The partial purification of the lecithinase of Clostridium Hemolyticum
by Donald Edward McRoberts

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Chemistry
Montana State University

Abstract:
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The lecithinolytic activity of the enzyme per milligram of nitrogen was used as a measure of its purity. Variables such as polymetaphosphate concentration, acidity, temperature, agitation, and acid contact time, which were thought likely to influence the amount of the precipitate, were investigated. The stability of the enzyme alone and in the presence of polymetaphosphate at certain pH values has been estimated in a number of chemical systems under various physical conditions.

The amount of the enzyme which can be recovered by acid precipitation from a solution with and without polymetaphosphate at various concentrations, pH values, and temperature has been evaluated.

An attempt has also been made to purify the crude lecithinase by separation on the N,N-Diethylaminoethylcellulose column. The amount of crude lecithinase adsorbed on a certain weight of DEAE cellulose was determined. TRIS (hydroxymethylamino) methane buffer was selected for ruse on the column. Calcium ion was found to stabilize the lecithinase during dialysis of the eluate.

Purification values for acid precipitation of the lecithinase -PO3 and DEAE cellulose column separation were determined. Acid purification of the crude lecithinase was typically about 22 fold for crude lecithinase which had been, purified about 10 fold in previous treatments; i.e., a total of 220 fold. Separation of the DEAE cellulose column gave about five fold purification. This value is estimated, since several lecithinase peaks were obtained on the graph when lecithinase activity was plotted against eluate tube number. It is thought that these several peaks may indicate that Clostridium hemoivticum elaborates more than one lecithinase.

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Dean, Graduate Division

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ABSTRACT

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The lecithinolytic activity of the enzyme per milligram of nitrogen was used as a measure of its purity. Variables such as polymetaphosphate concentration, acidity, temperature, agitation, and acid contact time, which were thought likely to influence the amount of the precipitate, were investigated. The stability of the enzyme alone and in the presence of polymetaphosphate at certain pH values has been estimated in a number of chemical systems under various physical conditions.

The amount of the enzyme which can be recovered by acid precipitation from a solution with and without polymetaphosphate at various concentrations, pH values, and temperature has been evaluated.

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Purification values for acid precipitation of the lecithinase $-PO_3$ and DEAE cellulose column separation were determined. Acid purification of the crude lecithinase was typically about 22 fold for crude lecithinase which had been purified about 10 fold in previous treatments; i.e., a total of 220 fold. Separation of the DEAE cellulose column gave about five fold purification. This value is estimated, since several lecithinase peaks were obtained on the graph when lecithinase activity was plotted against eluate tube number. It is thought that these several peaks may indicate that Clostridium hemolyticum elaborates more than one lecithinase.

INTRODUCTION

Immunization of cattle against bacillary icterohemoglobinuria, or "red water" disease, for periods longer than six months has not been achieved. This disease is caused by the bacterium Clostridium hemolyticum (1, 2) which produces a lethal hemolytic toxin, shown by Jasmin (3) to be a lecithinase. Swingle (4) has shown that the enzyme has lecithinolytic activity since it produces neutral fat and phosphoryl choline. The action of the enzyme is similar, then, to the Lecithinase A elaborated by Clostridium perfringens (Mac Farlane and Knight)(5).

As a first step towards developing an improved immunizing agent, an attempt was made to isolate this lecithinase in greater purity than had hitherto been achieved.

Previous attempts (6) at this isolation, using ammonium sulfate fractionation, nucleic acid precipitation, or adsorption on resin such as IRC-50 or on magnesium oxide, failed to yield a product of desired purity. In this study, two additional methods for isolation of the lecithinase were investigated: acid precipitation of the lecithinase in the presence of a protective agent, and the separation of crude lecithinase on an N,N-diethylaminoethylcellulose column.

Acid Precipitation of Lecithinase:

Turpin, Raynaud and Relyveld (7) were able to concentrate effectively the toxins and toxoids of tetanus, diphtheria, and staphylococci, and the toxins of Clostridium perfringens, Clostridium sordellii, and Clostridium botulinum by acid precipitation in the presence of sodium polymetaphosphate or ethylenediaminetetraacetic acid. Their studies did not include a consideration of Clostridium hemolyticum lecithinase. Since Swingle (4) has

shown that the lecithinase of Clostridium hemolyticum is rapidly inactivated by acid, the possibility of its being protected by an agent such as sodium polymetaphosphate before acid precipitation was investigated. Even though such protection was not in general achieved, a number of observations were made regarding the stability of the enzyme under various conditions, its reactions with sodium polymetaphosphate, and its precipitation by acids.

The first separation of crude lecithinase on the diethylaminoethyl-cellulose column showed that lecithinase activity occurred in more than one series of eluate tubes. When a profile curve was plotted showing lecithinase activity against eluate tube number, several peaks of lecithinase activity appeared on the curve. It was then assumed that these peaks represented the action of different species of lecithinase protein. As such, an effort was then made to demonstrate that two or more lecithinases were elaborated by Clostridium hemolyticum bacteria. No further effort was made to isolate the lecithinase from the crude lecithinase mixture by the procedure of acid precipitation.

HISTORICAL

Acid Precipitation of Bacterial Toxins:

Much research has been done on the possibility of concentrating toxins or toxoids by acid precipitation. Schmidt, Hansen, and Kjaer (8) in 1931 used hydrochloric acid on diphtheria toxin and toxoid, but observed a destruction of the active principle of the toxin or toxoid. Burnet and Freeman (9) purified the toxin of staphylococci by precipitation with acetic acid. Nelis (10) et al. studied the action of strong acids on

staphylococcal toxin. With H_2SO_4, the authors observed partial destruction of the toxin at room temperature. Jacobs and Behan (11) concentrated the antitoxin of tetanus with trichloroacetic acid and eliminated 99 per cent of the impurities. In 1951 Jacobs and Gillis (12) used metaphosphoric acid for the concentration of diphteria toxoid to eliminate 97 per cent of the protein nitrogen.

Turpin, Raynaud and Relyveld (7) improved the yield of toxin or toxoid separated by acid precipitation by adding a protective agent. A partial history of one of their protective agents, sodium polymetaphosphate, is given in the next section.

Polymetaphosphate and Protein:

Metaphosphoric acid of soda (13), so named in the early literature, has been recognized (13) since 1833 as an agent which will precipitate proteins (13). Because of the efficacious action of polymetaphosphoric acid as a protein precipitant, many workers have attempted to determine the specific action by which the protein in the presence of an acid solution of the polymer becomes insoluble.

According to Briggs, (14) Schofield, (15) Perlman, (16, 17) Herrmann and Perlmann, (18, 19) and Ebel and Colas, (20) a reaction occurs between polymetaphosphate and the nitrogen of a nitrogenous compound or protein. Briggs (14) has shown by dialysis that at neutral pH values the polymetaphosphate can be dialyzed from horse serum albumin, but at acid pH values this is not possible. Perlmann (17) precipitated egg albumin with metaphosphoric acid. The complex formed crystals which was taken as evidence that denaturation did not occur.

Briggs, (14) using horse serum albumin in the presence of polymetaphosphate, stated that at low pH values the ionization of the carboxyl group is repressed, and the positive charge on the amino group is completely neutralized. The overall effect is a shift of the isoelectric point in the acid direction.

Putnam and Neurath, (21) working with egg albumin and sodium dodecylsulfate, stated that precipitation may result from a combination of oppositely charged electrostatic forces; that is, an anion detergent will precipitate protein in the cationic form, but precipitation ceases somewhat above the isoelectric point, even though combination between detergent and protein (22) does occur. Just above the isoelectric point, (21) the protein-detergent complex precipitates completely and remains in the solid state for immediately lower pH values. The amount of protein precipitating in the presence of detergent depends on (first), the protein: detergent concentration ratio, (second) the pH of the solution, (third) the temperature, and (fourth), the ionic strength of the solution.

N,N Diethylaminoethyl Cellulose Column Chromatography:

Sober and Peterson (23, 24) first prepared this special cellulose ion exchanger by treating strongly alkaline cellulose with 2-chlorotriethylamine. They then used the product in a column for separation of serum proteins. Column material of this derivative is said by the authors (23, 24) to have the following properties: good mechanical strength, insolubility in water, and, as an anion exchanger, the ability to adsorb protein effectively at pH values above the isoelectric point of most proteins. Hence, elution of the bound protein can be accomplished by increasing the

ionic strength of the medium or decreasing the pH of the medium or both.

According to Guthrie and Bullock (25) DEAE cellulose[1] has a maximum capacity for protein of 0.7 meq. per g., and the pH at half capacity is 7.7. Boman and Westlund (26) believe that with an anion exchange column, best results in separation of protein will occur with a cation buffer such as TRIS (see also ref. 27). These authors state that DEAE cellulose is the preferred cellulose derivative for the separation of proteins, enzymes, and some hormones.

It should be mentioned that one disadvantage of using DEAE for separation of the lecithinase mixture rests in the fact that the material is an anion exchanger. On addition of protein to the column the pH of the eluate increases. If the protein is alkali labile, this procedure can be used only with caution.

Very few lecithinases have been isolated on a DEAE cellulose column. Of importance to this investigation is the work of Boman and Kaletta (27) who used DEAE cellulose in TRIS (hydroxymethylamino) methane buffer to separate three different forms of phosphodiesterases from phospholipase A amino acid oxidase.

Sober and Peterson (23) in 1954 reported the separation of two distinct amidase activities from a lyophilized kidney fraction which differed in their relative rates toward leucine and alanine amides.

1/ Diethylaminoethyl cellulose

MATERIALS AND METHODS

General Analytical Methods

Determination of Lecithinase Activity

The determination of the lecithinolytic activity of Clostridium hemolyticum was performed according to the method of Turner, Swingle and Strickfaden (28) as follows:

Reagents:

Borate Buffer 2/

Sodium chloride	5.3 g.
Anhydrous calcium chloride	2.2 g.
Boric acid	6.2 g.
Water to	1 liter

The pH was adjusted to 7.4 with 40% sodium hydroxide.

Standard Solution of Lecithinase in Glycerol:

A quantity of the culturel filtrate (prepared as described on 10-12) was dialyzed in cellophane tubing at room temperature against three daily changes of glycerol. The glycerol solution of the protein was then standardized by injecting graded amounts into the lateral tail veins of 35 g. mice and noting death or survival within 24 hours. If, for instance, 0.5 ml. of toxin diluted 1:50 killed all mice but 0.4 ml. of the same toxin did not kill any, then the original toxin solution was labeled 100 mouse minimum lethal dosage (MLD) per ml. The lecithinase solution which was used in column work was not standardized by mouse injection.

2/ Also referred to as isotonic borate buffer

Egg Yolk Substrate:

One egg yolk was uniformly suspended in 2.5 l. of borate buffer which was preserved with three or four large crystals of thymol or 1 part Roccal [3] to 375,000 parts of buffer. Roccal did not protect the substrate from decomposition for as long as thymol. The mixture was allowed to stand for one day at room temperature, centrifuged, filtered through a mat of Super-Cel [4] diatomaceous earth, and allowed to stand for another day. The suspension was then refiltered until the turbidancy of the solution as read against a water blank falls between 0.04 and 0.15 at 650 mμ as read with a round tube of 1.9 cm. diameter in a Coleman spectrophotometer. The substrate was then standardized against the standard solution of toxin in glycerol by the procedure described below.

Procedure:

A quantity of the enzyme solution in glycerol was added to borate buffer, and dilute base was added if necessary to bring the pH to 7.4. The solution was then diluted to 5.00 ml. with isotonic borate buffer. Five ml. of egg yolk substrate was then added and the mixture incubated at 37°C. for exactly 20 minutes. The turbidity which developed in the suspension was read in a spectrophotometer at 650 mμ against a blank containing 5.0 ml. of isotonic borate buffer and 5.0 ml. of egg yolk substrate. An almost linear relationship existed between the quantity of lecithinase and the turbidity developed.

3/ Roccal - Registered trade mark, Benzalkonium chloride (alkyldimethyl-ammoniumchloride).

4/ Super-Cel, Registered trade mark.

The activity of the unknown was converted to MLD from the standard curve which was plotted daily for the particular batch of substrate used in the assay.

The variance between successive identical lecithinase assays was found to be 0.02 MLD.

Calcium ion, which is essential for the action of the lecithinase on its substrate, is not only precipitated by the polymetaphosphate (34), but the precipitate itself contributes to the measured turbidity of the system. When the amount of PMP introduced into the assay tube was less than 0.1 mg., only a small error resulted in the lecithinase analysis. Swingle (4) found that maximum lecithinase activity occurs in a calcium ion range between 0.006 M to 0.04 M. In precipitates of relatively high specific activity to phosphate ratios, the polymetaphosphate interference was negligible.

Determination of Nitrogen or Protein

Acid Precipitation Studies:

Nitrogen was determined by the manometric method of Van Slyke (29). The only modification employed was that, after digestion and boiling down to the first appearance of white fumes, 5 ml. of water was added to insure the decomposition of the potassium peroxydisulfate.

Crude Lecithinase Placed on the Diethylaminoethylcellulose Column:

Crude lecithinase was first analyzed for protein content by the Biuret method (30). The Biuret color intensities were read on the Coleman or Beckman spectrophotometer at 540 mμ. A curve, Curve 1, was then plotted of absorbance vs. milliliters of protein solution.

According to Gornall, (30) 1 mg. of protein dissolved in 3 ml. of water in a 1 cm. cell will give an absorbance of 0.151. From Curve 1, the volume of protein solution giving an absorbance of 0.151 was determined, and the concentration of the solution in mg. of protein per ml. calculated. Then appropriate volumes of the protein solution were treated with the Lowry reagents, (31) and the absorbance obtained was used to plot Curve 2 (absorbance vs. ml. of protein solution). From Curve 2, the volumes of protein solution required for an absorbance of 0.1, 0.2, 0.3, and 0.4 were read. Then the weight of protein in these solution volumes was calculated from the Biuret value of mg. of protein per ml. and a new curve, Curve 3, was plotted using the absorbance values of Curve 2 against mg. or µg. of protein contained in these volumes of solution. This standard curve was then used to convert absorbance of a sample of eluate protein solution from the column, as determined by the Lowry method, (31) to the weight of protein which the solution contained.

Diethylaminoethylcellulose Column Eluates:

Protein content in column eluates was determined by the method of Warburg and Christian, (32) and Lowry, (31). In the Warburg method (32) absorbance of the protein solution at 260 mµ and 280 mµ was converted to mg. of protein by the use of a monograph.

Miscellaneous Analyses

Nitrogen in DEAE Cellulose and some protein determinations was determined by standard Kjeldahl (33) procedures. Sodium polymetaphosphate

was precipitated as barium polymetaphosphate and hydrolyzed to orthophosphate according to the method of Jones (34). The phosphate was then determined by the modified method of Fiske and Subbarow (35). Organic phosphate in the crude lecithinase was hydrolyzed with hydrogen peroxide or perchloric acid and determined by the modified method of Fiske and Subbarow.

Crude Lecithinase:

Culturel filtrate protein, separated by the ammonium sulfate precipitation, was analyzed for nitrogen and phosphorus according to the methods already mentioned (29, 33, 35). Protein-bound hexose was determined by the method of Lustig and Langer, (36) and Weimer and Moshin (37). The presence of nucleic acids in the protein solution was indicated after consideration of values obtained in the Warburg ultraviolet absorption procedure and by paper chromatography. The latter procedure involved developing the spot on paper with Pabst solvents (38) and then viewing the chromatogram with a SL Mineral light, Model V-41, which provides light at 253.7 mμ.

The presence of lipoprotein in crude lecithinase was shown by treating a paper chromatogram with Sudan black (39).

PREPARATION OF MATERIALS

Crude Lecithinase

Culture Medium and Toxin Production:

The crude lecithinase which was used for acid precipitation of lecithinase was prepared from the culturel filtrate of Clostridium hemolyticum (Montana Veterinary Research Laboratory, strain number 4473). The bacteria were grown in the peptic digest medium of Jasmin (3) modified by Swingle and Smith (40).

