Relative degree of	
Buffer	*p*H	mg. P hydrolysed	Hydrolysis	Turbidity	mg. P hydrolysed	Hydrolysis	Turbidity
Borate	7·1	0·031	1·0	1·0	0·041	1·3	1·4
Acetate	5·9	0·027	0·8	0·7	0·036	1·2	1·1
Borate	9·3	0·019	0·6	0·3	0·028	0·9	0·6

to the reaction at *p*H 7·1 for 20 min. as unity, shows that at *p*H 7·1 and 5·9 the rate of hydrolysis and the development of turbidity are almost parallel, while at *p*H 9·3 the hydrolysis is considerably faster than the increase in turbidity. It seems probable that the hydrolysis of the phospholipin is the primary action and that the aggregation of fat globules to which the turbidity is due is dependent on this hydrolysis but influenced by the physical conditions of the mixture.

Hydrolysis of egg lecithin by Cl. welchii *toxin*

Preparation of 'lecithin'. Three egg-yolks were washed, emulsified in 50 ml. 0·9 % NaCl and extracted 4–6 times with an equal volume of ether by shaking at room temperature and centrifuging. The ethereal solution was evaporated to 100 ml. and treated with 2 volumes of acetone; the precipitate was washed with acetone and taken up in 50 ml. ether; after centrifuging, the clear solution was again treated with acetone and the precipitate washed with acetone and dried in a vacuum desiccator over H_2SO_4. The product (1·5–2·0 g.) contained generally 3·7–3·9 % P and 1·9–2·1 % N and was completely soluble in ether; when freshly prepared it gave a stable emulsion with water which showed no change on standing at 0° for periods up to a week.

Course of hydrolysis. A typical hydrolysis was carried out as follows: 830 mg. lecithin (31 mg. P), emulsified in H_2O, 1·5 ml. 0·1 *M* $CaCl_2$ and 5 mg. dry *Cl. welchii* toxin (W.E. 6), were mixed in a total volume of 15 ml., adjusted to *p*H 7·4 with 0·1 *N* NaOH and placed in a thermostat at 37°; a further 2 mg. toxin were added after 5 hr. and the mixture was titrated back to *p*H 7·4 with 0·1 *N* NaOH at intervals until no further liberation of acid took place, by which time a layer of fat had risen to the surface. The fatty material was then removed from the aqueous portion by shaking 4 times with ether and separating the ethereal solution by centrifuging.

Identification of products. (*a*) *Phosphocholine.* Analysis of the aqueous solution showed that it contained practically all the original lipin P in an organic form, and an equivalent amount of N, but no free choline. A sample of the solution was treated at *p*H 8·5 with a bone phosphatase preparation free from diesterase; the organic P was rapidly and completely hydrolysed to inorganic phosphate, indicating that the compound was a monophosphoric ester, pre-

sumably of choline. The compound was slowly hydrolysed by N HCl at 100°, $k = 0{\cdot}15 \times 10^{-3}$; the hydrolysis curve agreed with that found by Plimmer & Burch [1937] for phosphocholine (phosphorylcholine), and the Florence test for choline was now positive in high dilutions.

The solution was evaporated to dryness and the organic P compound extracted from the salts with absolute alcohol. The alcoholic solution was evaporated to 16 ml. and 4 ml. 0·5 M $CaCl_2$ added. 186 mg. crystals were obtained in the first two crops, containing 70 % of the original lipin P; the composition of these crystals, analysed without recrystallization, agreed with that of the crystalline Ca compound of phosphocholine, $C_5H_{13}O_4NPClCa, 4H_2O$ [Plimmer & Burch, 1937]:

	% P	% N	% Cl	% Ca	% H_2O
Crop 1	9·75	4·1	10·2	12·2	18·5
Crop 2	8·7	3·5	11·8	10·7	22·3
Required	9·46	4·25	10·75	12·16	21·86

Without careful recrystallization this compound is apt to contain traces either of carbonate or of the acid salt.

(*b*) *Neutral fat.* The ethereal solution was evaporated to dryness and taken up in acetone leaving a small amount of acetone-insoluble material. The acetone-soluble material (574 mg.) was a neutral fat (acid value 1·1) with a saponification value of 172 (calc. for stearyloleylglyceride 176). The fatty acids were extracted from the saponified and acidified mixture with ether, and separated into saturated and unsaturated acids on the basis of the solubility of the Pb salts in 95 % alcohol. The nature of the fatty acids was not further determined, since it depends solely on the constitution of the original material. The aqueous portion of the saponification mixture was freed from H_2SO_4 with baryta and evaporated to dryness. The residue was extracted with an ether-alcohol (1 : 3) mixture which gave on evaporation 74 mg. syrup. The content of glycerol was estimated by refluxing with 2 ml. acetic anhydride and 0·5 g. Na acetate, and saponification of the resulting triacetin.

