A LYTIC PHENOMENON IN *PSEUDOMONAS FLUORESCENS*

By

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A LYTIC PHENOMENON IN PSEUDOMONAS FLUORESCENS

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CHAPTER I

INTRODUCTION

Ethylenediaminetetraacetate (EDTA) sensitizes certain bacteria to the action of lysozyme. Repaske (1958) first described the EDTA sensitization reaction and observed that Pseudomonas aeruginosa cells lysed following EDTA treatment and the lytic activity was more pronounced with young cultures. Lytic studies using lysozyme and EDTA indicated that neither lysozyme nor EDTA alone caused appreciable lysis of Escherichia coli or Azotobacter vinelandii, but when combined they caused immediate and rapid lysis. Aging apparently causes some changes in the cell wall because older cells or washed cells of E. coli, aged at 0 C for several hours, required higher concentrations of EDTA alone but lysozyme had no effect. However, lysozyme and EDTA together caused almost complete lysis. Liberation of nucleic acids from the lysed cells was indicated by an increase in viscosity which could be decreased by the addition of Mg^{++} and RNase to the lysate.

MacGregor and Elliker (1958) reported that EDTA may accelerate the action of quaternary ammonium compounds (QAC), at least with QAC-resistant strains of P. aeruginosa, by increasing the permeability of the cell to QAC. The EDTA effect appears to be similar to that of lipase in disrupting the QAC-resistant cell wall layer which suggests the possibility of a lipid or lipid-protein complex bound together by multivalent inorganic cations. The requirement for EDTA, a divalent

metal-chelating agent, indicates that metals associated with the cell surface may interfere with the action of lysozyme.

The extent of lysis is dependent upon the pH of the medium. As the pH of tris(hydroxymethyl)aminomethane (Tris) buffer is raised from 7.2 to 8.6 the rate of lysis increases, and at pH 8.6 lysis is almost complete within the first minute.

Another parameter, which is critical to the degree of lysis, is the physiological age of the culture. Correlation of susceptibility to lysis with the physiological age of the cells indicates that modifications of the cell surface accompany growth. In the late lag or early logarithmic phase of growth, cells are most resistant to lysis after which they abruptly increase in susceptibility reaching a maximum in the stationary phase. Thereafter, the susceptibility to lysis gradually decreases. It should also be noted that exposure of E. coli or A. vinelandii cells to EDTA does not produce permanent damage. Washed EDTA treated cells do not lyse unless EDTA is included in the lysing medium. This indicates that EDTA action is transient and does not permanently condition the cell for lysis (Repaske, 1958).

Brown, Sandine and Elliker (1962) observed lysis of twelve gram-negative organisms by EDTA and reported a striking difference in the rates of lysis when compared to the rates reported by Repaske (1958). All twelve organisms lysed slowly necessitating a 2-hour lysing period (Brown et al., 1962). Repaske (1958), however, reported almost complete lysis of four gram-negative bacteria in 6 minutes.

Gray and Wilkinson (1965) reported that EDTA was bacteriocidal rather than bacteriostatic. Tests using 0.0001 M EDTA at pH 9.2 showed that EDTA had a high antibacterial activity for cells of P. aeruginosa,

the reduction in viable count exceeding 99.99%. The action was rapid and the viable count reached a minimum within two minutes and remained constant for up to 30 minutes. Further evidence suggested that EDTA was active against P. aeruginosa by a mechanism involving chelation. Therefore, if chelation is involved, the bacteriocidal activity should decrease as the pH is reduced. Results showed that the number of organisms surviving the action of 0.0034 M EDTA at pH 7.1 was about two hundred times greater than the number surviving the action of 0.0001 M EDTA at pH 9.2.

Gray and Wilkinson (1965) also offered evidence that EDTA acted at the cell wall and not the cell membrane by testing P. aeruginosa in the presence of high concentrations (0.05 M) of non-penetrating solutes such as sucrose and NaCl. These solutes completely inhibited cellular leakage induced by EDTA indicating that the permeability characteristics of the membrane were not impaired thereby preventing lysis. Sodium hexametaphosphate and compounds related to EDTA are bacteriocidal against P. aeruginosa due to their ability to form highly stable chelates. Assuming that such chelates are formed and are not themselves toxic, the bacteriodical nature of the action suggests that the metal cation involved is not free in solution but bound to sites on the bacterium. Thus, the action of EDTA in causing leakage of cell solutes and loss of viability of cells of P. aeruginosa appears to involve the removal or displacement of a metal cation which is probably present in the cell wall and is essential to the structural integrity of the cell.