The peptic digest medium (4) was prepared by placing 500 g. of ground lean beef, 500 g. of beef liver, and 750-1000 g. of ground fresh pig stomach in 2000 ml. of water. The pH of the mixture was brought to 2 with hydrochloric acid and 10-20 g. of pepsin added. The mixture was digested at 50°C. for 12 hours, heated on the steam bath at 80-90°C. to stop peptic action, refrigerated overnight, and filtered through a mat consisting of 1 cm. of Super-Cel (diatomaceous earth). The pH of the filtrate was adjusted to about 7.3 with 5 N sodium hydroxide and 0.0375 per cent calcium chloride (W per V) was added, the solution heated at 90°C. for 30 minutes, and the precipitate filtered off through a mat of Super-Cel. The filtrate was the peptic digest, abbreviated PD.

Two volumes of PD were diluted with 1 volume of water. Then proteose peptone, 1 per cent, and glucose, 0.5 per cent, were added and the solution brought to a pH of 7.6 with sodium hydroxide. The medium was then sterilized in an autoclave.

An inoculum of Clostridium hemolyticum was transferred from the stock culture in Hall's medium 5/ into a tube containing about 20 ml. of the complete PD medium. This was incubated for about 6 hours at 37°C. and poured into a small flask containing about 200 ml. of fresh medium. After incubating for the same time interval, this culture was poured into a large flask three-fourths filled with medium, which had been recently heated and then cooled to 37°C. After a growth time of about 6 hours at 37°C., the pH of the medium was adjusted to 7.6 with 5 N sodium hydroxide and the liquid

5/ Hall's medium is probably similar to Holman's cooked meat medium with modification.

cooled to about 4°C. The refrigerated culture was centrifuged at an RCF 6/ of about 2000 until clear, and the supernatant filtered through a mat of Super-Cel. The filtrate is referred to as culturel filtrate.

The crude lecithinase used in the more recent work with the DEAE cellulose column was prepared according to the modified procedure of Claus (42). This medium was prepared as follows: equal weight of finely ground liver and water were mixed in a Waring blendor. Concentrated hydrochloric acid was added to a pH of 2-2.5. Then 0.50 g. of Difco pepsin per 100 g. of liver was stirred in the suspension, and the mixture placed in a water bath at 50°C. for 15 hours. After cooling the suspension to 15°C., it was centrifuged at moderate speed and the supernatant filtered through a mat, 1 cm. thick, of Super-Cel. The pH of the filtrate was then adjusted to 7.3 with 5 N sodium hydroxide and calcium chloride dihydrate added to a concentration of 0.5 mg. per ml. of solution. The solution was steamed to 90°C. for 30 minutes, cooled, and filtered through Super-Cel. An equal volume of water was added, plus trypticase to 1 per cent and soluble starch to 0.5 per cent. The pH of the solution readjusted to 7.3 with 5 N sodium hydroxide, after which the medium was autoclaved.

After growth of the bacteria and preparation of the culturel filtrate, the liquid, if passed through a Seitz filter, is referred to as the Seitz filtrate.

6/ Relative centrifugal force (a 1 pound mass spun at an RCF of 1500 would weigh 1,500 pounds.)

Precipitation and Dialysis of Toxin:

The crude lecithinase was precipitated from the cultural filtrate with ammonium sulfate. The filtrate was first made to 16 per cent by weight with ammonium sulfate, and solids, if any, were removed by centrifugation, RCF of 1000 and discarded. Additional ammonium sulfate was then added to bring the solution to the final content of 31 per cent by weight. The precipitated lecithinase was collected by centrifugation and dialyzed in a Vistex 7/ membrane against running distilled water at 10°C. In some cases tap water at 3-5°C. was used for 3-4 days until a chemical test for the ammonium ion or a conductivity test showed the near absence of ammonium sulfate in the dialysate.

At this stage the nucleic acid separation of Van Heyninger and Bidwell (43) was used for the protein which was to be placed on the DEAE cellulose column. The procedure for the Van Heyninger and Bidwell (43) separation was as follows: the dialysee in the Vistex membrane was centrifuged for 10 minutes at an RCF equals 1500 after which the precipitate was discarded. The pH of the supernatant was adjusted to 7 with 5 N sodium hydroxide and the solution cooled to near 0°C. Ice cold 1 per cent ribose nucleic acid, in the amount of 4.7 ml. per 100 ml. of lecithinase solution, was added with stirring, and the pH of the solution adjusted to 4.5 with 6 N acetic acid. The solution was allowed to stand for 1 hour at this temperature. Centrifuge cups were then removed from the deep freeze, and the precipitate was quickly centrifuged from the acetic acid solution at an RCF of 1500. Only a small precipitate was obtained. This precipitate

7/ Vistex, Registered trade mark.

was saved, and the supernatant was readjusted to pH 7 with sodium hydroxide. Additional 1 per cent ribose nucleic acid solution was added in the amount of 14.1 ml. per 100 ml. of lecithinase solution. The pH of the solution was then adjusted to 4.5 with 6 N acetic acid, and the suspension allowed to stand for 1.5 hours at 3-5°C. after which a heavy percipitate was obtained. The precipitate was separated from the liquid by centrifugation at RCF equals 1500 and the two precipitates combined in about 100 ml. of ice cold water which contained 2 drops of 5 N sodium hydroxide to dissolve the precipitate. To remove the protamine nucleate, the pH of the solution was adjusted to neutrality with dilute hydrochloric acid, and ice cold 1 per cent protamine sulfate added drop by drop until no further precipitate of protamine nucleate formed. After removing the precipitate by centrifugation at RCF equals 1500, 1 per cent ribose nucleic acid was added to just precipitate any excess protamine sulfate. The solution was then recentrifuged at an RCF equals 1500.

The final solution was light straw-colored, faintly opalescent and kept frozen at -15°C. until used. Almost one year passed before the protein separation was made on the DEAE cellulose column.

In the acid precipitation part of this investigation, the protein solution was lyophilized after dialysis, without the nucleic acid purification step.

Sodium Polymetaphosphate:

The polymer was either prepared by the fusion method (34) of Jones

or purchased as "Calgon".[8] (The analysis is described on page 9 and 10).

N, N-Diethylaminoethylcellulose:

The Eastman compound number 7392 was used. This DEAE cellulose was found to be somewhat hygroscopic and in purified form contained about 0.1 per cent less nitrogen than Sober's product (23).

If proteins in the column eluate are to be determined by using the Lowry method, any decomposition of DEAE cellulose must be avoided since the Lowry reagents will develop color with N,N-diethylamine. Moore and Lee (45) found that Eastman DEAE cellulose would yield nitrogenous products to the column eluate. The DEAE cellulose used in this investigation was purified according to their procedure, which involves treating the DEAE cellulose with 1 N sodium hydroxide, washing with water to a supernatant pH of 7, then washing with ethanol and finally with ether.

Buffers:

TRIS (hydroxymethylamino) methane (2-amino-2-hydroxylmethyl-1,3-propanediol) buffer (46) has been used on the DEAE cellulose column (27).

The product used in this investigation was labeled Sigma 7-9. The compound was twice crystalized from water. The final product absorbed in the ultraviolet in agreement with the value obtained by Boman and Westlund (26). (0.05-0.06 for a 0.5 M solution).

8/ The commercial preparation, Calgon, is a glass with a ratio of $Na_2O:P_2O_5$ of 1.1:1. It is made by fusing food grade phosphoric acid and commercial soda ash and is commonly called hexametaphosphate. The unadjusted form used in this study does not contain sodium carbonate.

Weights of constituents in 0.1 M buffer as used in acid precipitation of lecithinase-PO_3 are:

2-amino-2-methyl-1,3 propanediol	10.5 g.
Glacial acetic acid	3.0 g.
Distilled water to	1.0 l.

ANALYSIS OF CRUDE LECITHINASE

A one ml. sample of crude lecithinase as prepared by ammonium sulfate precipitation, dialysis of the precipitate, and centrifuging off any insoluble material was found to contain 12.4 mg. of solids per ml. of solution. Of this weight, 1.07 mg. was protein-bound hexose. The Biuret protein value calculated according to Gornall (30) was 2.2 mg. The calculated protein value using a factor of 6.25 for the Kjeldahl nitrogen was 3.8 mg. of protein. Very likely other nitrogen-containing compounds are present.

Lipoproteins were shown by a qualitative test involving Sudan black (39).

Electrophoresis of the crude lecithinase at a pH near 8 in the Perkin Elmer apparatus showed a large number of different proteins, so many proteins, in fact, that a photograph of the pattern showed broad heavy bands which gave no useful information.

Light absorption by the Seitz filtrate [9] at 260 mμ was intense, indicating the presence of rather large quantities of nucleic acids. Paper chromatograms of the protein viewed under ultraviolet light further indicated the presence of nucleic acids.

9/ Medium after growth of Clostridium hemolyticum centrifuged and run through diatomaceous earth twice, then through a Seitz filter.

PART I: ACID PRECIPITATION OF THE LECITHENASE -PO_3 COMPLEX AS MEANS OF INCREASING THE SPECIFIC ACTIVITY OF THE LECITHINASE

Since one intent of this investigation was to obtain lecithinase with a high specific activity [10], it was necessary to determine optimum physical or chemical conditions for retention of lecithinase activity in lecithinase-PO_3 [11] systems. This determination of the probable maximum specific activity of lecithinase as obtained in the precipitate with optinum conditions requires at least as empirical consideration of the nitrogen, lecithinase and PMP [12] equilibria existing between soluble and insoluble components in the various systems at certain acid contact times.

Swingle (4) has determined the stability of the impure enzyme in various systems under certain types of stresses, such as temperature, agitation, and adverse hydrogen ion concentration. The lecithinase-PO_3 complex was not investigated.

A. EXPERIMENTAL PROCEDURES

1. Stability of the Lecithinase in the Presence of Polymetaphosphate in Acid Solutions:

A weighed quantity of the crude lecithinase was taken up in several drops of 0.5 N sodium hydroxide solution and the slightly turbid solution diluted with buffer to a protein concentration of 0.5 per cent. Five meq. [13] of SPMP per gram of protein were added to half this solution. The remainder was used for controls. Three ml. aliquots were taken from

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10/ Specific activity refers to the MLD per mg. of nitrogen ratio in the protein mixture.

11/ A designation for the protein-polymetaphosphate complex. Perlman (17) considers the reaction between PO_3 and albumin.

12/ PMP, Sodium polymetaphosphate.

13/ 1 meq. of PMP equals 0.102 g. of the polymer.

both the SPMP solution and the control. The pH of the aliquots was adjusted with 0.5 N or 2 N sulfuric acid (the 0.5 N acid was added first and, if necessary, the 2 N acid was used). After the pH was adjusted the solutions were diluted with water to a final volume of 4.5 ml. One ml. portions of the 4.5 ml. solutions were measured into tubes marked at 2 ml. and the tubes allowed to stand for the indicated acid contact times at room temperature. The precipitate was then dissolved in 0.5 N sodium hydroxide. Results are presented in Figure 1, 2 and 3.

2. The Effect of pH on the Stability of Lecithinase-PO_3:

Crude lecithinase (0.5 per cent) plus one-half its weight of SPMP was dissolved in the buffer at pH 8-9. The protein alone in the buffer gave a suspension, but on addition of the SPMP the liquid cleared. Five protein-PMP solutions were adjusted to the intended pH values (1-5), and one ml. of each solution was placed in a calibrated tube. After the indicated time interval, sodium hydroxide (0.5 N) was added to bring the pH to 7-8, and sufficient isotonic borate buffer [14] was added to bring the solution to volume. The lecithinase activity was determined for 5 pH values, two acid contact times, and two temperatures (3-5°C. and room temperature). A second set of readings was taken for the pH range 5-12.1 with acid contact times of 4 hours at room temperature. The results are shown in Figure 4.

14/ Borate buffer, calcium chloride and sodium chloride solution at the same concentrations as used in the lecithinase determination (see page 6).

3. Lecithinase Purification as a Function of Acid Contact Time:

The lecithinase precipitates were obtained as follows: crude lecithinase, specific activity about 75, plus one-half its weight of SPMP was dissolved in 0.1 M, 2-methyl-2 amino-1, 3 propanediol buffer. The solution was divided into three parts. The pH of the solutions was then adjusted to 2.37, 3.72 or 5.0 with 0.5 N sulfuric acid. Aliquots of the solutions were then allowed to stand at 3-5°C. At a specified acid contact time, one tube was centrifuged (RCF equals 1500), the supernatant discarded, the precipitate washed twice with distilled water, and then dissolved within 45 seconds by mixing with several drops of 0.4 N sodium hydroxide. Isotonic borate buffer at a pH of 7.5-8.0 was immediately added and lecithinase activity determined in the solution. The nitrogen content of the precipitate was determined by the method of Van Slyke (29). Lecithinase by the method of Swingle (4). The values [15] have been plotted in Figure 5.

4. The pH at Which the Lecithinase-PO_3 Precipitate Forms for one Ratio of PMP and Protein:

In the experiment in which an aqueous solution was used, bacterial filtrate containing 1.0 per cent of unadjusted Calgon, (analyzing 75 per cent polymer) and 24 per cent sodium chloride was adjusted to the desired pH values with 0.5 N sulfuric acid and the solution allowed to stand at -15°C. for three and one-half hours. The tube was then centrifuged, the precipitate separated from the supernatant, dissolved, taken up in borate buffer and the lecithinase activity determined by the method of Swingle (4).

15/ The purification factor is the ratio of the specific activity of the final material to that of the starting material.

The supernatant solution was then used for the next separation at a lower pH value. (See Figure 6).

5. Purification of Lecithinase in Bacterial Filtrate at Acid pH Values:

Bacterial filtrate containing 1.0 per cent PMP, 25 per cent sodium chloride, and 12 per cent (v/v) glycerol was swirled at -15°C. during the 15 minute acid contact time. For the first precipitation, the pH was adjusted with 1.0 N sulfuric acid; on subsequent precipitations of the previous precipitate, 0.24 N acid was used. The precipitate was dissolved in borate buffer containing 25 per cent (v/v) glycerol and an aliquot of the solution analyzed for lecithinase and nitrogen by the same methods as were used in the aqueous solution. The data for the aqueous experiment are shown in Figure 6. Figure 7 presents the data as obtained in the glycerol experiment.

6. Concentration of Components in the Crude Lecithinase-PO_3 Precipitate as a Function of Concentration of Polymetaphosphate and the pH of the Solution:

Crude lecithinase, about 15 MLD per mg. of protein, specific activity of 94 (protein factor 6.25), was suspended in 2-methyl-2-amino-1, 3 propanediol-acetic acid buffer (each at 0.1 M) at a pH near neutrality. Then several drops of 1.0 N sodium hydroxide were added to bring the pH to near 8.5. (In this adjustment, care must be taken that higher pH values are not used, since the lecithinase is more susceptible to inactivation at pH values above 8.5). The solution cleared to a faint turbidity. Weighed solid PMP (95.6 per cent polymer) was then added and the solution was rapidly swirled for several minutes for complete solution of the PMP. The

solution became crystal clear. Aliquots of the solution were then adjusted to the desired pH with 0.5 N sulfuric acid which was added drop by drop with swirling. After an acid contact time of 10 hours at room temperature or 110 hours at 3-5 °C., the precipitate was recovered by centrifugation at RCF equals 1500 and the supernatant liquid discarded. The drained precipitate was dissolved in 0.5 N sodium hydroxide, taken up in isotonic buffer, pH 7.4, and the lecithinase activity, nitrogen content, and phosphorus determined. Lecithinase units are given in MLD per mg. of nitrogen; however, a primary standard was not available. Results and calculated ratios are shown in Table I. Statistical data in the form of an analysis of variance for F distribution significance are presented in Table II. The data have been plotted in Figures 8 to 14.

B. RESULTS

The area between Curves 1 and 2, Figures 1, 2, and 3, decreases from an acid contact time of 4 hours to an acid contact time of 72 hours in the pH range 2 to 4. In general, lecithinase recovery in a solution having a pH below 4 decreased with an increase in acid contact time. In each figure it is seen that Curve 1 intersects Curve 2 between pH 4 and 5. With increasing acid contact time, the point of intersection approaches pH 4. At pH 5 recovery in the PMP solution becomes less with increasing acid contact time.