A balance sheet for two hydrolyses of this kind is shown in Table 4. The yields obtained are calculated on the basis that the whole of the lipin P was

Table 4. *Products of the hydrolysis of lecithin by* Cl. welchii *toxin*

	Exp. 1		Exp. 2	
mg. Lecithin	550		830	
mg. P	21·0		31·0	
Hydrolysis products:	Found	% of calc.	Found	% of calc.
(*a*) m.-mol. acid	0·73	108	0·92	93
(*b*) Water-soluble product:	mg.		mg.	
P	19·5	93	29	94
N	9·3	96	13	93
Phosphocholine	187	74	226	69
(*c*) Ether-soluble product:				
Neutral fat	350	82	574	92
(*d*) Saponification products of (*c*):				
Glycerol	46·4	74	68	74
Saturated fatty acids	131	68	233	82
Unsaturated fatty acids	148	77	294	104

present as a stearyloleyl derivative of glycerophosphocholine, since the substitution of other fatty acids makes no significant difference to the results. It will be seen that the data agree substantially with the decomposition of more

than 90 % of the original phospholipin, with liberation of one acidic group, into phosphocholine and a diglyceride containing one molecule each of glycerol, a saturated and an unsaturated fatty acid. The phospholipin used was not specifically free from kephalin or sphingomyelin. The P : N ratio and the yield of crystalline phosphocholine indicate that at least 70 % of the P was present as lecithin, but the possibility that the toxin decomposes other phospholipins and in particular kephalin is not excluded.

Characteristics of Cl. welchii *lecithinase*

The enzymic activity under various conditions was estimated by the rate of formation of acid-soluble P from an aqueous emulsion of lecithin by the glycerinated toxins K 178–180 and K 188, the general technique being as follows: 0·1 ml. toxin (approximately 40 M.L.D.) was diluted with water to 30·0 ml. and transferred to a stoppered 50 ml. conical flask, samples being removed with an Ostwald pipette. Uniform thin-walled test tubes were used and the test mixtures were made up with water to a total volume of 6 ml. including 1 ml. Palitzsch's borate buffer, *p*H 7·1; the tubes were placed in a thermostat at 37° and the reaction stopped by the addition of 1 ml. 20 % trichloroacetic acid. The solution was filtered from the flocculated lecithin within 15 min. through a No. 30 or 44 Whatman paper, and re-filtered if necessary; it is essential that the filtrate should be crystal clear to exclude the presence of unchanged lecithin. Total phosphate was determined in aliquots (usually 3 ml.) of the filtrate, the amounts being of the order 0·005–0·15 mg. P. It was found that no appreciable hydrolysis of lecithin took place in absence of toxin either at *p*H 7·1 at 37° or in trichloroacetic acid at room temperature for ½ hr. Blank values for the acid-soluble P contained in the toxin and lecithin were obtained by adding the trichloroacetic acid before the addition of lecithin.

Enzyme specificity. None of the toxins examined hydrolysed diphenyl-, monophenyl- or β-glycero-phosphate or nucleic acid; the enzyme is therefore a lecithinase, as distinct from a phosphodiesterase, although the linkage is of a diester type.

Stability. The concentrated glycerinated toxin preparations have remained unchanged in activity (M.L.D. and L+/5 value) for over 9 months when kept at 0°. Dilute solutions in saline used for these enzyme studies, containing 5 M.L.D./ml. or less, are stable at 18° for a few hours if kept with a small surface exposed to the air. The diluted enzyme is however very rapidly inactivated by the exposure of a shallow layer, by shaking or by bubbling air through it. This inactivation is very probably due to surface denaturation and not oxidation, since the enzyme was unaffected by treatment with a 1 : 100 dilution of '30 vol.' H_2O_2, while bubbling with N_2 which had been passed through alkaline $Na_2S_2O_4$ inactivated it.

In a bubbling experiment air was drawn uniformly through a dilute toxin solution so that good foaming occurred, and samples were withdrawn at intervals and tested for activity by the egg-yolk reaction and on mice. The mice were injected intravenously with a volume of diluted toxin calculated from the results of the egg-yolk reaction to contain just above and just below one M.L.D. The mouse deaths agreed reasonably well, n view of the few mice and levels of dose employed, with the expected toxicity The percentage inactivation of the toxin shown by the animal test (col. 4, Table 5) was calculated from these figures.

