



Hydrolysis of lecithin and sphingomyelin by preparations of *Clostridium perfringens* type A culture filtrates

by George Lyman Card

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Bacteriology

Montana State University

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Abstract:

Since Macfarlane (1948) demonstrated that preparations of *C. per-fringens* alpha-toxin hydrolyzed sphingomyelin, it has been assumed, but neirer demonstrated, that one enzyme was responsible for the hydrolysis of both lecithin and sphingomyelin, The experiments reported in this paper were designed to demonstrate the presence of a sphingomyelinase, distinct from the lecithinase, in alpha-toxin preparations.

Alpha-toxin preparations were obtained by ammonium sulfate precipitation, acetone precipitation, and by dialyzing culture filtrate against glycerine. The ratio of sphingomyelinase activity to lecithinase activity was about the same for each preparation. If two enzymes were present, the relative concentrations of each enzyme remained constant when precipitated by ammonium sulfate or acetone.

After preparations of alpha-toxin had been inactivated by treatment with cysteine, heat, and antitoxin they were tested for their sphingomyelinase and lecithinase activity. In each experiment the inhibition of sphingomyelin hydrolysis corresponded to the inhibition of lecithin hydrolysis, The inhibition experiments provided no evidence for the presence of two enzymes in preparations of alpha-toxin.

The hydrolysis of sphingomyelin and egg lecithin was measured in the presence of borate buffer saturated with chloroform. The presence of chloroform increased the rate of hydrolysis of lecithin and inhibited the hydrolysis of sphingomyelin. Treatment of enzyme alone with chloroform had no effect on sphingomyelin hydrolysis. It is not known whether the effect of chloroform is on the substrate or the enzyme-substrate complex; there is no effect on enzyme alone.

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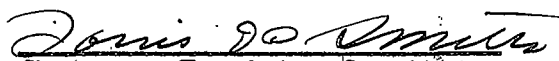
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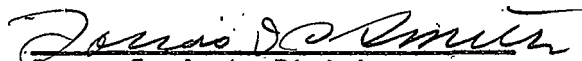
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ABSTRACT

Since Macfarlane (1948) demonstrated that preparations of C. perfringens alpha-toxin hydrolyzed sphingomyelin, it has been assumed, but never demonstrated, that one enzyme was responsible for the hydrolysis of both lecithin and sphingomyelin. The experiments reported in this paper were designed to demonstrate the presence of a sphingomyelinase, distinct from the lecithinase, in alpha-toxin preparations.

Alpha-toxin preparations were obtained by ammonium sulfate precipitation, acetone precipitation, and by dialyzing culture filtrate against glycerine. The ratio of sphingomyelinase activity to lecithinase activity was about the same for each preparation. If two enzymes were present, the relative concentrations of each enzyme remained constant when precipitated by ammonium sulfate or acetone.

After preparations of alpha-toxin had been inactivated by treatment with cysteine, heat, and antitoxin they were tested for their sphingomyelinase and lecithinase activity. In each experiment the inhibition of sphingomyelin hydrolysis corresponded to the inhibition of lecithin hydrolysis. The inhibition experiments provided no evidence for the presence of two enzymes in preparations of alpha-toxin.

The hydrolysis of sphingomyelin and egg lecithin was measured in the presence of borate buffer saturated with chloroform. The presence of chloroform increased the rate of hydrolysis of lecithin and inhibited the hydrolysis of sphingomyelin. Treatment of enzyme alone with chloroform had no effect on sphingomyelin hydrolysis. It is not known whether the effect of chloroform is on the substrate or the enzyme-substrate complex; there is no effect on enzyme alone.

INTRODUCTION

Clostridium perfringens, like the other clostridia, is essentially a saprophyte. The pathogenic properties of the organism depend on its ability to produce a variety of toxins. It is on the basis of toxin production that C. perfringens is separated into six different toxigenic types (Table I).

Table I. Toxigenic types of Clostridium perfringens.

Toxin	Toxigenic type					
	A	B	C	D	E	F
Alpha	+	+	+	+	+	+
Beta	-	+	+	-	-	+
Gamma	-	+	+	-	-	+
Delta	-	+	+	-	-	-
Epsilon	-	+	-	+	-	-
Theta	+	+	+	+	-	-
Iota	-	-	-	-	+	-
Kappa	+	+	+	+	-	-
Lambda	-	+	-	+	+	-
Mu	+	+	-	+	-	-
Nu	+	+	+	+	+	+

Clostridium perfringens type A is the most widely distributed of the pathogenic clostridia and the most important cause of human gas gangrene. As shown in Table I, C. perfringens type A produces alpha, theta, kappa, mu, and nu toxins. Alpha-toxin, which is the most important toxin produced by type A, is hemolytic, necrotic, and lethal. The first evidence suggesting that alpha-toxin might possess enzyme activity was provided by Nagler (1939). He reported that when C. perfringens was grown in a liquid medium containing human serum, first an opalescence developed in the medium and on continued

incubation a layer of fat formed on the surface of the medium. The same result was obtained by using culture filtrates; the reaction was inhibited by C. perfringens antitoxin. Macfarlane, Oakley, and Anderson (1941) showed that the opalescence in human serum was due to the activity of alpha-toxin. They found that a similar but more rapid and more sensitive reaction was obtained with egg-yolk emulsions. Macfarlane et al. concluded that free fat was released from lipoprotein complexes by the action of alpha-toxin. Either human serum or egg-yolk emulsion could be used as an indicator for toxin-antitoxin neutralization tests. The neutralization values obtained with egg-yolk tests corresponded to those obtained with hemolytic or lethal tests. They also observed that hemolysis and the serum or egg-yolk reaction were dependent on the presence of ionized calcium.

Subsequently, Macfarlane and Knight (1941) demonstrated that alpha-toxin was a lecithinase C which released phosphorylcholine and a diglyceride from an aqueous emulsion of lecithin. The enzyme retained 50 per cent of its activity after boiling for ten minutes, had an optimum pH range of 7.0-7.6, and its action was dependent on the presence of ionized calcium (optimum conc. 0.01 M Ca^{++}). The lecithinase was inhibited by the specific antitoxin and by fluoride, citrate, and phosphate. In neutralization tests with antitoxin the lethal dose and lecithinase activity were not strictly parallel although they were sufficiently close to suggest that the lecithinase corresponded to the predominant lethal factor. Other lethal factors were probably present in the crude toxin preparation, particularly theta-toxin. Although it was not definitely established, Macfarlane and Knight considered the lecithinase to be identical with alpha-toxin.

Considering the wide-spread occurrence of lecithin in tissue and in the stroma of red blood cells, the enzyme activity of the toxin was sufficient to account for its biological activity. This was the first example of a bacterial toxin which possessed enzyme activity and represented a major break through in the study of infectious diseases. For the first time the pathogenesis of an infection could be studied at the biochemical level, and the morphological and pathological changes which occurred in infected tissue could be related to the action of a specific enzyme on a specific substrate.

Lecithin was the only substrate which Macfarlane and Knight found to be hydrolyzed by the lecithinase. Glycerophosphates and nucleic acids were not attacked. Later, however, in a short note Macfarlane (1942) reported that sphingomyelin was hydrolyzed, although slowly, by C. perfringens lecithinase.

Zamecnik et al. (1947) studied the properties of the enzyme using a manometric technique. They reported that sphingomyelin was not hydrolyzed by C. perfringens lecithinase although it inhibited the hydrolysis of lecithin. They found the enzyme also inactive on 3 per cent suspensions of phosphatidylserine, ox brain cerebrosides, glycerophosphorylcholine, soybean phosphatides, and lysolecithin. Slight activity was found with phosphatidylethanolamine; however, they attributed this to the presence of a small amount of lecithin.

Macfarlane (1948) suggested that the low concentration of enzyme and the short incubation time which Zamecnik et al. used in their experiments, may have been responsible for their failure to detect hydrolysis

of sphingomyelin. Using an ether-insoluble fraction from an unreported natural material as a source of sphingomyelin, Macfarlane confirmed her earlier report. Sphingomyelin was hydrolyzed although the rate of hydrolysis was much slower than with egg lecithin. The hydrolysis of sphingomyelin, like that of lecithin, was dependent on Ca^{++} ions and was inhibited by sodium fluoride. Although the fatty acids in the hydrolysis products could not be identified, phosphorylcholine was recovered quantitatively.

Some question might be raised concerning the purity of the sphingomyelin used in Macfarlane's experiments. The preparation was supplied by Dr. O. Roxenheim and, as an ether-insoluble fraction, was considered lecithin free. However, according to Thannhauser *et al.* (1946) an ether-insoluble fraction is not necessarily free of lecithin. They reported that sphingomyelin prepared from brain, lung and spleen was contaminated with hydrolecithin (a saturated dipalmitolecithin). Previous methods of sphingomyelin preparation [including the method described by Rosenheim (1910)] yielded a product which contained 30 to 40 per cent hydrolecithin. The difficulty of separation resulted from the similar physical properties of the two materials, especially the fact that both were insoluble in ether.

Nevertheless, the presence of up to 40 per cent hydrolecithin could not account for Macfarlane's observation that 60 to 90 per cent of the organic phosphorus was converted to an acid soluble form. If both hydrolecithin and sphingomyelin were present in the preparation then apparently both were hydrolyzed.

Based on the slow rate of hemolysis of sheep red blood cells compared to human red cells, Macfarlane (1950) suggested that sheep red cells

probably contained sphingomyelin rather than lecithin in the cell membrane. This was later demonstrated by Turner (1957 & 1958) who reported that lecithin was absent from red cells of goats, sheep, oxen, and cows. Sphingomyelin was present in all red cells tested. Similar observations have been made by Matsumoto (1961 a) and de Gier et al. (1961). All these workers used chromatographic methods.

Macfarlane (1942 & 1948) and Zamecnik et al. (1947) were unable to detect hydrolysis of cephalin; however, from more recent studies on substrates of C. perfringens lecithinase it appears that under proper conditions cephalin is also hydrolyzed by the enzyme. Matsumoto (1961 b) reported cephalin extracted from sheep red cells was hydrolyzed by C. perfringens lecithinase. The rate of cephalin hydrolysis was much slower than the rate of either sphingomyelin or lecithin hydrolysis. From the work of de Geir and de Haas (1961) it appears that hydrolysis of cephalin will occur only when another phospholipid is present. They reported that while pure cephalins were not hydrolyzed, cephalins which were mixed with lecithin or sphingomyelin were hydrolyzed yielding phosphorylethanolamine. Matsumoto used sheep red cell extract as a source of cephalin and his preparations probably contained other phospholipids.

Contrary to Macfarlane's (1948) observation that sphingomyelin was hydrolyzed slowly, Matsumoto found that the rate of hydrolysis of sphingomyelin was only slightly less than that of lecithin. The differences between the results of Matsumoto and Macfarlane might have been the result of different enzyme preparations or different substrate preparations.

Lecithins, sphingomyelins, and cephalins are not single compounds.

The nature of the fatty acid component depends on the source from which they are isolated, and, at least with lecithin, affects the rate of hydrolysis by C. perfringens lecithinase. Long and Maguire (1953 & 1954) reported that only lecithins containing unsaturated fatty acids were hydrolyzed by C. perfringens lecithinase. Hydrogenated naturally-occurring lecithins and synthetic lecithins containing only saturated fatty acids were not acted upon by the enzyme. Hanahan (1954) suggested that lack of contact between enzyme and substrate in water suspension was responsible for the failure of C. perfringens lecithinase to hydrolyze saturated lecithins, for the saturated lecithins do not form uniform emulsions as do unsaturated lecithins. By suspending the lecithins in an ethanol-ether medium Hanahan found both saturated and unsaturated lecithins to be completely hydrolyzed. Similar results have been obtained by van Deenen et al. (1961) with water suspension of synthetic, saturated lecithins. They found that L- α -(didecanoyl) lecithin which formed a uniform emulsion was completely hydrolyzed while L- α -(ditetracosanoyl) lecithin, which did not form an emulsion, was not hydrolyzed.

Whether the presence of unsaturated fatty acids is an important factor in the hydrolysis of sphingomyelin has not been reported. Macfarlane (1948) analyzed the hydrolysis product from sphingomyelin and found a compound approximately the composition of lignocerylsphingosine (lignoceric acid is a saturated fatty acid). Matsumoto analyzed his sphingomyelin preparation and found the unsaturated nervonic acid and four saturated fatty acids, palmitic, stearic, arachidic, and lignoceric.

Most of the work with C. perfringens lecithinase has been done with

ammonium sulfate precipitates or glycerinated culture filtrate preparations of the enzyme. The fact that these preparations are by no means 'pure' lecithinase raises a question as to whether one enzyme is responsible for the hydrolysis of lecithin and sphingomyelin or whether both a lecithinase and a sphingomyelinase are present in preparations of alpha-toxin.

The ideal method of demonstrating the presence of more than one enzyme in the C. perfringens lecithinase preparations would be to isolate the different enzymes. There are, however, several other methods which might provide the same information.

Smith and Gardner (1950) found that C. perfringens lecithinase was inactivated by reducing agents. They further observed that inhibition of lecithinase activity did not correspond to loss of toxicity or hemolytic activity. It is possible that if two enzymes (for example lecithinase and sphingomyelinase) had been present they may not have been equally susceptible to inhibition by the reducing agents. After the lecithinase was completely destroyed the 'sphingomyelinase' could still be able to hydrolyze the sphingomyelin in the red cells. If only one enzyme were involved it would be expected that inhibition of lecithin hydrolysis would parallel inhibition of sphingomyelin hydrolysis. Several procedures other than treatment with reducing agents can be used to inhibit lecithin hydrolysis. Macfarlane and Knight (1941) found that lecithinase was inactivated by surface denaturation, heat, and detergents.