Lecithinase recovery in 2-methyl, 2-amino-1, 3 propanediol-acetic acid buffer as plotted in Figure 4 shows the dependency of lecithinase stability on the pH of the solution. In the acid region, curves for lecithinase recovery for different acid contact times and solution temperatures were well separated. Least lecithinase recovery occurred at pH 5-5.5 and near 12 and at acid pH values at room temperature. Good recovery occurred at pH 7 to 10 and at acid pH values. Curve 3 with an acid contact time of 24 hours at 3-5°C. shows a lecithinase recovery of over 100 per cent.

Lecithinase purification as a function of acid contact time in Figure 5 shows the highest purification factor at pH 3.72, the lowest factor occurred in the most acid solution.

In Curves 1 and 2, Figure 6, it is seen that nitrogen in the precipitate attains a peak at one pH value and the maximum purification factor shows a peak at a pH value about 0.5 pH units lower.

Figure 7 shows a relatively large decrease in the nitrogen content of the precipitate as formed by reprecipitation of one precipitate. The lecithinase purification factor increased to precipitation number 3 after which there was a decrease and then an increase in the factor. The amount of precipitate in the last precipitations made the determination of nitrogen difficult. These values being somewhat in doubt, are indicated with a dotted line on the graph. The lecithinase in these precipitates contained less than one per cent of the total starting material.

In Figure 8 the specific activity was at a maximum for pH 4.5 and 2 meq. of PMP per g. of protein. A slight rise for the same polymer concentration also occurred at pH 3.5. The best over all specific activity was obtained at 3-5°C., Figure 9. Good recovery occurred over a wider range of PMP (almost 0 to 2) at a pH value of 4.5.

The MLD/mg. P curve at room temperature, Figure 10, showed a maximum at pH 4.5 and with 3 meq. of PMP per g. of protein. The ratio of MLD/mg. P increased at a lower temperature, Figure 11, and the maximum was obtained with a smaller amount of polymer (2 meq. of PMP per g. of protein at pH 4.5). Figures 9 and 11 show a rough similarity in the general shape of the curves. The same observation can be made for Curves 8 and 10.

Figure 12 supports the general observation that size of the precipitate increases with a decrease in pH of the solution. In the intermediate acid pH range, more nitrogen appeared in the precipitate at 3-5°C. than at 25°C.

In Figure 13, the cross over between the curves for the two temperatures occurs at pH 3.5.

Figure 14 shows that the P/N ratio at room temperature varied with the amount of polymer per g. of protein.

Table I

The concentration of lecithinase, nitrogen, and phosphorus in precipitates obtained at three pH values and various concentrations of polymetaphosphate, (PMP) at room temperature and at 3-5°C.

pH	PMP* (meq./g. protein)	Lecithinase recovery (MLD's/ppt.)	Lecithinase recovery (per cent)	N (mg./ppt.)	P (mg./ppt.)	MLD's per mg.N	MLD's per mg.P	Molar ratio of P:N
			Acid contact time 10 hours at room temperature					
2.5	5.0	440	37	1.06	0.63	415	700	0.27
3.5		440	37	0.97	0.33	454	1330	0.15
4.5		325	27	0.48	0.12	678	2710	0.11
2.5	4.0	352	32	1.58	0.50	222	704	0.14
3.5		415	37	1.06	0.34	392	1220	0.14
4.5		345	31	0.49	0.13	704	2650	0.12
2.5	3.0	340	30	1.47	0.61	196	557	0.16
3.5		435	38	0.99	0.42	438	1035	0.19
4.5		290	25	0.30	0.09	967	3190	0.14
2.5	2.0	345	32	1.40	0.66	246	523	0.21
3.5		380	35	0.71	0.47	536	810	0.30
4.5		310	28	0.21	0.16	1510	1940	0.34
2.5	1.0	330	34	0.82	0.61	404	542	0.33
3.5		355	36	1.20	0.48	297	740	0.18
4.5		310	32	0.57	0.17	548	1820	0.14
2.5	0.10	335	32	0.66	0.25	506	1340	0.17
3.5		365	35	0.72	0.22	505	1650	0.14
4.5		330	31	0.49	0.13	676	2540	0.12
2.5	0.01	54	5	0.22	0.04	246	1350	0.08
3.5		270	26	0.20	0.06	143	4500	0.14
4.5		250	24	0.33	0.06	758	4170	0.08
2.5	0.00	86	8	0.02		5500		
3.5		88	8	0.01		7000		
4.5		48	5	0.01		3380		

* One meq. of sodium polymetaphosphate per gram of protein equals 0.102 grams of polymer per gram of protein.

Table I continued

pH	PMP (meq./g. protein)	Lecithinase recovery (MLD's/ppt.)	Lecithinase recovery (per cent)	N (mg./ppt.)	P (mg./ppt.)	MLD's per mg.N	MLD's per mg.P	Molar ratio of P:N
			Acid contact time 110 hours at 0°C.					
2.5	10.00	368		2.10	0.50	175	734	0.11
3.5		386		0.94	0.34	412	1120	0.16
4.5		266		0.34	0.14	784	1860	0.19
2.5	5.0	545	55	0.99	0.83	552	657	0.38
3.0		540	55	1.11	0.61	484	887	0.25
3.5		565	57	0.47	0.43	1200	1310	0.42
4.0		355	36	0.28	0.22	1260	1610	0.36
4.5		78	8	0.06	0.04	1300	1950	0.30
2.5	4.0	545	62	1.61	0.82	338	665	0.23
3.0		655	74	1.68	0.70	391	936	0.19
3.5		590	67	0.55	0.46	1070	1280	0.38
4.0		370	42	0.30	0.20	1240	1850	0.30
4.5		69	8	0.01	0.03	-	2300	0.14
2.5	3.0	575	64	1.04	0.97	554	594	0.40
3.0		610	69	1.70	0.88	338	694	0.24
3.5		610	69	1.52	0.65	402	938	0.19
4.0		340	39	1.08	0.36	315	945	0.15
4.5		52	6	0.46	0.07	113	744	0.68
2.5	2.0	680	86	1.83	0.89	371	764	0.22
3.0		610	77	1.76	0.74	348	825	0.19
3.5		585	74	1.43	0.49	411	1190	0.15
4.0		580	74	0.64	0.23	904	2520	0.16
4.5		168	21	0.08	0.02	2100	-	0.11
2.5	1.0	580	66	1.76	0.97	329	598	0.25
3.0		560	63	2.04	0.96	274	584	0.21
3.5		550	62	1.57	0.56	350	982	0.16
4.0		575	65	0.90	0.32	642	1790	0.16
4.5		320	36	0.16	0.09	2000	3550	0.25
2.5	0.10	193	21	0.79	0.22	244	878	0.13
3.5		405	44	0.68	0.22	598	1840	0.14
4.5		400	44	0.19	0.16	2100	2500	0.38

Table I continued

pH	PMP (meq./g. protein)	Lecithinase recovery (MLD's/ppt.)	Lecithinase recovery (per cent)	N (mg/ppt.)	P (mg/ppt.)	MLD's per mg.N	MLD's per mg.P	Molar ratio of P:N
2.5	0.01	205	26	0.50	0.12	410		0.11
3.5		590	75	0.49	0.11	1200		0.10
4.5		600	76	0.42	0.10	1420		0.11
2.5	0.00	14	2	0.11	0.0010	1270		0.0041
3.0		28	3	0.05	0.0006	560		0.0054
3.5		103	12	0.10	0.0010	1030		0.0045
4.0		210	25	0.13	0.0008	1610		0.0028
4.5		238	28	0.21	0.0010	1130		0.0021

Table II

Analysis of variance of the data from Table I
Summary of F distribution significance

Source of Variation	Lecithinase (MLD's/ppt.)	N (mg./ppt.)	P (mg./ppt.)	MLD's per mg. N	MLD's per mg. P	P:N
	At room temperature					
pH	*	**	**	-	-	-
Polymetaphosphate	**	**	**	**	*	**
σ_E	0.1656	0.4396	0.4140	0.7184	0.4152	0.5179
Linear regression	-	**	**	-	**	-
Quadratic regression	-	-	-	-	-	-
Control vs. rest	**	**	**	**	*	**
	At 3-5°C.					
pH	-	**	**	*	-	-
Polymetaphosphate	-	-	-	-	*	*
σ_E	0.5269	0.5847	0.6638	1.0590	2.3670	0.5419
Linear regression	-	**	**	*	-	-
Quadratic regression	-	-	-	-	-	-
Control vs. rest	*	*	*	-	-	-

* Significant at the 5 per cent level.

** Significant at the 1 per cent level.

σ_E Estimate of total error.

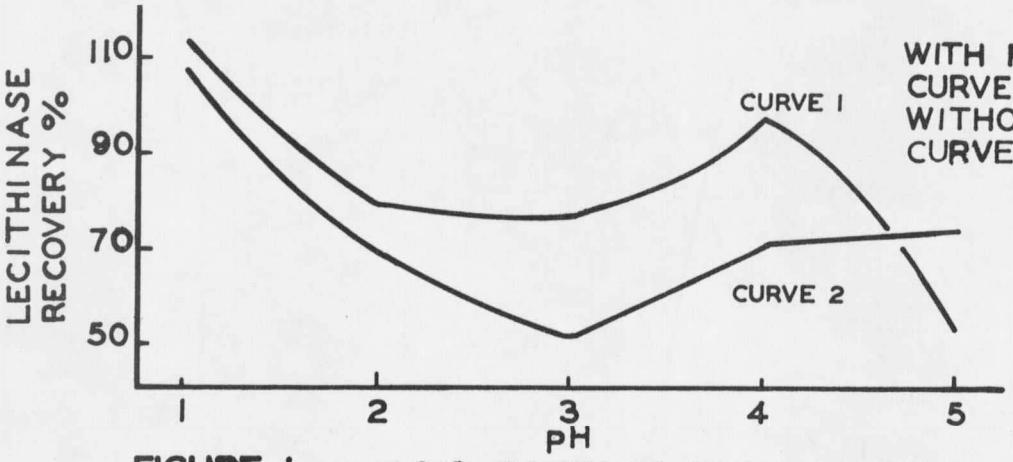

FIGURE 1 ACID CONTACT TIME 4 HOURS

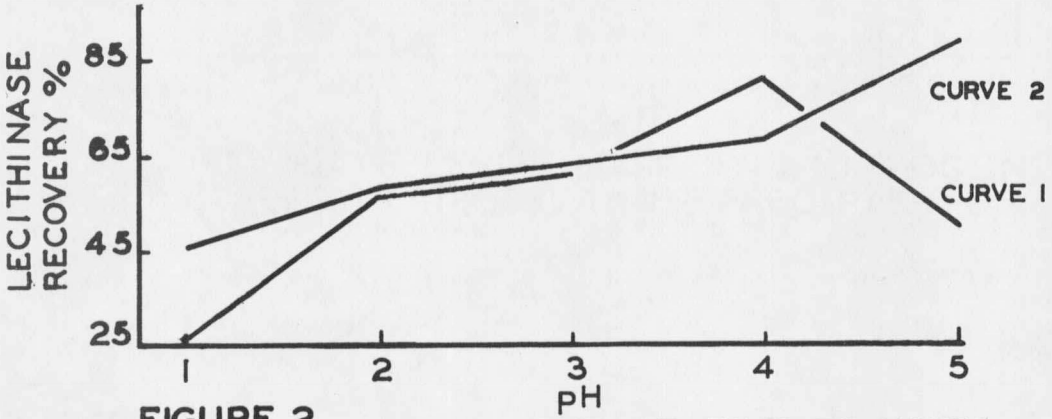

FIGURE 2 ACID CONTACT TIME 72 HOURS

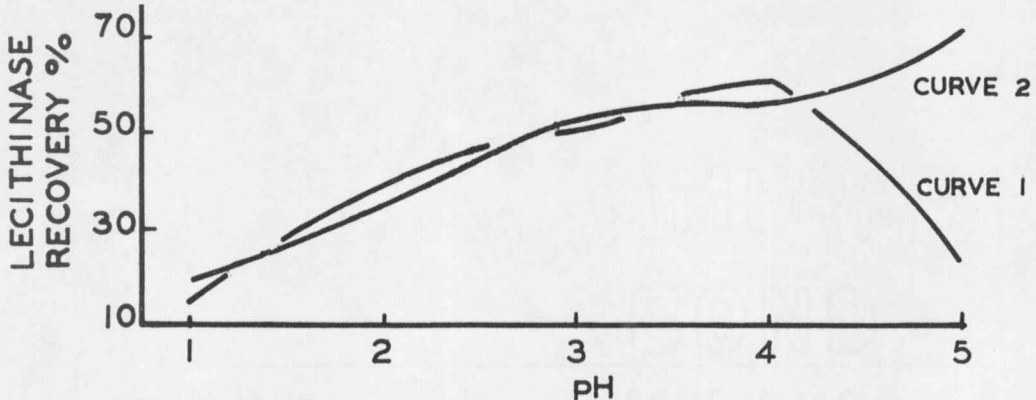

FIGURE 3 ACID CONTACT TIME 180 HOURS

FIGURES 3, 4 AND 5
LECITHINASE RECOVERY (%) IN SOLUTIONS AT ACID pH VALUES WITH AND WITHOUT PMP

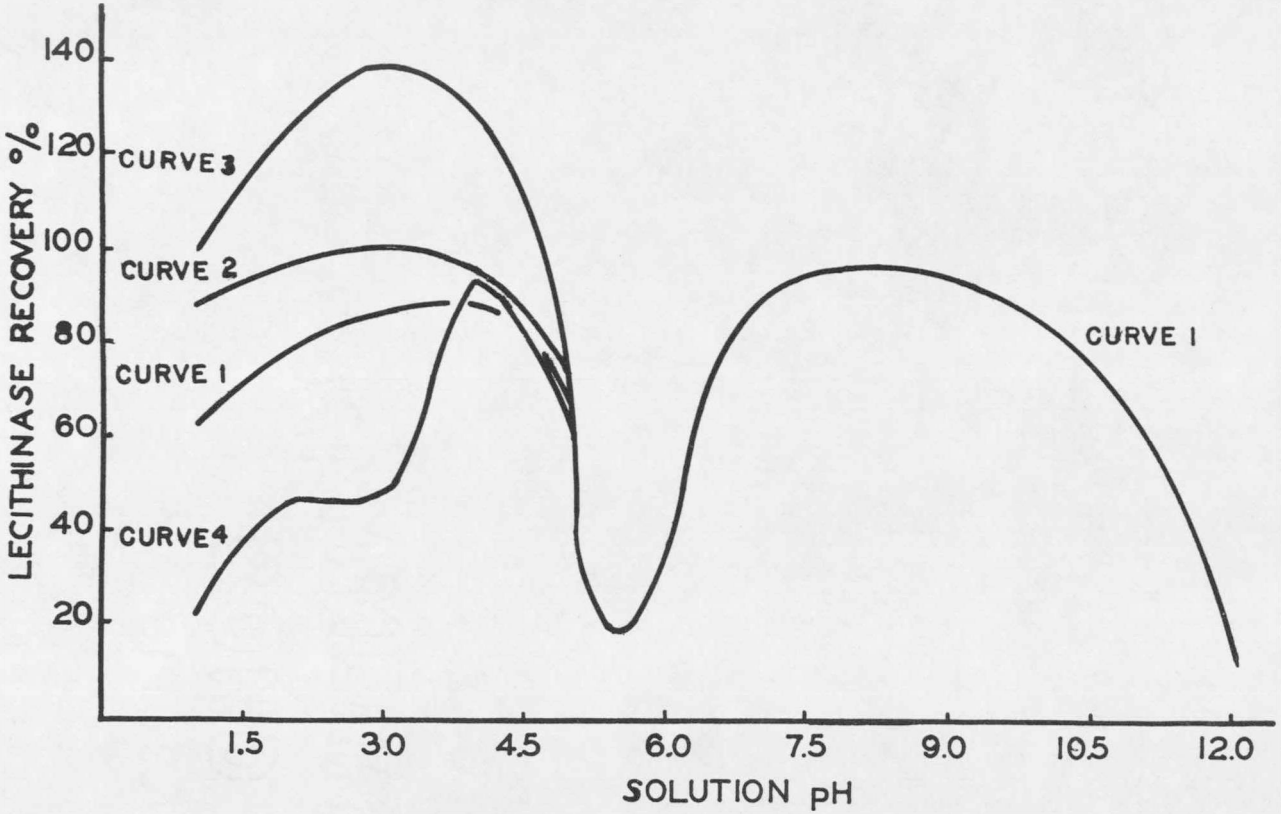

FIGURE 4 ENZYME RECOVERY IN 2-METHYL-2-AMINO 1, 3 PROPANEDIOL- ACETIC ACID BUFFER (1) ACID CONTACT TIME 4 HOURS AT 25 °C (2) AC TIME 4 HOURS AT 3-5 °C (3) AC TIME 24 HOURS AT 3-5 °C (4) AC TIME 24 HOURS AT 23-25 °C