A similar more accurate test showed 35 ± 5 % inactivation of the lecithinase and 37 ± 10 % inactivation of the toxin as measured in mice. Because of the very great ease of surface denaturation it was essential to adopt uniform methods

Table 5. *Inactivation of lecithinase and toxin by bubbling*

Time of bubbling min.	M.L.D./ml. by egg-yolk reaction	Percentage of initial activity by	
		Egg-yolk reaction	Mouse test
0	4·5	100	(100)
15	3·8	84	100
30	2·8	62	—
40	2·7	60	66
60	1·8	45	<66 >25
90	0·8	17	—

for the dilution of the toxin and the addition of reagents, and to mix carefully without shaking, in order to get reproducible results with dilute solutions of the toxin.

The enzyme is comparatively heat-stable, 45 % of the activity remaining after heating in borate buffer *p*H 7·6 for 10 min. at 100° in a sealed ampoule; it is more rapidly inactivated in acid than in alkaline solution at this temperature (Table 6). It is possible that a large part of this apparent heat-inactivation is due to surface denaturation during the heating process.

Table 6. *Heat stability of lecithinase of toxin K 188 at 100°*

The toxin was heated at a dilution of 1 in 80 in the medium stated.

Medium	A water	B borate	C borate	D acetate
*p*H	*ca.* 7·0	7·6	7·1	6·0
	Percentage activity			
Time min.				
10	8	45	35	14
30	0	12	8	5

p*H range*. The enzyme is active over a wide *p*H range, with a broad optimum, 7·0–7·6, in borate buffer; the activity at *p*H 9·3 and 5·2 in borate and acetate buffers respectively was 64 and 66 % of the maximum. Acetate and veronal buffers have a slight and phosphate buffer a marked inhibiting effect compared with borate at the same *p*H.

Substrate concentration and activation by Ca^{++} ions. The activity of the enzyme is greatly affected by the presence of Ca^{++} ions, the activation being most marked with very small amounts of toxin or with low concentration of lecithin. The enzyme is also activated by low concentrations of Mg salts but inhibited in concentrations of 0·01 *M* or over. Table 7 shows the effect of variation in the

Table 7. *Effect of substrate concentration on the hydrolysis in 15 min. at 37° by 2·0 ml. 1/300 dilution of toxin K 188*

Conc. of Ca^{++}	Concentration of lecithin (*M*)				
	0·011	0·008	0·0053	0·0027	0·0011
	mg. acid-soluble P formed				
Nil	—	0·132	0·103	0·050	0·016
0·008 *M*	0·199	0·189	0·162	0·121	0·077

substrate concentration on the rate of hydrolysis with and without the addition of Ca. For the quantity of toxin used the velocity was almost maximal in 0·01 *M*

lecithin. Half this maximal velocity was reached at a substrate concentration of $0{\cdot}002\,M$ in presence of Ca, but only at $0{\cdot}005\,M$ in its absence; that is to say, the affinity for lecithin is more than twice as great in the presence of Ca.

Table 8 shows the activating effect of Ca^{++} on the hydrolysis of $0{\cdot}0005\,M$ lecithin under the same conditions as in Table 7. A concentration as low as $1{\cdot}6 \times 10^{-4} M$ Ca^{++} had a striking effect on the activity which was vanishingly

Table 8. *Effect of Ca^{++} on rate of hydrolysis*

1/300 dilution of toxin K 178–180 ml.	Conc. lecithin *M*	Conc. of added Ca^{++}, $\times 10^{-3} M$: Nil	0·16	0·32	0·8	1·6	2·4	3·2	8·0	16
		mg. acid-soluble P formed								
2·0	0·0005	0·005	0·026	0·033	0·035	0·040	0·045	—	—	—
3·0	0·0053	0·095	—	—	—	0·163	—	0·210	0·214	0·214
0·5	0·0053	0·011	—	—	—	0·022	—	0·027	0·036	0·037

small in the absence of added Ca^{++}. With higher substrate concentrations, in which an appreciable hydrolysis took place without the addition of Ca^{++}, the concentration of Ca^{++} needed to reach the maximum velocity varied with the amount of enzyme, being lowest with the highest amounts of enzyme (Table 8). It is possible that the partial activity of the enzyme without addition of Ca^{++} in high substrate concentrations was due to traces of Ca or Mg in the lecithin; analysis of one sample of lecithin showed that it contained less than 0·2 % Ca, but a content of 0·05 % of Ca or Mg would have an appreciable effect. The activity was practically maximal in $0{\cdot}01\,M$ $CaCl_2$ for amounts of enzyme between 0·5 and 3·0 M.L.D.