Leive (1965a) reported that brief treatment of E. coli with low concentrations of EDTA showed an increase in permeability associated with little or no change in viability, growth rate or normal RNA and

protein synthesis. These findings suggest a selective action of EDTA on the cell surface. Also, brief exposure of E. coli to EDTA resulted in the rapid release of approximately one-half of the lipopolysaccharide cell wall layer. This loss was very rapid as was the permeability change caused by EDTA. The action is somewhat specific, since loss of several other cell components is apparently negligible. No correlation was drawn between lipopolysaccharide release and permeability change, but the rapidity and apparent specificity of the lipopolysaccharide loss suggests that it is a direct result of the action of EDTA on the cell surface. If a metal such as Mg^{++} participates in the non-covalent bonds holding the various layers of the cell wall, binding of the cation by EDTA might produce rapid and extensive changes in both function and composition of the cell surface, simultaneously causing both altered permeability and loss of the lipopolysaccharide cell wall layer. Wolin (1966) reported that much of the cell protein and almost all of the nucleic acids were released from Vibrio succinogenes during EDTA lysis. However, lysis by EDTA or lysozyme occurred only if the cells were suspended in a hypotonic environment and at an alkaline pH.

Leive (1965a; 1965b) reported that treatment with 0.001 M EDTA for two minutes produced greater permeability to actinomycin, and in general, permitted entry of other unrelated compounds. Moreover, by adjusting the conditions of EDTA treatment, it was possible to produce the permeability change without altering the growth rate or the viability of the cells. Thus, EDTA treatment may alter the permeability barrier of the cell without altering the function of specific transport systems.

Eagon and Carson (1965) and Eagon, Simmons and Carson (1965) reported that Ca^{++}, Mg^{++}, and Zn^{++} were components of the cell wall of

P. aeruginosa strain OSU 64. They concluded that the binding of divalent cations was essential for the integrity of the cell wall of this organism and inferred that these cations were present in the lipopolysaccharide fraction of the cell wall. Asbell and Eagon (1966) reported that osmoplasts, osmotically fragile rods obtained by EDTA plus lysozyme treatment in hypertonic sucrose solution, were restored to an osmotically stable state by the addition of a variety of multivalent cations as evidenced by the absence of lysis when the restored cells were removed from hypertonic sucrose and suspended in water. Di- or trivalent cations restored the cells to an osmotically stable state regardless of whether the metal was a normal constituent of the cell wall. Variations in the atom diameters of the multivalent cations did not appear important to the restoration process. However, monovalent cations were not able to restore osmoplasts even when used in high concentrations. Therefore, they proposed that the lipopolysaccharide is composed of subunits cross-linked via divalent cations. The lipopolysaccharide layer may also be cross-linked to other components of the cell wall via multivalent cations.

Spheroplasts are spherical bodies formed after partial loss of the bacterial cell wall. They are osmotically fragile and undergo lysis in media of low osmotic pressure. On the other hand, protoplasts are characterized by complete loss of the cell wall (Brenner et al., 1958; McQuillen, 1960). McQuillen further reported that lysis of E. coli could be prevented and the action arrested at a stage of spherical, osmotically shockable protoplasts in the presence of 0.5 M sucrose. Later, Voss (1964) reported that treatment of gram-negative bacteria with the lysozyme-EDTA-Tris buffer system may yield osmotically fragile

cells in which the destruction of the rigid cell wall was not sufficient to cause the production of true spheroplasts.

Birdsell and Cota-Robles (1967) reported that physiologically young cells plasmolyzed with 0.5 M sucrose in 0.01 M Tris buffer (pH 7, 8 or 9) were quantitatively converted to plasmolyzed osmotically sensitive rods after lysozyme treatment. Although such cells were osmotically sensitive, a 1:1 dilution in Tris buffer was necessary for conversion of rods into spheroplasts. Addition of EDTA resulted in the rapid conversion of the plasmolyzed spheroplasts into spherical structures devoid of a plasmolysis vacuole. The standard technique that has evolved for production of spheroplasts involves treatment of exponential or stationary phase cultures with EDTA and lysozyme at an alkaline pH in the presence of a stabiliizing solute, usually sucrose. The concentration of sucrose regulated the extent of plasmolysis, and removal of sucrose either before or after sphering led to complete lysis and the formation of spherical ghosts. They also reported that EDTA induced the rupture and subsequent coiling of the outer unit membrane component of the cell wall. More recently, Birdsell and Cota-Robles (1968) reported that the susceptibility of E. coli to detergent lysis also required treatment with EDTA.

Further evidence that EDTA alters the cell wall of gram-negative organisms was offered by Protass and Korn (1966) who studied the effect of EDTA on bacteriophage adsorption. They noted that EDTA impaired adsorption of temperate phage as a consequence of an interaction with the bacterial cell wall rather than by affecting the phage itself or by altering the cation composition of the adsorption medium. Two reasonable hypotheses are given to explain this interaction: either some

labile wall component required specifically for temperate phage adsorption was removed, or EDTA specifically altered the configuration of temperate phage binding sites in such a way that irreversible adsorption could not occur.

Best (1965) reported that vancomycin could complex with cell walls via electrostatic or ionic linkages to free anions present in the cell wall. Both muramic acid and C-terminal amino acids in the peptide of the cell wall could contribute an ionized carboxyl group for adsorption, while the phosphoryl linkages of the teichoic acids would be expected to carry negative charges that might also be involved in adsorption of vancomycin.