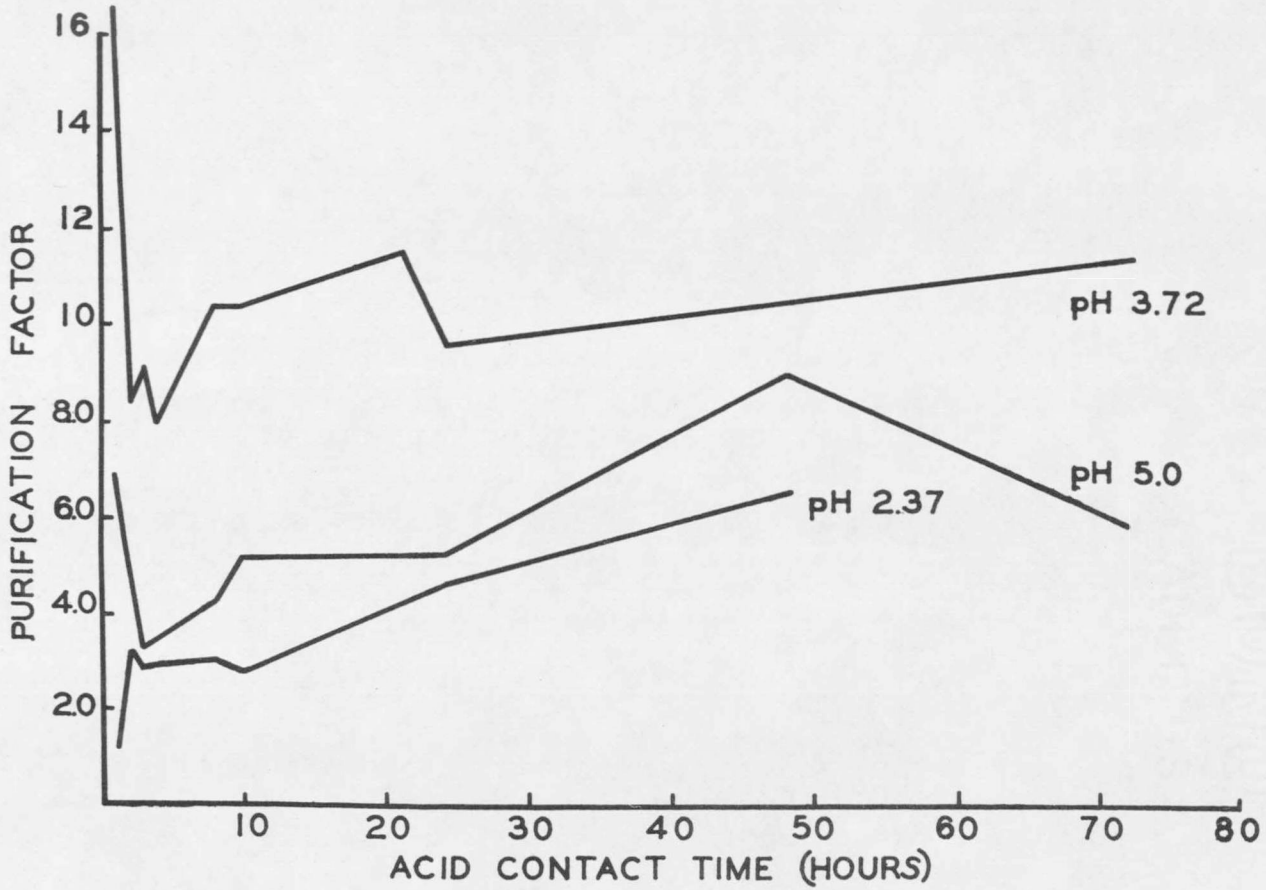

FIGURE 5 LECITHINASE PURIFICATION IN THE SODIUM POLYMETAPHOSPHATE PRECIPITATE AS A FUNCTION OF ACID CONTACT TIME AT 3-5 °C

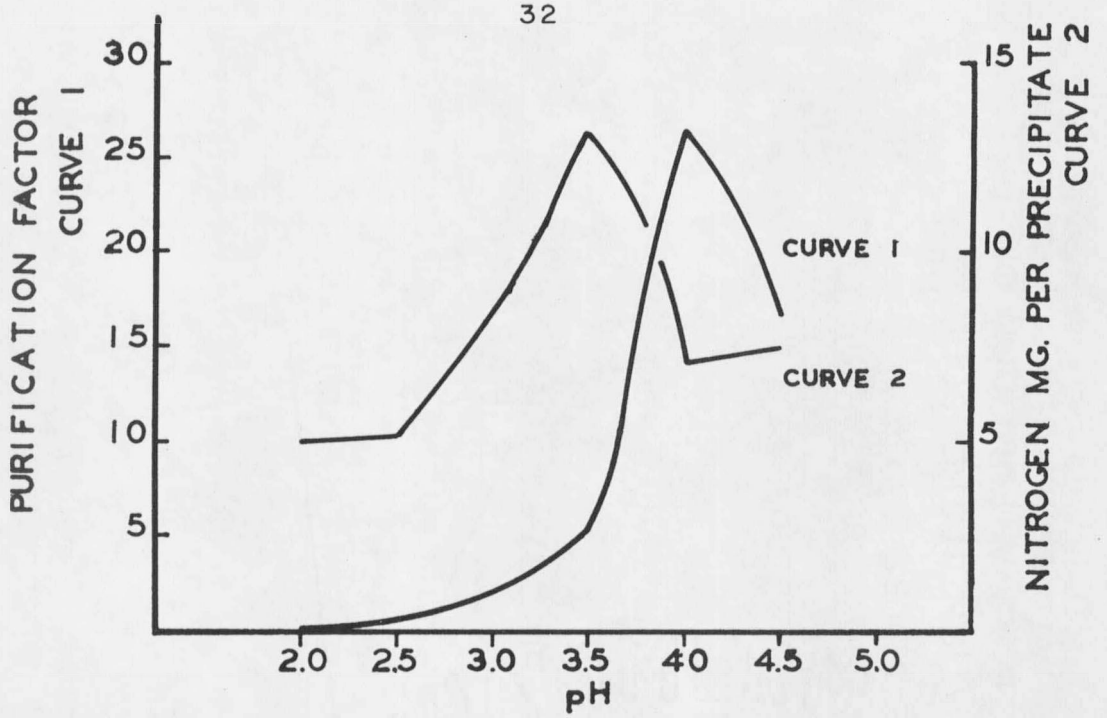

FIGURE 6 LECITHINASE AND NITROGEN RECOVERY IN PRECIPITATES

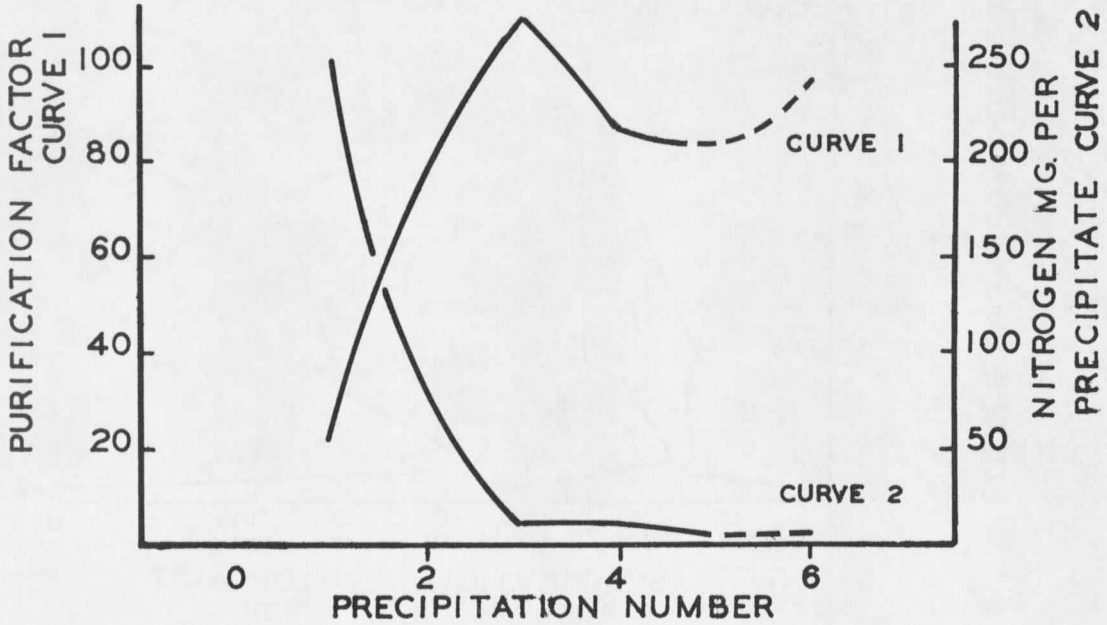

FIGURE 7 RECOVERY OF LECITHINASE BY REPEATED PRECIPITATION IN A GLYCEROL SOLUTION AT pH 2.8

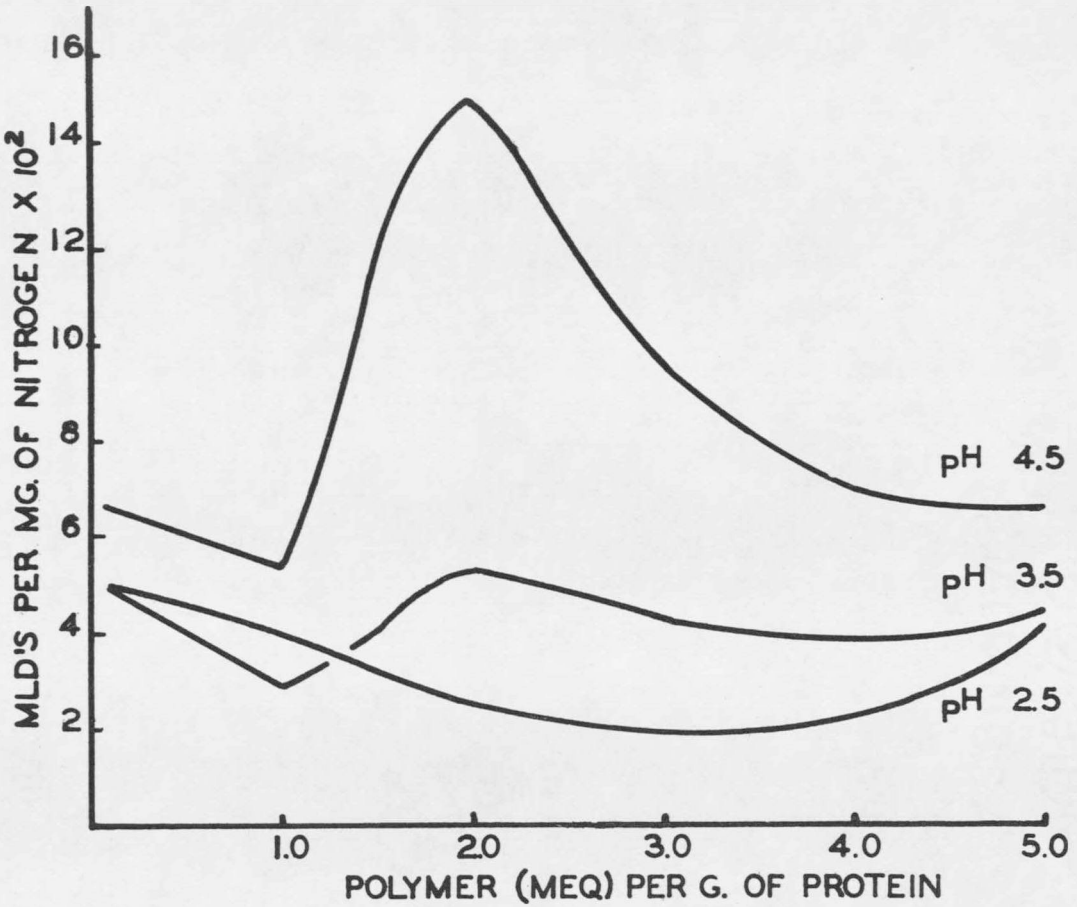

FIGURE 8 LECITHINASE ACTIVITY PER MG. OF NITROGEN AS A FUNCTION OF CONCENTRATION OF SPMP AT A CONSTANT pH AND ROOM TEMPERATURE