Examination of the hydrolysis curves (Table 9) shows that with low concentrations of toxin there is a marked induction period in the absence of Ca^{++} before any measurable hydrolysis takes place; this is reduced or abolished by

Table 9. *Effect of Ca^{++} on induction period of hydrolysis*

mg. P hydrolysed in total volume 25 ml. containing 1·9 mg. lecithin P.

ml. of 1/300 dilution of toxin K 178–180	5·0		1·0		0·5	
Conc. Ca^{++} (*M*)	Nil	0·008	Nil	0·008	Nil	0·008
Time (hr.)						
0·5	0·19	0·48	0·05	0·12	0·03	0·06
1·0	0·44	0·82	0·05	0·22	0·03	0·13
1·5	0·58	1·10	0·05	0·29	0·04	0·18
2·0	0·61	1·23	0·08	0·37	0·06	0·22
5·0	1·02	1·39	0·23	0·65	0·11	0·34
24	1·32	—	0·34	—	0·17	—

the addition of Ca^{++}. Since the velocity of the hydrolysis is also influenced by Ca^{++} and in any case falls off rapidly after a few hours, presumably owing to the gradual inactivation of the enzyme, the presence or absence of Ca^{++} has a profound effect on the total amount of hydrolysis by a given amount of toxin. The importance of this fact in estimations of the haemolytic activity of the toxin is discussed later.

Enzyme concentration. As a result of the foregoing experiments the following conditions were adopted for estimating the lecithinase activity: 0·2 ml. $0{\cdot}3\,M$ $CaCl_2$; 1 ml. borate buffer *p*H 7·1; toxin, any other reagent and H_2O to 5·0 ml.; 1 ml. 2·5 % aqueous lecithin. The reaction was stopped after 15 min. at 37° by the

addition of 1 ml. 20% trichloroacetic acid, and the total phosphate in the filtrate estimated as before, with the appropriate blanks. Table 10 shows that in these conditions the hydrolysis proceeds with almost linear velocity for 15 min. with the highest amount of toxin used, but falls off rapidly when more

Table 10. *Hydrolysis rate for 3·0 ml. of a 1/300 dilution of toxin K 178–180*

Total lecithin P = 1·0 mg.

Time min.	mg. P hydrolysed	Velocity constant $k \times 10^3$	% hydrolysis
10	0·140	7·4	15·7
15	0·195	7·0	21·4
20	0·237	6·6	26·3
30	0·309	6·1	34·2

Table 11. *Variation of hydrolysis with enzyme concentration*

1/300 dilution of toxin K 178–180 ml.	mg. P hydrolysed				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean
3·0	0·198	0·213	0·207	0·180	0·198
2·0	0·140	0·148	0·147	0·125	0·140
1·0	0·073	0·076	0·065	0·069	0·071
0·5	0·031	0·036	—	0·033	0·033
0·2	0·008	—	0·009	0·016	—

than 20% of the substrate has been decomposed. Table 11 shows that the hydrolysis is proportional to the enzyme concentration when the actual hydrolysis is of the order 0·04–0·20 mg. P, and that the values for a given toxin are reproducible with an accuracy of approximately ±6%.

A lecithinase unit was arbitrarily defined as that quantity of enzyme which under the conditions prescribed above produced 0·1 mg. acid-soluble P from lecithin in 15 min. at 37°. The relationship between the minimum lethal dose, the egg-yolk unitage and the lecithinase activity of toxins K 178–180 and K 188 is shown in Table 12.

Table 12. *Titres of* Cl. welchii *toxins*

Toxin	Units/ml.		
	M.L.D.	Egg-yolk	Lecithinase
K 178–180	400	200	210
K 188	450	266	290
Ratio toxin $\frac{\text{K 178–180}}{\text{K 188}}$	0·89	0·75	0·73

Effect on lecithinase activity of other factors in Cl. welchii *toxins*

Toxoid. The toxoid prepared for the routine immunization of horses had no appreciable lecithinase activity in quantities equivalent to 15–20 lecithinase units of toxin. The activity of 2 lecithinase units of toxin was not significantly altered by the presence of an excess of toxoid.

β- and δ-toxins. We are indebted to Dr Oakley for a specimen of dry *Cl. welchii* type C toxin (NX 17) containing only traces of α-toxin; 1 ml. of a 1% solution of this toxin contained β-toxin equivalent to 55 β-units and δ-toxin equivalent to 1·85 δ-units. We found that a 1% solution contained 1·8 lecithinase units per ml.; a mixture of 0·5 ml. of this solution and 1·0 ml. of type A toxin hydrolysed 0·145 mg. P under standard conditions, the sum of the separate activities being 0·150 mg. P. There was therefore no significant inhibition of the lecithinase activity by the β-toxin and δ-toxin present.