Several procedures have been described for the partial purification of C. perfringens lecithinase. Although none of these procedures would be expected to completely separate the two enzymes they might yield a product

which contained more of one enzyme than the other. For example, if after precipitation with acetone, the lecithinase activity increased while the sphingomyelinase activity decreased it would provide evidence that two enzymes were present. This approach was used by Knight et al. (1963) to demonstrate the existence of two polynucleotide phosphorylase enzymes of C. perfringens.

A similar approach would be to measure the rates of hydrolysis with enzymes produced by different strains of C. perfringens type A. Again if only a single enzyme were involved the hydrolysis of lecithin should parallel the hydrolysis of sphingomyelin. If two or more enzymes were involved it would seem odd that they were always produced in the same proportion by every strain of C. perfringens. A difference in the ratio of lecithinase to sphingomyelinase might be an explanation for the difference in the rate of sphingomyelin hydrolysis obtained by Macfarlane (1948) who used strain 107 and the rate of hydrolysis obtained by Matsumoto (1962 b) who used strain BP6K. Matsumoto's preparation may have contained more 'sphingomyelinase' than Macfarlane's preparation.

MATERIALS AND METHODS

A. Enzyme substrates

The soybean lecithin and sphingomyelin used in these experiments were obtained from Nutritional Biochemical Co., Cleveland, Ohio. The egg lecithin was extracted from fresh chicken eggs by the following two procedures.

Batch I lecithin: The first batch of lecithin was prepared by extracting egg yolks with ether and precipitating with acetone. The yolks of six eggs were separated and homogenized with 50 ml of water in a Waring blender. One hundred ml of ether were added to the egg yolk suspension in a separatory funnel and the mixture shaken vigorously. The resulting emulsion was broken by centrifuging at 5,000 r.p.m. for five minutes. The upper layer of ether was siphoned off and the lower layer was extracted with ether. The ether from five such extractions was collected in a suction flask and the volume reduced to 50-75 ml. The ether was added to 100 ml of cold acetone (0-5 C) and the precipitate removed by centrifugation at 5,000 r.p.m. for ten minutes. The precipitate was redissolved in ether and the ether solution centrifuged at 5,000 r.p.m. for ten minutes. The precipitate was discarded and the lecithin was again precipitated with cold acetone. This process was repeated until the acetone was clear and colorless after centrifugation and no precipitate remained after dissolving the acetone precipitate in ether. The final product was completely soluble in ether and 95 per cent alcohol and insoluble in acetone. It was white

and had the consistency of vaseline. After 24 hours it became slightly yellow.

Batch II lecithin: The second batch of lecithin was prepared according to Deverell's (1957) modification of the method described by Pangborn (1951). The yolks of 12 eggs were separated and beaten with a stirring rod until a homogenous suspension was obtained. Approximately 600 ml of acetone were added and the predipitate filtered off in a Buchner filter. The precipitate was repeatedly suspended in acetone and filtered until the acetone was colorless, and the precipitate was a fine, white powder. The extracted powder was then suspended in 800 ml of 95 per cent ethanol. The alcohol suspension was allowed to stand at room temperature for 60 minutes, then filtered through a Buchner filter. The precipitate was discarded and 20 ml of 50 per cent cadmium chloride were slowly added to the alcohol extract. The suspension was stored at 5 C for one hour then filtered through a Buchner filter. The filter cake was washed once with 100 ml of acetone. The filtrate was discarded and the precipitate redissolved in 100 ml of chloroform. The chloroform solution was slowly poured into 700 ml of 95 per cent ethanol and shaken vigorously. The precipitate was filtered, redissolved in chloroform, and reprecipitated with ethanol. This was repeated until the ethanol extract was crystal clear. The precipitate was then suspended in 200 ml of petroleum ether and the suspension shaken with 500 ml of 80 per cent ethanol containing 0.5 ml of cadmium chloride. The cadmium salt of lecithin is insoluble in either petroleum ether or 80 per cent ethanol, but it is soluble in 80 per cent ethanol saturated with petroleum ether. The petroleum ether was repeatedly extracted until a

total of 1,500 ml of ethanol were collected. The ethanol solution was freed from petroleum ether under vacuum, and stored over night at 5 C. The ethanol solution was filtered in a Buchner filter and the precipitate dissolved in 200 ml of chloroform. Cadmium chloride was removed by shaking the chloroform solution with 30 per cent ethanol. The emulsion which formed was broken by centrifugation at 5,000 r.p.m. for five minutes. After each extraction the ethanol was tested for chloride by adding a few drops of silver nitrate to about 5 ml of the extract. A positive test was indicated by the appearance of a white precipitate of silver chloride. The chloroform solution was repeatedly extracted until a negative test for chloride was obtained. The chloroform solution was then evaporated to dryness and the residue redissolved in ether. The ether solution was centrifuged and the lecithin precipitated with acetone. The acetone precipitate was again dissolved in ether and centrifuged. At this point there was no precipitate after centrifugation. The ether was evaporated to dryness and the residue dissolved in 95 per cent ethanol. The ethanol solution was adjusted to contain 2,000 μ g of phosphorus per ml and stored at 5 C. The final product was transparent and had the consistency of vaseline. The lecithin suspensions were made up by evaporating one ml of the ethanol solution to dryness and suspending the residue in 20 ml of distilled water. This suspension contained 100 μ g of phosphorus per ml.

B. Toxin production

The toxin used in all experiments was produced in the following medium suggested by Dr. L. DS. Smith.

One pound of lean round steak was ground and added to a liter of distilled water containing 25 ml of one normal sodium hydroxide. The meat suspension was brought to a boil, cooled, and the liquid poured into a large beaker. The liquid was allowed to stand at 5 C until the fat had congealed and then filtered.

The toxin medium was made up by adding the following materials to the meat extract: 2.0 per cent trypticase, 0.02 per cent magnesium sulfate, 0.5 per cent potassium phosphate, 0.5 per cent yeast extract, and 1.0 per cent starch. The pH was adjusted to 7.5 and the broth boiled to dissolve the starch.

The extracted chopped meat was added to a liter flask to about one third full. The flask was filled to about one half with the broth and autoclaved. The remaining broth was autoclaved separately. After sterilization the flask containing the broth-meat mixture was filled with the remaining broth. The toxin medium was cooled to 40 C and inoculated from a rapidly growing (4 to 6-hour) culture of C. perfringens type A (strain BP6K). After over night incubation at 37 C the culture fluid was clarified by centrifugation, and stored at -15 C if not used immediately.

C. Toxin purification

No attempt was made to obtain a preparation of 'pure' alpha-toxin. The toxin was prepared by several methods and the enzyme activity of each preparation was tested. If only one enzyme is involved in the hydrolysis of the different phospholipids then the relative rates of hydrolysis should be the same, regardless of how the enzyme was prepared.

Ammonium sulfate precipitation: An ammonium sulfate precipitate was prepared by saturating two liters of culture fluid with ammonium sulfate and allowing the saturated solution to stand over night at 5 C. The precipitated protein formed a scum on the surface which was skimmed off and dissolved in distilled water. This solution was dialyzed over night against distilled water and clarified by centrifugation at 9,000 r.p.m. for 20 minutes. The protein was again precipitated with ammonium sulfate. The precipitate was dissolved in distilled water, dialyzed over night, and centrifuged as before. Glycerol was then added to a final concentration of ten per cent. The mixture was pipetted into 13 mm x 100 mm screw cap tubes and stored at -15 C. Once a tube had been thawed it was never refrozen. If all the material was not used immediately after thawing the remainder was discarded.

Acetone fraction: Toxin was purified by acetone precipitation by a modification of the method described by Ellner (1961).

Two liters of culture fluid were clarified by centrifugation (5,000 r.p.m. for 30 minutes) and saturated with ammonium sulfate. The crude toxin formed a scum on the surface after standing at 5 C for several hours. The scum was dissolved in about 200 ml of 0.005 M ethylenediaminetetraacetic acid and dialyzed over night at 5 C against 0.03 M borate buffer (pH 6.0). Two volumes of acetone which had been cooled to -15 C were slowly added to the dialyzed solution, and the mixture stored over night at -15 C. As much acetone was siphoned off as could be removed without losing any precipitate and the remaining removed by centrifugation in the cold (0 C). The white precipitate was dissolved in 0.02 M borate buffer (pH 6.0) and

dialyzed over night at 5 C. The dialyzed solution was centrifuged and glycerine added to a final concentration of ten per cent. The partially purified toxin was stored at -15 C in 4 ml quantities. Once the toxin solution had been thawed it was not refrozen.

Glycerinated culture fluid: Alpha-toxin in the untreated culture fluid was concentrated and stabilized by dialyzing the fluid against glycerine.

Phosphate was removed from the culture fluid by dialyzing the fluid against distilled water for 96 hours at 5 C. The water was changed about every 12 hours. The dialysis tube was then placed in a 250 ml graduated cylinder and dialyzed against pure glycerine for three hours. At this time the volume was reduced to about one-half. The glycerinated fluid was distributed in 13 mm x 100 mm screw cap tubes and stored at -15 C.

D. Acid soluble phosphorus

Reagents: 1. Five normal sulfuric acid. Prepared by slowly adding 135 ml of concentrated sulfuric acid (sp. gr. 1.84) to 865 ml of distilled water.

2. Ten per cent trichloroacetic acid. Prepared by dissolving 50 gm of crystalline trichloroacetic acid in 500 ml of distilled water.

3. Ammonium molybdate solution. Prepared by dissolving 12.5 gm of ammonium molybdate in 500 ml of distilled water.

4. Fiske-Subbarow reagent. A stock reagent was prepared by mixing 2.5 gm of 1,2,4-aminonaphtholsulfuric acid, 142.5 gm of sodium bisulfite, and 5 gm of sodium sulfite. The reagent was then made up by dissolving

1.2 gm of the stock powder in 20 ml of distilled water. The solution was discarded if it was not used within a week.

Procedure: All of the experiments described here were set up so that the final volume of each enzyme-substrate mixture was 4.0 ml. Acid insoluble material was precipitated by the addition of 6 ml of ten per cent trichloroacetic acid to each sample and the precipitate removed by centrifugation (9,000 r.p.m. for 15 minutes) followed by filtration through Whatman no. 42 filter paper. The acid soluble phosphorus content of the clarified filtrate was then determined by the following procedure: Eight ml of the filtrate were mixed with 5 ml of 5 N sulfuric acid and digested on a Lindberg (model H-2) hot plate. Digestion was continued until only a dark black residue remained. The digestion flasks were cooled and three or four drops of 30 per cent hydrogen peroxide added to each. The flasks were returned to the hot plate and digestion continued. If the sample was not completely clear when the hydrogen peroxide had boiled away it was again cooled and a few more drops of hydrogen peroxide were added. This was repeated until the sample was completely clear and colorless. After cooling, about ten ml of distilled water were added and the solution boiled for two or three minutes to remove any hydrogen peroxide.

The digested solution was cooled and quantitatively transferred to a 25 ml volumetric flask. The sample was mixed well with 2.5 ml of the ammonium molybdate reagent. One ml of the Fiske-Subbarow reagent was then added and the solution shaken vigorously. The volume was brought up to the 25 ml mark with distilled water and the solution transferred to a 20 mm test tube.

When all the samples had been processed in this manner the tubes were placed in a water bath and held at 90 C for ten minutes. After cooling to room temperature the samples were pipetted into 10 x 75 mm Coleman cuvettes and read in a Coleman (model 6-A) spectrophotometer at 660 m μ . For all readings, including the standards, distilled water was used for the zero reading.

EXPERIMENTAL

A. Standard phosphate curves

A standard curve for acid soluble phosphorus was run for each experiment.

For the standard phosphate solution 4.8928 gm of monobasic potassium phosphate, which had been dried over calcium chloride in a vacuum desiccator for one week, was dissolved in distilled water in a 100 ml volumetric flask. This solution contained 10 mg of phosphorus per ml. Ten ml of this solution (100 mg phosphorus) was transferred with a volumetric pipette into a liter volumetric flask and diluted to the mark with distilled water. This was the standard phosphate solution and contained 100 μ g of phosphorus per ml.

Phosphate determinations were made by digesting 0.10 ml, 0.20 ml, 0.40 ml, 0.60 ml, 0.80 ml, and 1.00 ml quantities of the standard solution with 5 ml of 5 N sulfuric acid. Digestion was continued until all the water had boiled away. Although no dark residue formed in the digestion flasks they were treated with a few drops of hydrogen peroxide in the same manner as described for the determination of acid soluble phosphorus.

In early experiments some difficulty was encountered in reproducing the standard curves. It was noted that as the developing tubes remained standing at room temperature for several hours the color became enhanced. In a few trial and error experiments it was found that a stable color was obtained by heating the developing solutions for ten minutes at 90 C. The

curves obtained by heating the samples were highly reproducible.

As shown in Figure 1, the color of an unheated sample gradually increased in intensity until it reached that of the heated sample, usually in about 24 hours.

Similar findings have been reported by Bartlett (1959). After heating the samples at 100 C for seven minutes he made readings at 830 m μ . He stated that there was little advantage in heating the samples if readings were made at 660 m μ . Apparently, it is not merely a matter of reaction time, as is suggested by these experiments, but that the final color of the heated sample is different than that of the unheated sample.