These free anions are not readily available in the cell wall of gram-negative bacteria as compared to the gram-positive organisms. Since EDTA functions as a chelating agent, EDTA treatment of gram-negative bacteria removes divalent metal ions from the cell wall. This removal facilitates the breakdown of the lipoprotein component of the cell wall and exposes more free ionic groups. Thus, the adsorption of vancomycin can be used as a criterion to assess the extent of cell wall degradation.

This thesis is concerned with the presence or absence of an autolytic enzyme which may be responsible for the increased lytic activity of P. fluorescens at 37 C. Growth studies and vancomycin adsorption experiments furnish much evidence which substantiates the presence of this enzyme. It is proposed that the cell wall of the sensitive organism is sufficiently altered by EDTA that the autolytic enzyme no longer recognizes it as "native" but rather considers it to be "foreign" cell wall material. Thus, EDTA acts as an initiator of

cell wall degradation which is completed by the autolytic enzyme. The isolation of an EDTA-resistant strain would offer further evidence that an enzyme is involved in this lytic phenomenon.

CHAPTER II

MATERIALS AND METHODS

Test Organisms

The principle microorganism used throughout this study has been tentatively identified as P. fluorescens (Keudell, 1967). It is a gram-negative, motile rod which forms smooth, raised colonies on nutrient agar. The organism exhibits a negative reaction for hydrogen sulfide production, indole production, and nitrate reduction, and produces acid but no gas in glucose. This organism also produces fluorescein and pyocyanin when grown on Bacto-Pseudomonas agar F and Bacto-Pseudomonas agar P, respectively. A second organism used in this study was isolated from the soil and tentatively identified as belonging to the genus Flavobacterium. Both organisms were obtained from Dr. Norman N. Durham, Oklahoma State University.

Stock cultures of P. fluorescens and the species of Flavobacterium were maintained on slants of either nutrient agar or glucose-salts agar and stored at 4 C.

Synthetic Medium

A glucose-salts synthetic medium was used in some phases of this study to measure recovery of organisms following EDTA treatment as compared to recovery in a complete medium such as nutrient broth. The synthetic medium had the following composition: K_2HPO_4, 7 g; KH_2PO_4,

3 g; and $(NH_4)_2SO_4$, 1 g, dissolved in 475 ml of glass distilled water. The solution was sterilized by autoclaving for 15 minutes at 121 C. After cooling, 25 ml of a sterile aqueous solution of 10% glucose was added. The medium was then supplemented with 0.5 ml of a sterile mineral salts solution which was autoclaved separately. The mineral salts solution contained the following compounds: $MgSO_4 \cdot 7\ H_2O$, 5 g; $MnSO_4 \cdot H_2O$, 0.1 g; $FeCl_3 \cdot 6\ H_2O$, 1 g; and $CaCl_2$, 0.5 g, per 100 ml glass distilled water. Two per cent agar was added when a solid medium was needed.

Chemicals

Ethylenediaminetetraacetate (EDTA) was purchased as the disodium salt from Fisher Scientific Company. Vancomycin was graciously supplied by Eli Lilly and Company. The potency of the antibiotic was 1015 units per mg. The concentrations of all chemicals used in this study are recorded in the text.

Effect of Temperature on EDTA Treatment

Growth studies were conducted to determine the effect of temperature on the response of P. fluorescens and the species of Flavobacterium to EDTA. The organisms were grown for 12-15 hours on nutrient agar or glucose-salts agar slants. The cells were harvested with sterile glass distilled water and suspended to an absorbance of 0.7 at 540 mμ using 18 x 150 mm test tubes in a Baush and Lomb "Spectronic 20" colorimeter. Nutrient agar or glucose-salts agar plates were inoculated with 0.5 ml of the suspension and incubated for 12-15 hours at 37 C. The organisms were harvested with 0.01 M potassium phosphate buffer (pH 7.0), washed

two times with cold distilled water by centrifugation at 10,000 x g, and suspended to an absorbance of 1.0 at 540 mμ. Samples (39 ml) were pipetted into 125 ml Erlenmeyer flasks and equilibrated for 10 minutes at 37 C or 2 C in a reciprocal shaking water bath. One ml of 0.04 M EDTA (pH 4.4) or 1 ml distilled water was added to bring the final liquid volume to 40 ml and give a final EDTA concentration of 0.001 M in the appropriate flasks. A 5 ml sample was withdrawn after 5 minutes and the absorbance read at 540 mμ to determine if lysis was taking place. The remainder of the cell suspension was incubated for an additional 10 minutes at which time two 10 ml samples were withdrawn from each flask, washed one time by centrifugation at 10,000 x g at 0 C and suspended in 10 ml of cold distilled water. The cells were added to 40 ml of fresh nutrient broth and incubated at 2 C and 37 C. Growth was followed for 2.5 hours by measuring the absorbance at 540 mμ at 30 minute intervals.

Studies were also conducted to determine the effect of vancomycin on growth. The procedure was identical to the above except vancomycin (150 μg/ml final concentration) was added to the nutrient broth growth medium.

