THE EDTA-PROMOTED LYSIS OF *PSEUDOMONAS FLUORESCENS*

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The ethylenediaminetetraacetic acid (EDTA)-promoted lysis of Pseudomonas aeruginosa was first reported by Repaske (1956). Since that time, a number of workers have studied the phenomenon in an attempt to determine the mechanism of this lysis. Although there have been reports of lysis of Vibrio succinogenes (Wolin, 1966) and two species of the genus Alcaligenes (Wilkinson, 1967) by EDTA, the EDTA-promoted lysis is primarily confined to the pseudomonads. Wilkinson (1967) tested forty-one strains representing twenty-one species of the genus Pseudomonas. He found that P. aeruginosa and P. fluorescens were among the sensitive species, and P. diminuta, geniculata, iodinum, maltophilia, pevonacea, and rubescens were resistant to EDTA. EDTA sensitivity has been used to differentiate between the fluorescent and non-fluorescent groups of pseudomonads (Stanier, Doudoroff, and Adelberg, 1970).

Treatment with EDTA affects the cell wall of gram negative bacteria. Leive (1965a) reported the release of a lipopolysaccharide-lipoprotein complex from Escherichia coli by EDTA treatment. The release of lipopolysaccharide (LPS) increases the permeability of the cell to various compounds (Hamilton-Miller, 1966; Leive, 1965b) and also changes the surface configuration of the cell (Protass and Korn, 1966). The release of LPS from E. coli is rapid, and an equilibrium is quickly established between the releasable and non-releasable LPS (Levy and Leive, 1968).

This suggests that there are two forms of LPS in the cell wall, only one of which is sensitive to EDTA. The release of LPS from E. coli does not produce osmotically sensitive cells (Leive, 1965b). Treatment of P. aeruginosa with EDTA also causes the release of LPS-lipoprotein complex (Cox and Eagon, 1968; Asbell and Eagon, 1966). Fensom and Meadows (1970) reported the occurrence of two regions in the LPS of P. aeruginosa. However, the release of LPS from P. aeruginosa does not produce osmotically sensitive cells.

The release of LPS is reportedly due to chelation of divalent metal ions by EDTA (Repaske, 1958; Leive, 1968). However, other chelating agents (Fe-3 specific versene, versenol, 8-hydroxyquinoline, O-phenanthroline, diethylenetriaminopentaacetic acid, and citric acid) will not replace EDTA in production of lysis in P. aeruginosa (Repaske, 1958; Eagon and Carson, 1965). Also, a brief treatment with detergents (benzylsulfonic acid, deoxycholate, and sodium dodecyl sulfate) will not substitute for the action of EDTA in producing lysis (Eagon and Carson, 1965).

The cell wall of P. aeruginosa contains Ca^{+2}, Mg^{+2}, and Zn^{+2} (Eagon, Simmons, and Carson, 1965). The addition of Mg^{+2} after treatment with EDTA will restore the cell to an osmotically stable form (Asbell and Eagon, 1966). In studies using freeze etched P. aeruginosa, electron micrographs show the reaggregation of lipoprotein units on the surface of the cell after the addition of Mg^{+2} (Gilleland et al., 1973). Brown and Melling (1969) reported that growth in conditions of limited Mg^{+2} produces cells which are resistant to EDTA. This EDTA resistance could be due to alterations in the attachment of the LPS to the cell or to changes in the chemical make-up of the cell surface (Ellwood and

Tempest, 1967; Elder, 1973; and Walker, 1973).

A current model for the molecular arrangement of the cell envelope of E. coli depicts an outer membrane composed of lipoprotein, phospholipid, and LPS. Magnesium stabilizes the association of the LPS and protein in this outer membrane by forming ionic bridges between the LPS and protein (Braun, Rehn, and Wolff, 1970; Schnaitman, 1971). The outer membrane is attached to an inner layer of mucopeptide by a trypsin sensitive linkage. Braun and Wolff (1970) have shown that this covalent linkage between the outer membrane layer and mucopeptide provides some structural integrity to the cell wall in E. coli.

Wilkinson (1970) reported that the cell walls of pseudomonads sensitive to EDTA contain LPS, protein, phospholipid, and mucopeptide. P. fluorescens does not contain a covalent linkage between the outer membrane and mucopeptide layer, and this might explain its sensitivity to EDTA (Heilman, 1972). However, the absence of a covalent linkage between the outer membrane and mucopeptide in other gram negative bacteria does not correlate with EDTA sensitivity in these organisms. Proteus vulgaris does not contain the covalent linkage, nor has EDTA sensitivity been reported for this organism (Braun, et al., 1970).

The LPS of the pseudomonads, although similar to the LPS of the Enterobacteriacae, is unusually rich in phosphorus (Chester, Gray and Wilkinson, 1972). The phosphorus level in the LPS varies among the different species of pseudomonads, and there is a correlation between the level of phosphorous and sensitivity to EDTA. The EDTA sensitive pseudomonads contain higher levels of phosphorus than the EDTA resistant pseudomonads (Wilkinson, Galbraith, and Lightfoot, 1973).

The restriction of lysis by EDTA within the pseudomonads, the

specificity of the requirement for EDTA to produce lysis, and the release of LPS from other bacteria without the subsequent lysis of the cell, suggests that either the action of EDTA on the cell wall is not the primary factor in producing lysis or that the LPS of the pseudomonads plays a unique role in producing structural integrity.

Haslam, Best, and Durham (1969) suggested that lysis was produced by the action of an autolytic enzyme(s) present in the pseudomonads. The presence of bacteriolytic enzymes in microorganisms has been extensively reviewed (Ghuysen, Tipper, and Strominger, 1966; Strominger and Ghuysen, 1967; Ghuysen, 1968). Most bacteriolytic enzymes attack the mucopeptide of the cell wall. They can be divided into three general classes: (1) those enzymes hydrolyzing the polysaccharide chains (glycosidases), (2) those splitting the peptide cross links (endopeptidases), or (3) those cleaving the junctions between polysaccharides and peptides (acetyl-muramyl-L-alanine amidases). Most autolytic enzymes have been isolated from gram positive organisms. These enzymes have been postulated to function in surface growth and cell division (Rogers, 1970). However, their precise roles in these processes are not known. Collins (1964) reported the isolation of an enzyme from aged cultures of P. aeruginosa which hydrolyzed the mucopeptide of the cell wall. The enzyme produced lysis of suspensions of P. aeruginosa, and its action was enhanced by EDTA.

This thesis is concerned with the isolation of an autolytic enzyme from P. fluorescens. Studies of the production of the enzyme under different growth conditions and characterization of the enzyme's substrate indicate that presence of the enzyme could account for the unusual EDTA sensitivity of the organism. It is proposed that EDTA

alters the cell wall of the organism, allowing the enzyme access to its substrate, which results in lysis. Thus, EDTA acts as an initiator of lysis which is completed by the autolytic enzyme.

CHAPTER II

MATERIALS AND METHODS

Test Organisms

The principal microorganism used throughout this study has been tentatively identified as Pseudomonas fluorescens (Montgomery, 1966). The organism was obtained from Dr. Norman N. Durham, Oklahoma State University. Bacillus subtilis W23, a prototrophic strain initially obtained from Dr. W. C. McDonald, Washington State University, was used for the preparation of protoplasts.

Stock cultures of P. fluorescens and B. subtilis W23 were maintained on slants of either nutrient agar or glucose salts and stored at 4 C.

Media

The phosphate base synthetic minimal salts medium used in this study had the following composition: 0.2 percent NaCl; 0.2 percent NH_4Cl; 0.32 percent KH_2PO_4; and 0.42 percent K_2HPO_4. Carbon sources were used in concentrations of 0.2 percent for succinate and asparagine or 0.5 percent for glucose. Succinate and asparagine were added directly to the minimal salts base; glucose was autoclaved separately and added aseptically to the sterile minimal salts base. The pH was adjusted to 7.0 with KOH prior to sterilization. Sterilization was achieved by autoclaving at 121 C with 15 pounds pressure per square inch for 15

minutes. After the medium had cooled to room temperature, 0.1 ml of a sterile mineral salts solution was added for each 100 ml of the medium. The mineral salts solution had the following composition: 5.0 g of $MgSO_4 \cdot 7H_2O$; 0.1 g $MnSO_4$; 1.0 g of $FeCl_3$; and 0.5 g of $CaCl_2$ in 100 ml of distilled water. Agar (Difco) was added to give a concentration of 2.0 percent when solid medium was desired. Unless otherwise stated, the minimal medium used in this study was phosphate base.

Certain experiments in this study were conducted using the Tris base minimal medium of Levinthal, Singer, and Fetherolf (1962). A complex medium composed of 0.5 percent yeast extract, 0.5 percent peptone, and 0.5 percent glucose (YPG) was also used in certain experiments in the study.

EDTA

Ethylenediaminetetraacetate (EDTA) was purchased as the disodium salt from Fisher Scientific Company. Unless otherwise stated in the text, EDTA was used at a final concentration of 1 mM and a pH of 7.0 throughout this study.

Growth of Cells

Sterile 250 ml flasks containing 100 ml of medium were inoculated from stock cultures and incubated at 37 C on a reciprocal shaker (100 excursions per minute). For growth experiments conducted in defined medium, the cells were first grown in the defined medium 10-12 hours, and an aliquot of these cultures used as an inoculum. Growth was determined by measuring the increase in absorbance of the culture at 540 nm on a Coleman Junior II spectrophotometer. Growth assays were performed

in 250 ml side-arm flasks.

For production of crude autolytic enzyme, the cells were grown in YPG medium. The cells were incubated for 24 hours and removed by centrifugation (10,400 x g for 30 minutes). The spent YPG medium was assayed for lytic activity and used for further purification of that activity.

Substrate for Determination of Lytic Activity

P. fluorescens was grown in YPG medium for 24 hours at 37 C. The cells were harvested by centrifugation (12,000 x g for 10 minutes) and washed 3 times with phosphate buffer (10 mM, pH 7.0). The washed cells were suspended in buffer to an approximate absorbance of 0.85 at 540 nm. This suspension was used as a substrate for the determination of lytic activity. In some experiments, Tris-HCl buffer (10 mM, pH 7.0) was substituted for the phosphate buffer.

Assay of Lytic Activity

To determine the units of lytic activity in a sample, the following were placed in 18 x 150 mm tubes: 4.0 ml of the substrate cell suspension, 1.0 ml of EDTA (6×10^{-3} M, pH 7.0), an appropriate volume of test sample, and phosphate or Tris buffer (10 mM, pH 7.0) to bring the final volume in the tubes to 6.0 ml. Controls for buffer, sample, and EDTA were run with each assay. The absorbance of each tube was determined at 0 time, the tubes incubated at 37 C without shaking, and the change in absorbance determined at regular time intervals.

A unit of lytic enzyme is defined as the amount giving a decrease

of .001 absorbance units at 540 nm per 30 minutes. The number of units per sample was calculated using the following formula:

$$\text{Units of Act.} = \frac{[(A_{0T}-A_{30T}) - (A_{0E}-A_{30E})]}{.001}$$

where A_{0T} is the absorbance of the test sample of 0 time, A_{30T} is the absorbance of the test sample after 30 minutes incubation, A_{0E} is the absorbance of the EDTA control at 0 time, and A_{30E} is the absorbance of the EDTA control after 30 minutes incubation.

Ammonium Sulfate Fractionation

Spent YPG medium was fractionated using ammonium sulfate precipitation. The spent medium was cooled in an ice bath to between 2 and 4 C. Dry, finely ground ammonium sulfate was slowly added to the spent medium with constant stirring to produce levels of saturation of 20, 40, 60, and 80 percent. The amount of ammonium sulfate necessary to produce each level of saturation was determined using the ammonium sulfate saturation nomagram of Kunitz (1952). The spent medium was allowed to stand at each level of saturation for 30 minutes to facilitate the precipitation of the protein. The precipitate was collected at each level of saturation by centrifugation (37,000 x g for 30 minutes). The pellet was washed once with 10 ml of ammonium sulfate at the appropriate percent saturation and dissolved in 5.0 ml of phosphate buffer (10 mM, pH 7.0). The ammonium sulfate fractions were dialyzed against 3 liters of phosphate buffer for 24 hours at 4 C. After dialysis, the ammonium sulfate fractions were centrifuged (10,000 x g for 10 minutes) to remove any particulate matter and stored at 4 C until needed.

Column Chromatography

DEAE-Sephadex (20 gm) was allowed to swell in an excess of Tris-HCl buffer (0.1 M, pH 7.0) for 24 hours. The excess buffer was decanted and the swollen gel used to pack an 80 x 2.5 cm column. The packed column was washed with 1 liter of Tris buffer prior to the application of the sample.

The sample used in the chromatography experiments was prepared by precipitation of 2.5 liters of spent YPG medium with 40 percent ammonium sulfate saturation, as previously described. The precipitate was dissolved in 50 ml of Tris buffer (0.1 M, pH 7.0) and dialyzed against 3 liters of Tris buffer for 24 hours. After dialysis, the sample was centrifuged (12,000 x g for 10 minutes) to remove any particulate matter and 45 ml was loaded onto the column.

The loaded column was washed with 200 ml of Tris buffer containing 0.5 M NaCl and developed with a gradient of 0.5 to 1.0 M NaCl in Tris buffer. The flow rate for all washings and development of the column was 0.25 ml per minute. Fractions of eluent, each containing 120 drops, were collected, and the protein concentration of each fraction was monitored by measuring the absorbance at 280 nm. Each fraction was tested for lytic activity as previously described. The fractions containing each protein peak were pooled and dialyzed against 3 liters of Tris buffer (10 mM, pH 7.0) for 48 hours. The buffer was replaced 3 times during the 48 hour period. After dialysis, the pooled peak which contained the lytic activity was precipitated with 40 percent ammonium sulfate, dissolved in 10 mM Tris buffer, and dialyzed against 3 liters of Tris buffer and stored at 4 C until needed.