Inhibition by Cl. welchii *antitoxic sera*

Measurement of inhibition. The inhibition of the lecithinase activity of the toxin by specific antitoxic sera was measured as follows: 2·0 ml. diluted toxin (1–2 units lecithinase) were allowed to react with a saline dilution of the serum in a total volume of 3·8 ml. for 15 min. at room temperature; 0·2 ml. 0·3 *M* $CaCl_2$, 1 ml. borate buffer *p*H 7·1 and 1 ml. 2·5 % lecithin were added and the amount of hydrolysis in 15 min. at 37° estimated in the usual way. A simultaneous estimation of the hydrolysis by the same quantity of toxin in conditions identical except for the omission of serum was always carried out. Table 13

Table 13. *Inhibition of varying amounts of lecithinase by antitoxin*

15 min. hydrolysis at 37°.

Toxin (1/300 dilution)	ml.	mg. P hydrolysed without antitoxin	Decrease in P hydrolysed with antitoxin: 0·01 unit mg.	%	0·03 unit mg.	%	0·05 unit mg.	%	0·10 unit mg.	%
K 178–180	3·0	0·239	0·018	7	0·068	29	0·133	56	0·205	86
,,	2·0	0·148	0·012	8	0·039	26	0·092	62	0·129	87
,,	1·0	0·076	0·011	14	0·025	33	0·046	60	0·068	89
K 188	2·0	0·205	0·021	10	0·077	38	0·134	65	0·192	94

shows that the combination of the enzyme with the anti-lecithinase does not take place by simple multiple proportions, the absolute decrease in the hydrolysis with a given amount of serum decreasing with decreasing concentration of enzyme. It will be seen that in this experiment the percentage inhibition of the activity by a given amount of serum is relatively almost independent of the enzyme concentration in the limited range tested, considering the experimental variation in tests made at different times. Slight variations in the absolute amount of hydrolysis due to inactivation of the enzyme or variation in the time or temperature of the reaction therefore do not appreciably affect the results if these are calculated as percentages of the uninhibited hydrolysis carried out simultaneously.

Titration of antisera. The experiments in Table 13 were carried out with the Elstree No. 1 standard antitoxin (*Cl. welchii* type A) containing 95 international units/ml. This serum has been taken as a standard and arbitrarily defined as containing 95 anti-lecithinase units/ml. Table 14 shows the percentage inhibition

Table 14. *Inhibition of lecithinase by antitoxin*

Antitoxin units	% inhibition	Mean	Antitoxin units	% inhibition	Mean
0·005	4, 8, 4	5	0·06	81, 74, 77, 77	77
0·01	10, 17, 11	13	0·07	85, 76, 84, 84	82
0·02	32, 24, 29, 29, 25	28	0·08	92, 82, 85, 86	86
0·03	46, 35, 44, 37, 38	40	0·09	96, 88, 94, 91	92
0·04	49, 52, 51, 48	50	0·10	94, 96	95
0·05	70, 72, 68, 65, 67	68	—	—	—

of the hydrolysis by 1·9 lecithinase units of toxin K 188 produced by amounts of this serum containing from 0·01 to 0·1 antitoxin units. From these values a curve was constructed, plotting the percentage inhibition against the number of anti-lecithinase units. The concentration of anti-lecithinase in other sera was found with an accuracy of approximately ± 10 % by reference to this curve, after

estimation of the percentage inhibition of this toxin produced by 2 or 3 different quantities of the unknown serum in the conditions prescribed above. It was usually necessary to make at least 1/1000 dilution of the serum, so that the amount of phosphate added in the serum was negligible in these cases. The lecithinase activity was not significantly altered by the addition of normal or diphtheria antitoxic horse sera in quantities 10 to 50 times greater than those of the *Cl. welchii* antitoxic sera used.

Table 15 shows the antitoxic units per ml. of a number of *Cl. welchii* antitoxic horse sera determined by different methods. It was found that the titres of the type A sera determined by the anti-lecithinase method ran parallel with the

Table 15. *Titres of* Cl. welchii *antitoxic horse sera*

	Antitoxin units/ml.		
Serum	L+ method*	Egg yolk	Anti-lecithinase
Type A antisera:			
Elstree Standard No. 1	95	95	95
'Aden'	550	500	600
'Berbera'	350	340	330
'Crete'	—	960	1000
'Eritrea'	350	240	420
'Massawa'	200	170	260
'Sollum'	530	400	440
Type C antisera	α-Antitoxin		
R 7157	85	—	80
R 7164	48	—	62

* The animal test values for the type A sera are taken from routine testing results which were done at 100 unit intervals. The measurements were not designed to test accurately the correspondence between the animal and *in vitro* tests.

egg-yolk titres; the latter have already been shown by Nagler [1939] and by Macfarlane *et al.* [1941] to run parallel with those obtained by the animal protection (L+) method. There seems little doubt that the two *in vitro* tests measure the α-antitoxin and for convenience the unitage of the standard serum Elstree No. 1 by the egg-yolk and lecithinase titrations has been arbitrarily fixed at the same numerical value as that found by the L+ method, i.e. 95 units/ml.