B. Enzyme substrates

In order to use comparable amounts of all substrates they were expressed in terms of their phosphorus content. Pure phospholipid contains one mole of phosphorus per mole of phospholipid, and the molecular weight can easily be determined by determining the total phosphorus content. However, it is doubtful that all, if any, of the substrates used in these experiments were pure. The presence of contaminants which did not contain phosphorus would give an artificially high molecular weight but probably would not otherwise interfere. On the other hand, if contaminants which contained phosphorus were present one mole of phosphorus would not represent one mole of substrate. Some idea of the purity of the different substrates can be obtained by determining the nitrogen to phosphorus ratio which should be 1:1 for lecithin and cephalin and 2:1 for sphingomyelin. This was not determined for the substrates used in these experiments. Based on the

Table II. Colorimeter readings of unheated standard phosphate solutions.

Micrograms of phosphorus	Reading (at 660 m μ) after developing			
	$\frac{1}{2}$ hr.	4 hr.	8 hr.	24 hr.
0	.005	.010	.010	.010
20	.072	.115	.136	.176
40	.130	.195	.241	.330
60	.178	.289	.347	.485
80	.215	.369	.449	.641
100	.241	.455	.560	.760

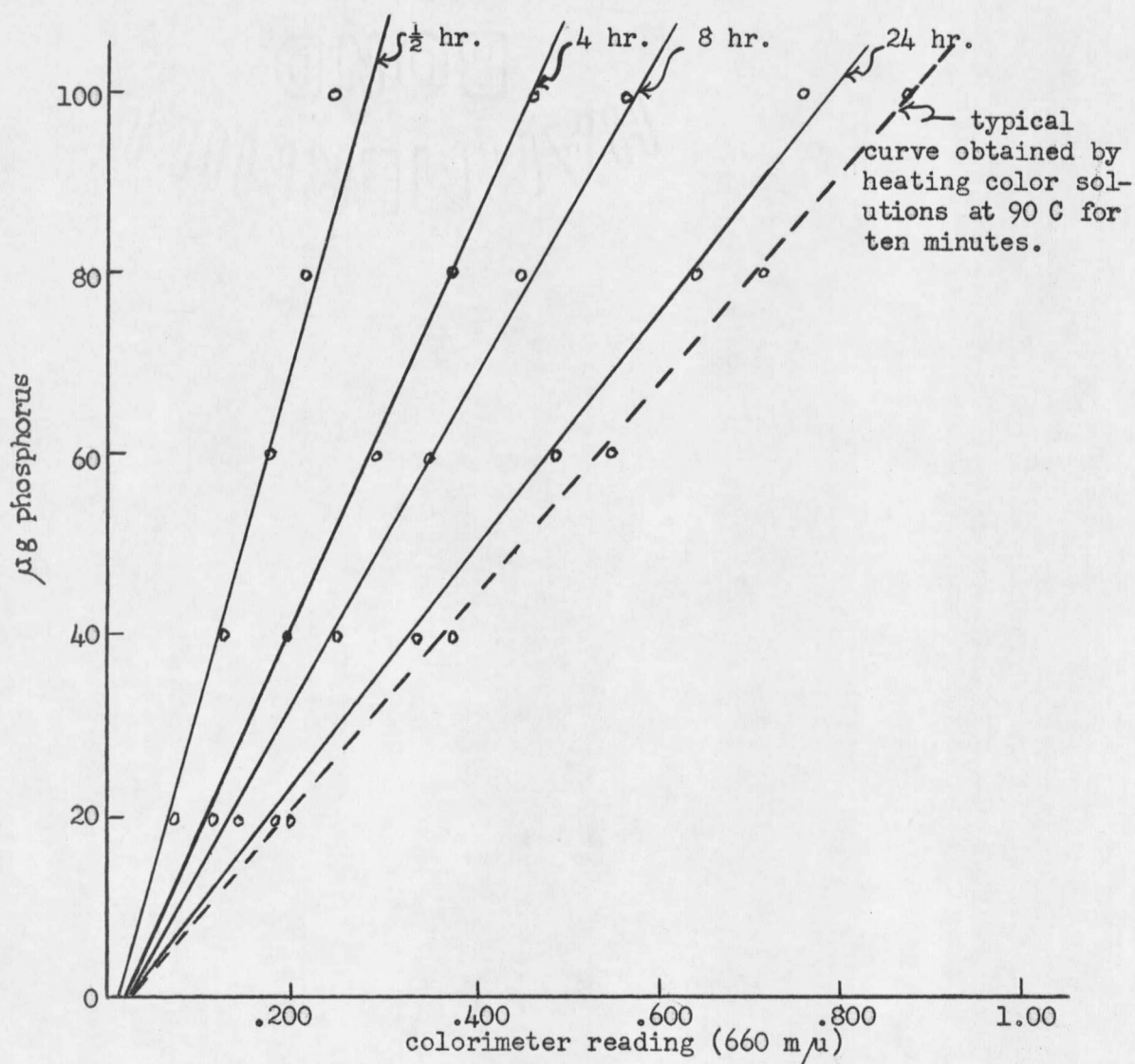


Figure 1. Change in color intensity of unheated standard phosphate solution.

homogenous appearance of the preparations and the fact that the content of phosphorus corresponded to previously reported values (3.0-4.0 per cent); it was assumed that any error introduced by contaminants would be small and one mole of phosphorus represented approximately one mole of phospholipid.

The total phosphorus content of each substrate was determined as follows: One hundred mg of phospholipid was suspended in 25 ml of distilled water. The phospholipid suspension was added to a series of digestion flasks in 0.10 ml, 0.20 ml, 0.40 ml, 0.60 ml, 0.80 ml, and 1.00 ml quantities. Five ml of 5 N sulfuric acid were added to each flask and the suspension was digested to a black residue. The phosphorus content was then determined as described for acid soluble phosphorus (page 14). The phosphorus content of the different substrates is given in Table III.

C. Enzyme activity

The enzyme unit of Macfarlane and Knight (1941) was taken as the standard for enzyme activity. One enzyme unit is defined as the amount of enzyme which will release 100 μ g of phosphorus from excess substrate in 15 minutes at 37 C. The enzyme activity for each toxin preparation was determined as follows.

The toxin solution was diluted in a series ($1/2$, $1/4$, $1/8$, - - $1/n$) with distilled water. Duplicate incubation tubes were set up for each toxin dilution by mixing one ml borate buffer (pH 7.5), one ml of calcium chloride (0.04 M), one ml of diluted toxin, and one ml of egg lecithin (Batch I preparation) containing 400 μ g of phosphorus. All the reagents had been previously heated to 37 C. The tubes were incubated in a 37 C water bath for

Table III. Phosphorus content of substrates.

Substrate	Source	Color	Percentage phosphorus
Egg lecithin	commercial* preparation	dark brown	2.9
Egg lecithin Batch I	ether extract of egg yolks	white	2.7
Egg lecithin Batch II	cadmium chloride precipitate of egg yolks	transparent	3.6
Animal lecithin	commercial preparation	dark brown	3.6
Vegetable lecithin	commercial preparation	pale yellow	2.9
Soybean lecithin	commercial preparation	pale yellow	3.0
Sphingomyelin	commercial preparation	pale yellow	3.4

* All the commercial preparations of phospholipids used in these experiments were obtained from Nutritional Biochemical Co., Cleveland, Ohio.

15 minutes. The reaction was stopped by adding 6 ml of ten per cent tri-chloroacetic acid to each tube and shaking vigorously.

After standing about 15 minutes the mixture was filtered through Whatman no. 42 filter paper. Acid soluble phosphorus was determined on an eight ml sample of the filtrate as described on page 14.

The activity curves for the different toxin preparations are shown in Figure 2. To prepare a toxin solution containing one enzyme unit per ml, the glycerinated culture fluid was diluted 1:25, the ammonium sulfate preparation was diluted 1:75, and the acetone preparation was diluted 1:17.5.

D. Inactivation with cysteine

One of the strongest pieces of evidence suggesting that more than one enzyme is involved in the hydrolysis of phospholipids comes from the report of Smith and Gardner (1950) on reducing agent inhibition. If a sphingomyelinase were present and more resistant to inhibition by a reducing agent than lecithinase it could account for the difference which they found between the inhibition of hemolytic activity and inhibition of lecithinase activity.

The following two experiments were set up to detect any differences in the hydrolysis of sphingomyelin and lecithin by an alpha-toxin preparation after treating it with cysteine.

In the first experiment, one ml of the ammonium sulfate preparation of toxin was incubated with one ml of freshly neutralized 0.06 M cysteine hydrochloride. After 15 minutes at room temperature the enzyme-cysteine

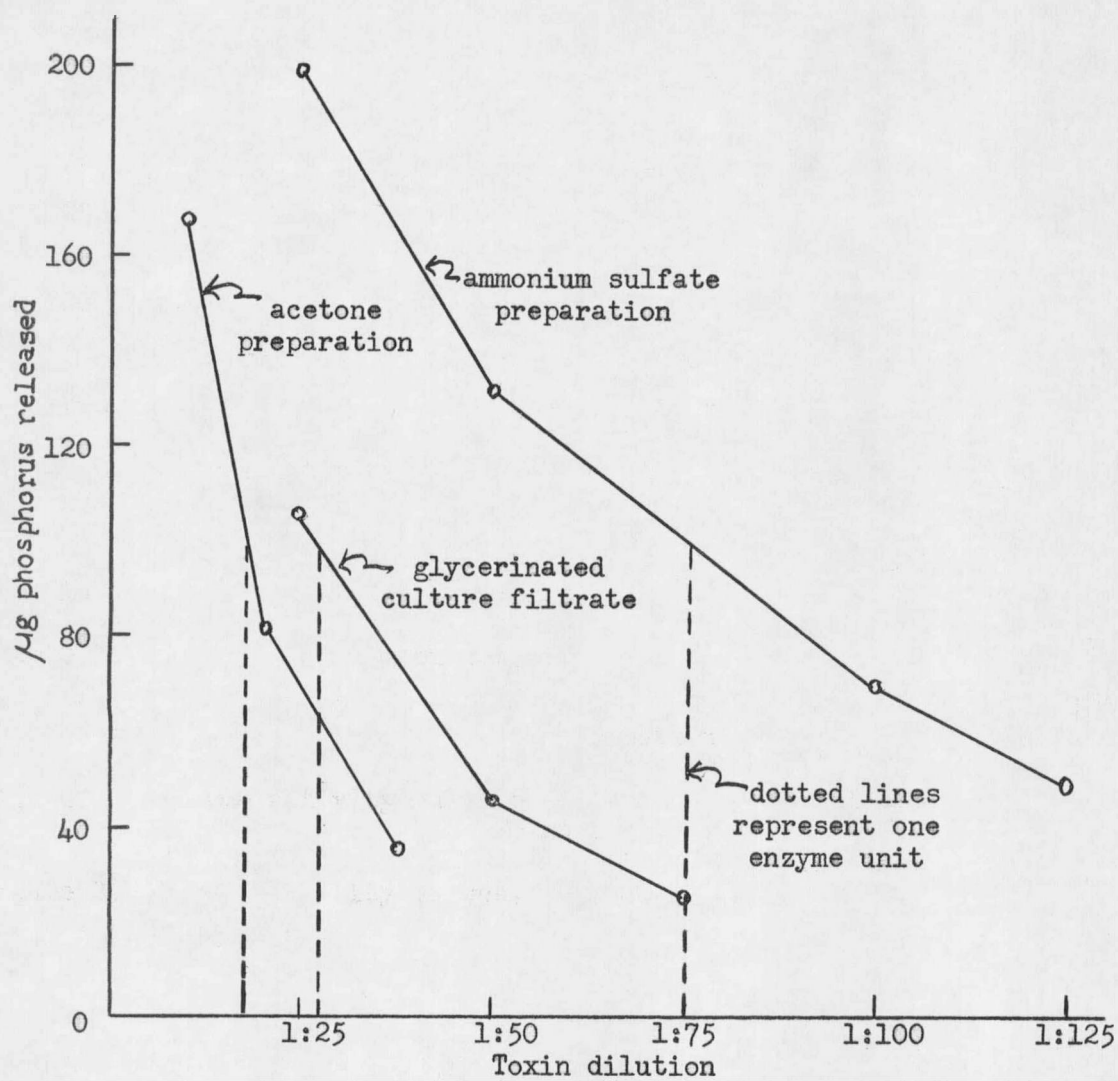


Figure 2. Enzyme activity curves.

mixture was diluted to one enzyme unit per ml (1:75 dilution). One ml of the diluted enzyme (one enzyme unit) was added to each of a series of test tubes containing one ml of borate buffer (pH 7.5) and one ml of 0.04 M calcium chloride. One ml of an emulsion of lecithin or sphingomyelin containing 100 μ g. phosphorus was then added to each tube. For the zero time sample, 6 ml of ten per cent trichloroacetic acid were added to the mixture before the addition of phospholipid. All tubes were incubated in a 37 C water bath and the reaction stopped at the desired time by the addition of 6 ml of ten per cent trichloroacetic acid. After vigorous shaking, each tube was allowed to stand about ten minutes and the contents were then filtered through S & S no. 589 filter paper. In order to get a clear filtrate it was necessary to centrifuge (9,000 r.p.m. for 15 minutes) the sphingomyelin suspension before filtering. Acid soluble phosphorus was determined on an eight ml sample of the filtrate as described on page 14. The results of this experiment are listed in Table IV and plotted in Figure 3. It is apparent from Figure 3 that inhibition of sphingomyelinase activity corresponded to inhibition of lecithinase activity.