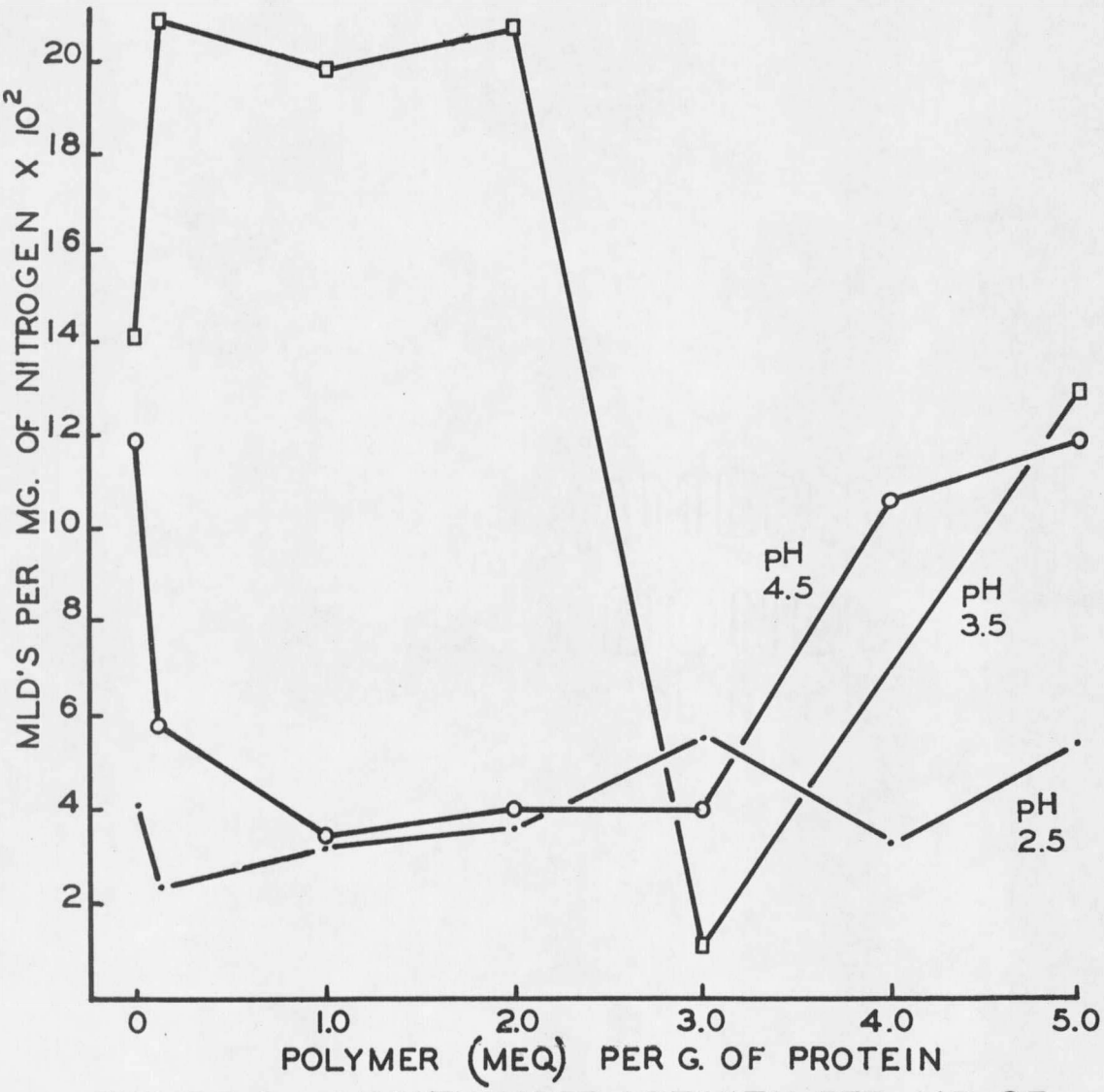

FIGURE 9 LECITHINASE ACTIVITY PER MG OF NITROGEN AS A FUNCTION OF CONCENTRATION OF SPMP AT CONSTANT pH AND 3 - 5 °C

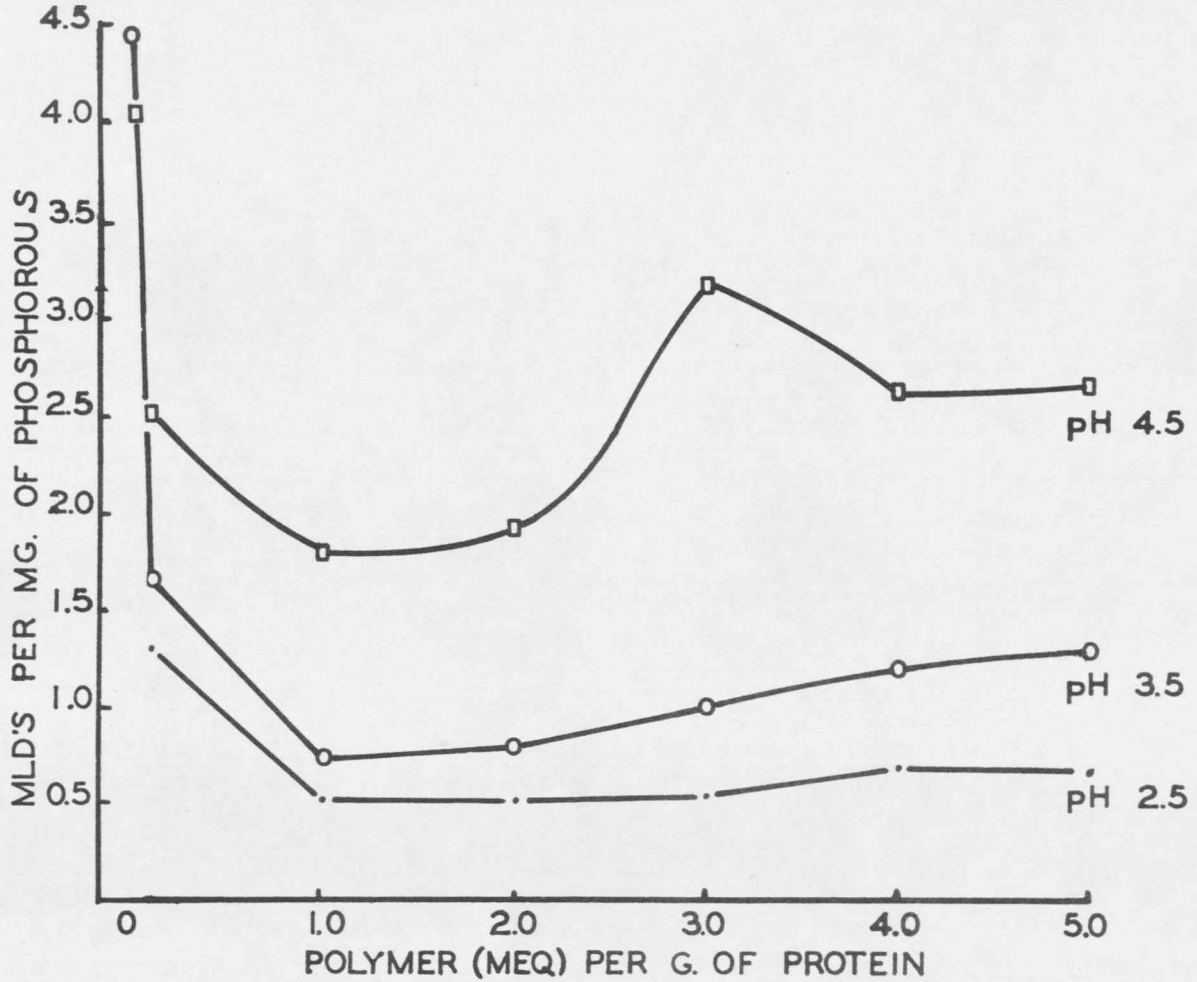

FIGURE 10 LECITHINASE ACTIVITY PER MG. OF PHOSPHOROUS AS A FUNCTION OF THE SPMP CONCENTRATION AT A CONSTANT pH AND ROOM TEMPERATURE

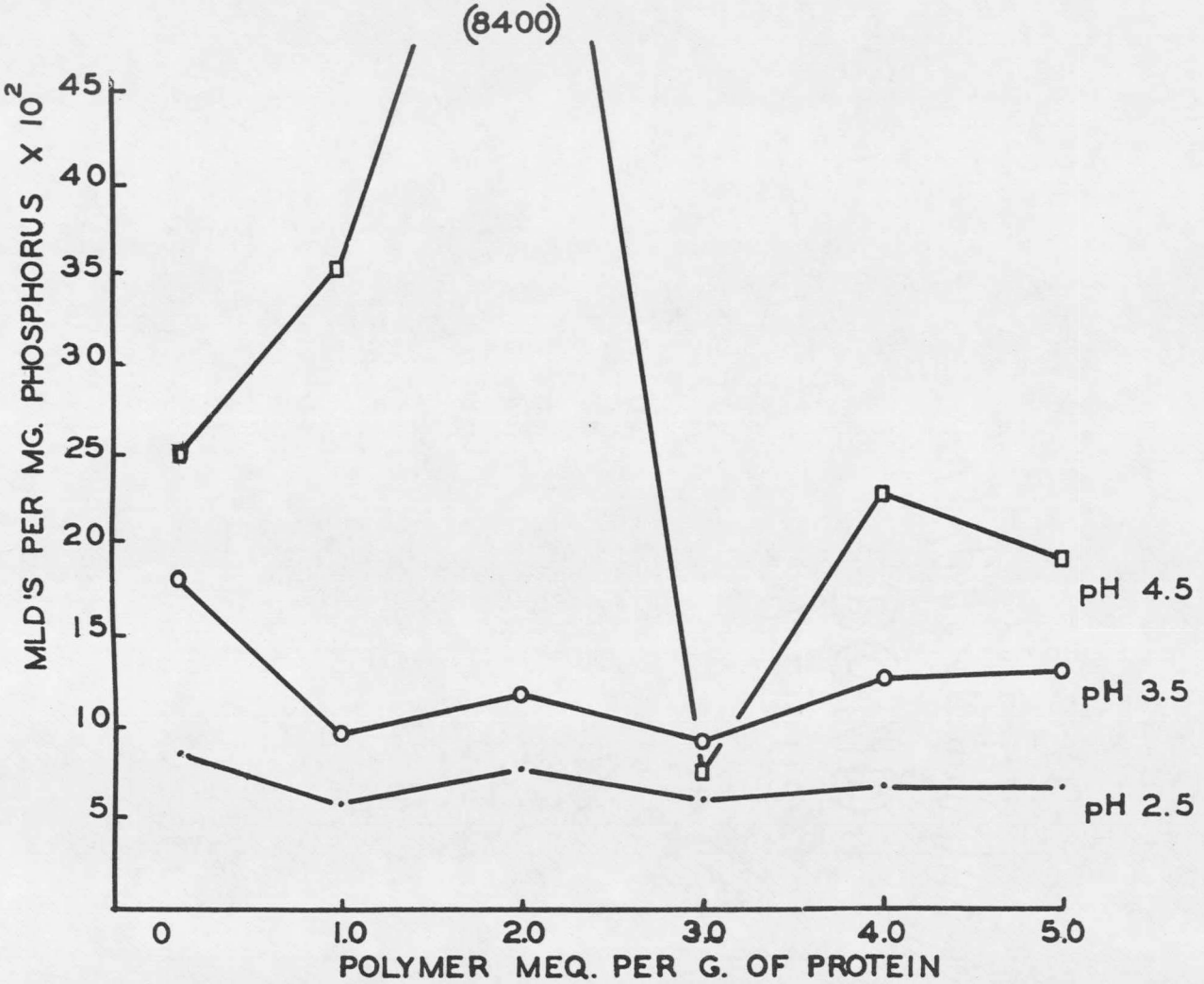

FIGURE 11 LECITHINASE ACTIVITY PER MG. OF PHOSPHORUS AS A FUNCTION OF CONCENTRATION OF SPMP AT CONSTANT pH AND 3-5 °C

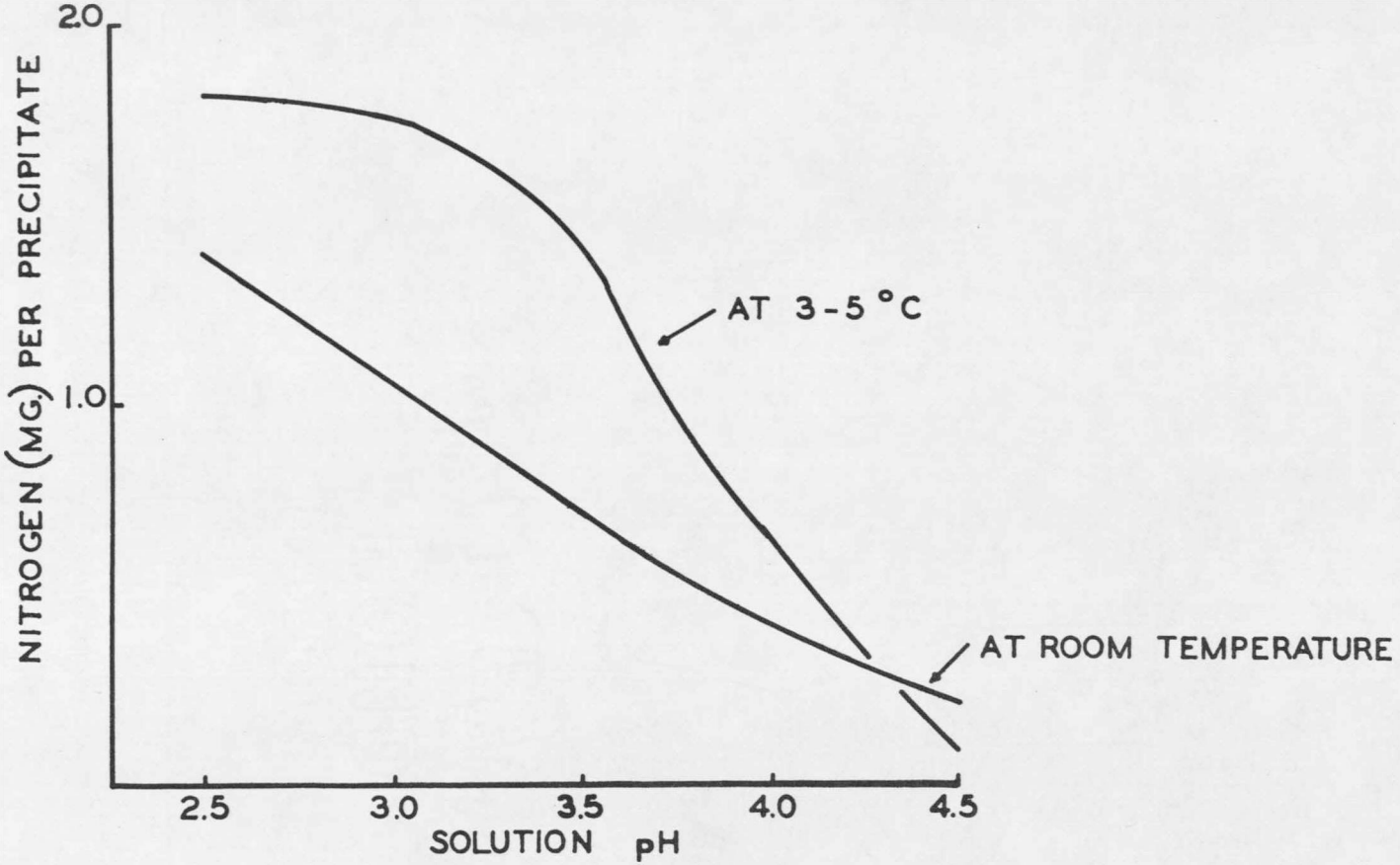

FIGURE 12 PROTEIN NITROGEN IN THE PRECIPITATE AS A FUNCTION OF SOLUTION pH IN THE PRESENCE OF 2 MEQ. OF POLYMETAPHOSPHATE PER G. OF PROTEIN