The sera R. 7157 and R. 7164, for which we are indebted to Dr Oakley, are type C sera with the following antitoxin units/ml.; α, 85 and 48; β, 3000 and 1000; δ, 0·5 and 80 respectively. Table 15 shows that the anti-lecithinase titres found by us agree well with the α-antitoxin titres obtained by a different method in a different laboratory. It seems probable that the anti-lecithinase titre is not affected by excess of β-antitoxin, and only slightly if at all by excess of δ-antitoxin.

Effect of Ca^{++} on the inhibition of lecithinase by antitoxic sera. When the lecithinase activity is measured in the presence of Ca^{++}, a larger amount of antitoxin is required for the neutralization (100 % inhibition) of a given amount of toxin than in absence of Ca^{++} (Table 16). Taking the formation of 0·005 mg. acid-soluble P in 15 min. as the lowest limit of appreciable hydrolysis (analogous with the estimation of a minimum haemolytic dose) the decrease in the hydrolysis to this amount in this experiment required the addition of only 0·1 unit of antitoxin in absence of Ca^{++} but of 0·19 unit in the presence of Ca^{++}. The regulation of the Ca^{++} concentration is therefore of great importance in any *in vitro* tests of the activity of the α-toxin or of α-antitoxins, including the haemolytic titre. Table 17 shows the effect of Ca^{++} on the minimum haemolytic

Table 16. *Effect of Ca^{++} on inhibition by antitoxin*

mg. P hydrolysed by 3·0 ml. toxin K 178–180 (1/300) in 15 min.

Conc. Ca^{++}	Antitoxin units								
M	Nil	0·01	0·02	0·03	0·04	0·05	0·10	0·15	0·20
Nil	0·110	0·096	0·081	0·065	0·052	0·030	0·001	Nil	Nil
0·01	0·239	0·221	0·197	0·171	0·136	0·106	0·034	0·007	0·005

Table 17. *Effect of Ca^{++} on haemolytic titre of toxin K 178–180*

Diluent	ml. toxin	
	M.H.D.	$L_h/5$ dose
'Analytical Reagent' NaCl	0·0006	0·080
'Analytical Reagent' NaCl + *M*/100 Ca^{++}	0·0002	0·069
'Commercial Pure' NaCl	0·0002	—

dose and on the $L_h/5$ dose of toxin K 178–180. It will be seen that the substitution of C.P. for A.R. sodium chloride in the saline used for dilution is sufficient to reduce the M.H.D. from 0·0006 to 0·0002 ml. presumably through addition of Ca^{++} or Mg^{++}, and that the $L_h/5$ dose of toxin was reduced from 0·08 to 0·07 ml. in presence of 0·01 *M* $CaCl_2$. The values in these particular tests do not necessarily give the full haemolytic titre, since no attempt was made to secure optimal conditions for other haemolysins present, but they confirm that one haemolysin present, presumably the α-toxin, is affected by Ca^{++}.

It was shown by Neill [1926] that a reversibly reducible haemolysin was present in *Cl. welchii* culture filtrates of which only the reduced form was haemolytic. It seems probable that the considerable variation in the M.H.D. which has been found on occasion in different laboratories for the same toxin [cf. Ipsen *et al.* 1939] may be due in part to variation in the Ca^{++} concentration and in part to the degree of oxidation of the reversibly reducible haemolysin in the different tests.

Combining power of toxin with antitoxin. An attempt was made to assess the combining power of the toxin with antitoxin, as in the estimation of the L+ dose of toxin, by finding the smallest amount of toxin which, after combination with 0·2 unit of antitoxin, still hydrolysed lecithin at a rate approximately equivalent to 1·0 M.L.D. of toxin; this rate was taken as a hydrolysis of 0·05 mg. P in 15 min. at 37°. The estimation of this dose is materially affected by the dilution at which the enzyme action is allowed to proceed (Table 18). The toxin

Table 18. *Lecithinase activity in presence of 0·2 unit antitoxin*

	ml. toxin K 188				
	0·032	0·028	0·024	0·020	0·016
Volume during hydrolysis	mg. P hydrolysed in 15 min.				
A 4 ml.	0·039	0·021	0·008	0·007	0·004
B 20 ml.	0·065	0·032	0·022	0·007	0·007

and antitoxin were allowed to combine for 15 min. at room temperature in a total volume of 2·4 ml.; the hydrolysis was then carried out in a final volume of 4 ml. in series A and 20 ml. in series B, the volumes of $CaCl_2$, borate buffer and lecithin being adjusted to give the same concentrations in both series. It will be seen that the amount of toxin required is greater in the smaller volume, the actual difference of approximately 0·003 ml. of toxin representing in this

case about $1\frac{1}{2}$ lethal doses. It seems probable that the increased lecithinase activity on dilution is due to dissociation of the toxin-antitoxin complex; it remains to be seen whether other differences in sera or serum fractions can be detected by the reaction.