In the second experiment the concentration of cysteine was varied and total hydrolysis after four hours was determined. In this experiment one ml of the acetone preparation of alpha-toxin was added to each of a series of tubes containing one ml of 0.02 M, 0.04 M, 0.06 M, 0.08 M, and 0.10 M cysteine. After 15 minutes at room temperature the toxin-cysteine mixture was diluted to one enzyme-unit per ml and the incubation tubes set up as before. The results of this experiment are shown in Figure 4. The data from this experiment were handled in the same manner as in the first

Table IV. Hydrolysis of egg lecithin and sphingomyelin by alpha-toxin treated with cysteine.

Incubation time (min.)	Tube no.	Colorimeter reading	Micrograms of phosphorus	(x 1.25) dil- ution factor	Average of duplicates
Egg lecithin					
0	1	.116	9	11.25	10.3
0	2	.098	7.5	9.4	
5	3	.132	11.2	14	13.9
5	4	.129	11	13.75	
10	5	.160	14.5	18.1	15.5
10	6	.122	10.3	12.9	
15	7	.142	12.5	15.6	16.2
15	8	.150	13.5	16.9	
30	9	.192	18.5	23.2	20.7
30	10	.160	14.5	18.1	
90	11	.500	53.5	67	61.7
90	11	.435	46	56.3	
150	12	.630	67	84	89.5
150	12	.700	76	95	
Sphingomyelin					
0	13	.225	22	27.5	23.5
0	14	.170	15.5	19.4	
15	15	.179	17	21.2	19.7
15	16	.159	14.5	18.1	
30	17	.250	25	31.2	24.7
30	18	.159	14.5	18.1	
90	19	.235	23.2	29	25.6
90	20	.192	18.5	23.2	
150	21	.330	34	42.5	40.4
150	22	.298	30.5	38.2	
240	23	.365	37	46.3	46.6
240	24	.370	37.5	46.8	

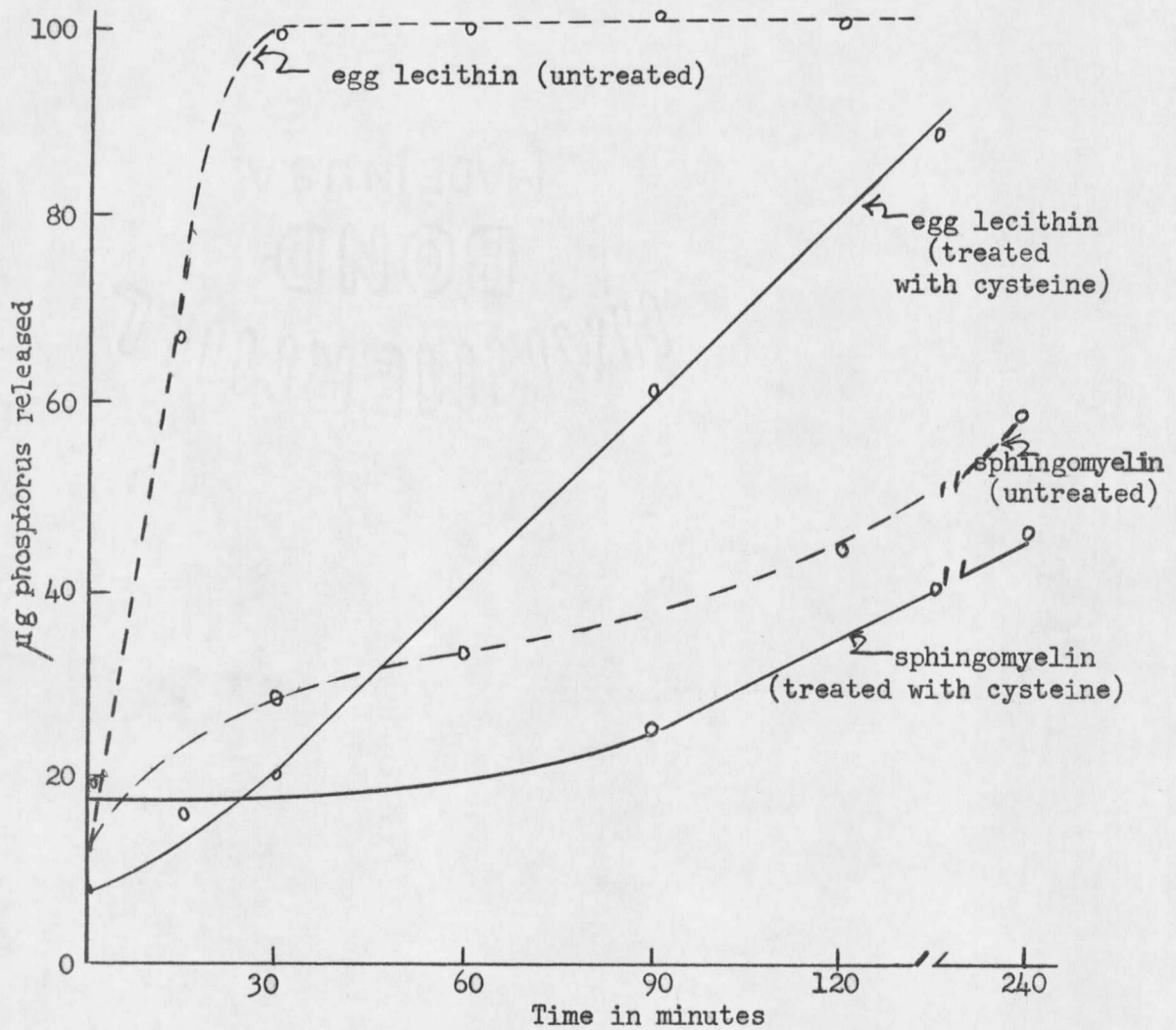


Figure 3. Hydrolysis of lecithin and sphingomyelin by alpha-toxin treated with cysteine.

Table V. Hydrolysis of sphingomyelin and lecithin by alpha-toxin treated with cysteine.

Molar concentration of cysteine	Per cent hydrolysis of lecithin	Per cent hydrolysis of sphingomyelin
untreated control	100	100
0.01 M	103	101
0.02 M	99	78
0.03 M	41	45.2
0.05 M	25.9	45

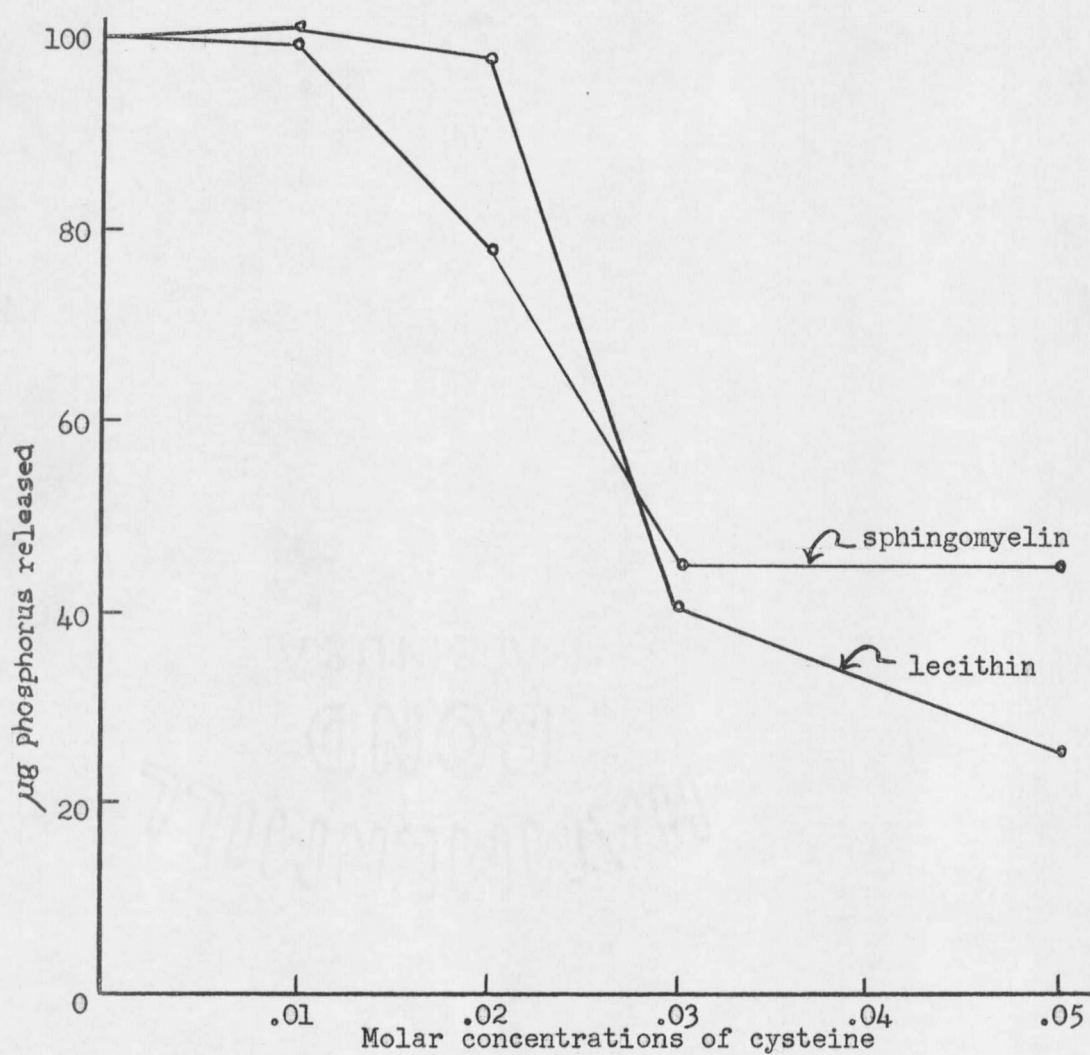


Figure 4. Hydrolysis of sphingomyelin and egg lecithin by alpha-toxin treated with cysteine.

experiment, therefore only the percentage hydrolysis is listed in Table V. In this experiment, as in all the others reported here, all samples were run in duplicate. Both of the experiments with cysteine inhibition suggest that if two enzymes were present they were equally susceptible to inactivation by cysteine.

E. Activity of the different toxin preparations

If only one enzyme were involved in the hydrolysis of lecithin and sphingomyelin then the relative rates of hydrolysis of the two phospholipids would be the same for the same concentrations of enzyme, regardless of how the enzyme was prepared. If, on the other hand, two enzymes were involved, then the ratio of one enzyme to the other might vary from one preparation to another. In this case the relative rates of hydrolysis of lecithin and sphingomyelin would also vary. This possibility was tested by measuring the rates of hydrolysis of sphingomyelin and lecithin by glycerinated culture filtrate, an ammonium sulfate precipitate, and an acetone precipitate of alpha-toxin. Each preparation was adjusted to a concentration of one enzyme unit per ml. Duplicate incubation tubes were prepared by mixing one ml of diluted toxin (one enzyme unit), one ml of borate buffer (pH 7.5), one ml of calcium chloride (0.04 M), and one ml of a suspension of substrate (100 μ g of phosphorus). For the zero time reading 6 ml of ten per cent trichloroacetic acid were added to the toxin-buffer-calcium chloride mixture before the addition of substrate. Acid soluble phosphorus was determined at the different time intervals as described on page 14.

As shown in Table VI and Figure 5 the acetone preparation appeared to have more sphingomyelin hydrolyzing activity than the ammonium sulfate preparation. The experiment with each preparation of toxin were run on separate days, using different substrate suspensions. It was possible, therefore, that the substrate suspension used with the acetone preparation may have contained more sphingomyelin (either the result of an error in weighting or an error in dilution) than the substrate suspension used with the ammonium sulfate preparation. This possibility was tested by repeating the experiments using the same suspension of sphingomyelin for both the acetone preparation and the ammonium sulfate preparation. The results of this experiment are shown in Table VIII and plotted in Figure 7.

It is apparent from Figure 7 that the differences between the rates of hydrolysis of sphingomyelin by the acetone preparation and by the ammonium sulfate preparation were not due to differences in substrate concentration. From Figure 6 it appears that the lecithinase activity of the ammonium sulfate preparation was equal to that of the acetone preparation (in fact the ammonium sulfate preparation appears to be slightly more active than the acetone preparation). However, egg lecithin is rapidly hydrolyzed (100 per cent within 30 minutes) while sphingomyelin is only about 60 per cent hydrolyzed after four hours. It seemed possible, therefore, that a slight difference in the enzyme concentration of the two preparations would not be apparent in the hydrolysis of egg lecithin, but would make a significant difference in the hydrolysis of sphingomyelin. For this reason the lecithinase activity of the two preparations was tested with soybean lecithin, which is hydrolyzed at about the same rate as sphingomyelin. The

experiment was set up in the same way as with egg lecithin and sphingomyelin. As shown in Figure 8, when lecithinase activity was determined with soybean lecithin rather than egg lecithin the difference in lecithinase activity of the two preparations corresponded to the difference in sphingomyelinase activity.

Table VI. Hydrolysis of sphingomyelin by different toxin preparations.

Time (minutes)	μ g phosphorus released*		
	Acetone preparation	Ammonium sulfate preparation	Glycerinated culture filtrate
0	8.25	15	11.5
30	45.9	28.4	38.0
60		33.5	39.4
90	56.2		
120		44.6	44.4
180	63.8		
240	68.9	59.0	51.1

* All values are the average of duplicate determinations.