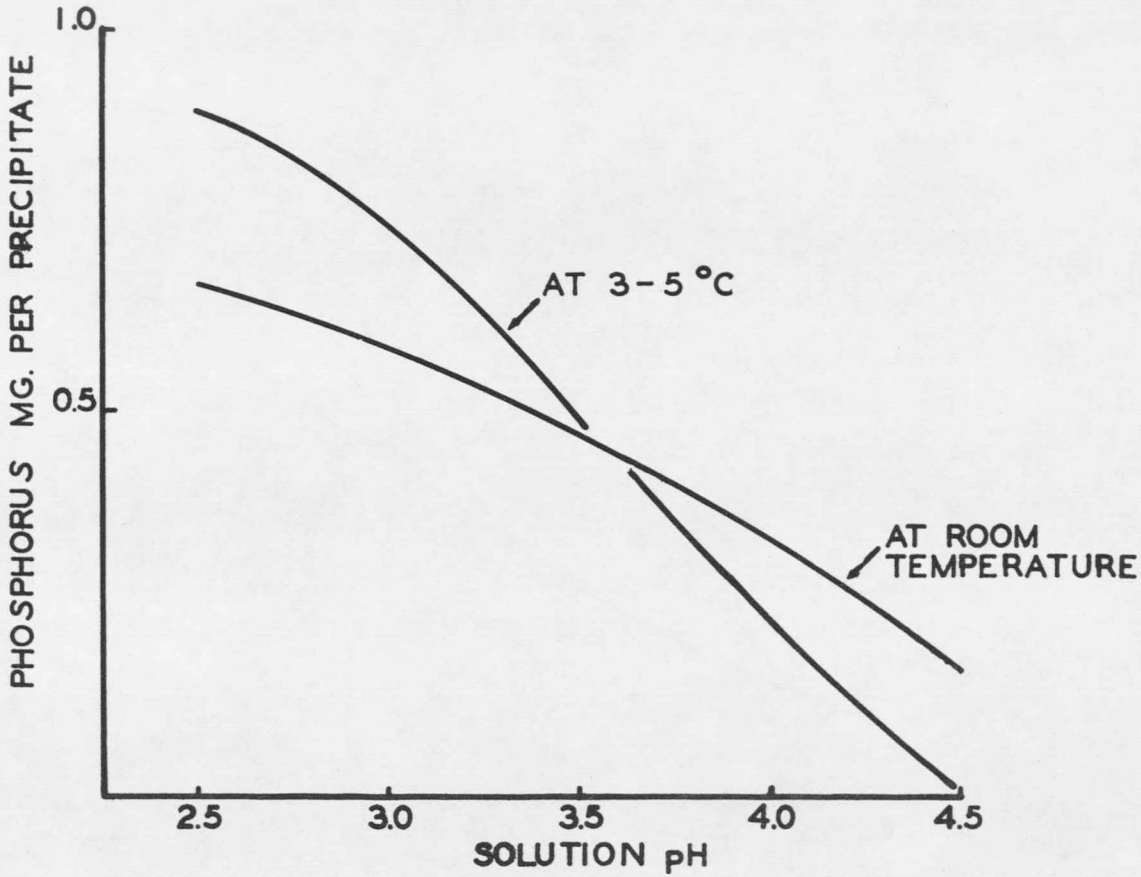

FIGURE 13 AMOUNT OF PHOSPHORUS IN THE PRECIPITATE AS A FUNCTION OF pH IN THE PRESENCE OF 2 MEQ OF PMP PER G. PROTEIN

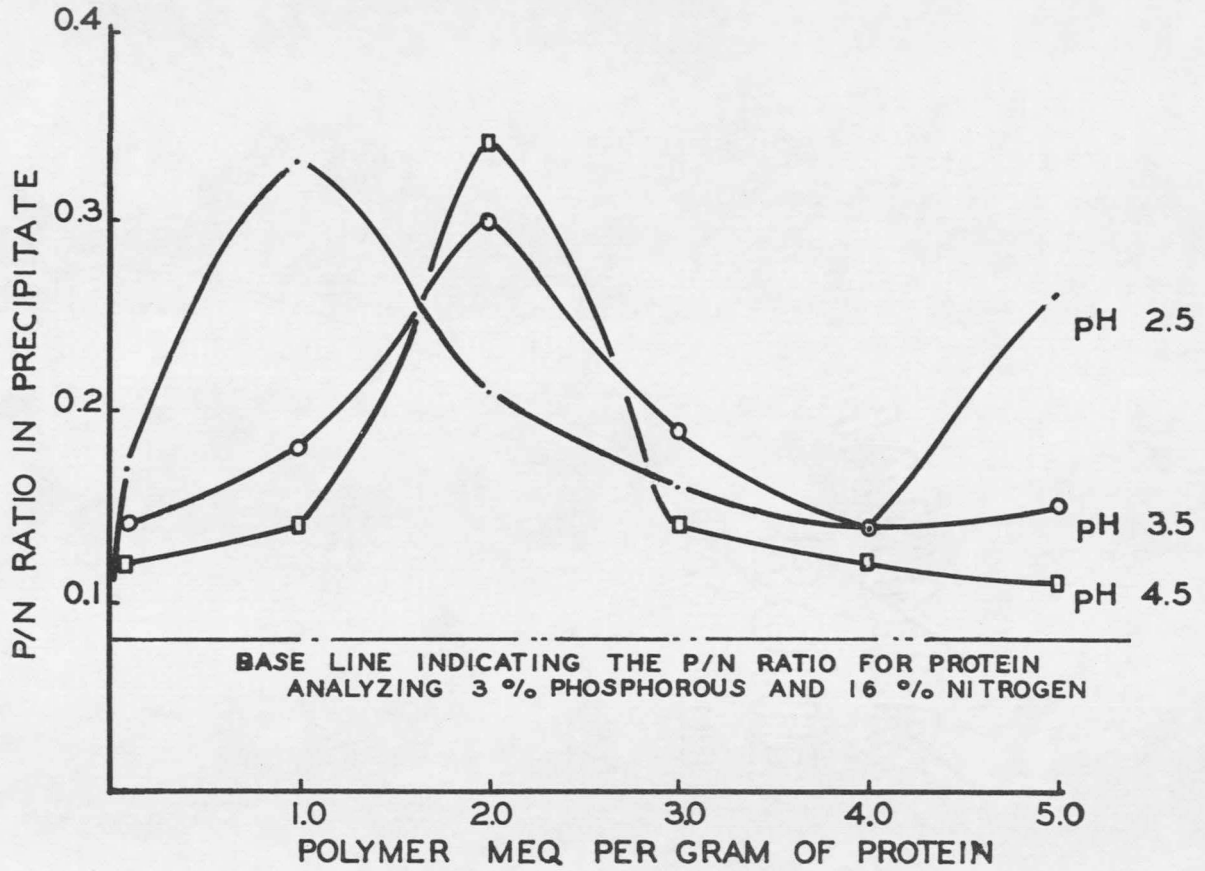

FIGURE 14 P/N RATIO AS A FUNCTION OF SPMP CONCENTRATION AT CONSTANT pH AND ROOM TEMPERATURE

C. DISCUSSION

The previously presented data offer an estimate of the possibilities and limitations for the acid precipitation method of purifying the lecithinase in the presence of PMP.

1. Protective Action of PMP on Lecithinase:

At four hours acid contact time, Figures 1, 2, and 3, PMP exerted a protective action on the lecithinase in acid solution; that is, the complex was more resistant to loss of biological activity and a precipitate of higher specific activity can be obtained by including PMP in the lecithinase solution. With increasing acid contact time, bonding between protein and PMP was probably decreased. Depolymerization of a PMP solution at room temperature and pH 2 was found to be less than one per cent after 10 hours of acid contact. At 96 hours, 15.5 per cent of the PMP depolymerized (at 0°C. pH of 1, 14.5 per cent of the PMP was lost after 120 hours). It was also determined that crude lecithinase did not demonstrate any phosphatase activity.

In this experiment only one concentration of PMP was investigated. Since an equilibrium exists between the protein-PO_3 complex and soluble PMP, increasing the amount of PMP in solution might extend the protective action of the PMP.

Addition of acid to the lecithinase solution initially at pH 5, Figures 1, 2, and 3 with or without PMP resulted in formation of a precipitate in the pH range 4 to 5. On this basis the isoelectric point of the lecithinase could be estimated at about 4.7. According to Briggs (14),

the isoelectric point of the lecithinase-PO_3 complex shifts to the acid side with increased PMP content. That the lecithinase-PO_3 is probably formed at pH values above this isoelectric point (4.7) is shown by the work of Putnam and Neurath, (22) who found that sodium dodecysulfate formed two complexes with albumin at pH 6.8. The low lecithinase recovery with PMP at pH 5 indicates that in these solutions the lecithinase-PO_3 complex is more susceptible to denaturation than is lecithinase alone.

2. The Stability of Lecithinase-PO_3 in 2-methyl-2-amino-1,3 propanediol Buffer:

Recovery of more than 100 per cent of the original lecithinase activity, Figure 4, when the enzyme in solution is allowed to stand at low temperatures is a frequently encountered phenomenon. This effect might be explained by assuming that removal of extraneous protein from biologically active centers on the lecithinase molecule occurs.

As would be expected, at high pH values the enzyme was least stable, as the pH of the solution approached that of physiological pH (7.33), lecithinase stability was near maximum. At pH 5.5 most of the protein was still in a soluble form, and without the stabilizing effect of insolubility inactivation occurred. As the pH of the solution was lowered, more of the lecithinase separated from the solution in insoluble form and became less susceptible to denaturation. Increasing the acidity gave more rapid denaturation indicating that the stabilizing effect of the precipitated form no longer balanced the denaturing action of the strong acid. The 2-methyl-2-amino-1,3 propanediol buffer, pK 8.78 (46), was included in the system to prevent the solution pH from becoming to basic while adjusting the pH of

lecithinase solution or dissolving the lecithinase-PO_3 precipitate.

3. Lecithinase Purification as a Function of Acid Contact Time:

The nitrogen content of the precipitate, Figure 5, between one and two hours of acid contact time increased six-fold, the lecithinase activity three-fold. Comparison of the amount of nitrogen in the precipitates formed from two to 72 hours showed a standard deviation of 0.044. The standard deviation for lecithinase content of the precipitate in per cent of the original for the same acid contact time is 6.1. Hence the rise occurring between four and eight hours for the solutions at pH 3.72 is probably the result of reactivation of the lecithinase. Thus within certain limits it could be expected that longer acid contact times would give a higher specific activity.

4. The Separation of Lecithinase Protein from Nonlecithinase Protein:

Figure 6 shows a good separation between lecithinase and total protein; hence, the possibility exists for increasing the specific activity of lecithinase-PO_3 by the method of acid precipitation. In this series of investigations, losses of lecithinase activity varied from about 15 to 36 per cent. Below pH 4 the left slope of the curve appears to be greater than that on the right side of the peak. This decrease in purification factor may result from denaturation because of the relatively longer acid contact time to which these solutions were subjected.

Putnam and Neurath (21) found less combination between bovine serum albumin and dodecylsulfate with more salt in the reaction mixture or with reduced temperature. Stabilizing action of PMP on lecithinase in the presence of 25 per cent salt might then be less because of reduced com-

bination between protein and PMP. Turpin et al, (7) repeatedly emphasize the necessity for use of low solution temperatures (-15°C. or less) in the purification of the toxins and antitoxins of certain bacteria. However, it would seem that the good recoveries which these authors obtained are certainly not dependent on complete saturation of all free amino groups in their proteins with the protective agent. Preliminary tests on crude lecithinase-PO_3 have shown little temperature inactivation to about 25°C. with an acid contact time of three and one-half hours.

5. Purification of Lecithinase by Reprecipitation:

It is seen that subjecting one precipitate, Figure 7, to a number of subsequent precipitations at first increases the specific activity and there then results a decrease in recovery of lecithinase. This method of increasing the specific activity of the precipitate is probably limited by the increased possibility of denaturation which results from alternately dissolving and precipitating the enzyme. Also agitation of the solution might be involved to a greater extent. Preliminary experiments indicated that in agitated solutions of high acidity the presence of PMP actually increased the relative loss of lecithinase activity. Agitation with PMP at 3-5°C. in the pH range 4-8 gave little or no loss of lecithinase activity. Agitation of the lecithinase with or without PMP at room temperature resulted in loss of lecithinase activity.

6. Influence of PMP Concentration on the Composition of the Acid Precipitate:

At room temperature, the amount of PMP present in the starting solution influenced both the amount of all listed components in the precipitate

and the calculated ratios. However, the ratio of MLD per mg. of phosphorus showed significance at the five per cent level only. For solutions at 3-5°C., the amount of PMP showed no significant relationship to the composition of the precipitate except for MLD per mg. of phosphorus and the P/N ratio (this at the five per cent level). The lack of significance at 3-5°C. for the relationship of concentration of initial polymer in the solution to phosphorus in the precipitate demonstrates that the point of equilibrium between PMP and protein is probably temperature controlled, Putnam and Neurath (21).

7. Influence of pH on the Amounts of Various Components in the Precipitate:

At both temperatures, the pH influenced the amount of nitrogen and phosphorus in the precipitate ($p \leq 0.01$). Lecithinase activity of the precipitate was significantly influenced by pH at room temperature ($p \leq 0.05$) as was the ratio of MLD per mg. of nitrogen at 3-5°C. ($p \leq 0.05$). All other pH relationships showed no significance at the 5 per cent level. A statistical analysis was not obtained for the influence of temperature on the amount of components in the precipitate.

8. Interpretation of the data in Tables I and II as Plotted in Figures 8 to 14:

Because statistical evaluation showed that the amount of PMP influenced the amount of various components in the precipitate in a highly significant manner for the solutions at room temperature, PMP (in meq. per g. of protein) was plotted against other components in Figures 8 to 14 for both temperature ranges investigated.

The influence of the amount of polymer on the MLD per mg. of nitrogen ratio at room temperature as shown in Figure 8 was apparently profound. This is indicated by the high peak for the specific activity ratio at 2 meq. of polymer per g. of protein for the solution at pH 4.5. A smaller peak occurred for the same polymer concentration for the solution at pH 3.5. The smaller peak might indicate a lap over in which lecithinase was linked by the PMP to material which was insoluble at this pH; if so, sharp separation of lecithinase from other proteins would be difficult by the method of acid precipitation. At pH 2.5 most of the lecithinase and non-lecithinase protein appeared in the precipitate. The slope of the curve probably indicates denaturation of lecithinase.

The data plotted in Figure 9 enable some evaluation of the same relationships at 3-5°C. These data show a less regular curve, with wide fluctuations. Two factors are probably influencing the MLD per mg. of nitrogen ratio: first, the possible unmasking of enzymatically active centers; and, secondly, the probable reduced combination of PMP with protein at lower temperatures.

9. The MLD per mg. of Phosphorus ratio as Influenced by the Amount of PMP:

In Figures 10 and 11 it is seen that in general the data for MLD per mg. of phosphorus vs. the amount of polymer in some respects seem to parallel that for MLD per mg. of nitrogen vs. the amount of polymer with respect to shape of curve. This might be expected, because the linkage in the protein-PO_3 complex is between free protein amino groups in cation form and the negatively charged PO_3 monomer, a 1:1 ratio.

10. Nitrogen and Phosphorus in the Precipitate as a Function of Solution pH:

Figure 12 shows that protein nitrogen once precipitated at a higher pH value remains insoluble as the acidity of the solution increases. To the left of the cross over pH where the two temperature curves intersect, more protein nitrogen appeared in the precipitate at 3-5°C. than in the solution at 25 °C. For 1 meq. to 3 meq. of PMP per g. of protein, the pH of the cross over point increased in a nonlinear manner. The solution pH values were initially adjusted at room temperature; hence, the actual acidity of the solutions at 3-5°C. would differ somewhat from the values shown in the figure. At these cross over pH points, the nitrogen content of the precipitate was independent of the temperature. In Figure 13, it is seen that the phosphorus content of the precipitate was independent of the temperature at pH 3.5. This may mean that the most stable linkage between protein and PMP occurs at these cross over pH points.

The next question is whether these cross over pH values correspond to the probable isoelectric point of the lecithinase-PO_3 complex; that is, the pH at which the lecithinase complex should be most stable. To adjust the isoelectric point of the lecithinase from about pH 4.7 to a more acid pH value should require progressively more PMP per g. of protein. The cross over pH point for 1 to 5 meq. of PMP per g. of protein (5 graphs) varied from pH 3.5 to pH 4.4 and did not show a regular change in cross over pH with increase in PMP concentration.

Two meq. of PMP per g. of protein does, however, seem to be a significant value. In Figure 14, the P/N ratio at room temperature at

pH 3.5 and 4.5 attained a maximum at 2 meq. of PMP per g. of protein. In Figure 8 and 9, the specific activity of the lecithinase was at or near maximum for 2 meq. of PMP per g. of protein. It is apparent that in certain ranges of PMP per g. of protein, a small change in PMP concentration results in a considerable change in the lecithinase content of the precipitate.

Saturation of the lecithinase with PMP in the sense that all available reacting sites on the protein are combined with PMP would differ from saturation of the protein with PMP in the sense that under certain conditions an equilibrium between complex and protein plus PMP favored the formation of free protein and PMP.

At room temperature with 5 meq. of PMP per g. of protein, only a fraction of the polymer added (one-fourth to more than one-half according to pH) appeared in the precipitate. With 2 meq. of polymer per g. of protein, the precipitate contained more phosphorus, natural and added, than was initially present in the PMP.

Phosphorus values in the precipitate on the controls at room temperature were not reported because of experimental difficulties.

D. CONCLUSIONS

A lecithinase precipitate of high specific activity can be obtained by using a ratio of 2 meq. of PMP per g. of protein in 2-methyl-2-amino-1, 3 propanediol-acetic acid buffer and an acid precipitating pH of 4.5 at a temperature of 3-5°C. A short or long acid contact time with a minimum of agitation could be used. With a long acid contact time, reactivation of the lecithinase occurs.

Starting with the bacterial filtrate, the first precipitation of lecithinase with ammonium sulfate can be expected to increase the specific activity after dialysis and lyophilization of the precipitate by a factor of about 10. The overall purification factor would be 220 fold. This value is in accord with that observed in the data obtained by acid precipitation of lecithinase in the presence of PMP from the bacterial filtrate. In these solutions purification factors between 27 and 221, depending on conditions were obtained.

It is evident that acid precipitation of lecithinase in the presence of PMP offers possibilities as a means of obtaining lecithinase protein of high specific activity.