Influence of EDTA on Vancomycin Adsorption

Vancomycin adsorption studies were conducted to determine the effect of EDTA treatment on cells of P. fluorescens and the species of Flavobacterium. The organisms were grown for 12-15 hours on nutrient agar or glucose-salts agar slants and harvested with sterile glass distilled water. The cells were suspended to an absorbance of 0.7 at 540 mμ. Nutrient agar plates were inoculated with 0.5 ml of the

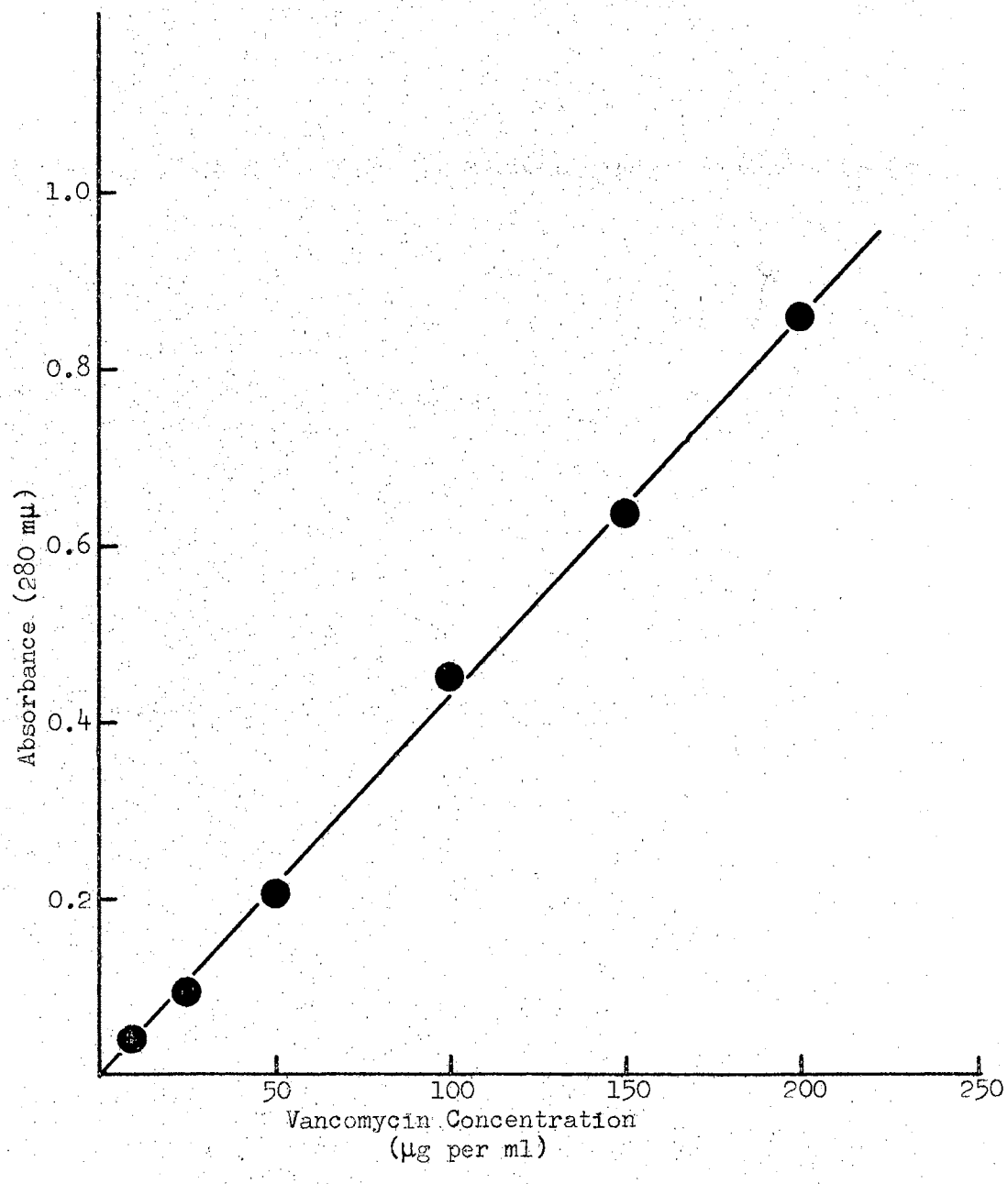
suspension and incubated for 12-15 hours at 37 C. The organisms were harvested with 0.01 M potassium phosphate buffer (pH 7.0), washed 2 times with cold distilled water by centrifugation at 10,000 x g, and suspended to an absorbance of 1.0 at 540 mμ. Samples (25 ml) were centrifuged and washed in the cold, suspended in 39 ml of distilled water and 1 ml of 0.04 M EDTA or 1 ml of water added to bring the final volume to 40 ml. The cells were incubated at 2 C or 37 C for 15 minutes. One 10 ml sample was withdrawn from each flask, washed one time and suspended in 3 ml of distilled water. Three ml vancomycin (300 μg/ml) was added to each tube (6 ml total volume) to observe vancomycin adsorption. Control tubes were run concommitantly containing 6 ml distilled water total volume. The cells were incubated for 10 minutes at room temperature to facilitate vancomycin adsorption and centrifuged at 14,400 x g for 10 minutes. The absorbance of the supernatant solution was read at 280 mμ on a Beckman DU spectrophotometer to determine the amount of vancomycin adsorbed by the cells. A standard curve for vancomycin adsorption is presented in Figure 1.