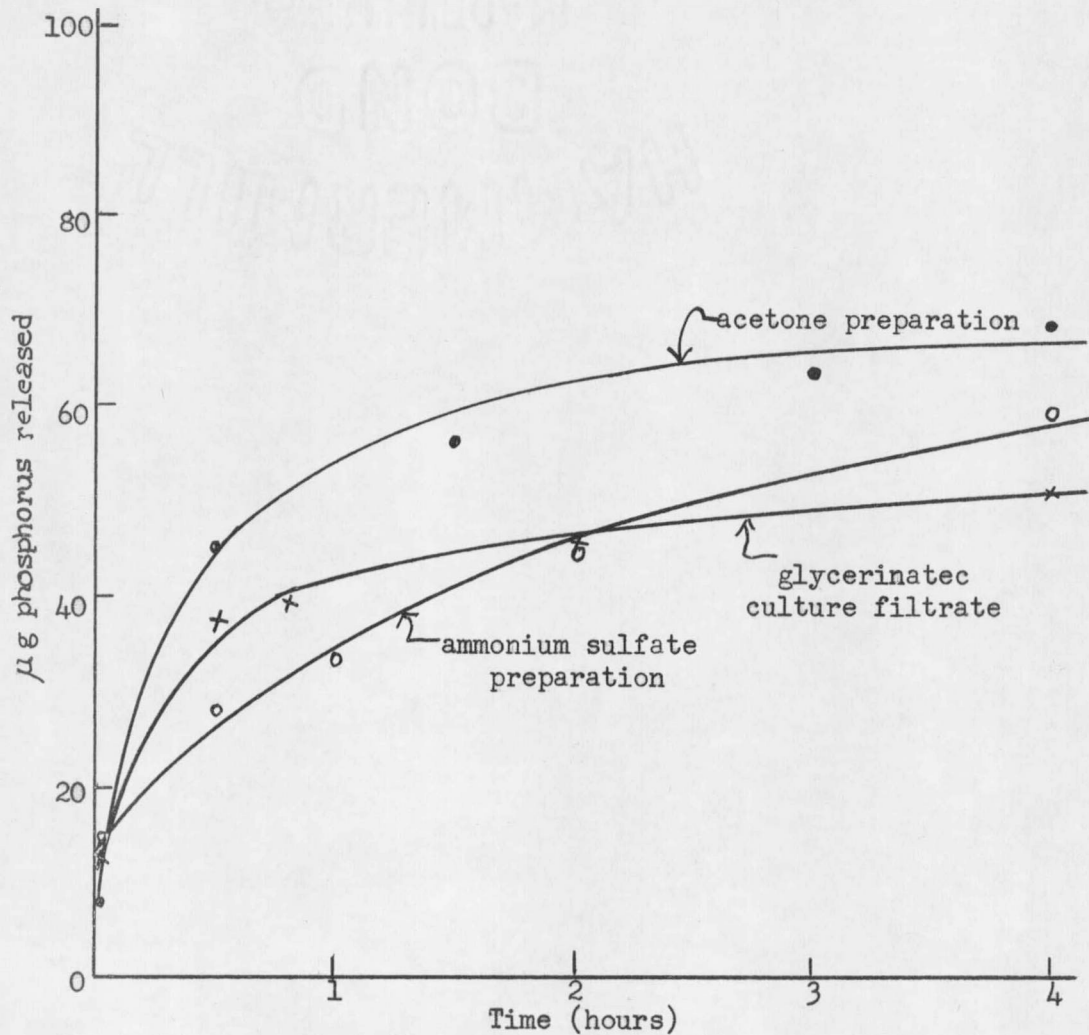


Figure 5. Hydrolysis of sphingomyelin by different preparations of alpha-toxin.

Table VII. Hydrolysis of egg lecithin by different toxin preparations.

Time (minutes)	μg phosphorus released* Acetone preparation	Ammonium sulfate preparation
0	.68	8.13
15	57.2	67.7
30	89.4	100
90	103.3	101.2
150	104.3	102.7

* All values are the average of duplicate determinations.

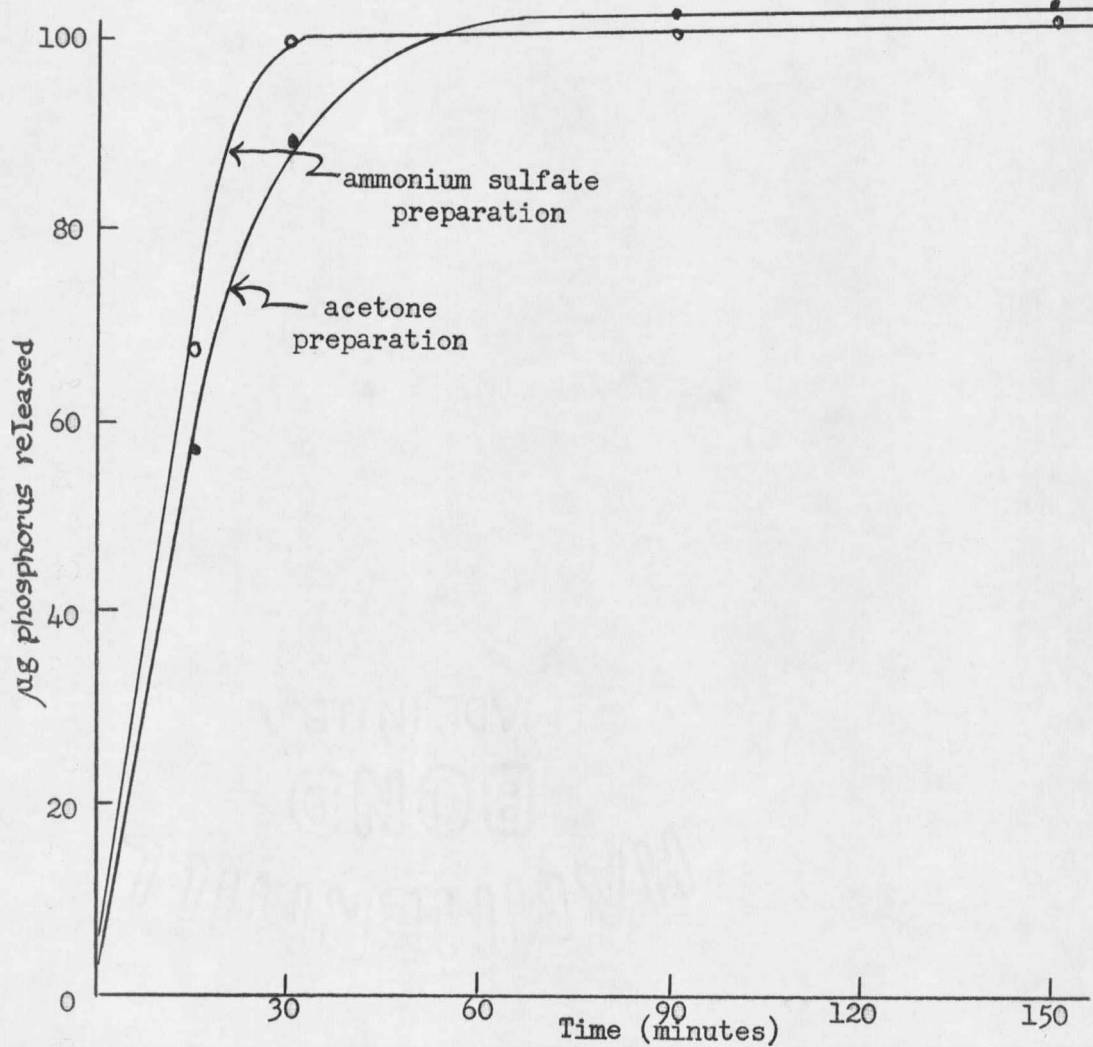


Figure 6. Hydrolysis of egg lecithin by different preparations of alpha-toxin.

Table VIII. Hydrolysis of sphingomyelin by the ammonium sulfate preparation and the acetone preparation of alpha-toxin.

Time (minutes)	μ g phosphorus released*	
	Acetone preparation	Ammonium sulfate preparation
0	15	13.5
30	34.4	25.6
90	41.5	34.2
180	44.4	35.8
240	50.6	40.1

* All values are the average of duplicate determinations.

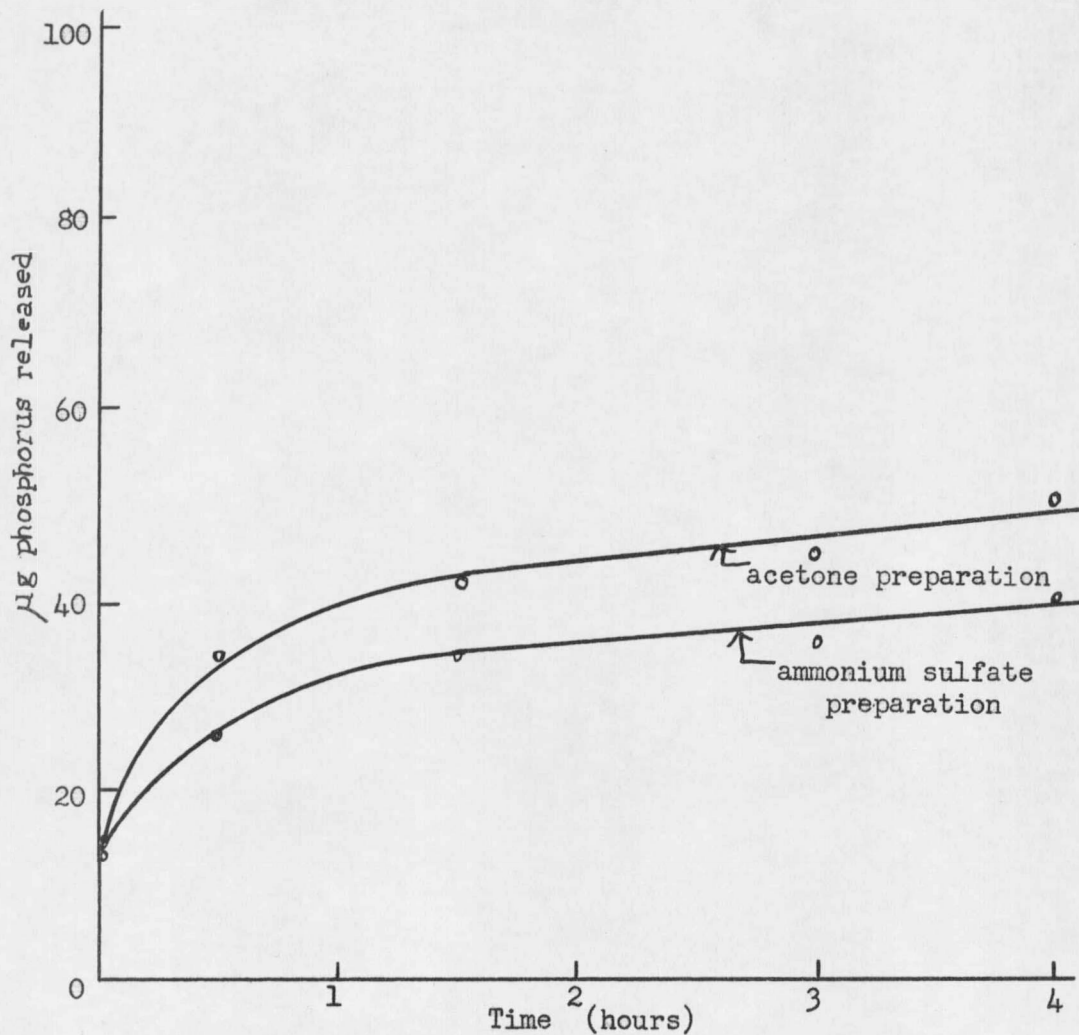


Figure 7. Hydrolysis of sphingomyelin by the ammonium sulfate and acetone preparations of alpha-toxin.

Table IX. Hydrolysis of soybean lecithin by the acetone preparation and the ammonium sulfate preparation of alpha-toxin.

Incubation time (minutes)	μg phosphorus released*	
	Acetone preparation	Ammonium sulfate preparation
0	12.3	10.6
30	60	46.9
90	71	65.1
180	82.2	75
270	74.2	71.9

* All values are the average of duplicate determinations.