PART II: THE SEPARATION OF CRUDE LECITHINASE ON THE DIETHYLAMINOETHYL CELLULOSE COLUMN

Preliminary determinations by the Lowry method (31) for protein in TRIS buffer have shown that the protein value is influenced by the amount of TRIS buffer in the reaction mixture; however, the TRIS buffer absorbed only slightly at the wave lengths used in the Warburg method (32) of determining protein. Separation of the buffer from the protein by dialysis would result in about a 22 per cent loss of lecithinase activity in the column eluates. This lecithinase loss can be tolerated if the activity of the protein placed on the column is of the order of that used in this experiment. It was further determined that saturation of DEAE with crude lecithinase protein will not occur below a ratio of about 9 mg. of protein per g. of DEAE.

A. EXPERIMENTAL PROCEDURE

1. The Column Material:

The column material was Eastman DEAE cellulose, catalog number 7392, which was purified according to the procedure of Moore and Lee (45). The purified DEAE analyzed approximately 1.3 per cent nitrogen. The DEAE (3.2 g.) was suspended in 0.005 M TRIS buffer at an initial pH of 7.75. The slurry was then poured into the 1x20 cm. glass tube. After each increment of slurry, the slowly forming column was packed under a pressure of about 5 psi with compressed air.

2. Eluent Buffers:

The composition of the eluent buffers is shown in Table III.

Table III

The composition of eluent buffers for the DEAE column.

Buffer Number	Number of tubes of Eluate Expected	Actual	TRIS* (Molarity)	Sodium Chloride (Molarity)	Final Volume (ml.)	pH
1	5	6	0.005	none	50	7.80
2	10	10	0.01	0.05	100	7.50
3	10	10	0.02	0.10	100	7.20
4**	10	10	0.05	0.15	100	7.00
5**	10	10	0.05	0.25	100	7.00
6**	15	15	0.05	0.50	150	7.00
					600	

* The Sigma 7-9 product was twice crystalized from water.

** Buffers 4, 5, and 6 were made 0.02 M with respect to calcium chloride to stabilize, as much as possible, lecithinase remaining in contact with DEAE cellulose for longer periods of time.

Before elution, protein from any bacterial growth in the buffers was determined by ultraviolet absorption at 260 and 280 mμ. All buffers except number 4 showed negligible absorption. Absorption of buffer number 4 showed a protein equivalent of 28 μg. per ml. Gitlin (50) reports the sensitivity of the UV method of protein determination as being 90 μg. per ml. Hence, this value of 28 μg. was initially taken as being insignificant.

3. Protein Placed on the Column:

The history of the crude lecithinase which was placed on the column is given in the Preparation of Materials section, pages 10-14, and in Table IV.

In Table IV lecithinase units as determined in column eluates refer to enzyme activity but are not to be taken as being synonymous with MLD. The lecithinase activity in the solutions which are mentioned in Table IV

was determined by antitoxin precipitation. A portion of the Seitz filtrate, mentioned in Table IV, was diluted with glycerol until the final glycerol solution contained 100 such units per ml. This glycerol standard was then used to determine the sensitivity of the egg yolk substrates which were used to evaluate lecithinase activity in the DEAE column eluates.

Table IV

The purification of the crude lecithinase previous to placing it on the DEAE column.

Lecithinase Fraction	Biuret Protein mg./ml.	Lecithinase* Units/ml.	Lecithinase Units/mg. N	Purification Factor (over previous step)
Seitz Filtrate	14.5	220	15.2	----
Dialysee**	12.8	1995-2090	160.	10.5***
Supernat-ant****	1.9	8407-8550	4450.	27.8

* By antitoxin precipitation.
** After dialyzing out ammonium sulfate.
*** Overall-includes gain by ammonium sulfate precipitation and loss due to dialysis. Estimated gain for ammonium sulfate precipitation, about 18 times.
**** From nucleic acid precipitation; referred to as Sol. 6.

It is seen that this protein sample has already been purified 292 times.

The crude lecithinase protein Sol. 6 (4.05 ml.), which contained a total of 7.61 mg. of crude lecithinase (according to the Biuret method) and 36,138 lecithinase units, was made 0.005 M to TRIS buffer and the pH of the solution adjusted to 7.75 with hydrochloric acid. The protein solution was placed on the column in the cold room (3-5°C.) by adding a portion of the solution to the free space above the DEAE and then forcing

the solution down with a hand bulb. When the solution level was just above the DEAE, several portions of 0.005 M TRIS buffer at pH 7.75 were used to wash the protein into the column material. A control sample of the protein was saved and this lecithinase subjected to the same temperature conditions as were encountered by the column lecithinase.

4. Connecting Tubing:

Tests showed that gum rubber connecting tubing would release compounds which would develop a color with Lowry's (31) reagent; hence, polyethylene tubing was used in making glass-to-glass connections.

5. Mixing Chamber:

A Mariotte mixing tube (24) of about 6 ml. volume was connected between the buffer reservoir and the DEAE column. This chamber was used to effect a smoother gradient elution between changes of buffer in the step-wise procedure. Figure 16 shows that the increase in ionic strength of the buffer was nearly linear to 150 ml. of column eluent. With addition of the calcium chloride solution the slope of the curve increased, but a nearly straight line was obtained from 155 ml. to 350 ml. of eluent.

6. Column and Dialysis Operation:

Column fractions were collected in volumes of about 10 ml. each. Pressure (1-2 pounds per square inch) was used on the column. The fraction collector in the cold room was set to change tubes every eight minutes. A total of 62 samples were collected over a time interval of about eight and one-half hours. From each fraction a 4 ml. sample was measured into a cellophane sack which was then string tied. The eluate

samples were dialyzed at 3-5°C. with stirring against 0.02 M calcium chloride solution for approximately 24 hours. The first 35 eluates were dialyzed against 6 liter portions of salt solution which was changed three times. The next 27 eluates were dialyzed against a comparable volume of calcium chloride in portions. The undialyzed eluate was frozen until the pH values could be obtained. The pH of the dialyzed samples was about 5.6. After completion of dialysis, the samples were frozen at -15 to -17°C. They were then thawed and analyzed for lecithinase activity and protein content at a later time. The protein content of the eluates was determined by the Warburg (32) and Lowry (31) procedures.

After the eluate fractions were collected, the DEAE cellulose in the column was removed and teated with 10 ml. of 1 N sodium hydroxide, the resin removed by centrifugation, and the ultraviolet absorption of the solution measured at 260 and 280 mμ.

B. RESULTS

Values obtained in analyzing the eluate fractions are given in Table V. The Warburg protein values for column eluates were not included because a graph of lecithinase activity vs. eluate tube number for the Warburg data seemed to show the presence of some interfering material, perhaps nucleic acids. Because of this interference, peaks on the graph were rounded and troughs filled in.

Graphs of pH, lecithinase activity, and protein are shown in Figures 15, 17, and 18. The graph of lecithinase activity vs. eluate tube number shows lecithinase activity in tubes 1-38 only. No lecithinase activity was found in tubes 39 to 62, and the graph was not extended. The control contained 30,678.7 units per 4.05 ml. at the time of stopping the fraction collector. At completion of lecithinase analysis, the control contained 21,991.5 units per 4.05 ml.

Calculation revealed that 18.6 per cent of the starting lecithinase activity remained in the dialyzed eluate fractions. Meanwhile the control in just standing at comparable temperatures lost 39.2 per cent of its lecithinase activity. It was estimated from dialysis experiments that the lecithinase should have lost 23 per cent of its activity. The loss of lecithinase activity is then 19.2 per cent more than expected.

When the DEAE cellulose was treated with 1 N sodium hydroxide, 1.2 mg. of protein were recovered as indicated by UV absorption. In the Lowry method, about 41 per cent of the starting protein was accounted for. Since the column at addition of the sodium hydroxide was still moist with TRIS buffer, a Lowry protein determination was not run.

Table V

Analysis of column eluates for lecithinase and protein

Fraction Number	Fraction Volume (ml.)	Fraction pH	Lecithinase (units per fraction)	Protein Lowry* (µg./fraction)
1	8.5	7.80	.1	0
2	7.4	8.00	0.1	.43
3	7.5	8.25	878	225
4	10.0	8.47	3900	200
5	8.0	8.00	680	107
6	12.5	7.93	95	25
7	12.0	7.62	.46	0
8	13.0	7.80	6.5	0
9	12.5	9.00	306	83
10	12.0	9.15	278	165
11	9.0	9.20	145	93
12	8.3	9.13	54	53
13	8.7	9.10	39	35
14	9.0	9.10	24	39
15	8.4	9.10	24	18.9
16	10.0	9.10	20	32
17	9.8	9.20	25	16.4
18	9.8	9.19	17	1.6
19	9.8	9.23	68	39
20	10.0	9.35	88	71
21	9.0	9.40	17	36
22	9.5	9.37	10	22
23	9.4	9.28	2.8	14
24	9.6	9.28	.93	16
25	9.0	9.19	1.4	1.5
26	7.5	8.15	.56	11.3
27	8.0	7.52	2.1	16
28	9.7	7.50	1.2	2.4
29	9.9	7.48	1.6	15
30	10.4	7.50	1.8	45
31	9.4	7.68	15	83
32	10.2	7.59	7.6	41
33	9.8	7.40	4.3	31
34	10.4	7.40	2.4	1.7
35	9.8	7.29	1.3	31
36	9.8	7.25	2.7	0
37	9.5	7.24	4.9	0
38	10.4	7.29	1.3	5.2
39	9.7	7.25	.62	8.1
40	10.0	7.25	.90	12.5
41	9.7	7.23	.40	42.2

Table V continued

Fraction Number	Fraction Volume (ml.)	Fraction pH	Lecithinase (units per fraction)	Protein Lowry* (ug./fraction)
42	10.2	7.23	.22	8.5
43	10.3	7.21	.86	5.2
44	10.0	7.19	1.0	0
45	9.3	7.15	1.0	7.9
46	9.7	7.15	1.0	0
47	10.2	7.13	.42	1.4
48	10.8	7.13	.22	1.8
49	10.5	7.13	.44	13.1
50	11.4	7.13	0	72
51	10.4	7.15	1.3	31
52	10.5	7.40	.48	32
53	9.4	7.40	0.10	20
54	10.8	7.40	0.24	9.1
55	9.8	7.29	0.21	19.6
56	10.3	7.22	0.11	15
57	10.0	7.20	0.10	23
58	10.8	7.19	0.23	1.8
59	10.0	7.18	0.10	1.3
60	10.5	7.18	0.22	0
61	9.0	7.15	0.20	4.5
62	6.5	7.16	0.27	.65
Summation values	607.3		6,737.6	1,907

* The Lowry standard curve for protein (absorbancy vs. ug.) was obtained by first plotting absorbancy vs. ml. of Sol. 6. Then a final curve was plotted in which ml. of protein were converted to ug. of protein by using the 1.88 mg. protein/ml. ratio.