Protein Determination

Protein concentration was determined using the procedure of Sutherland et al. (1949). Bovine serum albumin was used as the standard in all protein determinations.

Polyacrylamide Gel Electrophoresis

Detergent (SLS) gel electrophoresis was performed in 10 percent acrylamide gels using the method of Weber and Osborn (1969) as modified by Grula and Savoy (1971).

Inorganic Phosphate Determination

The concentration of inorganic phosphate in YPG medium was determined using the method of Ames and Dubin (1960).

Protoplast Formation

Protoplasts of B. subtilis W23 were prepared by growing the cells 10-12 hours on 5 glucose-salts agar plates. The cells were harvested by adding phosphate buffer (10 mM, pH 7.0) to the plates and scraping with a glass rod. The cells were pelleted by centrifugation (6,000 x g for 10 minutes) and washed twice with phosphate buffer. The pellet was suspended in 30 ml of 0.6 M sucrose containing lysozyme (400 μg per ml) and allowed to sit at room temperature for 2 hours. Protoplast formation was confirmed by phase-contrast microscopy. The protoplasts were further purified by a slow speed centrifugation (2,000 x g for 5 minutes) to eliminate whole cells or cell ghosts from the preparation.

Preparation of Labeled Cell Walls

P. fluorescens cells were inoculated into 1 liter of glucose minimal salts medium containing either 10 μCi D-glucosamine-U-^{14}C (192.4 mCi per mmole) or 10 μCi N-acetyl-1-^{14}C-D-glucosamine (41.7 mCi per mmole). The cells were incubated for 18 hours at 37 C, harvested by centrifugation (12,000 x g for 10 minutes), and washed once with Tris buffer (10 mM, pH 7.0). The washed cells were suspended in Tris buffer containing 1 percent sodium lauryl sulfate (SLS). The cell suspension was heated in a boiling water bath for 1 hour and pelleted by centrifugation (54,000 x g for 30 minutes). The supernatant was decanted and replaced with fresh Tris-SLS buffer. The heating was continued for 1 hour intervals until a clear, gelatinous pellet was obtained. The pellet was washed 3 times with 10 ml of sterile distilled water and lyophilyzed. The lyophilyzed walls were resuspended in distilled water to a concentration of 1.0 mg per ml, divided into 1.0 ml aliquots and frozen. The specific activity of the radioactive walls was 3.5 nCi per mg for N-acetylglucosamine labeled walls and 1.1 nCi per mg for glucosamine labeled walls.

Assay for Muramidase Activity

Labeled cell wall (4.0 mg) was suspended in 10 ml of Tris buffer (10 mM, pH 7.0) and equilibrated to 37 C in a shaking water bath. One tenth mg of either crude enzyme or lysozyme was added to the reaction mixture at 0 time. Samples (0.5 ml) were removed from the reaction mixture at regular time intervals, filtered through Millipore filters (0.45 μ pore size), and the filtrate collected. All filters were washed twice with 1.0 ml of distilled water and the washings were combined with

the filtrate. The filter and combined filtrate from each sample were placed in separate scintillation vials. The filter was allowed to dry overnight at room temperature. Ten ml of Aquasol counting fluid (New England Nuclear) were added to each vial and the radioactivity determined using a Nuclear-Chicago Model 722 scintillation spectrometer. The percent of the total counts which were released into the filtrate was used as a measure of muramidase activity.

Assay for Esterase Activity

Samples were screened for esterase activity by placing a small drop of the sample on tributyrin agar plates. The plates were incubated 12 hours at 37 C and checked for clearing.

The esterase activity was quantitated using a 2 percent v/v suspension of tributyrin in phosphate buffer (10 mM, pH 7.0). The suspension was produced by sonification (three 30 second bursts) using a Bronsen Sonifier followed by incubation at 37 C for 1 hour. Five ml of the suspension was placed in tubes (18 x 150 mm). An appropriate volume of test sample and enough phosphate buffer to bring the total volume to 6.0 ml was added to each tube. The absorbance at 540 nm was determined at 0 time and at regular time intervals during incubation at 37 C.

A unit of esterase activity is defined as the amount causing a decrease of .001 absorbance units per 30 minutes at 37 C.

Proteolytic Enzyme Assay

Proteinase activity was determined using a modification of the procedure described by Rinderknecht et al. (1968). Samples to be tested were incubated in 6.0 ml of phosphate buffer (10 mM, pH 7.0) containing

10 mg hide powder azure (Cal-Biochem) at 37 C for 1 hour. The supernatant was collected by filtration through Millipore filters (0.45 μ pore size) and the absorbance determined at 595 nm. Controls of phosphate buffer and trypsin at concentrations of 100, 80, 60, 40, and 10 μg/ml were run with each assay.

A unit of proteinase activity is defined as the amount giving an increase of .001 absorbance units per hour at 37 C.

CHAPTER III

RESULTS AND DISCUSSION

Influence of EDTA Concentration on Lysis

Experiments were conducted to determine if lysis of P. fluorescens cells was dependent upon the concentration of EDTA used to treat the cells.

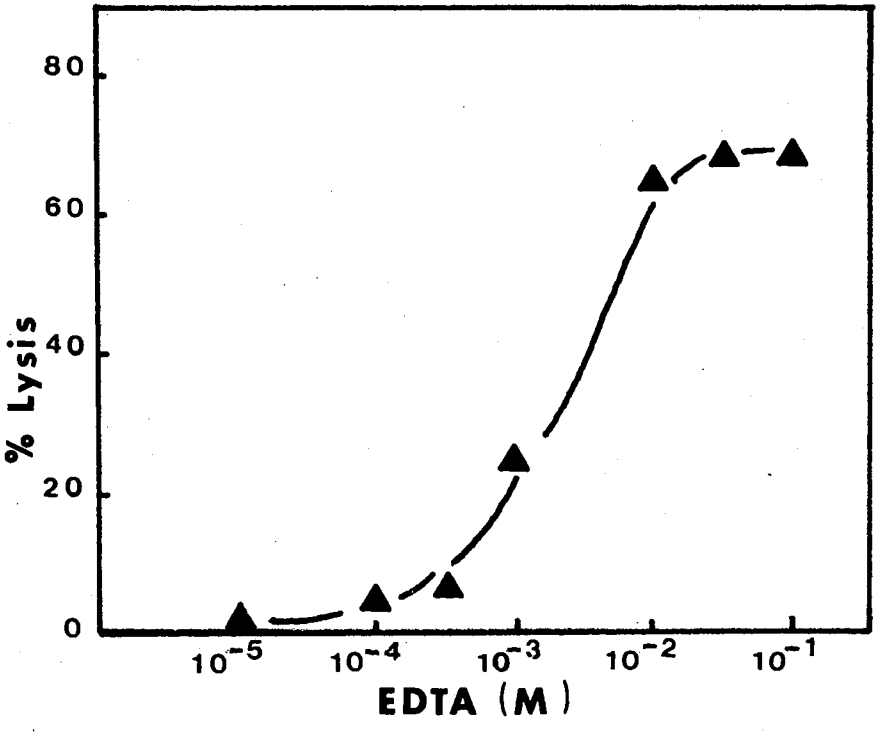
Cells were grown in asparagine minimal salts medium for 12-15 hours. The cells were harvested and washed in phosphate buffer (10 mM, pH 7.0) and suspended to an absorbance of 0.4 at 540 nm in 75 ml of phosphate buffer. Various concentrations of EDTA (0 to 0.1 M) were added to 5 ml aliquots of this suspension. The cells were incubated at 37 C for 30 minutes, and the percent lysis calculated from the change in absorbance after 30 minutes incubation.

The results of these experiments (Fig. 1) indicate that lysis is dependent upon the concentration of EDTA between 10^{-4} and 10^{-2} M. Concentrations of EDTA below 10^{-4} M do not produce lysis, nor does an increase in concentration above 10^{-2} M increase the percent lysis.

Influence of Temperature on Lysis

Haslam (1969) reported that P. fluorescens treated with EDTA at 37 C and then incubated at 0 C did not lyse. He suggested that the inhibition of lysis was due to the inhibition of enzymatic activity at the low temperature. Studies were conducted to examine this suggestion.

Figure 1. Lysis of P. fluorescens Cells in Different Concentrations of EDTA.


80
60
40
20
0
% Lysis
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
EDTA (M)

Cells were grown in asparagine minimal salts medium for 12-15 hours, harvested by centrifugation (5,900 x g for 10 minutes), washed 3 times with phosphate buffer (10 mM, pH 7.0), and suspended in 10 ml of phosphate buffer. This suspension was used to inoculate tubes containing 5.0 ml of phosphate buffer to an absorbance of 0.5 at 540 nm. Two tubes were allowed to equilibrate at each of a series of incubation temperatures for 15 minutes. Buffer or EDTA was added to each tube at the different temperatures. The absorbance at 540 nm was determined at 0 time after the addition of EDTA, and the change in absorbance measured at 10 minute time intervals. The results are reported as percent lysis of treated cells after 30 minutes of incubation.

The EDTA-promoted lysis is temperature sensitive (Fig. 2). The maximum lysis occurred at approximately 37 C and the amount of lysis decreased as the temperature of incubation was increased or decreased. This type of curve is characteristic of an enzymatic reaction. An enzyme is not active at low temperatures, and as the temperature is raised the activity increases to a maximum and then decreases as the enzyme is denatured at higher temperatures.

Lysis can also be inhibited by incubation at high temperatures prior to treatment with EDTA (Fig. 3). Cells were incubated at either 60 C or 37 C for 30 minutes. The cells were then equilibrated to 37 C. EDTA was added to the cells and incubation at 37 C continued. The absorbance at 540 nm was determined at 0 time and at regular time intervals during incubation.

Cells incubated at 60 C prior to treatment with EDTA do not lyse when incubated at 37 C. These results support the suggestion that lysis is due to enzymatic activity. An enzyme would be denatured by the heat

Figure 2. Lysis of P. fluorescens Cells in 1 mM EDTA at Different Temperatures.

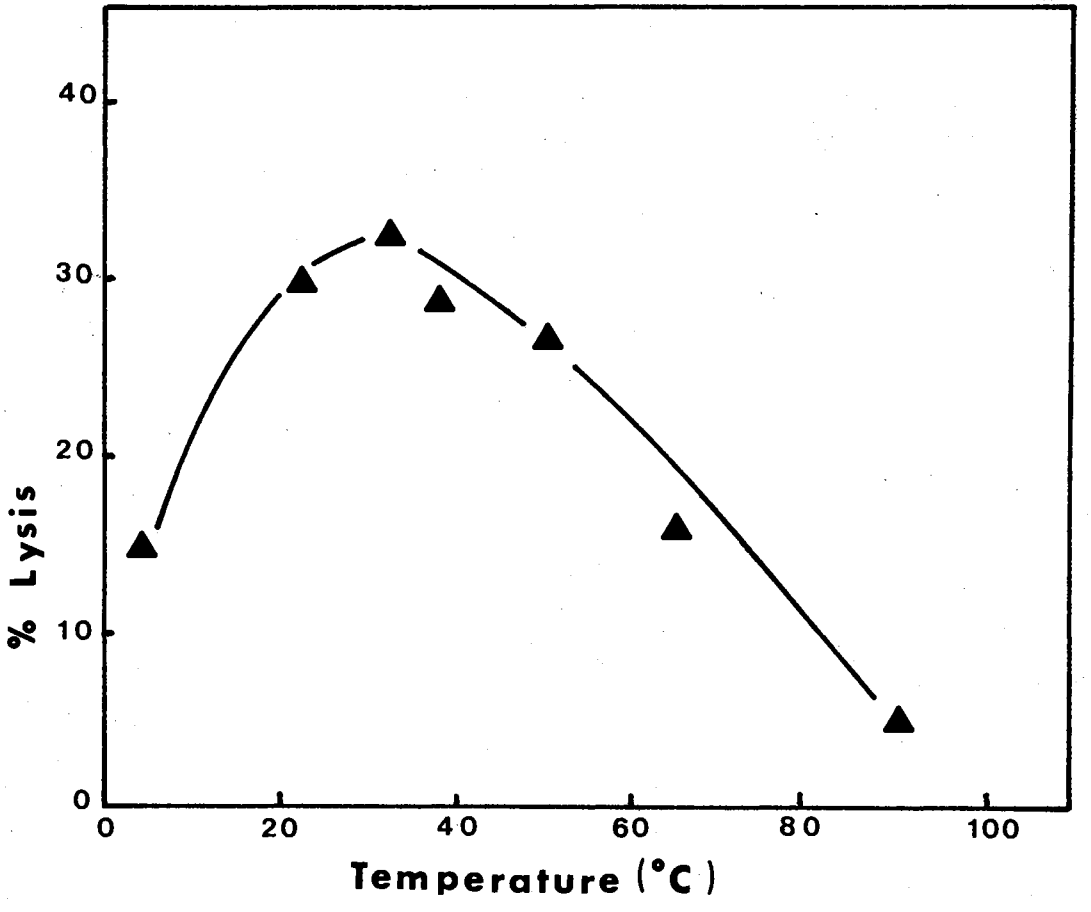
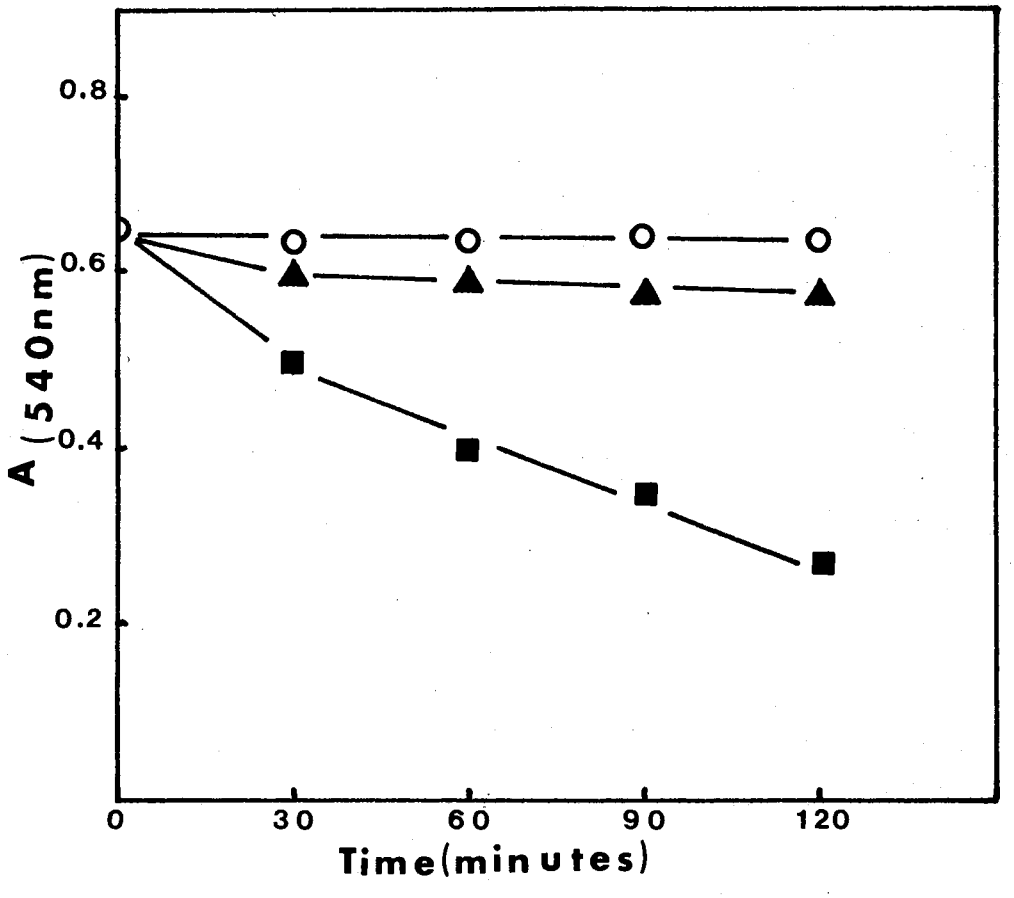