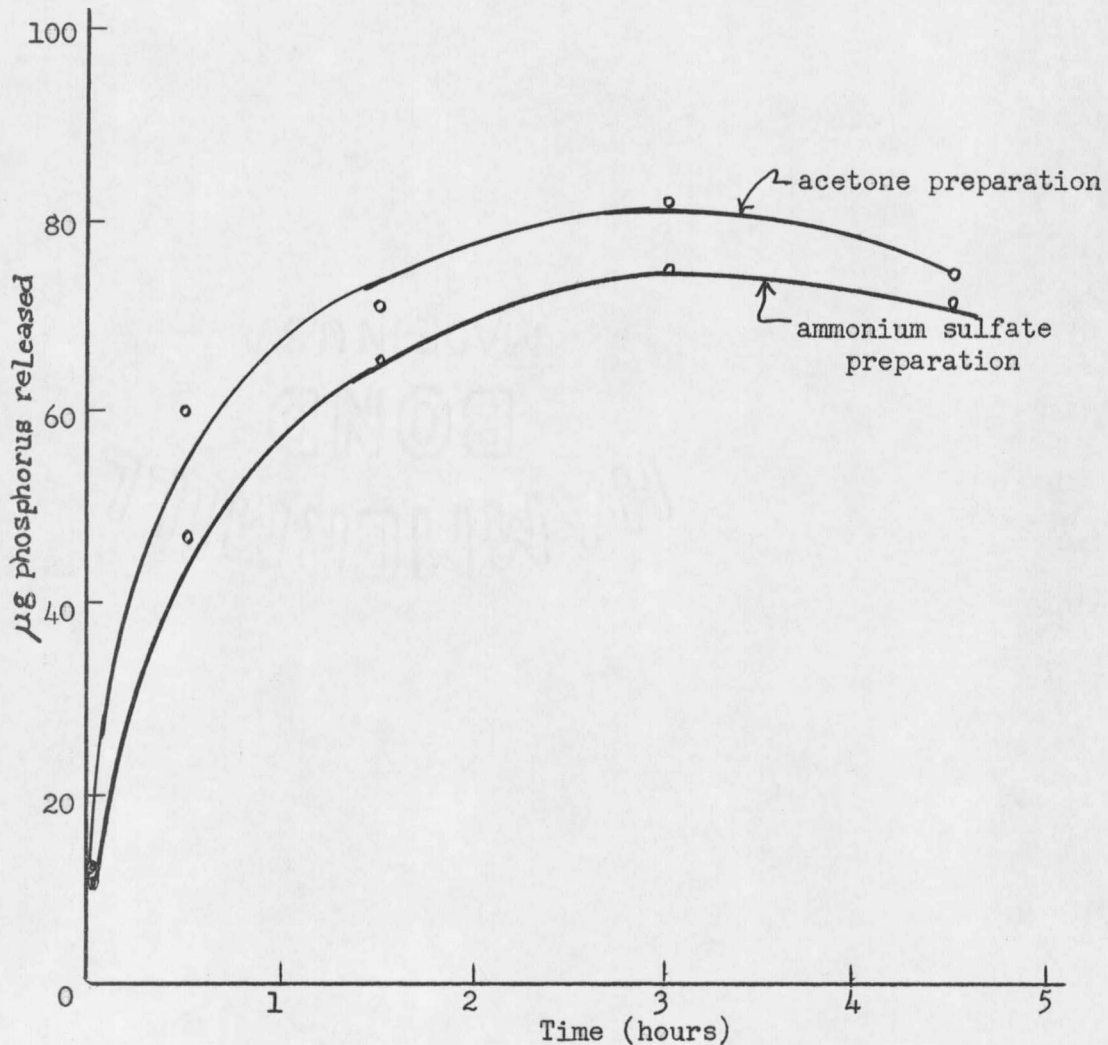


Figure 8. Hydrolysis of soybean lecithin by ammonium sulfate and acetone preparations of alpha-toxin.

F. Heat inactivation

The following experiment was set up to detect any differences in the resistance of the lecithinase and the sphingomyelinase to heat denaturation.

Acetone precipitated toxin was diluted to one enzyme unit per ml in distilled water. The diluted toxin was heated for ten minutes at 50 C, 60 C, 70 C, 80 C, and 90 C. Timing was started when a tube containing distilled water and a thermometer reached the desired temperature. After heating for ten minutes the tubes were immediately placed in a 25 C water bath. Duplicate incubation tubes for each temperature were set up by mixing one ml of the diluted toxin (one enzyme unit), one ml of calcium chloride (0.04 M), one ml of borate buffer (pH 7.5), and one ml of substrate (100 μ g phosphorus). After four hours incubation at 37 C the reaction was stopped by adding six ml of ten per cent trichloroacetic acid and acid soluble phosphorus was determined as described on page 14. The results of this experiment are listed in Table X and plotted in Figure 9. If two enzymes were present they were equally susceptible to heat inactivation. The inhibition of lecithinase activity corresponded to the inhibition of sphingomyelinase activity.

G. Antitoxin neutralization

If two enzymes were involved in the hydrolysis of lecithin and sphingomyelin it would seem odd if antitoxin always contained the same ratio of anti-lecithinase to anti-sphingomyelinase. It would be more likely that the ratio of one anti-enzyme to another would vary, depending on the

source and method of preparing the antitoxin. This possibility was tested in the following experiments.

Type A antitoxin: A serial dilution of type A antitoxin was made in distilled water. One ml of the ammonium sulfate preparation of toxin was added to one ml of each dilution of antitoxin and the mixture was incubated at 37 C for 30 minutes. The toxin-antitoxin mixture was then diluted (1:75) to contain one enzyme unit of toxin per ml. Duplicate incubation tubes were set up for each antitoxin concentration by mixing one ml of the diluted antitoxin-toxin mixture (one enzyme unit), one ml of borate buffer (pH 7.5), one ml of 0.04 M calcium chloride, and one ml of substrate suspension (100 μ g. phosphorus). The tubes were placed in a water bath and incubated four hours at 37 C. After incubation six ml of ten per cent trichloroacetic acid were added to each tube and the precipitate removed by centrifugation (9,000 r.p.m. for ten minutes) followed by filtration through Whatman no. 42 filter paper. Acid soluble phosphorus was determined on an eight ml sample from each tube as described on page 14. As shown in Table XI and Figure 10 inhibition of sphingomyelinase activity corresponded to inhibition of lecithinase activity.

Antilecithinase: The same experiment was repeated using a different preparation of antitoxin. The antitoxin preparation was labeled only as containing 500 units of antilecithinase per ml. The results of this experiment are shown in Figure 11. In both of the antitoxin experiments the inhibition of sphingomyelinase activity corresponded to inhibition of lecithinase activity.

Table X. Hydrolysis of lecithin and sphingomyelin by alpha-toxin heated for ten minutes at different temperatures.

Temperature heated	/ug phosphorus released*	
	Lecithin	Sphingomyelin
unheated control	114	50.0
50 C	115.1	47.3
60 C	113.8	41.4
70 C	34.9	14.4
80 C	85	19.4
90 C	114.3	27.6

* All values are the average of duplicate determinations.

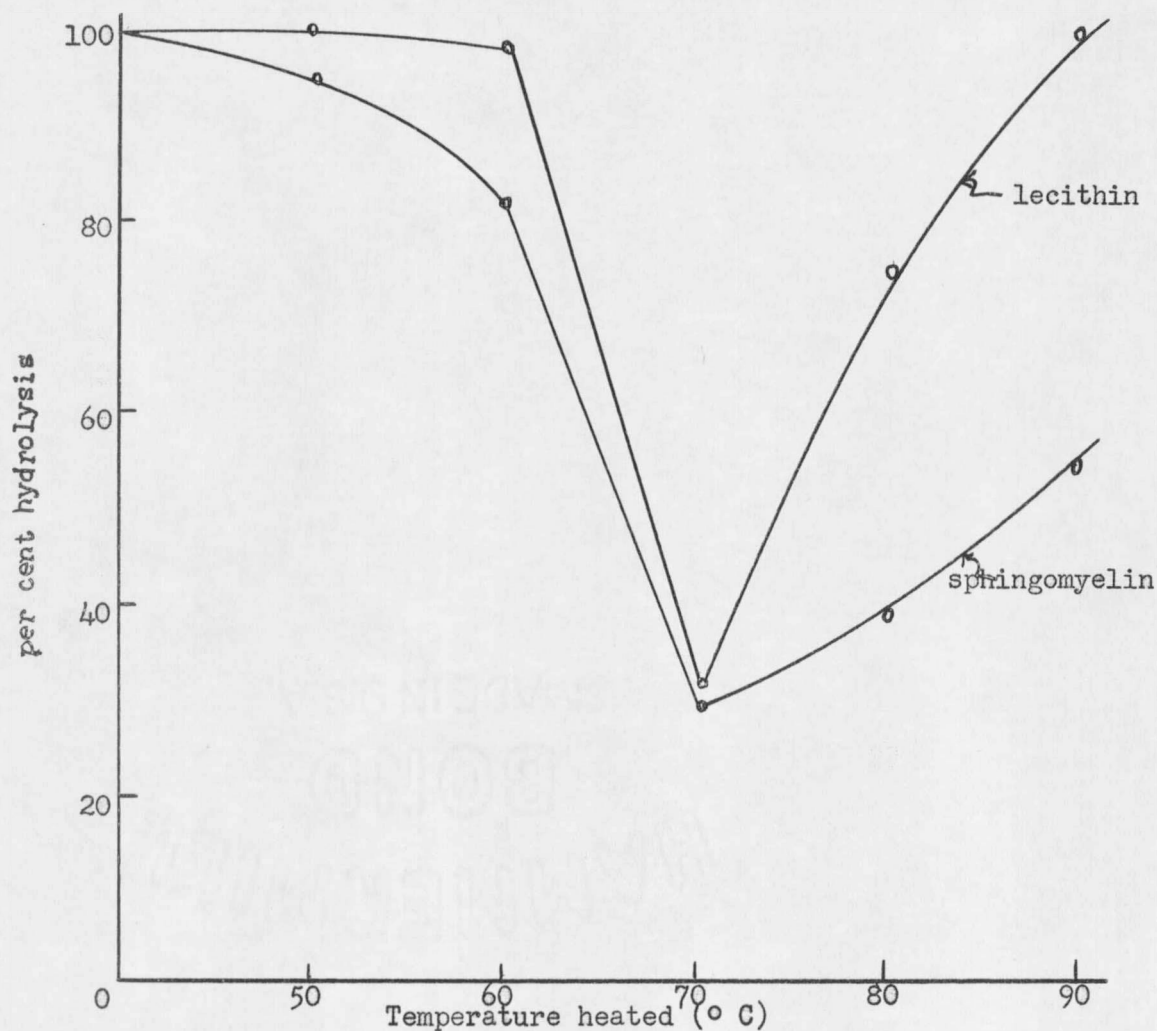


Figure 9. Hydrolysis of egg lecithin and sphingomyelin by heat-treated alpha-toxin.

Table XI. Hydrolysis of sphingomyelin and egg lecithin with alpha-toxin treated with type A antitoxin.

Units of antitoxin	μ g phosphorus released* Lecithin	Sphingomyelin
0	104.5	49.7
0.9	104	48.3
1.8	106	49.8
3.6	99.5	40.8
7.2	48.1	26.8

* All values are the average of duplicate determinations.

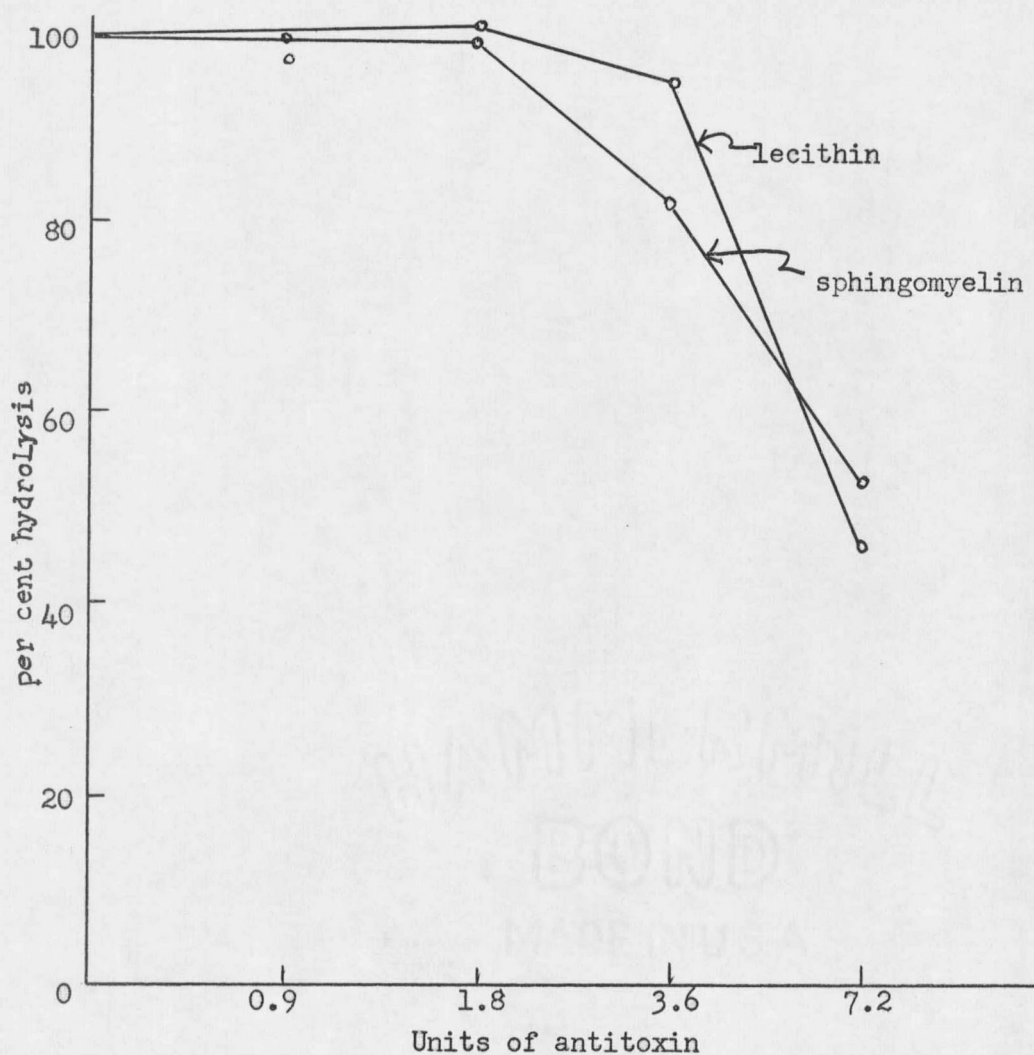


Figure 10. Hydrolysis of egg lecithin and sphingomyelin by alpha-toxin treated with type A antitoxin.

Table XII. Hydrolysis of egg lecithin and sphingomyelin by alpha-toxin treated with antilecithinase.