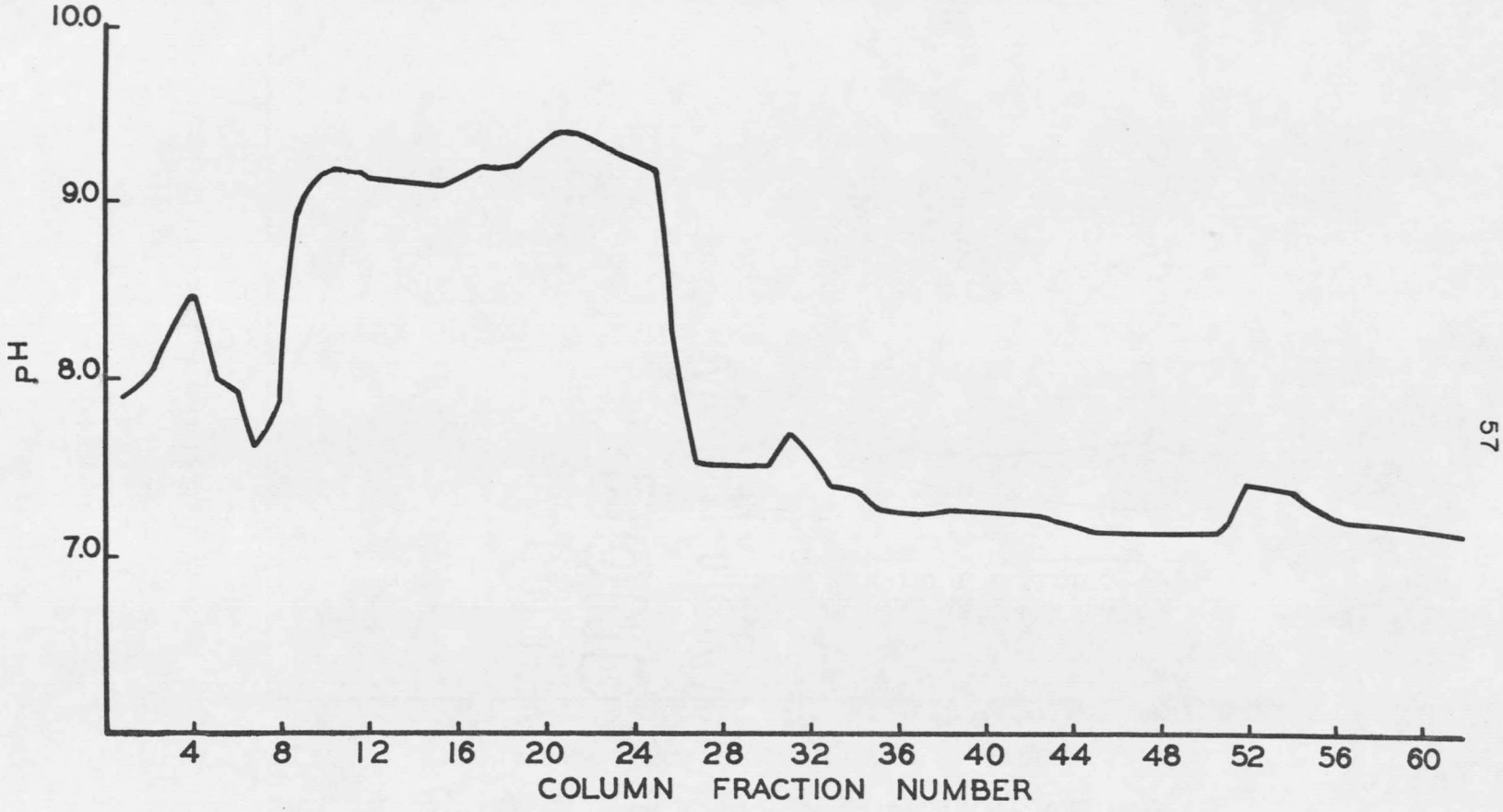

FIGURE 15 HYDROGEN ION CONCENTRATION AS pH IN THE ELUATE FRACTION

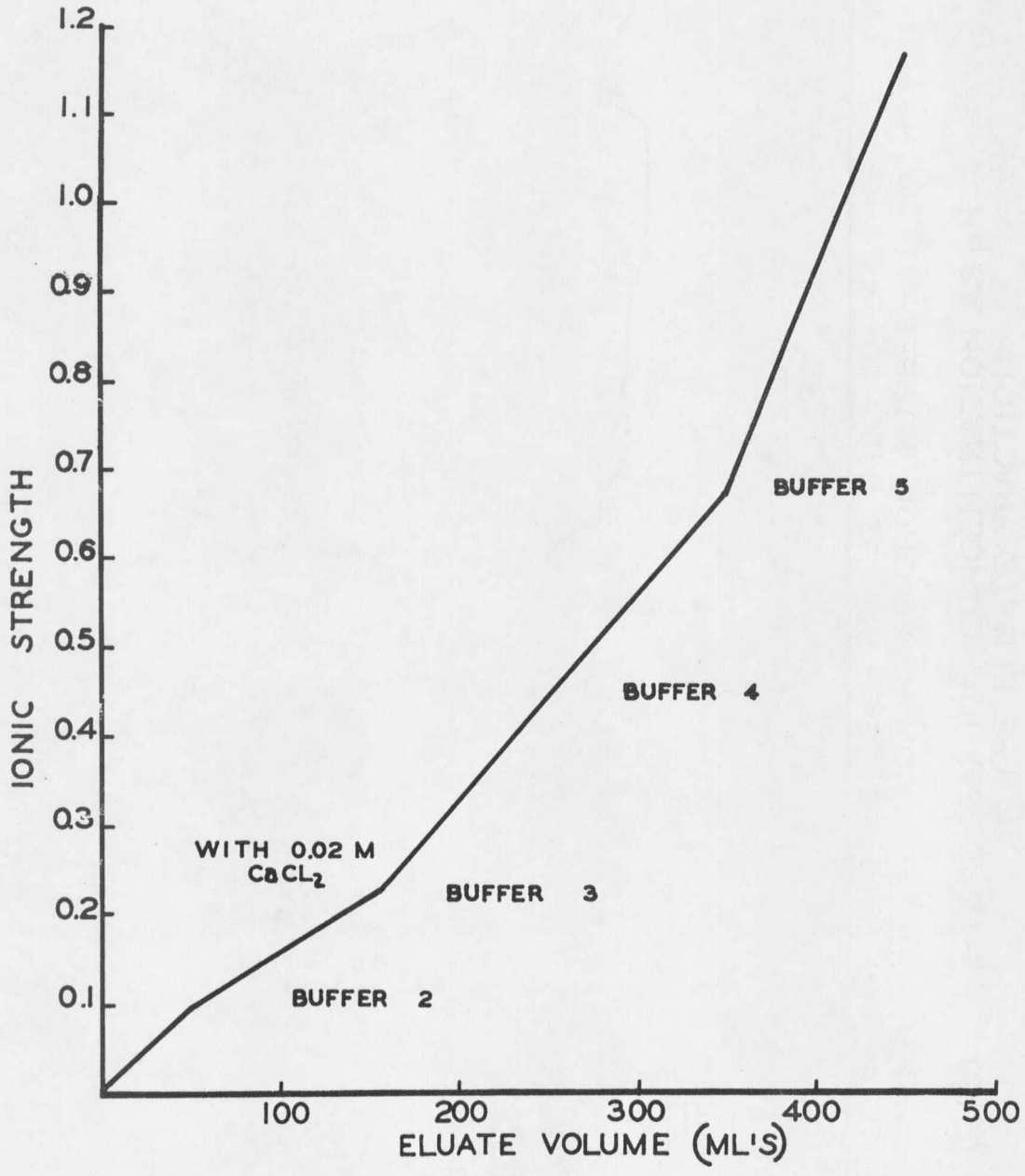

FIGURE 16 IONIC STRENGTH OF COLUMN ELUATES

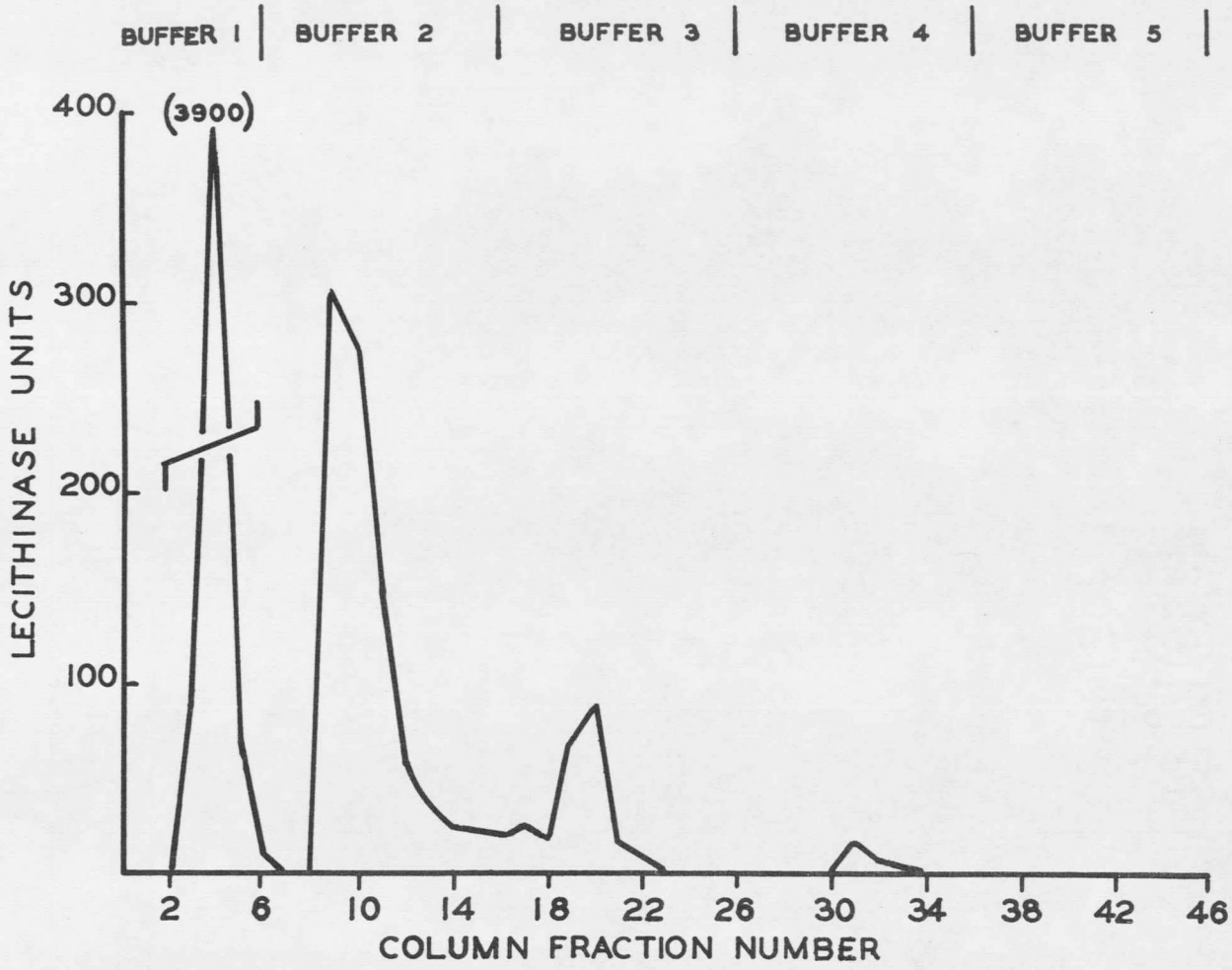

FIGURE 17 ANALYSIS OF COLUMN ELUATES FOR LECITHINASE ACTIVITY

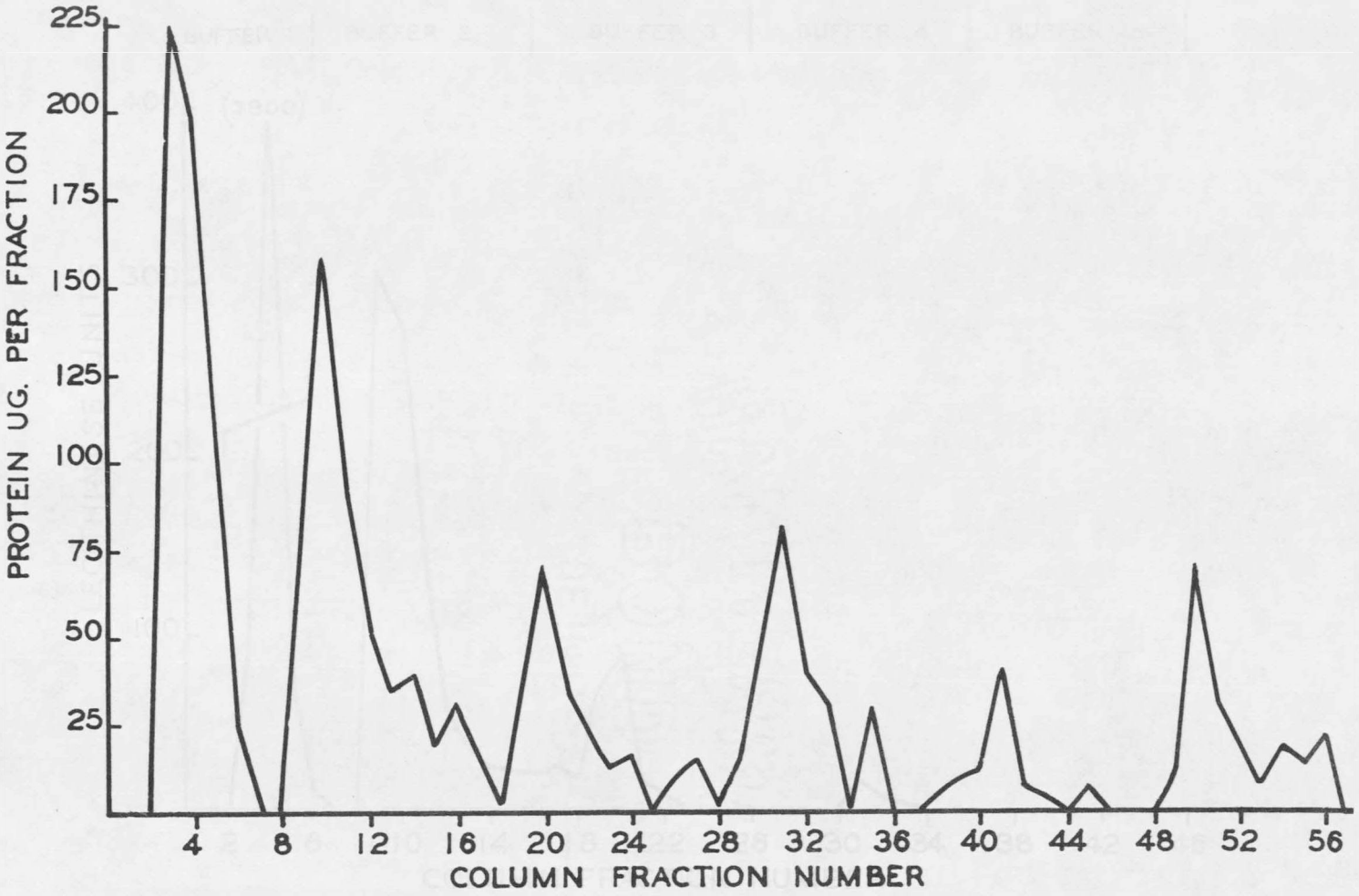

FIGURE 18 LOWRY PROTEIN CONCENTRATION IN COLUMN ELUATES

C. DISCUSSION

The discrepancy between amount of protein placed on the column and the amount of protein determined in the dialyzed eluate by the two methods of analysis was great.

Since there is no accurate method of determining protein concentration in a mixture such as is encountered in the crude lecithinase, it was thought that analysis of the eluate by the ultraviolet procedure of Warburg (32) and by the Lowry method (31) would enable a more accurate interpretation of protein content than one based on a single method of protein determination. With regard to the ultraviolet method of determining protein content of a solution, it has been observed in previous work that protein concentration curves (absorbancy vs. mg. of protein solution) obtained on dialyzed crude lecithinase usually failed to intersect the zero point on the curve. This fact is an indication of protein denaturation. Glazer and Smith (51) have determined the effect of protein denaturation on ultraviolet absorption of protein before and after denaturation. They found that "in addition to difference peaks due to tyrosine and tryptophane side chains at 278 mμ. and 285 mμ. and 293 mμ., respectively, denaturation was invariably associated with the appearance of a far more prominent peak at 230 to 235 mμ."

As quoted by Nielands and Stumpf (52), a ratio of absorbancy 280/absorbancy 260 of 1.6 or larger indicates the absence of appreciable amounts of nucleic acids (nucleoproteins). Values for this ratio on column eluates were frequently less than one; hence, the concentration of protein as read from the Warburg monograph would be in error with excess nucleic acids.

It would be expected that denaturation of the protein or the presence of nucleic acids would change the protein value of a sample as determined by the two methods. That the extent of the change should be the same for both methods is not probable.

It is apparent that peaks of lecithinase activity occurred in fractions number 4, 9, 20, and 31. From the protein values obtained by the Lowry method on column eluates, protein peaks occurred in factions number 3, 10, 20, 31, 41, and 50. A rough estimate of the areas under the respective curves indicates a purification factor of 2-3 for the first three lecithinase peaks.

The results suggest that there are three separate lecithinases. The plot of lecithinase units versus column fraction number shows three definite peaks and a possible fourth. It is noticed that the height of these peaks decreases as the fraction number increases. It is possible that a portion of the lecithinase which should have appeared in one peak was displaced for some reason to another peak. However, the fact that lecithinase concentration in intermediate tubes approaches zero would seem to nullify this assumption. The decrease in peak height could result from inactivation of lecithinase with increased DEAE contact time, or the second and third lecithinases could be present in the starting material in lesser amounts, or their activity could be less. Figure 17 shows that these peaks of lecithinase activity occur about two or three tubes after a change of buffer. The column was operated under a pressure a little above atmospheric pressure. During a change of eluent, the compressor was dis-

connected from the column and the column was then at atmospheric pressure for a time interval of about 45 seconds. Cassidy (53) states that column elution once started cannot be interrupted without changing the shape of the profile curve. It is possible, then, that each change of eluent solution resulted in more enzyme of the same species appearing in the eluate. The complete lack of trailing between Peak 1 and Peak 2 (Figure 17) supports the contention that two different lecithinases are present. Trailing was encountered between Peaks 2 and 3; hence, the same enzyme might be involved in tube 9 and tube 20. There was no trailing between Peaks 3 and 4.

An approximate calculation of the ionic strength of the six buffered solutions which were used to elute lecithinase from the DEAE column shows, as plotted in Figure 16, a nearly linear relationship between the ionic strength of Buffers 1, 2, 3. To insure that lecithinase would be removed from the column as rapidly as possible in the second half of the elution, the ionic strength of Buffers 4, 5, and 6 was increased above this linear relationship. These buffers contain calcium ion (.02 M) to reduce inactivation of lecithinase on the DEAE.

The calculated activity of the starting protein was 36, 138 units (Vol. of solution X Units/ml.). A recovery of 6,737 lecithinase units indicates a loss of 81.4 per cent. It is felt that this loss is excessive and that better recoveries should be possible.

D. CONCLUSIONS

The rate of migration of crude lecithinase on the DEAE cellulose column differs for several lecithinase fractions. The assumption is made that this indicates the presence of two or more different lecithinases which are elaborated by the Clostridium hemolyticum bacteria. It seems probable that the best application of the DEAE column with crude lecithinase will be to separate these lecithinase fractions.

GENERAL CONCLUSIONS

The separation of crude lecithinase protein on the DEAE cellulose column does not give a large purification factor. A comparison of the area occupied by the curve as obtained by plotting lecithinase activity against tube fraction number and the curve obtained by plotting total protein against tube fraction number, as shown in Figures 17 and 18, typically shows about a three fold increase in the purity of the lecithinase. Acid precipitation of the lecithinase in the presence of PMP will give an increase in specific activity of about 220 fold. A second disadvantage involved in the use of the DEAE cellulose column is the amount of eluent required to make the separation; hence, the lecithinase is diluted, and removal of the excess liquid is time consuming and will probably result in the denaturation of some of the lecithinase. It is suggested that a quick procedure for concentrating the lecithinase in the eluate from the DEAE cellulose column would be to use the procedure of acid precipitation of the lecithinase in the presence of PMP. This procedure would probably work best with eluate fractions which do not have a high salt concentration, and would probably increase the specific activity of the lecithinase.

Further work should be done in characterizing the several lecithinases as separated on the DEAE cellulose column. A comparison should be made between these lecithinases to determine if there is a difference in lethal or lecithinolytic, activity and the ability to lyse red blood cells. It would also be interesting to find out if these eluate lecithinases differ in the ability to cause an antibody response in animals, or in wave pattern in electrophoresis, or ultracentrifugation.

Most especially it would be of value to know how many lecithinases are elaborated by Clostridium hemolyticum. The author is currently investigating the relatively new method of immunoelectrophoresis in which electrophoresis is used to separate the various proteins in an agar covered slide.

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