40
30
20
10
0
% Lysis
0
20
40
60
80
100
Temperature (°C)

Figure 3. EDTA-Promoted Lysis of P. fluorescens Cells After Incubation as 37 C and 60 C. ○, 37 C Control; 60 C Control; ▲, 60 C Incubated EDTA Treated; ■, 37 C Incubated EDTA Treated.


0.8
0.6
0.4
0.2
A (540nm)
0
30
60
90
120
Time(minutes)

prior to treatment with EDTA. The subsequent incubation at 37 C, although at a temperature permissive to lysis, would not produce lysis because the enzyme(s) had been inactivated by denaturation. These results might also indicate that a change in the substrate of the enzyme had occurred due to heating.

Influence of Growth in Different Media on Lysis

Repaske (1958) and Haslam (1969) reported that EDTA sensitivity varied with the medium in which the cells were grown. In an attempt to maximize lysis, cells were grown in different media and the EDTA sensitivity tested. It was assumed that the most sensitive cells would contain the largest amount of any enzymes responsible for lysis. These cells could then be used as a rich source for the isolation of these enzymes.

Side-arm flasks containing 100 ml of different media were inoculated from a stock culture of P. fluorescens. The flasks were incubated at 37 C until the cells entered the exponential phase of growth. The cells from each flask were harvested by centrifugation and inoculated into flasks of fresh medium. Incubation was continued and growth determined by measuring the absorbance at 540 nm. Aliquots (5.0 ml) were removed from the flasks at various points on the growth curve and placed in tubes. EDTA was added to each tube and the change in absorbance measured at regular time intervals after the addition of EDTA.

Cells growing in either YPG or asparagine minimal salts medium (Fig. 4) are sensitive to EDTA. Cells growing in succinate or glucose minimal salts medium (Fig. 5) are resistant to EDTA. Thus, it is

Figure 4. EDTA-Promoted Lysis of P. fluorescens Cells Grown in Different Carbon Sources. ○, Untreated YPG Cells; ●, Treated YPG Cells; △, Untreated Asparagine Cells; ▲, Treated Asparagine Cells.

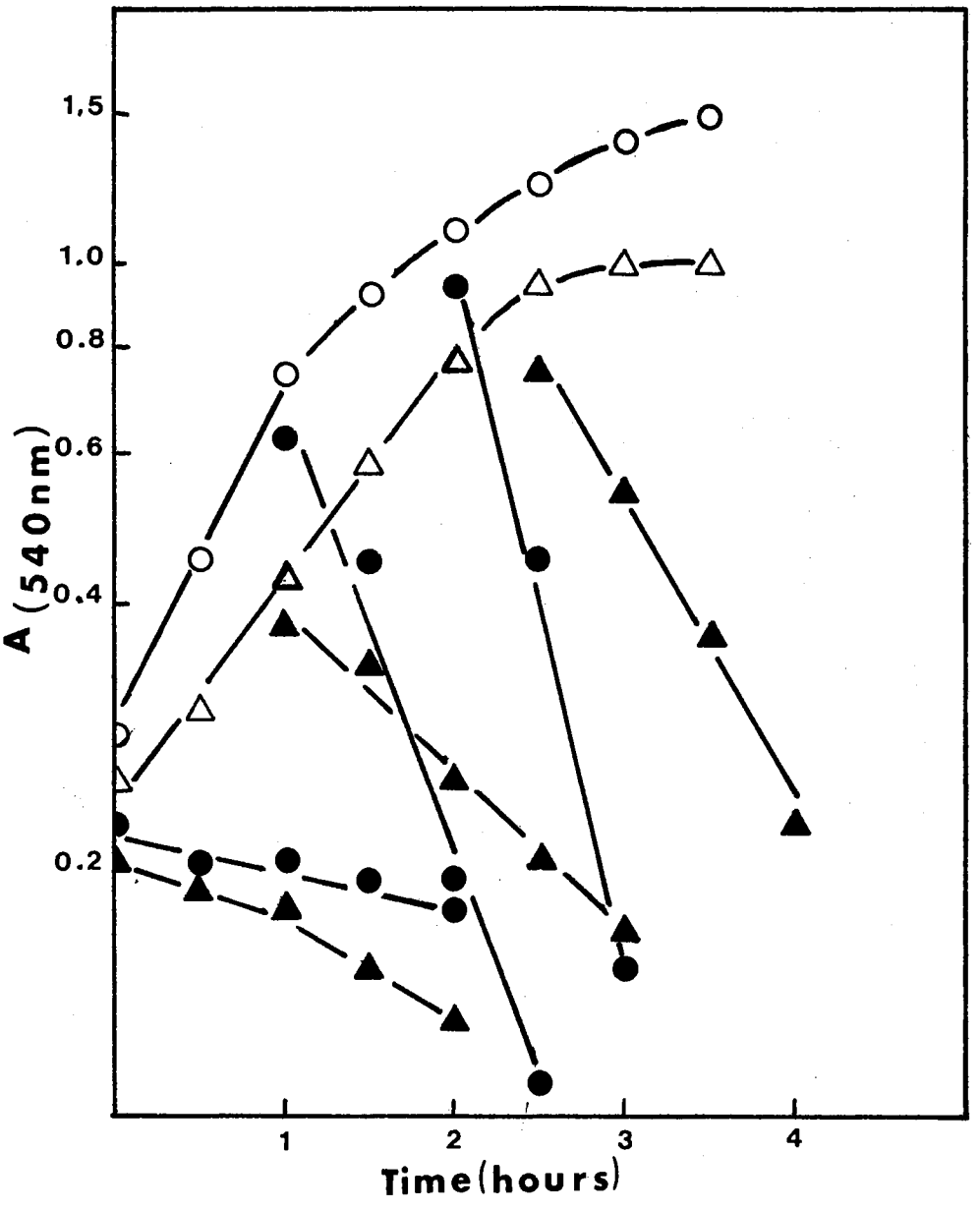
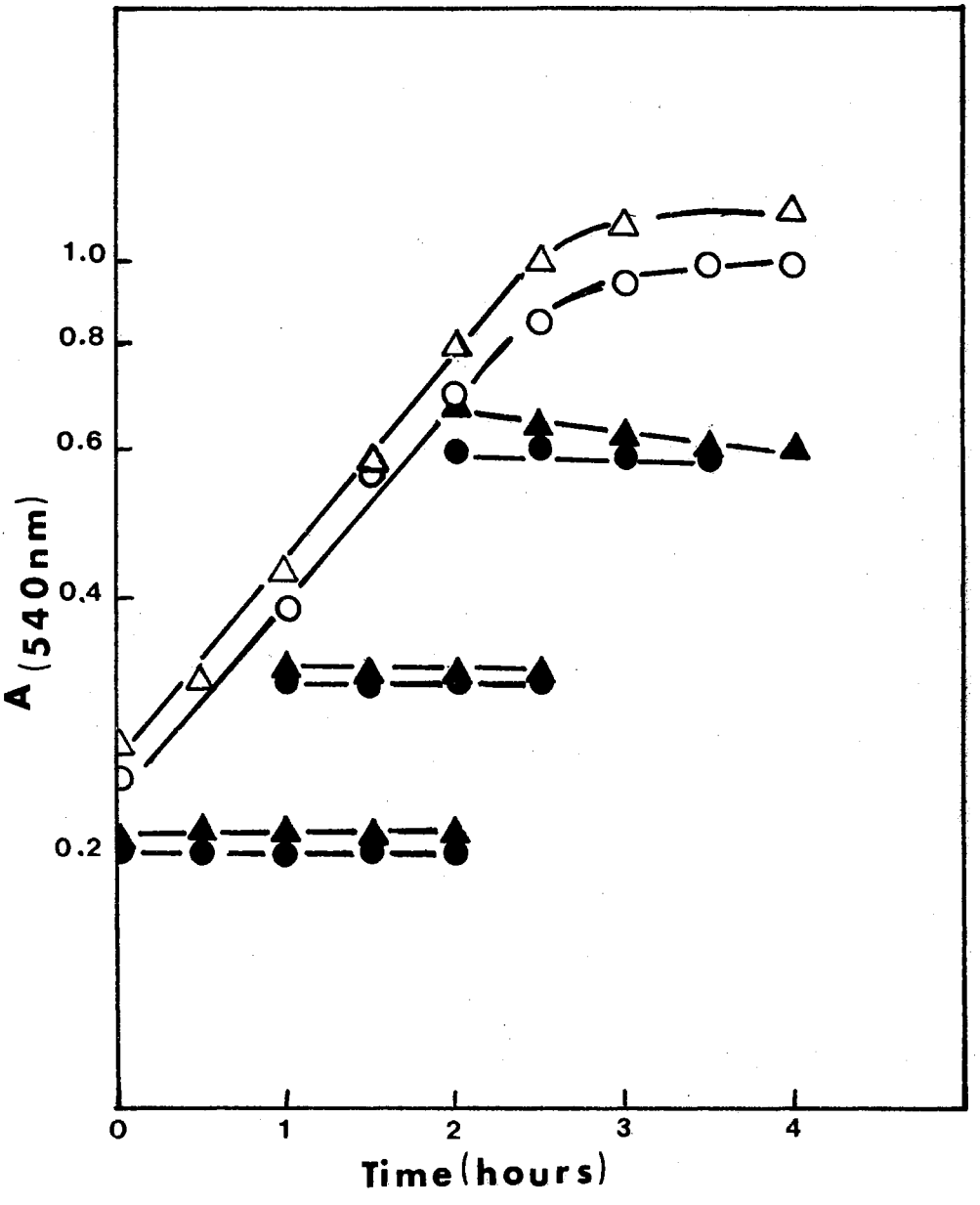

1.5
1.0
0.8
0.6
0.4
0.2
A (540nm)
1
2
3
4
Time (hours)

Figure 5. EDTA-Promoted Lysis of *P. fluorescens* Cells Grown in Different Carbon Sources. △, Untreated Glucose Cells; ▲, Treated Glucose Cells; ○, Untreated Succinate Cells; ●, Treated Succinate Cells.


1.0
0.8
0.6
0.4
0.2
A (540nm)
0
1
2
3
4
Time (hours)

apparent that the growth medium alters the sensitivity of the cells to EDTA.

The resistance of glucose and succinate grown cells could be due to alterations in the quantity of LPS produced by the cells. If cells grown in glucose and succinate produced excess LPS, then treatment with EDTA might not cause enough damage to the cell periphery to produce lysis. Walker (1973) reported that P. fluorescens produced higher amounts of LPS when grown in a medium with glucose as a carbon source than when grown in a medium with succinate as a carbon source. Smith (1973) reported higher amounts of LPS produced by cells grown in a glucose medium when compared to cells grown in asparagine medium. The amount of LPS in succinate and asparagine grown cells reported by these workers was comparable. Thus, resistance to EDTA does not directly correlate with higher amounts of LPS production by the cell.