Autolysis of P. fluorescens in Buffer

Experiments were performed to determine the optimal autolytic conditions. P. fluorescens cells were grown for 12-15 hours on nutrient agar slants, harvested with sterile glass distilled water and adjusted to an absorbance of 0.7 at 540 mμ. Nutrient agar plates were inoculated with 0.5 ml of the suspension and incubated at 37 C for 12-15 hours. The cells were harvested with cold 0.01 M potassium phosphate buffer (pH 7.0), washed and suspended in cold water so that a final absorbance of 0.5 at 540 mμ was obtained when 1 ml of cells was added to 19 ml of

Figure 1. Standard Curve for Vancomycin Adsorption at 280 mμ

Vancomycin (free base) adsorption at 280 mμ was measured by dissolving various concentrations of the antibiotic in distilled water and reading the absorbance on a Beckman DU spectrophotometer using 10 mm cuvettes.


1.0
0.8
0.6
0.4
0.2
Absorbance (280 mμ)
50
100
150
200
250
Vancomycin Concentration
(μg per ml)

various buffers ranging in pH from 4.4 to 10.4. The buffers were citrate-phosphate (pH 4.4-7.0), barbital (pH 7.4-9.0) and glycine (pH 9.4-10.4) as described by Gomori, 1955. The flasks were agitated at 37 C in a reciprocal water bath for 15 minutes at which time a 5 ml sample was withdrawn from each tube and the absorbance read at 540 mμ to determine the degree of clearing.

Experiments using divalent cations to determine their effect upon lysis differed from the above procedure only in the fact that 18 ml of buffer was used and 1 ml of the appropriate concentration of the cation was added.

Osmoplast Stabilization

P. fluorescens cells were converted to osmoplasts by treatment with EDTA in the presence of 0.5 M sucrose. Cells were grown on nutrient agar as previously described, harvested with 0.01 M potassium phosphate buffer, washed and suspended in cold water to an absorbance of 1.0 at 540 mμ. Aliquots (40 ml) were washed by centrifugation at 14,400 x g and suspended in 9 ml of cold water. The suspended cells were added to flasks containing 0.001 M EDTA (pH 7.4) and 0.5 M sucrose in a total volume of 40 ml previously equilibrated to 37 C. Appropriate controls were run concommitantly. The flasks were incubated with gentle shaking for 15 minutes at which time the absorbance at 540 mμ was read to determine the degree of lysis. The cells were then diluted 1:5 in distilled water and read at 540 mμ, correcting for the dilution factor, to ascertain if true osmoplasts were formed.

Treatment With Mutagens

Experiments were conducted in an attempt to isolate an EDTA-resistant organism. Ultraviolet light was used as a mutagenic agent. P. fluorescens cells were inoculated into 6 ml of nutrient broth and grown for 5 hours at 37 C on a reciprocal shaker. The cells were washed and suspended in sterile saline to contain approximately 1×10^8 cells per ml. Samples (5 ml) of the cell suspension were placed in petri dishes and irradiated for 15 seconds with constant agitation at a distance of 40 cm using a 15 watt Sylvania germicidal lamp. Irradiation under these conditions kills approximately 99.99% of the organisms (Bruce, 1965). Each irradiated cell suspension was added to an equal volume of nutrient broth and incubated for 4 hours in the dark at 37 C to allow for phenotypic lag. The cells (0.1 ml) were spread on nutrient agar plates and overlaid with soft agar containing 0.01 or 0.001 M EDTA. Colonies were picked and maintained on nutrient agar slants containing 0.01 or 0.001 M EDTA. Growth was slow and the colonies were quite small.

CHAPTER III

RESULTS AND DISCUSSION

Effect of Temperature on EDTA Activity

Two gram-negative organisms, P. fluorescens and a species of Flavobacterium, were used throughout this study to determine the possible presence of an autolytic system. Both organisms appeared to possess some type of autolytic mechanism. Both organisms adsorb vancomycin, and a quantitative difference in adsorption can be related to susceptibility to the antibiotic. Treatment with EDTA alters the susceptibility of gram-negative organisms by chelating metal ions and disrupting the cell wall to a certain extent. The species of Flavobacterium was used in vancomycin adsorption studies since this organism adsorbed virtually no vancomycin unless the cells were first treated with EDTA. Since untreated P. fluorescens cells adsorb vancomycin to a greater extent, the Flavobacterium assay system was used in the vancomycin adsorption aspect of this study to determine the extent of cell wall damage following EDTA treatment. P. fluorescens cells were used in all other aspects of this study.

