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
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Abstract:
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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.

HYDROLYSIS OF LECITHIN AND SPHINGOMYELIN BY PREPARATIONS OF *CLOSTRIDIUM PERFRINGENS* TYPE A CULTURE FILTRATES

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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.

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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*.

	Toxigenic type					
Toxin	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 neutraliaztion 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 yiedling 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-a-(didecanoyl) lecithin which formed a uniform emulsion was completely hydrolyzed while L-a-(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 dorrespond 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 existance 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 stiring 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 /ug 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 /ug 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 potossium 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 crystaline 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 sulfurid 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 mμ. 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 µg 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/u. He stated that there was little advantage in heating the samples if readings were made at 660 m/u. 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