In sensitive cells (Fig. 4), the rate of lysis is slow at the early stages of exponential growth. As the cells continue exponential growth, the rate of lysis increases rapidly. This is particularly apparent in YPG medium. The change in the rate of lysis indicates a change in EDTA sensitivity. This change in sensitivity could be due to an increase in the level of an enzyme involved in EDTA-promoted lysis. The change in EDTA sensitivity also suggests that the amount of LPS produced by the cell is not the primary factor in EDTA resistance. If the amount of cellular LPS was the primary factor, then the rate of lysis should be lowest when the level of LPS is highest. Walker (1973) and Smith (1973) reported that the level of cellular LPS was highest in late exponential or early stationary phases of growth. The rate of lysis is most rapid during these phases of growth.

Both Walker and Smith reported qualitative changes in the LPS of P. fluorescens during growth of the cells. Thus, it is possible that the change in EDTA sensitivity could be due to qualitative changes in the constituents of the cell periphery. Changes in the LPS of the envelope could affect the cell's sensitivity to EDTA, as well as alter the substrate of any enzyme responsible for lysis.

Influence of Chloramphenicol on EDTA-Promoted Lysis

Pooley and Shockman (1969) reported that the inhibition of protein synthesis inhibited autolysis in Streptococcus faecalis. Since it was observed that P. fluorescens exhibited a shift in EDTA sensitivity during early exponential growth, studies were conducted to determine if chloramphenicol (CAP) affected this shift.

P. fluorescens cells were inoculated into YPG medium to an absorbance of 0.05 at 540 nm. The cells were incubated at 37 C until they reached the early exponential phase of growth. Samples were removed and various concentrations of CAP added to the samples and incubation at 37 C continued for 30 minutes. The cells were harvested by centrifugation (12,000 x g for 10 minutes), suspended in Tris buffer (10 mM, pH 7.0), and EDTA added to each sample. The change in absorbance at 540 nm was measured at regular time intervals during continued incubation at 37 C after the addition of EDTA.

Incubation with CAP prior to the addition of EDTA inhibits both growth and the EDTA-promoted lysis of the cells (Table I). The inhibition exhibits a dose response relative to the incubation concentration of CAP. These results indicate that the inhibition of protein synthesis

TABLE I

EDTA-PROMOTED LYSIS OF P. FLUORESCENS CELLS AFTER INCUBATION IN CHLORAMPHENICOL

Concentration of CAP µg/ml	Initial Absorbance 540 nm	Decrease in Absorbance* 540 nm	Percent Lysis	Percent Inhibition of Lysis
0	0.50	0.17	34	0
25	0.50	0.14	28	18
50	0.50	0.09	18	47
75	0.50	0.08	16	53
100	0.50	0.05	10	71

*After 20 minutes incubation at 37 C in 1 mM EDTA

inhibits the shift in EDTA sensitivity, thus supporting the suggestion that the shift in EDTA sensitivity is mediated by a protein. Increased levels of CAP or increased incubation time will not totally inhibit lysis, indicating that the cells contain a certain level of the protein mediating lysis at the time of CAP addition.

The CAP inhibition of EDTA-promoted lysis could also indicate that CAP interferes with the action of EDTA on the cells. The addition of CAP at the same time as the addition of EDTA to sensitive cells (Fig. 6) does not support this suggestion. CAP will not inhibit the EDTA-promoted lysis of cells unless the cells are allowed to grow in its presence. This supports the suggestion that the inhibition of lysis is due to the inhibition of protein synthesis rather than any interaction between EDTA and CAP.

The inhibition of protein synthesis inhibits growth. It is therefore possible that the inhibition of lysis is the result of growth inhibition rather than protein synthesis inhibition. Table II shows the results of a comparison of the inhibition of growth and EDTA-promoted lysis by sodium azide and CAP. These results indicate that although incubation with sodium azide inhibits growth, EDTA-promoted lysis is not affected by this growth inhibition. Growth and EDTA-promoted lysis are both inhibited by incubation with CAP. Thus, the CAP inhibition of EDTA-promoted lysis is not the result of a simple inhibition of growth.

Influence of Culture Age on EDTA-Promoted Lysis

Collins (1964) reported the cell wall of P. aeruginosa varies with

Figure 6. EDTA-Promoted Lysis of P. fluorescens Cells in the Presence of Chloramphenicol. ○, No EDTA; ▲, 100 µg/ml CAP; ■, 25 µg/ml CAP; ●, No CAP.

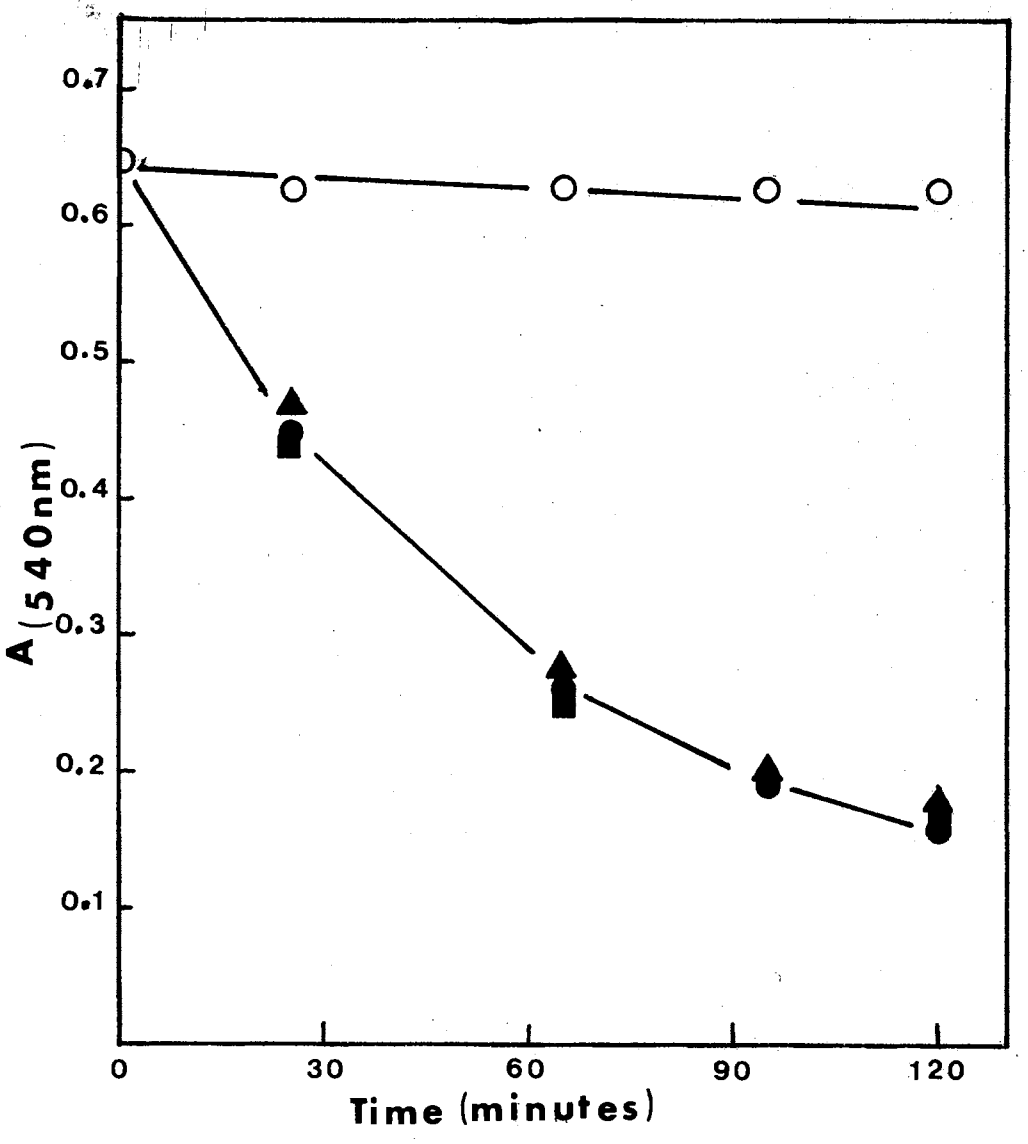

0.7
0.6
0.5
0.4
0.3
0.2
0.1
A(540nm)
0
30
60
90
120
Time (minutes)

TABLE II

INHIBITION OF GROWTH AND EDTA-PROMOTED LYSIS OF *P. FLUORESCENS** BY SODIUM AZIDE AND CHLORAMPHENICOL

Inhibitor	Concentration (μg/ml)	Percent Growth** Inhibition	Percent Lysis*** Inhibition
Sodium Azide	100	69	0
	75	57	0
	50	37	9
	25	16	5
Chloramphenicol	100	92	82
	75	88	77
	50	66	63
	25	50	55

*Cells were incubated in presence of inhibitors for 1 hr prior to treatment with EDTA

**$100-\left[\frac{\text{Amount of Growth in presence of inhibitor}}{\text{Amount of growth in control cells}} \times 100\right]$

***$100-\left[\frac{\text{Amount of EDTA-promoted lysis of cells grown in presence of inhibitor}}{\text{Amount of EDTA-promoted lysis of control cells}} \times 100\right]$

the age of the culture. He also reported the isolation of a wall lytic enzyme from the growth medium of aged cells. Studies were conducted to determine if the age of the culture affected EDTA sensitivity in P. fluorescens.

Cells were inoculated into YPG medium and harvested by centrifugation (12,000 x g for 10 minutes) after 24 hours of growth. The cells were washed 3 times with phosphate buffer (10 mM, pH 7.0) and suspended in phosphate buffer to an absorbance of 0.85 at 540 nm. Aliquots of the suspension (4 ml) were treated with combinations of EDTA, the spent YPG medium the cells were grown in, fresh YPG medium, and phosphate buffer.

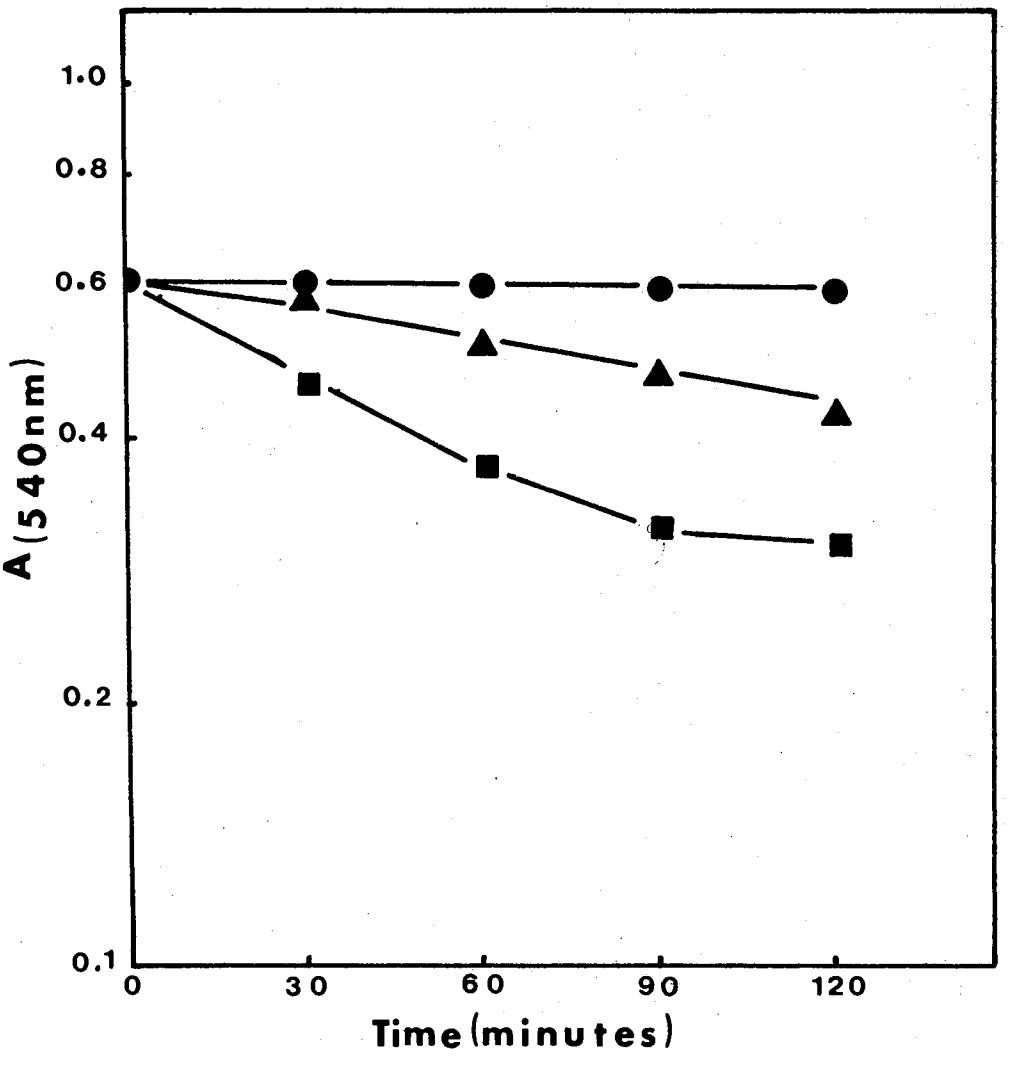
Cells grown for 24 hours in YPG medium lyse only slightly when treated with EDTA (Fig. 7). The amount of lysis can be increased by treating the cells with spent medium in combination with EDTA. Combination of EDTA and fresh YPG medium will not increase the lysis.

The ability of spent medium to overcome the EDTA resistance of aged cultures suggests the presence of a lytic enzyme in the spent medium. It also points out that the reported changes in the wall are not sufficient to produce EDTA resistance. The cells are still capable of lysis but lack some factor essential to produce lysis.