Growth studies using nutrient agar grown P. fluorescens were conducted to determine the influence of temperature upon lysis following EDTA treatment. The ability of treated cells to recover and grow in nutrient broth was used as one criterion to determine the extent of cellular damage and lysis. Cells were treated with EDTA at 2 C and

37 C for 15 minutes, washed and suspended in nutrient broth at 2 C and 37 C to observe growth.

Cells treated with EDTA at 2 C and incubated at 37 C showed a 30 minute lag period followed by exponential growth corresponding to the control indicating that there was no profound reduction in the number of viable cells (Figure 2). Cells treated with water or 0.001 M EDTA at 2 C showed no indication of lysis when placed in nutrient broth equilibrated to 2 C. On the other hand, cells treated with EDTA at 37 C exhibited some lysis with little subsequent growth during the 2.5 hour growth period (Figure 3). Control cells held at 2 C in water and then placed in nutrient broth for growth at 37 C showed no deleterious effects (Figure 2) since growth was comparable to cells held at 37 C in water and grown in nutrient broth at 37 C (Figure 3). Control cells placed at 2 C in nutrient broth following HOH or EDTA treatment at 37 C demonstrated no lysis during the 2.5 hour incubation period (Figure 3). The results suggest that the extent of lysis following EDTA treatment and subsequent ability to recover and grow in nutrient broth was largely dependent on the temperature during EDTA treatment.

The lysis resulting from EDTA treatment appears to be more pronounced as the temperature of treatment is raised above 2 C. Since the temperature should not significantly affect the chemical activity of EDTA, a two step reaction may be involved in the degradation of the wall and subsequent lysis initiated by EDTA. A possible explanation of this phenomenon could involve the presence of an autolytic enzyme. This would explain the apparent reduction in lysis following treatment with EDTA at 2 C since an enzyme would have much less activity at this low temperature. At 37 C EDTA partially degrades the wall by removing

Figure 2. Growth of P. Fluorescens at 2 C and 37 C Following Treatment With EDTA at 2 C

The cells were treated with 0.001 M EDTA (pH 4.4) at 2 C and inoculated into nutrient broth previously equilibrated to the desired temperature. Appropriate controls were run concurrently. ▲, HOH treated, grown 37 C; ●, EDTA treated, grown 37 C; ○, HOH treated, grown 2 C; △, EDTA treated, grown 2 C.