Units antitoxin	μ g phosphorus released* Lecithin	Sphingomyelin
0	104.9	65
3.125	103.6	63.3
6.25	101.7	48.7
12.50	69.3	35.2
25	5	14

* All values are the average of duplicate determinations.

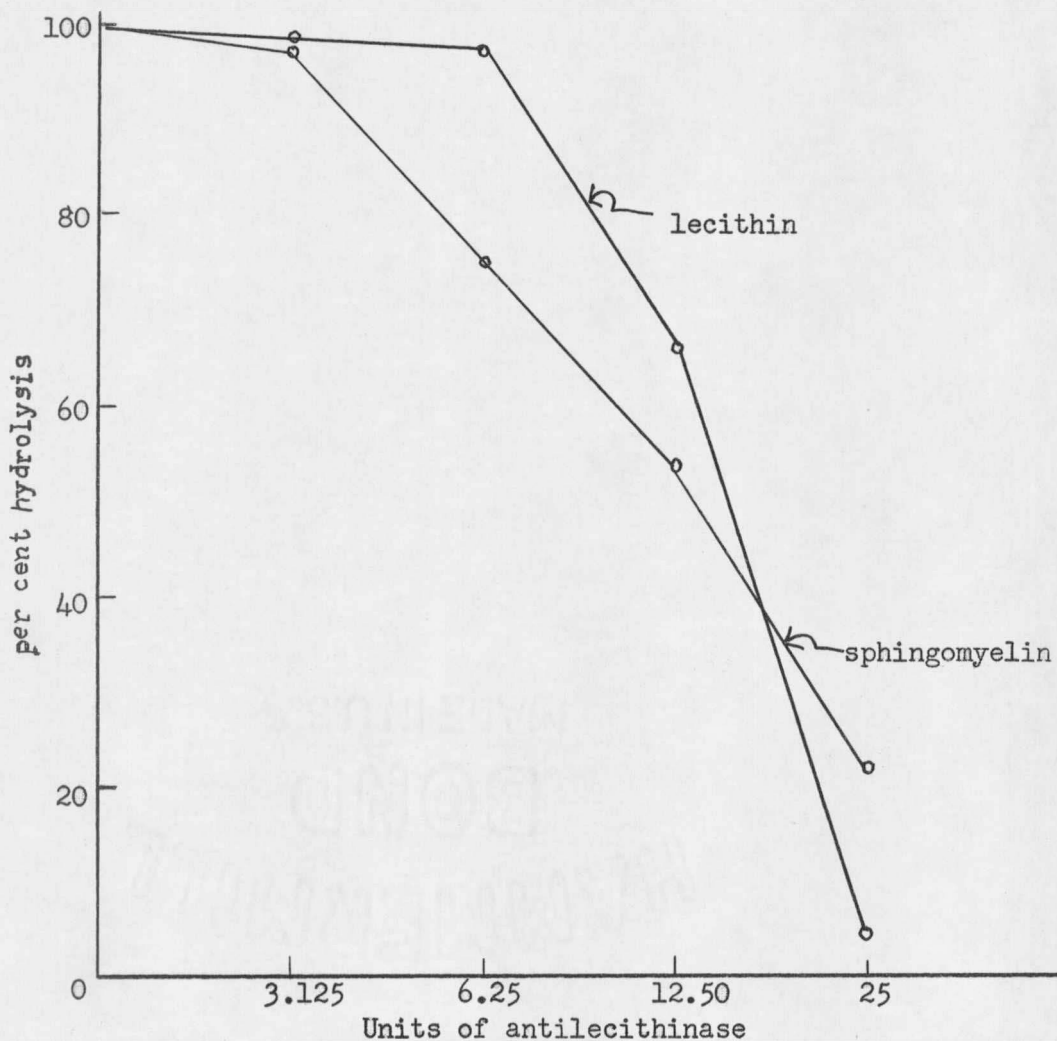


Figure 11. Hydrolysis of egg lecithin and sphingomyelin by alpha-toxin treated with antilecithinase.

G. Effect of chloroform on the rate of hydrolysis

One difficulty encountered in comparing hydrolysis of lecithin and hydrolysis of sphingomyelin results from the difference in their rates of hydrolysis. For example, in the experiment on heat inactivation the two curves are comparable except for the highest temperature. It appears that although the lecithinase was completely reactivated, the sphingomyelinase was only partially reactivated. Actually, the difference between the two curves probably reflects differences in the rates of hydrolysis of the two substrates.

In previous experiments it was noted that the sphingomyelin tended to flocculate after about 30 minutes incubation. Whether this affects the rate of hydrolysis of sphingomyelin is unknown. Nevertheless, after observing that the presence of a small amount of chloroform prevented this flocculation the following experiment was performed.

The borate buffer solution was saturated with chloroform by adding 1.5 ml of chloroform to 100 ml of buffer solution. The mixture was shaken and allowed to stand at room temperature for several hours. Incubation tubes were set up by mixing one ml of diluted toxin (one enzyme unit), one ml of the chloroform-buffer solution (pH 7.5), one ml of 0.04 M calcium chloride, and one ml of substrate suspension (100 μ g phosphorus). The tubes were incubated in a 37 C water bath and the reaction stopped at the desired time by the addition of ten per cent trichloroacetic acid. Acid soluble phosphorus was determined as described on page 14. As shown in Figure 12 rather than increase the rate of hydrolysis the chloroform

markedly inhibited the hydrolysis of sphingomyelin. The experiment was repeated in the same manner using egg lecithin with the result shown in Figure 13. The rate of hydrolysis of egg lecithin was slightly increased by the addition of chloroform.

The immediate question was whether the effect of chloroform was on the enzyme or on the sphingomyelin. The effect of chloroform on the enzyme was determined as follows.

Three ml of an acetone preparation of toxin were added to one tube containing three ml of borate buffer saturated with chloroform and to another tube containing untreated borate buffer. The toxin-buffer mixtures were incubated at room temperature for one hour. To remove the chloroform both tubes were placed in a suction flask and the volume of each was reduced by about one fourth. The toxin-buffer mixtures were then diluted to one enzyme unit per ml with distilled water. Duplicate incubation tubes for both the chloroform treated and the untreated toxin were set up by mixing one ml of diluted toxin (one enzyme unit), one ml of borate buffer (pH 7.5), one ml of 0.04 M calcium chloride, and one ml of substrate suspension (100 μ g phosphorus). As shown in Figure 14 treatment of the toxin with chloroform had no effect on sphingomyelinase activity.

Table XIII. The effect of chloroform on the hydrolysis of sphingomyelin and egg lecithin.

Incubation time (minutes)	μg phosphorus released* Sphingomyelin	Egg lecithin
0	15.2	0
15		90.1
30	22.7	104
60		107.5
90	27.9	111
150	31.4	
270	35.3	

* All values are the average of duplicate determinations.

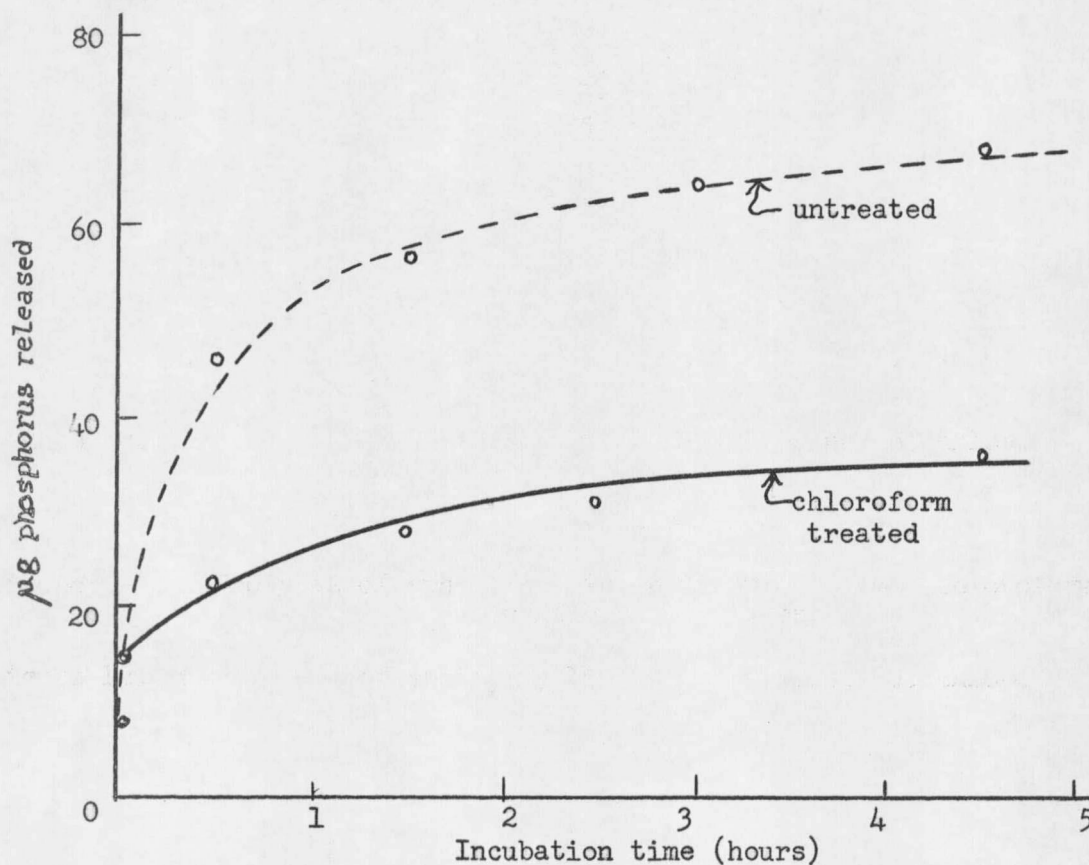


Figure 12. Hydrolysis of sphingomyelin in the presence of chloroform.

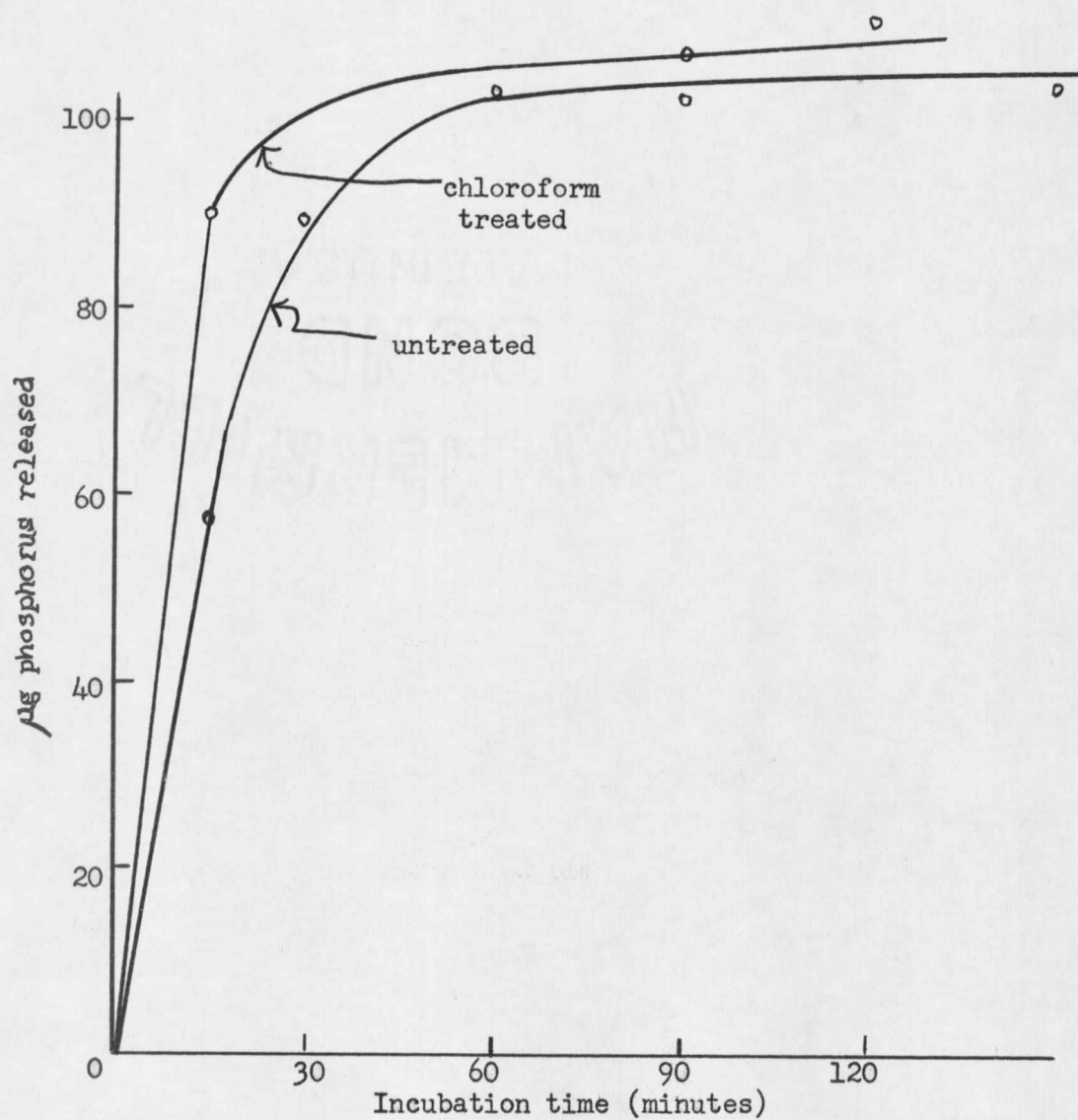


Figure 13. Hydrolysis of egg lecithin in the presence of chloroform.