Treatment with spent medium without EDTA will not cause lysis of aged cells (Fig. 7). This suggests that the cell periphery must be damaged prior to the action of the enzyme and supports the suggestion of Haslam et al. (1969) that lysis of P. fluorescens is a two stage process.

Two important conclusions were drawn from these studies. (1) Aged YPG grown cells could be used as a substrate for quantitation of the

Figure 7. Lysis of P. fluorescens Grown in YPG Medium for 24 Hours. ●, Phosphate Buffer, Spent Medium, Fresh Medium; ▲, Phosphate Buffer + EDTA, Fresh Medium + EDTA; ■, Spent Medium + EDTA.


1.0
0.8
0.6
0.4
0.2
0.1
A(540nm)
0
30
60
90
120
Time(minutes)

lytic activity in isolation studies. This was important since heat treatment of cells to inhibit lysis appears to destroy the substrate of the lytic enzyme, making it unsuitable for assay purposes. (2) Spent YPG medium could be used as a source material for isolation of the lytic enzyme.

Determination of Extracellular Lytic Activity

The presence of lytic activity in the spent YPG medium suggested an extracellular enzyme might be involved in the EDTA-promoted lysis phenomenon. Whiteside and Corpe (1969) reported the isolation of extracellular enzymes from a species of Pseudomonas capable of lysing heated envelopes of Chromobacterium violaceum. Liu (1964) reported the production of extracellular hemolysin, lecithinase, and proteinase by P. aeruginosa. He also reported that the phosphate concentration in liquid medium was the most critical factor influencing the production of extracellular enzymes by P. aeruginosa. The extracellular hemolysin, lecithinase, and proteinase are only produced if the phosphate concentration is low. Thus, studies were conducted to determine if the lytic factor was produced as an extracellular enzyme and if phosphate influenced its release.

Cells were inoculated into side-arm flasks containing 100 ml of YPG medium and the flasks incubated at 37 C. Samples (5 ml) were removed from the flasks at various time intervals during growth. The samples were centrifuged (12,000 x g for 10 minutes) to separate the cells from the growth medium. The cells from each sample were suspended in phosphate buffer (10 mM, pH 7.0) and treated with EDTA. The growth medium

from each sample was tested for lytic activity against 24 hour old YPG grown cells. The concentration of inorganic phosphate in each sample of medium was determined.

The level of lytic activity in the growth medium increased as the cells entered the stationary phase of growth (Fig. 8). The rate of increase in lytic activity did not coincide with the rate of increase in the mass of cells. The level of an extracellular enzyme should increase as the cell mass in the culture increases, since more cells should produce more enzyme. The level of lytic activity does not increase until the cells are well into the stationary phase of growth, suggesting that the lytic activity is not released from the cell in the same manner as an extracellular enzyme.

EDTA sensitivity (Fig. 8) is highest in cells in the exponential phase of growth. It decreases rapidly as the cells enter the stationary phase of growth. The EDTA sensitivity of the cells is lowest when the lytic activity in the medium is highest. This suggests that the cells may be releasing the factor responsible for their sensitivity into the medium when they enter the stationary phase of growth.

The phosphate concentration decreased rapidly as the cell mass increased. It was lowest when the lytic activity was highest, which could be coincidental, depletion of phosphate due to growth not necessarily related to the appearance of lytic activity. It could also be, as suggested by Liu (1964), the reason for the sudden appearance of lytic activity in the spent medium. Studies on the release of the lytic factor from cells grown in YPG medium supplemented with inorganic phosphate (Table III) demonstrate that the release of the lytic factor is not influenced by high concentrations of phosphate in the medium. This

Figure 8. Change in EDTA Sensitivity in P. fluorescens as a Function of Growth in YPG Medium. ●, Absorbance of Cell Culture; ⬢, Percent Lysis of Cells in EDTA; ▲, Units of Lytic Activity in Medium; ■, Concentration of Phosphate in Medium.

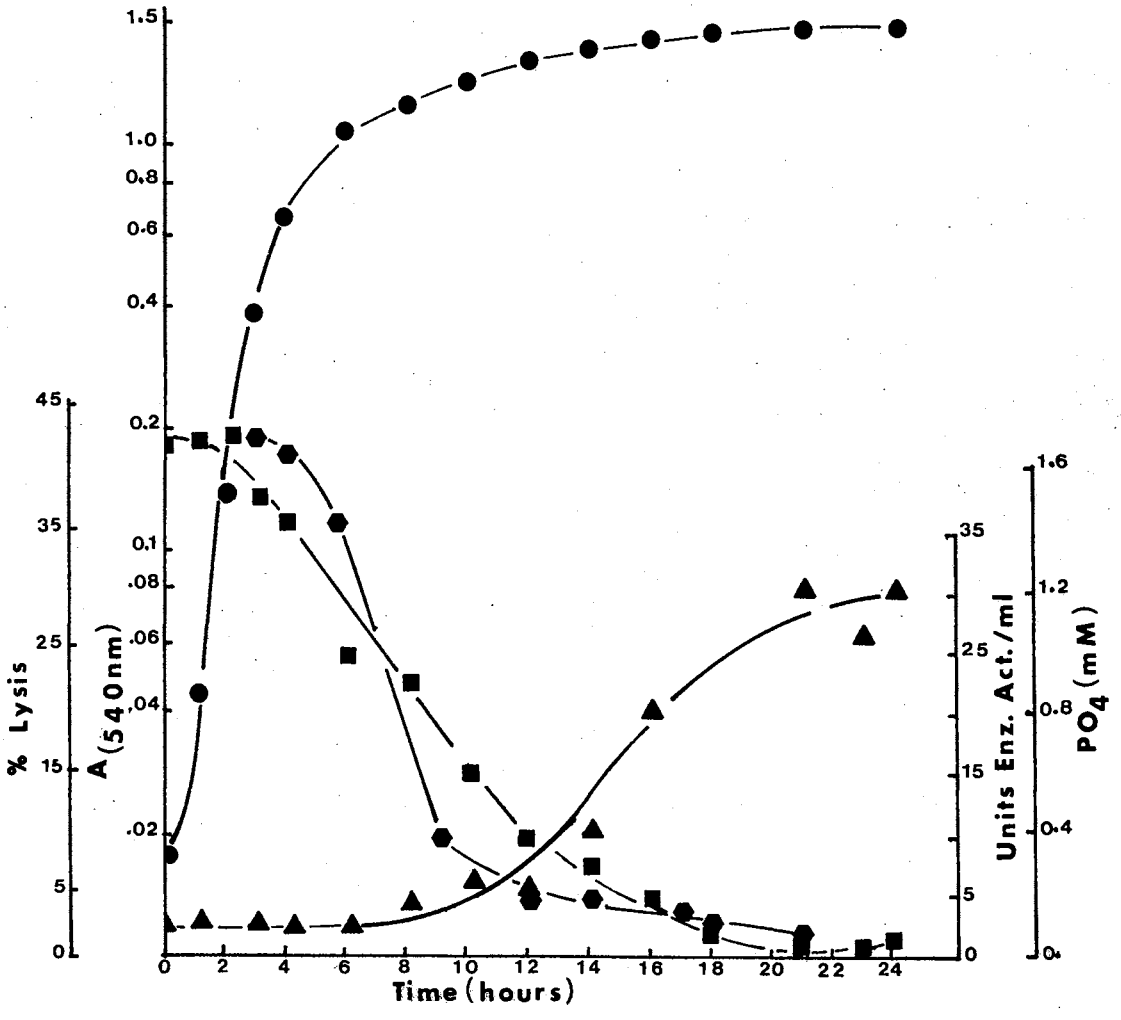

% Lysis
A(540nm)
Units Enz. Act./ml
PO4 (mM)
Time (hours)
0
2
4
6
8
10
12
14
16
18
20
22
24

TABLE III

EFFECT OF PHOSPHATE CONCENTRATION ON PRODUCTION OF LYTIC FACTOR BY P. FLUORESCENS GROWN IN YPG MEDIUM

Phosphate Concentration (mM)	Culture Age (Hr.)	Lytic Activity*
1.6	5	0
	7	0
	24	60
10.0	5	0
	7	0
	24	70
80.0	5	0
	7	0
	24	70

*Expressed as units of lytic activity/ml of spent medium

indicates that the correlation between lytic activity and phosphate concentration in the medium (Fig. 8) is coincidental.

The decrease in EDTA sensitivity of the cells also coincided with the decrease in phosphate concentration in the medium. Studies were conducted to determine if the level of phosphate in YPG medium affected the EDTA sensitivity of the cells. Table IV indicates that the change in EDTA sensitivity occurs in high as well as in low concentrations of phosphate. However, cells grown in YPG medium containing a high concentration of phosphate appear to be less sensitive to EDTA.

Earlier studies (Fig. 4 and 5) indicated that cells grown in a phosphate base (80 mM PO_4) minimal medium with succinate or glucose as a carbon source were resistant to lysis by EDTA, whereas when asparagine was used as the carbon source the cells were sensitive to EDTA. Studies were therefore conducted to determine if the resistance of the succinate and glucose grown cells was due to the phosphate concentration of the medium. These studies were conducted using the Tris base minimal medium of Leventhal, et al. (1962) supplemented with KH_2PO_4. Table V shows that cells grown in Tris base medium are sensitive to EDTA regardless of the carbon source and that high concentrations of phosphate tend to decrease the sensitivity. However, growth in Tris buffer containing 80 mM phosphate with glucose or succinate as the carbon source did not totally inhibit lysis of the cells by EDTA as had been previously shown (Fig. 5). This suggests that although the phosphate concentration of the medium plays a role in EDTA sensitivity, it is not the only component of the cell's environment affecting EDTA sensitivity.

TABLE IV

EFFECT OF PHOSPHATE CONCENTRATION ON EDTA-PROMOTED LYSIS OF *P. FLUORESCENS* GROWN IN YPG MEDIUM

Phosphate Concentration (mM)	Culture Age (Hr.)	% Lysis of Cells*
1.6	5	39
	7	16
	24	0
10.0	5	25
	7	12
	24	0
80.0	5	17
	7	9
	24	0

*Percent decrease in absorbance at 540 nm during 30 min incubation at 37 C in Tris buffer in presence of 1 mM EDTA.

TABLE V

EFFECT OF PHOSPHATE CONCENTRATION ON EDTA-PROMOTED LYSIS OF P. FLUORESCENS GROWN IN MEDIUM* CONTAINING DIFFERENT CARBON SOURCES

Carbon Source	Phosphate Concentration (mM)	Percent Lysis** of Cells in exponential phase of growth
Glucose	1.6	54.7
	10.0	59.0
	80.0	33.0
Succinate	1.6	82.7
	10.0	65.7
	80.0	43.9
Asparagine	1.6	87.0
	10.0	90.0
	80.0	57.0

*Tris base minimal medium of Leventhal, et al. (1962).

**Percent decrease in absorbance at 540 nm during 60 min incubation at 37 C in Tris buffer in presence of 1 mM EDTA

Isolation of the Lytic Factor

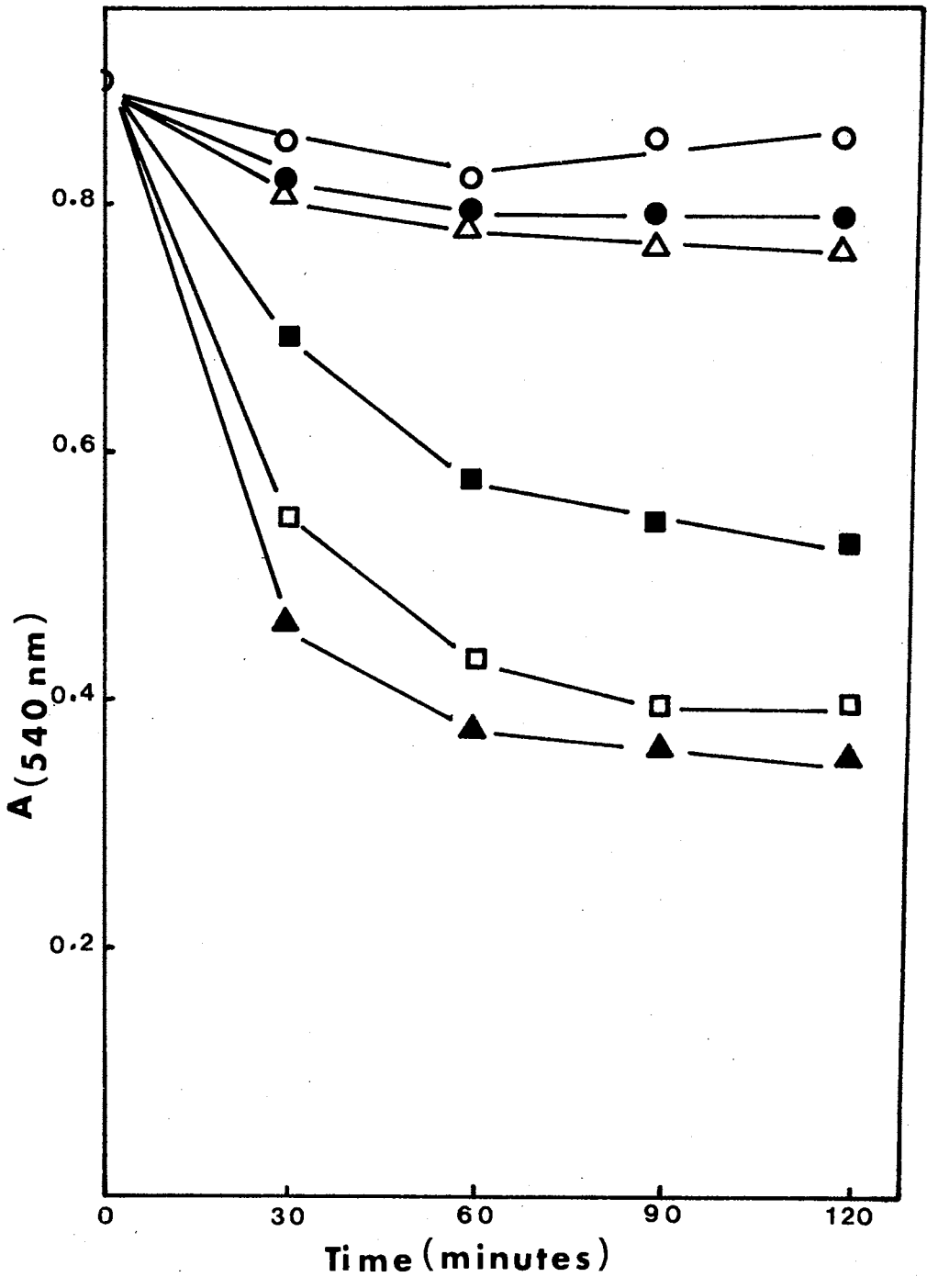
Studies were conducted to isolate the lytic factor from spent YPG medium from a 24 hour old culture of P. fluorescens. Spent YPG medium (100 ml) was sequentially fractionated using ammonium sulfate. The protein precipitated by each level of ammonium sulfate saturation was dissolved in phosphate buffer and dialysed against phosphate buffer. After dialysis, the ammonium sulfate fractions were tested for the ability to increase the EDTA-promoted lysis of 24 hour old YPG grown cells. The results of one such study are shown in Fig. 9.