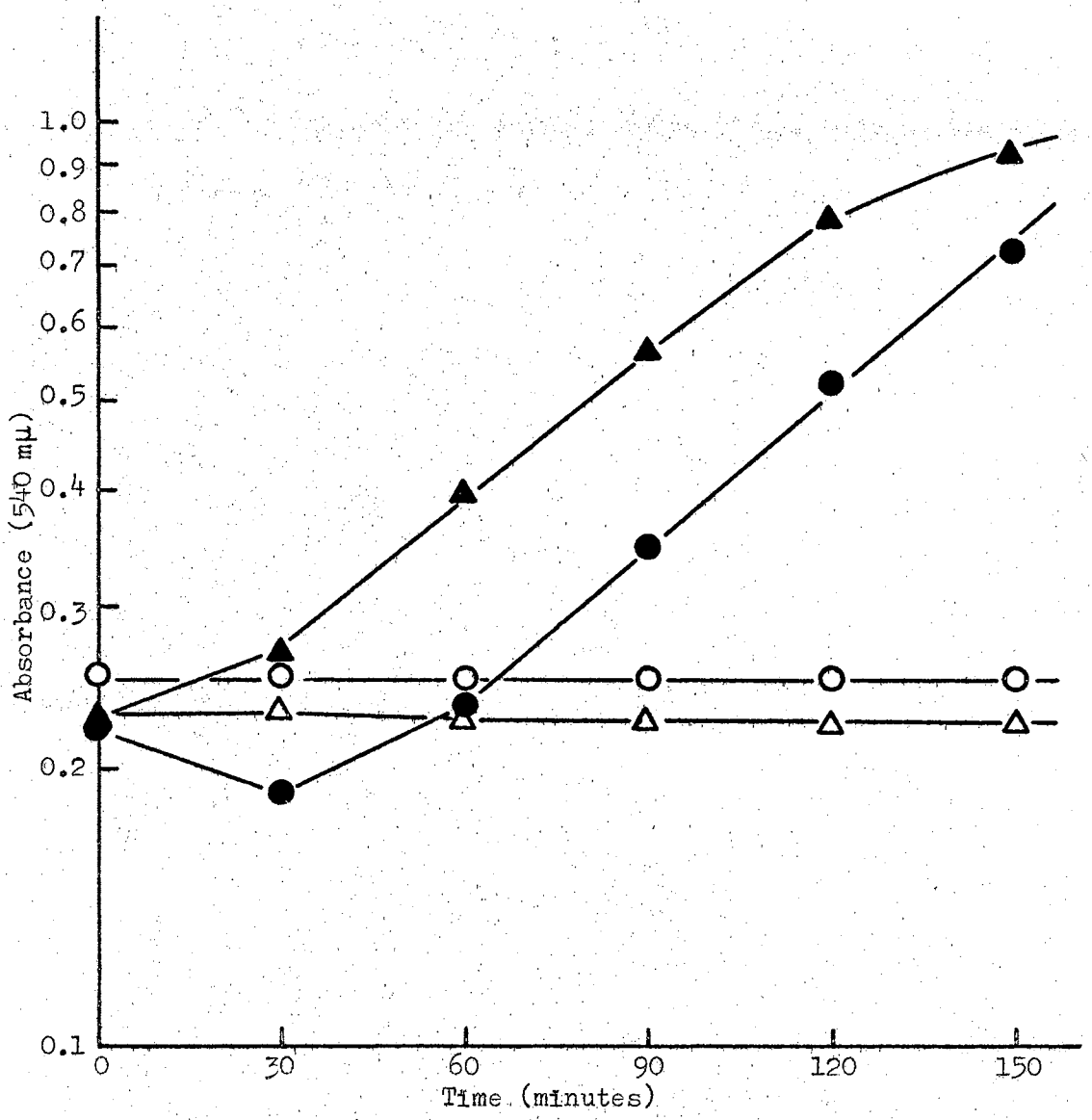
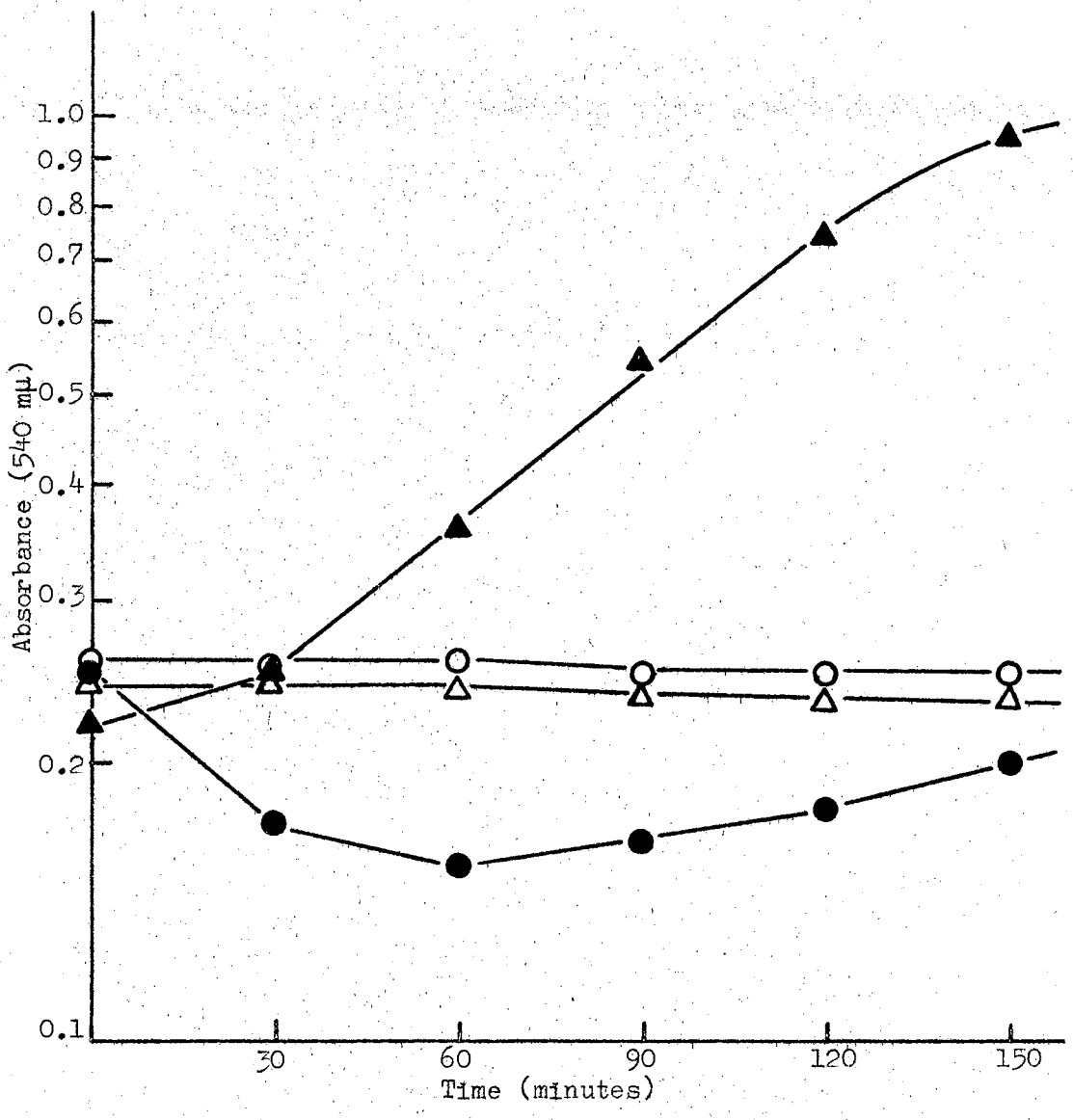