Inhibition by non-specific agents

Calcium precipitants. The enzymic activity is reduced by the presence of fluoride, citrate and phosphate. The concentration of these salts necessary to produce complete inhibition is dependent on the Ca^{++} concentration in the test mixtures; it appears that the inhibition is due to the reduction of Ca^{++} below a certain threshold value.

Antiseptics etc. The effects of a number of other substances are shown in Table 19; the substances were carefully mixed with the diluted toxin and allowed to stand 10 min. at room temperature before the addition of lecithin in the usual test.

Table 19. *Effect of various substances on lecithinase activity*

Substance	Conc. %	Enzyme activity %	Substance	Conc. %	Enzyme activity %
m-Cresol	0·25	150	Eserine	0·15	95
,,	0·025	130	Prostigmine	0·04	100
Euflavine	0·1	80	Urea	2·5	95
Proflavine	0·1	78	Na dodecyl sulphate	0·25	0
Sulphanilamide	0·2	97	,,	0·03	5
,,	0·025	100	Na oleate	0·08	50
Sulphapyridine	0·05	100	Na taurocholate	0·08	90
Glycerol	0·1	100	Digitonin	0·25	60

Sulphanilamide and sulphapyridine in final concentration of 0·2 and 0·05 % respectively had no appreciable effect, while only a slight inhibition was produced by 0·1 % of proflavine or euflavine, and the activity was actually increased in presence of *m*-cresol. Prostigmine and physostigmine (eserine) which inhibit the action of choline esterase in high dilution had no effect on the lecithinase in a final concentration of 0·04 %. The most effective inhibiting agent was Na dodecyl sulphate with which inhibitions of 80–100 % were obtained in concentrations as low as 0·025 %.

Discussion

The enzymic decomposition of lecithin into phosphocholine and a diglyceride has not previously been well established. According to Udagowa [1935] an enzyme of this type is present in takadiastase but the reaction products were not isolated. In the present case the absence of phosphomono- and di-esterase and lipases from the toxin enabled the quantitative isolation of the reaction products to be made without difficulty. The lecithinase present in snake venoms, which decomposes lecithin into an unsaturated fatty acid and lysolecithin, was designated by Belfanti *et al.* [1936] as 'Lecithase A'; that present in tissues, rice bran and other sources, which splits off both fatty acids from lecithin, was classified by these authors as 'Lecithase B'. The enzyme in *Cl. welchii* toxins, which decomposes lecithin with formation of phosphocholine and a diglyceride may, therefore, be termed a lecithinase C. Like the other lecithinases and the simple lipases, this enzyme is activated by Ca^{++} ions. Apart from its distinction from phosphodiesterase, its specificity has not yet been investigated.

From the pathological point of view the chief interest of the lecithinase in *Cl. welchii* toxins is its probable identity with the α-toxin, the lethal, haemolytic

and necrotic factor of this toxin. It has been shown by Nagler [1939] and Macfarlane *et al.* [1941] that the activity of the toxin in producing an opalescence in human serum or egg-yolk is a fair measure of the content of α-toxin. We have found that the development of opalescence is accompanied, and in certain circumstances preceded, by the decomposition of the lecithin present, and that like the former phenomenon this decomposition is activated by Ca^{++} and inhibited by citrate, fluoride and phosphate. The lecithinase of the toxin is inhibited by specific antitoxic sera, the inhibitory action running parallel in various type A antisera with the protective power of the serum *in vivo*, while in two type C sera the antilecithinase activity was in good agreement with an independent assay of the α-antitoxin content. The evidence is, therefore, strongly suggestive of the identity of this lecithinase with the α-toxin, though the ultimate proof of this is dependent on the isolation of a homogeneous substance with the requisite biochemical and pathogenic properties. It is, of course, inherently probable that this lecithinase would confer haemolytic and necrotic powers on the toxin, since it would have a disintegrating effect on any cell membrane or intracellular lipoprotein complex whose integrity depended on the presence of lecithin. Moreover, the lecithinase activity of the toxins examined is so high that it may well account for the pathological effect; an amount roughly equivalent to one lethal dose for a mouse could at its maximum velocity hydrolyse the whole of the blood lecithin of the animal in 2 or 3 hr. It seems possible that the inhibitory action of this toxin on the aerobic oxidation of succinate observed by Wooldridge & Higginbottom [1938] may have been due to a disintegration of cell structure leading to a disorientation of the various enzyme systems concerned rather than to a specific inhibition of any one enzyme.