The 40, 60, and 80 percent ammonium sulfate fractions enhanced the EDTA promoted lysis of the cells, indicating the lytic factor could be precipitated with ammonium sulfate. The presence of lytic activity in the three ammonium sulfate fractions is probably due to the inefficiency of the precipitation procedure rather than to the presence of more than one lytic factor. This is supported by the comparison of lytic activity contained in each ammonium sulfate fraction based on the protein concentration. When the amount of lytic activity in each fraction is compared on the basis of protein concentration, the 40 percent fraction is shown to contain the majority of the lytic activity. Thus, fractionation of the lytic activity can also be accomplished using ammonium sulfate.

Characterization of Ammonium Sulfate Fractions

Studies were conducted to characterize the enzyme activity contained in the different ammonium sulfate fractions. The protein concentration in each fraction was determined, and the fractions were

Figure 9. Lysis of P. fluorescens by Ammonium Sulfate Fractions From YPG Medium. ○, All Controls; Phosphate Buffer, 30 Percent Ammonium Sulfate Fraction, 40 Percent Ammonium Sulfate Fraction, 60 Percent Ammonium Sulfate Fraction, 80 Percent Ammonium Sulfate Fraction; ●, EDTA Treated; △, 30 Percent Ammonium Sulfate Fraction + EDTA; ▲, 40 Percent Ammonium Sulfate Fraction + EDTA; ■, 60 Percent Ammonium Sulfate Fraction + EDTA; □, 80 Percent Ammonium Sulfate Fraction + EDTA.


0.8
0.6
0.4
0.2
A(540 nm)
0
30
60
90
120
Time (minutes)

assayed for lytic activity, proteinase activity, and esterase activity. The results of the enzyme assays are expressed as units of activity per mg protein (Table VI).

The 30 percent ammonium sulfate fraction contained no lytic or proteinase activity. It did contain 23 percent of the total esterase activity. The 40 percent ammonium sulfate fraction contained 80 percent of the total lytic activity. It contained no proteinase activity and 26 percent of the esterase activity. The 60 percent ammonium sulfate fraction contained 18 percent of the total lytic activity, 60 percent of the total proteinase activity, and 37 percent of the esterase activity. The 80 percent ammonium sulfate fraction contained 2 percent of the total lytic activity, 40 percent of the total proteinase activity, and 14 percent of the total esterase activity.

These results indicate that the EDTA-promoted lysis is probably not due to the action of a proteolytic enzyme, but could be due to the action of a lipolytic enzyme. Whiteside and Corpe (1969) reported the release of a number of esterase enzymes by a species of Pseudomonas. A similar release by P. fluorescens could explain the presence of esterase activity in the fractions containing little lytic activity.

Purification of Lytic Activity

Purification of the lytic factor was attempted using ion exchange chromatography. The 40 percent ammonium sulfate fraction was loaded onto a column of DEAE Sephadex. The sample was eluted with a gradient of NaCl and fractions collected. The concentration of protein in the eluent was monitored by measuring the absorbance at 280 nm. Each fraction was tested for lytic activity.

TABLE VI

ENZYMATIC ACTIVITIES OF AMMONIUM SULFATE FRACTIONS

Ammonium Sulfate Fraction	Protein mg/ml	Lytic	Specific Activity* Proteinase	Esterase
30%	0.3	0	0	1333
40%	0.55	363	0	1514
60%	0.6	83	1333	2110
80%	1.0	20	930	733

*Specific Activity: Units of Activity/mg Protein

The elution pattern (Fig. 10) indicates that the protein comes off the column in two peaks. The first peak contains no lytic activity. The lytic activity in the second peak was concentrated by precipitation with 40 percent ammonium sulfate. The purification of the lytic factor was checked using acrylamide gel electrophoresis.

The densitometer scans of the acrylamide gels (Fig. 11) indicate the presence of 3 peaks (those designated 6, 7, and 8) common to the samples with lytic activity. These peaks were very small in the DEAE peak I scan, and this peak did not contain any detectable lytic activity.

The amount of purification achieved by each fractionation step is shown in Table VII. The initial precipitation with 40 percent ammonium sulfate produced the greatest amount of purification (133 fold purification and 75 percent recovery). The DEAE column chromatography increased the purity of the lytic factor, but also resulted in a large decrease in the amount of lytic factor recovered. The precipitation of the lytic factor contained in DEAE peak II showed a 179 fold increase in purity of the lytic activity with a 15 percent recovery of the total units of activity. This fraction was designated as crude enzyme.

Lysis of Cell Walls by Crude Enzyme

Enzymes which degrade the mucopeptide of the cell wall produce lysis (Fleming, 1922). Studies were conducted to determine if the crude enzyme degraded the cell wall of P. fluorescens.

Isolated ^{14}C-labeled cell walls of P. fluorescens were incubated with the crude enzyme. Samples were removed from the incubation mixture at regular time intervals, filtered through Millipore filters, and the supernatant collected. The filter and supernatant were placed in

Figure 10. Elution Pattern of DEAE Sephadex Column. ▲, Absorbance; ●, Lytic Activity.

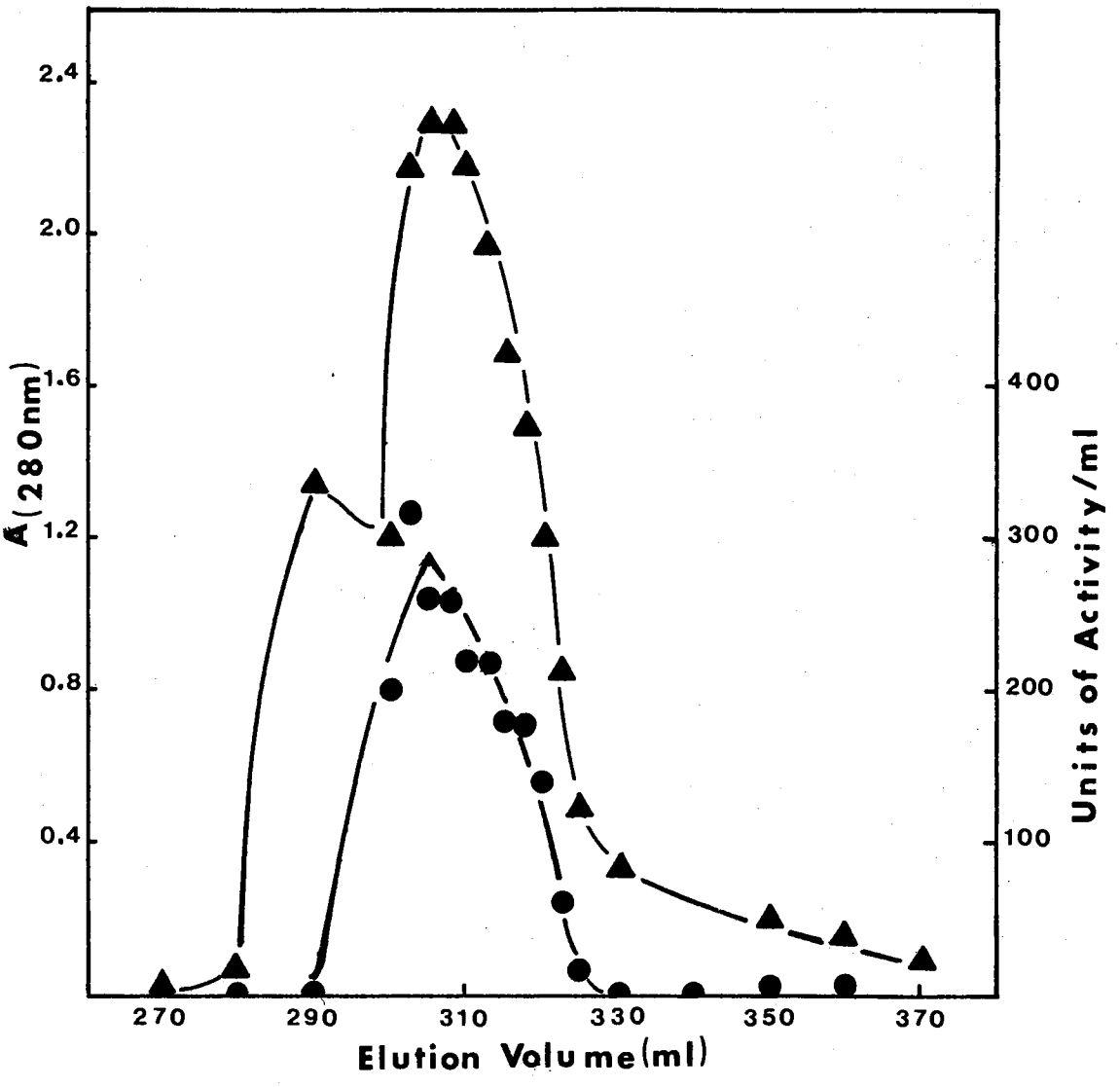

2.4
2.0
1.6
1.2
0.8
0.4
A(280nm)
400
300
200
100
Units of Activity/ml
270
290
310
330
350
370
Elution Volume(ml)

Figure 11. Densitometer Scans of Acrylamide Gels of Fractionation Samples. A, 40 Percent Ammonium Sulfate Fraction; B, DEAE Peak I; C, DEAE Peak II; D, 40 Percent Ammonium Sulfate Fraction of DEAE Peak II.

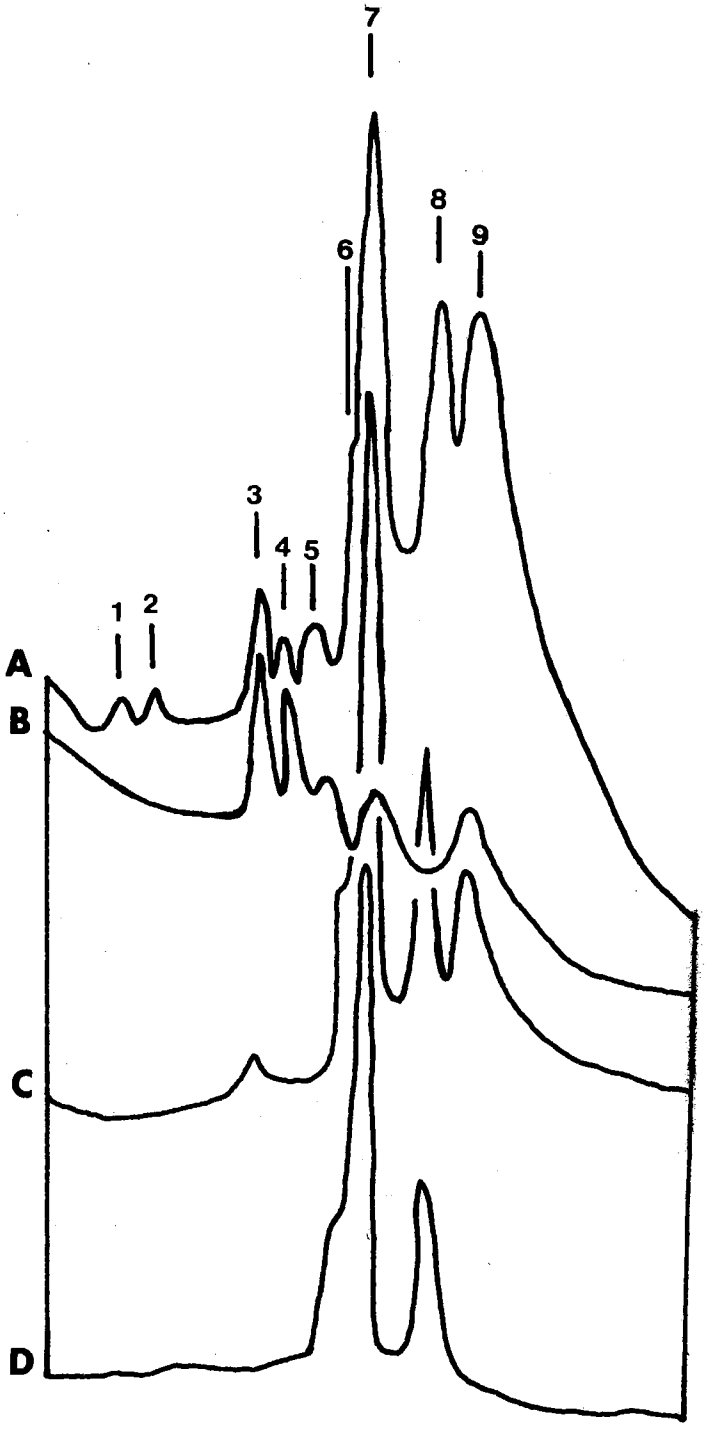

7
8
9
6
3
4
5
1
2
A
B
C
D

TABLE VII

PURIFICATION OF LYTIC ACTIVITY FROM SPENT YPG MEDIUM

	Protein mg/ml	Lytic units/ml	Volume	Total Units	% Recovery	Specific Activity	Purification
Spent YPG Medium	2.9	40	2500	100,000	-	14	-
40% Ammonium Sulfate	0.8	1500	50	75,000	75	1875	133
DEAE Peak II	0.4	800	30	24,000	24	2000	142
40% Ammonium Sulfate Peak II	0.6	1500	10	15,000	15	2500	179

scintillation vials, Aquasol (10 ml) added to the vials, and the radioactivity in each determined. The percent of the total radioactivity released into the supernatant was used as a measure of wall degradation. Controls of lysozyme (100 µg per ml of assay mixture) and Tris buffer (10 mM, pH 7.0) were run to check the validity of the assay procedure.