Table XIV. Effect of chloroform on sphingomyelinase activity of alpha-toxin.

Incubation time (minutes)	μ g phosphorus released*	
	Treated enzyme	Untreated enzyme
0	20	21.9
60	42.3	38.1
90	46	41.9
180	51.3	49.4
270	53.1	51.3

* All values are the average of duplicate determinations.

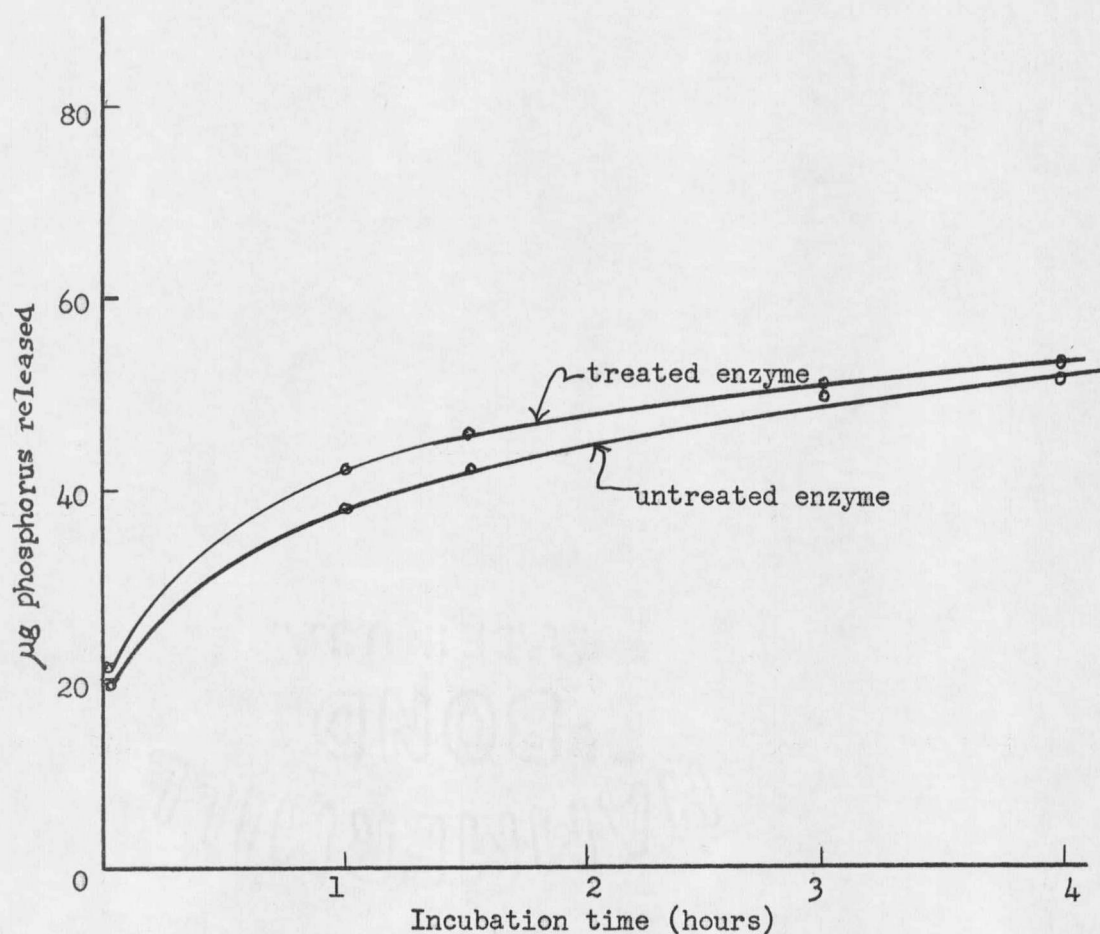


Figure 14. Hydrolysis of sphingomyelin by alpha-toxin treated with chloroform.

DISCUSSION

The question of whether one or two enzymes are involved in the hydrolysis of lecithin and sphingomyelin is important from a practical as well as a theoretical point of view. The practical implications of the problem can be seen from a consideration of the toxemia which develops in cases of gas gangrene. Briefly, the problem may be summarized as follows [for a complete discussion see MacLennan (1962)]: C. perfringens type A produces five toxic substances; alpha-toxin (lecithinase), theta-toxin (a hemolysin), kappa-toxin (collagenase), mu-toxin (hyaluronidase), and nu-toxin (deoxyribonuclease). A consideration of the biochemical properties of these toxins is sufficient to account for most (if not all) of the histological changes which occur in muscle tissue infected with C. perfringens type A. However, when death occurs in a case of gas gangrene it is not caused by the tissue destruction at the site of infection but by the resulting toxemia. The toxic substances (or substance) which cause the toxemia have never been identified.

There is little question that alpha-toxin is the most important factor in the pathogenesis of C. perfringens type A infections. For example, Evans (1945) found a general relationship between the ability of a strain of C. perfringens type A to produce a fatal infection in guinea-pigs and its capacity to produce alpha-toxin in-vitro. He further observed that the virulence of a strain was independent of its ability to produce theta-toxin or hyaluronidase. Nevertheless, repeated attempts

to detect alpha-toxin in wound exudates or in the circulating blood have been unsuccessful, and as MacLennan (1962) has stated: "It must not be forgotten that toxemia means the presence of toxin in the blood; and at once it may be stated, not only that direct evidence for the presence of a circulating toxin has never been obtained, but also that, if some such agent is involved, then it is almost certainly not the lecithinase."

It is concerning the methods used to detect alpha-toxin that the possible existence of a sphingomyelinase, distinct from the lecithinase, becomes important. As far as this author knows, these methods (usually the lecithovitellin test) have been designed only to detect lecithinase activity; therefore, if two separate enzymes exist the sphingomyelinase might have been present but not recognized. However remote this possibility may be, as long as the question of sphingomyelinase separate from a lecithinase remains unanswered, it would seem wise to test for sphingomyelinase activity as well as for lecithinase activity.

Unfortunately no definite conclusions can be drawn from the experiments reported here. The most that can be said is that if two enzymes were present in the alpha-toxin preparation their physical and chemical properties are similar.

The differences which Smith and Gardner (1950) found between inhibition of hemolytic activity and lecithinase activity were apparently not the result of a sphingomyelinase. If both enzymes were present they were equally susceptible to inhibition with cysteine.

It appears from the data in Table X and Figure 9 that alpha-toxin was inactivated more at 70 C than at 80 C and 90 C. Smith and Gardner (1950)

studied this anomalous heat inactivation and found that the phenomenon was dependent on the presence of calcium or magnesium ions. On the basis of centrifugation studies they concluded that a complex was formed at 65 C. which was dissociated at 100 C. From the experiment reported here it is apparent that if two enzymes were present in the alpha-toxin preparation they were equally susceptible to heat inactivation and to complex formation.

As shown in Figures 4, 9, 10, and 11, the percentage hydrolysis curves obtained with partially inactivated alpha-toxin are not strictly parallel. The hydrolysis of sphingomyelin was always slightly less than the hydrolysis of lecithin. These differences are most easily accounted for by considering the difference in the rates of hydrolysis of the two substrates. Egg lecithin is 100 per cent hydrolyzed in 30 to 60 minutes. If 20 per cent of the enzyme were inactivated by treatment with an inhibitor the remaining 80 per cent would probably be sufficient to hydrolyze 100 per cent of the egg lecithin in the four-hour incubation time. On the other hand, sphingomyelin is only about 60 per cent hydrolyzed in four hours and a 20 per cent decrease in enzyme activity would probably result in a corresponding decrease in the percentage hydrolysis.

When the different preparations of toxin were adjusted to one enzyme unit of lecithinase activity, using egg lecithin, the acetone preparation appeared to have more sphingomyelinase activity than the ammonium sulfate preparation. However, when soybean lecithin, which is hydrolyzed at about the same rate as sphingomyelin, was used to determine lecithinase activity the two preparations were comparable. If both a sphingomyelinase and a lecithinase were present the relative concentration of each enzyme remained

constant when precipitated by acetone or ammonium sulfate.

As mentioned previously, the hydrolysis of lecithin is dependent, in part at least, on the amount of unsaturation of the fatty acid component. In addition to reporting that saturated lecithins were not hydrolyzed, Long and Maguire (1954) also found an almost linear relationship between the iodine number and the rate of hydrolysis of lecithin preparation. The more unsaturated the lecithin the faster it was hydrolyzed by alpha-toxin. In view of these findings, and the report of Levene and Rolf (1926) that soybean lecithin was highly unsaturated, the relatively slow rate of hydrolysis of soybean lecithin (Figure 8) is somewhat puzzling. The ratio of unsaturated to saturated fatty acids is about 3:1 for soybean lecithin compared to a ratio of 1:1 for egg lecithin (Deuel 1951).

The iodine numbers for the egg and soybean lecithins used in these experiments were not determined; however, if they corresponded to previously reported values then some factor (or factors) in addition to unsaturated fatty acid content must be important in the hydrolysis of different lecithins. Failure to form a uniform water emulsion, as suggested from the work of Hanahan (1954) and van Deenen et al. (1961) also does not apply in this case. The soybean lecithin formed uniform and stable water emulsions.

The only experiments in which sphingomyelin hydrolysis differed significantly from lecithin hydrolysis were those in which chloroform was added to the enzyme substrate mixtures. The hydrolysis of sphingomyelin was inhibited while the hydrolysis of egg lecithin was slightly increased. Since treatment of the toxin preparation alone had no effect on sphingomyelin hydrolysis, it can only be concluded that the effect of chloroform

is either on the substrate or on the enzyme-substrate complex. The increased rate of hydrolysis of egg lecithin in the presence of chloroform is in accord with the results of Burley and Kushner (1962), who found that chloroform increased the rate of hydrolysis of both lecithin and lipovitellin solutions.

In conclusion it must be emphasized that the experiments reported here were not designed to demonstrate the presence of only one enzyme in alpha-toxin preparations. They were set up to demonstrate differences in the behavior of separate enzymes. The fact that sphingomyelinase activity corresponded to lecithinase activity in each experiment does not necessarily indicate that only one enzyme was present. It is possible that two separate enzymes with very similar physical and chemical properties were present in the alpha-toxin preparations. Although it can not be conclusively demonstrated that only one enzyme is present, each experiment in which sphingomyelinase activity corresponds to lecithinase activity increases the probability that only one enzyme is responsible for the hydrolysis of the two substrates.

SUMMARY

1. An ammonium sulfate preparation of alpha-toxin was treated with 0.03 M cysteine and the rates of sphingomyelin and lecithin hydrolysis by the partially inactivated toxin were measured. The rate of hydrolysis of sphingomyelin was inhibited to about the same extent as was the hydrolysis of lecithin.

2. An acetone preparation of alpha-toxin was treated with 0.1 M, 0.2 M, 0.3 M, and 0.5 M cysteine, and the total hydrolysis of lecithin and sphingomyelin was determined after four hours incubation. Inhibition of sphingomyelinase activity corresponded to inhibition of lecithinase activity. Inactivation of alpha-toxin with cysteine provided no evidence for the existence of a sphingomyelinase distinct from the lecithinase.

3. Three preparations of alpha-toxin; an ammonium sulfate preparation, an acetone preparation, and a glycerinated culture filtrate preparation, were adjusted to one enzyme unit (the amount of enzyme releasing 100 μ g of phosphorus from excess egg lecithin in 15 minutes at 37 C) and tested for their sphingomyelinase activity. A slight difference was observed between the sphingomyelinase activity of the acetone preparation and sphingomyelinase activity of the ammonium sulfate preparation; however, the same difference was found with the hydrolysis of soybean lecithin. It was concluded from this experiment that if two enzymes were present the relative concentration of each enzyme remained constant when precipitated by ammonium sulfate or acetone.

4. An acetone preparation of alpha-toxin was inactivated by heating at 50 C, 60 C, 70 C, 80 C, and 90 C for ten minutes. The hydrolysis of lecithin and sphingomyelin was then determined. The inhibition of sphingomyelinase activity corresponded to inhibition of lecithinase activity. If two enzymes were present they were equally susceptible to heat inactivation.

5. One ml of the ammonium sulfate preparation of alpha-toxin was incubated for 30 minutes with 0.9, 1.8, 3.6, and 7.2 units of type A antitoxin. The hydrolysis of lecithin and sphingomyelin was then determined. It was found that inhibition of sphingomyelinase activity corresponded to inhibition of lecithinase activity. The same experiment was repeated using a different preparation of antitoxin and the same results were obtained. Neutralization with antitoxin provided no evidence for the presence of a sphingomyelinase distinct from a lecithinase.

6. In an effort to increase the rate of hydrolysis of sphingomyelin, chloroform was added to the enzyme-substrate mixture. It was observed that although chloroform increased the rate of hydrolysis of egg lecithin, the hydrolysis of sphingomyelin was inhibited. The effect of chloroform on the enzyme alone was determined by mixing the acetone preparation of toxin with borate buffer saturated with chloroform. After incubating for one hour the chloroform was removed under vacuum and the hydrolysis of sphingomyelin by the chloroform treated and untreated toxin was determined. This treatment had no effect on the sphingomyelinase activity. Apparently the effect of chloroform is on the substrate or on the enzyme-substrate complex; it has no effect on the enzyme alone.

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Gydrolysis of lecithin and
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