Though the parallelism between the lethal dosage, the egg-yolk unitage and the lecithinase activity in the type A toxins examined was sufficiently close to indicate that the latter activity corresponded with the predominant lethal toxin, it was not quite so close as would be expected if the lecithinase were the sole agent in the biological effect. There are, however, two other factors at least which must be taken into account, hyaluronidase [McClean & Hale, 1941] and the second haemolytic factor, θ-toxin [see Macfarlane *et al.* 1941]. The contributions of all these various factors to the biological effect can hardly be gauged until they are obtained free from each other. Nevertheless the lecithinase activity of type A toxins is a guide to their biological potency which can at least effect a considerable economy in animal tests.

The regulation of the calcium concentration is of great importance both in the estimation of lecithinase and in that of the haemolytic titre of the toxin. It has been shown in this paper that the effect of Ca^{++} is to increase the affinity of the enzyme for lecithin and that the effect on the amount of lecithin hydrolysed is very marked when the concentrations of toxin and lecithin are low, as is the case in haemolytic tests. If, as is probable, the lecithinase of *Cl. welchii* toxins is haemolytic the mechanism of haemolysis is in interesting contrast to that of the lecithinase of snake venom. In the latter case, the phosphoric acid group of the decomposed lecithin is still present as a lipin, lysolecithin, which is in itself a highly haemolytic substance. The decomposition of lecithin by *Cl. welchii* toxin constitutes a more drastic change in physical properties, since the phosphoric acid group is now present as a water-soluble compound, but the products of the decomposition are innocuous. The pharmacological action of phosphocholine has been shown [Beznák & Chain, 1937] to be surprisingly weak, less than that of choline, and it seems improbable that its formation has any secondary consequences in the pathology of *Cl. welchii* infections.

Most of the bactericidal and other drugs examined had little effect in concentrations of less than 1 % on the lecithinase activity of the toxin. The failure to obtain any inhibition of the lecithinase by sulphanilamide may be specially mentioned; this is in agreement with the findings of Osgood & Powell [1938] but contrary to those of Carpenter & Barbour [1939] who found that this drug had a protective effect in a concentration of one in a million. The most effective inhibitory agent found was Na dodecyl sulphate and in view of the ease with which the toxin is inactivated by surface denaturation, it will be of interest to examine the properties of the toxin by surface film techniques.

The estimation of the lecithinase activity of a toxin has the advantage that the results can be expressed in absolute units independent of any particular toxin, but since it involves a microestimation of total P it is more laborious than the measurement of relative turbidities necessary in the egg-yolk technique devised by van Heyningen. The latter is on this account preferable as a rapid routine method of the assay of this activity in toxic culture filtrates by direct comparison with a toxin of known activity. The absolute amount of turbidity developed by a stable toxin however was found to vary considerably with different emulsions of egg-yolk and from day to day with the same egg-yolk emulsion. This difficulty was doubtless due in part to variation in the substrate concentration and could be largely overcome by diluting the emulsions of egg-yolk to a constant content of phospholipin and adding the optimum amount of Ca^{++}. The emulsions however tended to become opalescent on standing at 0° even if sterile, and this progressive change appeared particularly to affect the antitoxin titrations, so that it was necessary to compare the inhibitory effect of the unknown sera with that of a standard antitoxin in every separate test. The titration of antitoxic sera by estimation of the anti-lecithinase activity for routine purposes is not numerically more accurate than the egg-yolk test, since the error due to variations in enzymic activity in individual tests in both cases is greater than that of the estimation of P or of turbidity; it is preferable in that it is based on a specific reaction with a purified substrate, and should be a convenient method for investigation of the toxin-antitoxin reaction.

Summary

1. *Cl. welchii* (type A) toxin contains a lecithinase (optimum *p*H 7·0–7·6) which decomposes lecithin into phosphocholine and a diglyceride.

2. This lecithinase is probably identical with the specific α-toxin which is the lethal, haemolytic and necrotic substance predominant in type A culture filtrates.

3. The lecithinase is relatively heat-stable but is readily inactivated by surface denaturation and by sodium dodecyl sulphate. The enzyme is activated by Ca^{++} and inhibited by fluoride, citrate and phosphate, and is specifically inhibited by *Cl. welchii* (type A) antitoxic sera.

4. Methods are described for the estimation of the lecithinase activity of toxins and the corresponding anti-lecithinase activity of *Cl. welchii* antitoxic sera. The anti-lecithinase activity of *Cl. welchii* (type A) antitoxic sera runs parallel with the animal protection titre (L+) and is a fair measure of the α-antitoxin.

5. The rate and amount of hydrolysis by the lecithinase, alone and in the presence of antitoxin, are greatly affected by the concentration of Ca^{++} present. The significance of these results is discussed in relation to the estimation of the haemolytic titre of *Cl. welchii* toxins.

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