Incubation with lysozyme released counts from the labeled walls (Fig. 12). The release of counts was essentially linear for the first 10 minutes of incubation and plateaued after 60 minutes incubation. This indicated that the isolated walls could be used as an assay for mucopeptide degradation.

Incubation with the crude enzyme (100 µg per ml of assay mixture) did not release counts from the labeled walls above that released by the Tris buffer control, indicating that the crude enzyme did not degrade the mucopeptide.

Lysis of Membranes by Crude Enzyme

The inability of the crude enzyme to degrade the cell wall suggested that degradation of the cell membrane might be the cause of cell lysis. Studies were conducted to determine if the crude enzyme would cause lysis of the cell membrane.

Protoplasts of B. subtilis were prepared. The protoplasts were suspended in 0.6 M sucrose and 60 µg of the crude enzyme was added to the suspension. The absorbance at 540 nm was determined at 0 time after the addition of the crude enzyme, and the change in absorbance measured at regular time intervals during incubation at 37 C. The crude enzyme lysed the protoplasts (Fig. 13), and this suggests that the degradation of the cell membrane is involved in lysis by the crude enzyme.

Figure 12. Degradation of Isolated ^{14}C-Labeled Walls of P. fluorescens. ○, Walls Labeled With Glucosamine-U-^{14}C; △, Walls Labeled With N-Acetyl-1-^{14}C Glucosamine; Closed Symbols, Crude Enzyme Treated; Open Symbols, Lysozyme Treated; 10^3 cts/min. Equals 100 Percent Release.

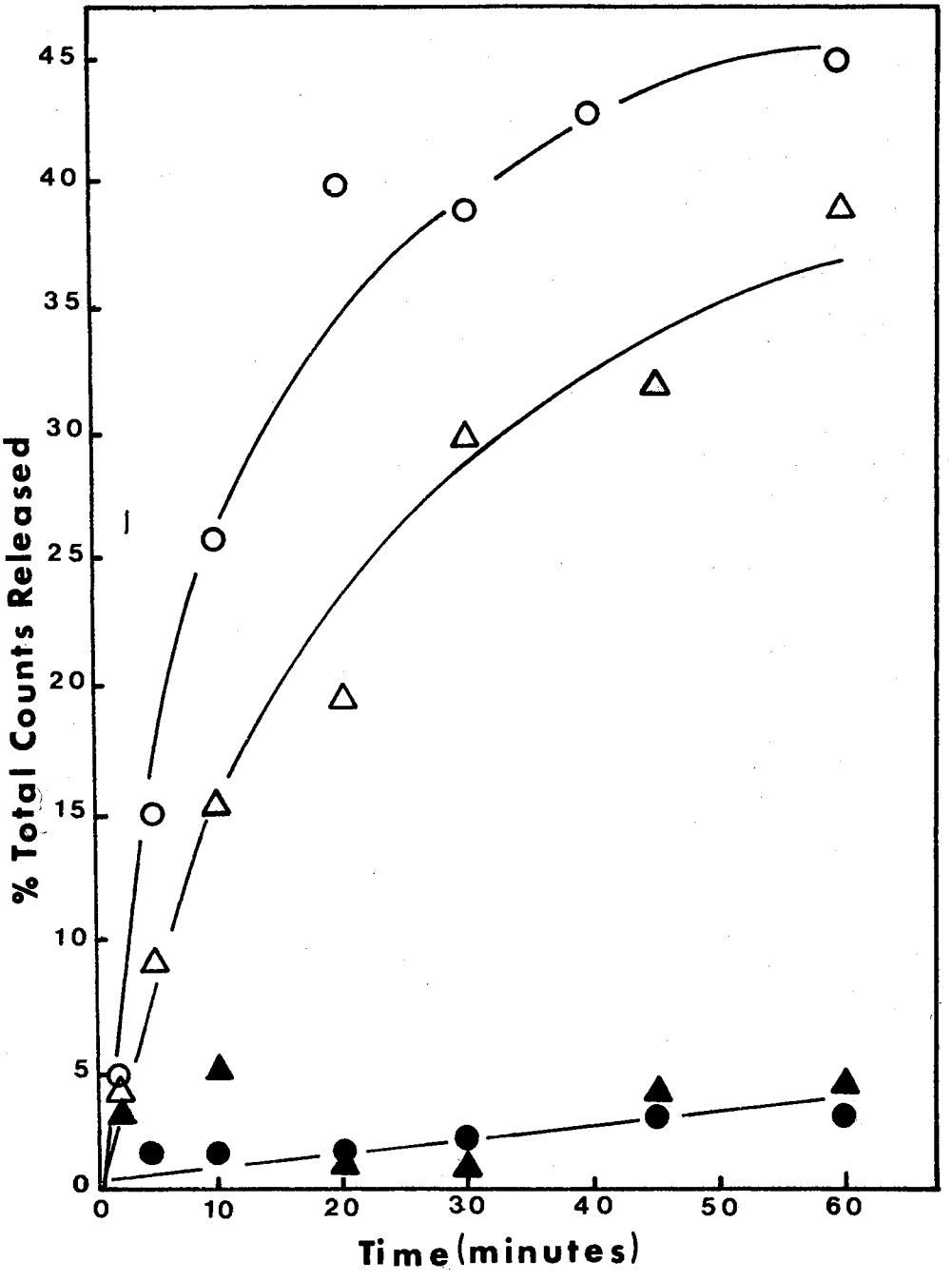
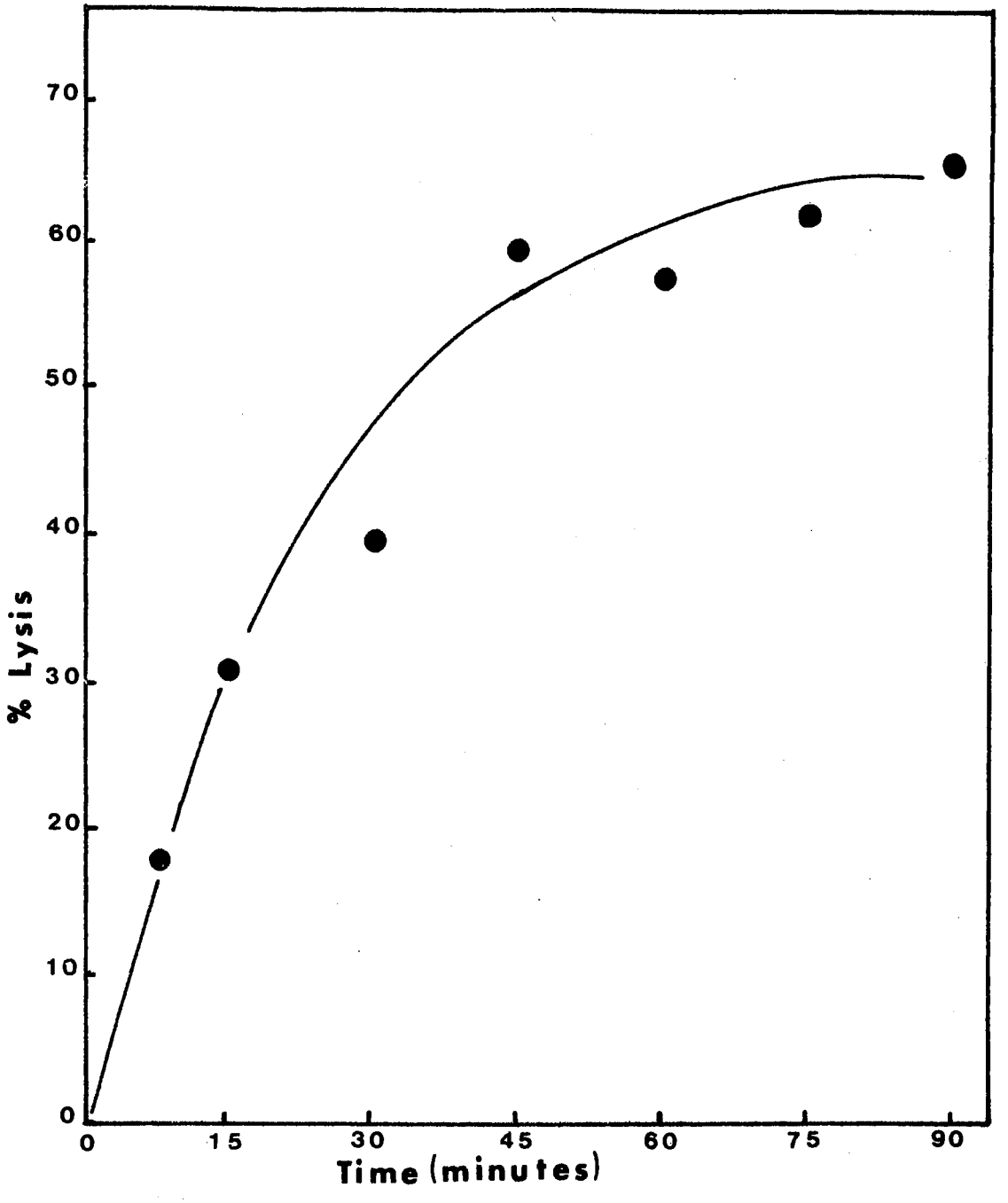

% Total Counts Released
0
5
10
15
20
25
30
35
40
45
0
10
20
30
40
50
60
Time (minutes)

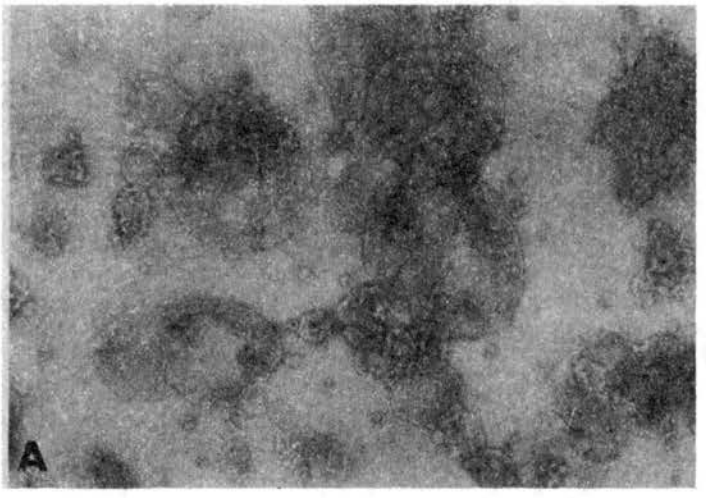
Figure 13. Lysis of Protoplasts of B. subtilis by Crude Enzyme.

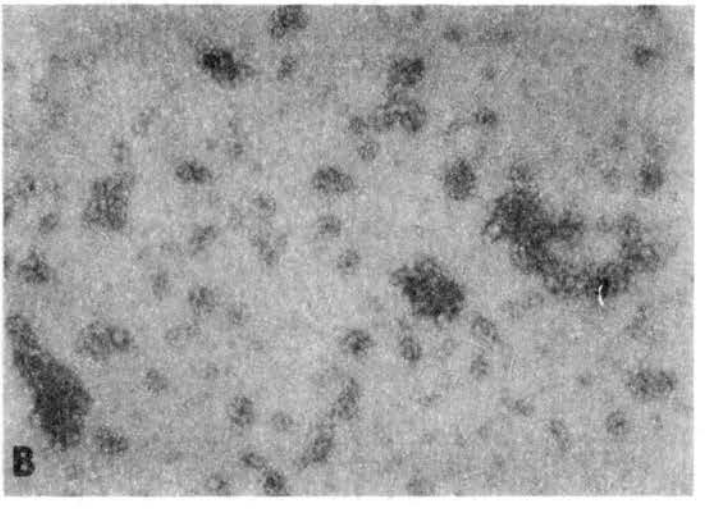

70
60
50
40
30
20
10
0
% Lysis
0
15
30
45
60
75
90
Time (minutes)

This finding is supported by studies on the effect of crude enzyme on isolated membranes of M. lysodeikticus. Electron micrographs of M. lysodeikticus membranes (Fig. 14) indicate the breakdown of the membrane by the crude enzyme. The control membranes (Fig. 14a) show a typical sheet-like structure. After incubation with the crude enzyme, the membranes appear as highly convoluted structures (Fig. 14b). This suggests that a portion of the membrane has been degraded by the crude enzyme. Gel electrophoresis of the treated membrane indicates that the proteins of the membrane have not been significantly altered, suggesting that the crude enzyme acts on the lipid portion of the membrane.

Whole cells of M. lysodeikticus and B. subtilis do not lyse when treated with the crude enzyme. This suggests that removal of the cell wall is necessary before the crude enzyme can attack its substrate.

Figure 14. Electron Micrographs of Membranes of M. lysodeikticus. A, Untreated Membranes; B, Membranes Treated With Crude Enzyme. 30,000 X Magnification.


A


B

CHAPTER IV

SUMMARY AND CONCLUSIONS

Studies on the physical parameters influencing the EDTA-promoted lysis of P. fluorescens indicate that the lysis is dependent on the concentration of EDTA used to treat the cells and the temperature of incubation following EDTA treatment. Experiments conducted on the lysis versus temperature of incubation dependency show results characteristic of an enzymatic process. The amount of lysis is optimum at approximately 37 C and decreases at either higher or lower temperatures of incubation. Incubation of the cells at high temperatures prior to treatment with EDTA at 37 C also inhibits the amount of lysis, providing another indication that lysis is an enzymatic process. The hypothesis that an enzyme(s) is involved in the EDTA promoted lysis was originally proposed by Haslam (1969), who showed that incubation of P. fluorescens cells at 0 C following treatment with EDTA at either 0 C or 37 C did not result in lysis of the cells.

The environment in which the cells are grown influences the EDTA-promoted lysis of P. fluorescens. Cells grown in phosphate base minimal medium with glucose or succinate as a carbon source are resistant to EDTA-promoted lysis. Cells grown in the same basal medium with asparagine as the carbon source are sensitive to EDTA-promoted lysis. Thus, there is a relationship between carbon source and EDTA sensitivity. Changes in the basal medium in which the cells are grown also affect the

EDTA sensitivity of the cells. P. fluorescens grown in Tris base minimal medium with either glucose, succinate, or asparagine as the carbon source are sensitive to EDTA. The addition of phosphate to Tris base minimal medium decreases the EDTA sensitivity of the cells regardless of the carbon source. However, the addition of phosphate to Tris base minimal medium at a concentration equivalent to the phosphate concentration of the phosphate base minimal medium will not totally inhibit lysis of glucose or succinate grown cells. Thus, it is apparent that the relationship between the growth environment and the EDTA-promoted lysis of cells is complex. The resistance to EDTA-promoted lysis of cells grown in different environments could be due to a change in the enzymatic constituents of the cell, or to changes in the constituents of the cell periphery, or to a combination of both.

Cells grown in YPG medium exhibit a shift in EDTA sensitivity during growth. Cells in early exponential phase of growth are only slightly sensitive to EDTA, and as the cells continue exponential growth, the EDTA sensitivity increases. This change in sensitivity to EDTA can be inhibited by incubation with chloramphenicol, indicating the involvement of protein synthesis in the change in EDTA sensitivity. Prolonged incubation in YPG medium to the stationary phase of growth produces cells which are again insensitive to EDTA. Concomitant with the loss of EDTA sensitivity by late stationary phase cells is the appearance of a factor in the spent medium which restores EDTA sensitivity to stationary phase cells. This lytic factor is not released from the cells as an exocellular enzyme, but occurs only after the cells enter stationary phase. Nor is the release of the lytic factor influenced by the phosphate concentration of the medium.

The lytic factor can be isolated from spent YPG medium using ammonium sulfate precipitation. The protein fraction precipitated by 40 percent saturation with ammonium sulfate contains approximately 90 percent of the lytic activity found in the spent medium. This fraction has no proteinase activity but does contain esterase activity. The lytic protein in this fraction can be partially purified by column chromatography using DEAE Sephadex. The lytic activity is recovered in a single peak by development of the column with a gradient of NaCl. Acrylamide gel electrophoresis of the DEAE peaks indicate the presence of three proteins, one major and two minor, in the sample containing all the lytic activity.

Experiments designed to determine the site of action of the lytic factor indicate that the lytic factor does not attack the mucopeptide of the cell wall. These experiments do indicate that the site of action of the lytic factor is the cell membrane. The lytic factor produces lysis of protoplasts of B. subtilis and degrades isolated membranes of M. lysodeikticus. Electron micrographs of the isolated membranes indicate that some portion of the membrane has been removed by the lytic factor resulting in the collapse of the membrane sheet and giving it a highly convoluted appearance. The proteins of the membrane are not altered; and this, coupled with the observed esterase activity in the sample, suggests that the lipids of the membrane are the site of action of the lytic factor.

This study provides evidence that, similar to other selected gram-negative bacteria, P. fluorescens has a lytic enzyme and the unusual sensitivity to EDTA exhibited by P. fluorescens is due to the presence of this enzyme. The lytic enzyme produces lysis, after an initial

disruption of the cell periphery by EDTA, by attacking the cell membrane. The lytic factor will not produce lysis of P. fluorescens in the absence of EDTA treatment. Thus, the initial effect of EDTA is necessary to uncover the site of action of the lytic enzyme. These conclusions are consistent with the two-step mechanism of EDTA-promoted lysis proposed by Haslam (1969).

LITERATURE CITED

Ames, B. N., and D. Dubin. 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. 235: 796.

Asbell, M. A., and R. G. Eagon. 1966. The role of multivalent cations in the organization and structure of bacterial cell walls. Biochem. Biophys. Res. Comm. 22: 664-671.

Braun, V., and H. Wolff. 1970. The murein-lipoprotein linkage in the cell wall of Escherichia coli. Eur. J. Biochem. 14: 387-391.

Braun, V., K. Rehn, and H. Wolff. 1970. Supramolecular structure of the rigid layer of the cell wall of Salmonella, Serratia, Proteus, and Pseudomonas fluorescens. Number of lipoprotein molecules in a membrane layer. Biochem. 9: 5041-5049.

Brown, M. R. W., and J. Melling. 1969. Loss of sensitivity to EDTA by Pseudomonas aeruginosa grown under conditions of Mg-limitation. J. Gen. Microbiol. 54: 439-444.

Chester, I. R., G. W. Gray, and S. G. Wilkinson. 1972. Further studies of the chemical composition of the lipopolysaccharide of Pseudomonas aeruginosa. Biochem. J. 129: 395-407.

Collins, F. M. 1964. Composition of cell walls of ageing Pseudomonas aeruginosa and Salmonella bethesda. J. Gen. Microbiol. 37: 379-388.

Cox, Jr. S. T., and R. G. Eagon. 1968. Action of ethylenediaminetetraacetic acid, tris(hydroxymethyl)-aminomethane, and lysozyme on cell walls of Pseudomonas aeruginosa. Can. J. Microbiol. 14: 913-922.

Eagon, R. G., and K. J. Carbon. 1965. Lysis of cell walls and intact cells of Pseudomonas aeruginosa by EDTA and by lysozyme. Can. J. Microbiol. 11: 193-201.

Eagon, R. G., G. P. Simmons, and K. J. Carbon. 1965. Evidence for the presence of ash and divalent metals in the cell wall of Pseudomonas aeruginosa. Can J. Microbiol. 11: 1041-1043.

Elder, M. J. 1973. Mechanism of action of a new hydroxybenzindazole on Pseudomonas fluorescens. M. S. thesis. Oklahoma State University.

Ellwood, D. C., and D. W. Tempest. 1967. Influence of growth conditions on the cell wall content and wall composition of Aerobacter aerogenes. Biochem. J. 105: 9P.

Fensom, A. H., and P. M. Meadow. 1970. Evidence for two regions in the polysaccharide moiety of the lipopolysaccharide of Pseudomonas aeruginosa 8602. FEBS Letters 9: 81-84.

Fleming, A. 1922. On a remarkable bacteriolytic element found in tissues and secretions. Proc. Roy. Soc. (London) Ser. B. 10: 226-236.

Ghuysen, J. M. 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bacteriol. Rev. 32: 425:464.

Ghuysen, J. M., D. J. Tipper, and J. L. Strominger. 1966. In: S. P. Colowick and N. O. Kaplan (ed.), Methods in Enzymology. Academic Press, Inc., New York.

Gilleland, J. D. Jr., J. D. Stinnett, I. L. Roth, and R. G. Eagon. 1973. Freeze-etch study of Pseudomonas aeruginosa: localization within the cell wall of an ethylenediaminetetraacetate-extractable component. J. Bacteriol. 113: 417-432.

Grula, E. A., and C. F. Savoy. 1971. A detergent-polyacrylamide gel system for electrophoretic resolution of membrane and wall proteins. Biochem. Biophys. Res. Comm. 43: 325-332.

Hamilton-Miller, J. M. T. 1966. Damaging effects of ethylenediaminetetra-acetate and penicillins on permeability barriers in gram-negative bacteria. Biochem. J. 100: 675-682.

Haslam, D. F. 1969. A lytic phenomenon in Pseudomonas fluorescens. M. S. thesis. Oklahoma State University.

Haslam, D. F., G. K. Best, and N. N. Durham. 1969. Activation of autolytic enzyme(s) in Pseudomonas aeruginosa by ethylenediaminetetra-acetate treatment. Bacteriol. Proc. 1969: 136.

Heilmann, H. D. 1972. On the peptidoglycan of the cell walls of Pseudomonas aeruginosa. Eur. J. Biochem. 31: 456-463.

Kunitz, M. 1952. Crystalline inorganic pyrophosphatase isolated from bakers yeast. J. Gen. Physiol. 35: 423-454.

Levy, S. B., and L. Leive. 1968. An equilibrium between two fractions of lipopolysaccharide in E. coli. Proc. Nat. Acad. Sci. U. S. A. 61: 1435-1439.

Levinthal, C., E. R. Singer, and K. Fetherolf. 1962. Reactivation and hybridization of reduced alkaline phosphatase. Proc. Nat. Acad. Sci. U. S. A. 48: 1230-1237.

Lieve, L. 1965a. Release of lipopolysaccharide by EDTA treatment of E. coli. Biochem. Biophys. Res. Comm. 21: 290-296.

Lieve, L. 1965b. A nonspecific increase in permeability in Escherichia coli produced by EDTA. Proc. Nat. Acad. Sci., U. S. A. 55: 745-750.

Lieve, L. 1968. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate. J. Biol. Chem. 243: 2373-2380.

Liu, P. V. 1964. Factors that influence toxigenicity of Pseudomonas aeruginosa. J. Bacteriol. 85: 1421-1427.

Montgomery, K. 1966. Influence of o-nitrobenzoic acid on the protocatechuate oxygenase system of Pseudomonas fluorescens. M. S. thesis. Oklahoma State University.

Pooley, H. M., and G. D. Shockman. 1969. Relationship between the location of autolysin, cell wall synthesis, and the development of resistance to cellular autolysis in Streptococcus faecalis after inhibition of protein synthesis. J. Bacteriol. 103: 457-466.

Protass, J. J., and D. Korn. 1966. Impairment of temperate bacteriophage adsorption by brief treatment of Escherichia coli with dilute solutions of EDTA. J. Bacteriol. 91: 143-147.

Repaske, R. 1956. Lysis of gram-negative bacteria by lysozyme. Biochim. Biophys. Acta. 22: 189-191.

Repaske, R. 1958. Lysis of gram-negative organisms and the role of versene. Biochim. Biophys. Acta. 30: 225-232.

Rinderknecht, H., M. C. Geokas, P. Silverman, and B. J. Haverback. 1968. A new ultrasensitive method for the determination of proteolytic activity. Clin. Chim. Acta. 21: 197-203.

Rogers, H. J. 1970. Bacterial growth and the cell envelope. Bacteriol. Rev. 34: 194-214.

Schnaitman, C. A. 1971. Effect of ethylenediaminetetraacetic acid, triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of Escherichia coli. J. Bacteriol. 108: 553-563.

Smith, J. A. 1973. Effect of carbon source on lipopolysaccharide production in Pseudomonas fluorescens. M. S. thesis. Oklahoma State University.

Stanier, R. Y., M. Doudoroff, and E. A. Adelberg. 1970. In: Microbial World. Prentice-Hall Inc., Englewood Cliffs, New Jersey.

Strominger, J. L., and J. M. Ghuysen. 1967. Mechanisms of enzymatic bacteriolyses. Sci. 156: 213-221.

Sutherland, E. W., C. F. Cori, R. Haynes, and N. S. Olson. 1949. Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. J. Biol. Chem. 180: 825-837.

Walker, C. A. 1973. The effect of carbon source on the sensitivity of Pseudomonas fluorescens to actinomycin D. Ph.D. thesis. Oklahoma State University.

Weber, K., and M. Osborn. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406-4412.

Whiteside, T. L., and W. A. Corpe. 1969. Extracellular enzymes produced by a Pseudomonas sp. and their effect on cell envelopes of Chromobacterium violaceum. Can. J. Microbiol. 15: 81-92.

Wilkinson, S. G. 1967. The sensitivity of Pseudomonads to ethylenediaminetetraacetic acid. J. Gen. Microbiol. 47: 67-76.

Wilkinson, S. G. 1970. Cell walls of Pseudomonas species sensitive to ethylenediaminetetraacetic acid. J. Bacteriol. 104: 1035-1044.

Wilkinson, S. G., L. Galbraith, and G. A. Lightfoot. 1973. Cell walls, lipids, and lipopolysaccharides of Pseudomonas species. Eur. J. Biochem. 33: 158-174.

Wolin, M.J. 1966. Lysis of Vibrio succinogenes by ethylenediaminetetraacetic acid or lysozyme. J. Bacteriol. 91: 1781-1786.

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