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance (540 mμ)
0
30
60
90
120
150
Time (minutes)

Figure 3. Growth of P. fluorescens at 2 C and 37 C Following Treatment With EDTA at 37 C

The cells were treated with 0.001 M EDTA (pH 4.4) at 37 C and inoculated into nutrient broth previously equilibrated to the desired temperature. Appropriate controls were run concurrently. ▲, HOH treated, grown 37 C; ●, EDTA treated, grown 37 C; ○, HOH treated, grown 2 C; △, EDTA treated, grown 2 C.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance (540 mμ)
30
60
90
120
150
Time (minutes)

divalent cations functioning as cross-linkages (Gray and Wilkinson, 1965). The alleviation of the EDTA effect by Mg^{++}, discussed later in this paper, suggests that EDTA functions primarily as a chelating agent in lysing P. fluorescens cells. The results suggest that the partial degradation of the cell wall, following EDTA treatment, may release an autolytic enzyme which further degrades the cell wall. The cell wall must be altered to such an extent that the autolytic enzyme no longer recognizes it as "native" wall material.

Influence of EDTA and Tris on Vancomycin Adsorption by Nutrient Agar Grown Cells

Since EDTA treatment has been shown to substantially increase the adsorption of vancomycin to gram-negative bacteria (Ferguson, 1967) and Tris has been reported to compound the lethal effects of EDTA (Eagon and Asbell, 1966), the effect of EDTA and Tris treatment at 2 C and 37 C was examined using the species of Flavobacterium. The purpose was to determine if the temperature of the incubation mixture during treatment influenced the amount of vancomycin adsorbed to the cells. Studies were also conducted in which Tris was used separately and in combination with EDTA to ascertain if Tris and EDTA had a similar effect on the wall.

Nutrient agar grown Flavobacterium cells were incubated in the presence of 0.001 M EDTA and/or 0.002 M Tris for 15 minutes, washed and suspended in 3 ml of distilled water. Three ml of vancomycin (300 µg/ml) was added to the appropriate tubes (6 ml total volume) to determine vancomycin adsorption. Control tubes were run concommitantly using distilled water. Cells treated with 0.001 M EDTA and/or Tris at 2 C

(Table I) did not show a pronounced increase in adsorption over the control cells unless treated for an extended period of time (90 minutes). This slight increase in wall damage with time would be expected if an enzyme(s) is involved since all enzymic activity is not stopped at low temperatures but does show a very slow rate. However, cells treated at 37 C for 15 minutes demonstrated a marked increase in adsorption of vancomycin (Table I). Although both additives enhanced adsorption, EDTA appears to be more effective than Tris in making available the sites to which vancomycin can adsorb. Prolonged treatment with EDTA or Tris at 37 C for periods up to 90 minutes showed little if any additional effect as measured by adsorption of vancomycin (Table I). Disruption and cellular damage by EDTA treatment at 2 C for 90 minutes was not sufficient to permit adequate evaluation. Treatment at 37 C for 90 minutes showed many uncontrolled variables which masked the true picture.

The results suggest that the action of both EDTA and Tris is rapid at 37 C and, since EDTA treatment increased adsorption approximately threefold compared to a twofold increase following Tris treatment (Table I), EDTA appears to be the more efficient for removing lipopolysaccharides (Leive, 1965c) from the cell wall.

Influence of EDTA on Vancomycin Adsorption by Nutrient Broth Grown Cells

Collins (1964) reported that the cell wall composition of P. aeruginosa varies with the age of the culture. Studies were conducted to determine if the medium and age of the cells influenced their response to EDTA or Tris treatment. Nutrient broth grown cells of the Flavobacterium were harvested during the exponential phase of growth

TABLE I

INFLUENCE OF TIME AND TEMPERATURE OF EDTA AND TRIS TREATMENT ON VANCOMYCIN ADSORPTION BY FLAVOBACTERIUM CELLS*

Additive and Temperature**	Vancomycin Adsorbed (μg)		Per Cent Adsorbed	
	Time of Treatment			
	15 min	90 min	15 min	90 min
Control (2 C)	60	72	6.6	8.0
EDTA (2 C)	72	108	8.0	12.0
Tris (2 C)	72	72	8.0	8.0
EDTA plus Tris (2 C)	72	114	8.0	12.7
Control (37 C)	72	60	8.0	6.6
EDTA (37 C)	204	258	22.7	28.7
Tris (37 C)	150	165	16.7	18.3
EDTA plus Tris (37 C)	306	270	34.0	30.0

The total quantity of vancomycin available for adsorption was 900 μg. The EDTA (pH 4.4) concentration was 0.001 M and the Tris (pH 7.0) concentration was 0.002 M.

*Nutrient agar grown cells (12-15 hours).

**Treatment time was 15 minutes.

Figure 4. Growth Curve for P. fluorescens in Nutrient Broth

Growth was measured by reading the absorbance at 540 mμ on a Bausch and Lomb colorimeter using 13 x 150 mm cuvettes. Samples were taken at the indicated times for use in the desired experimentation.

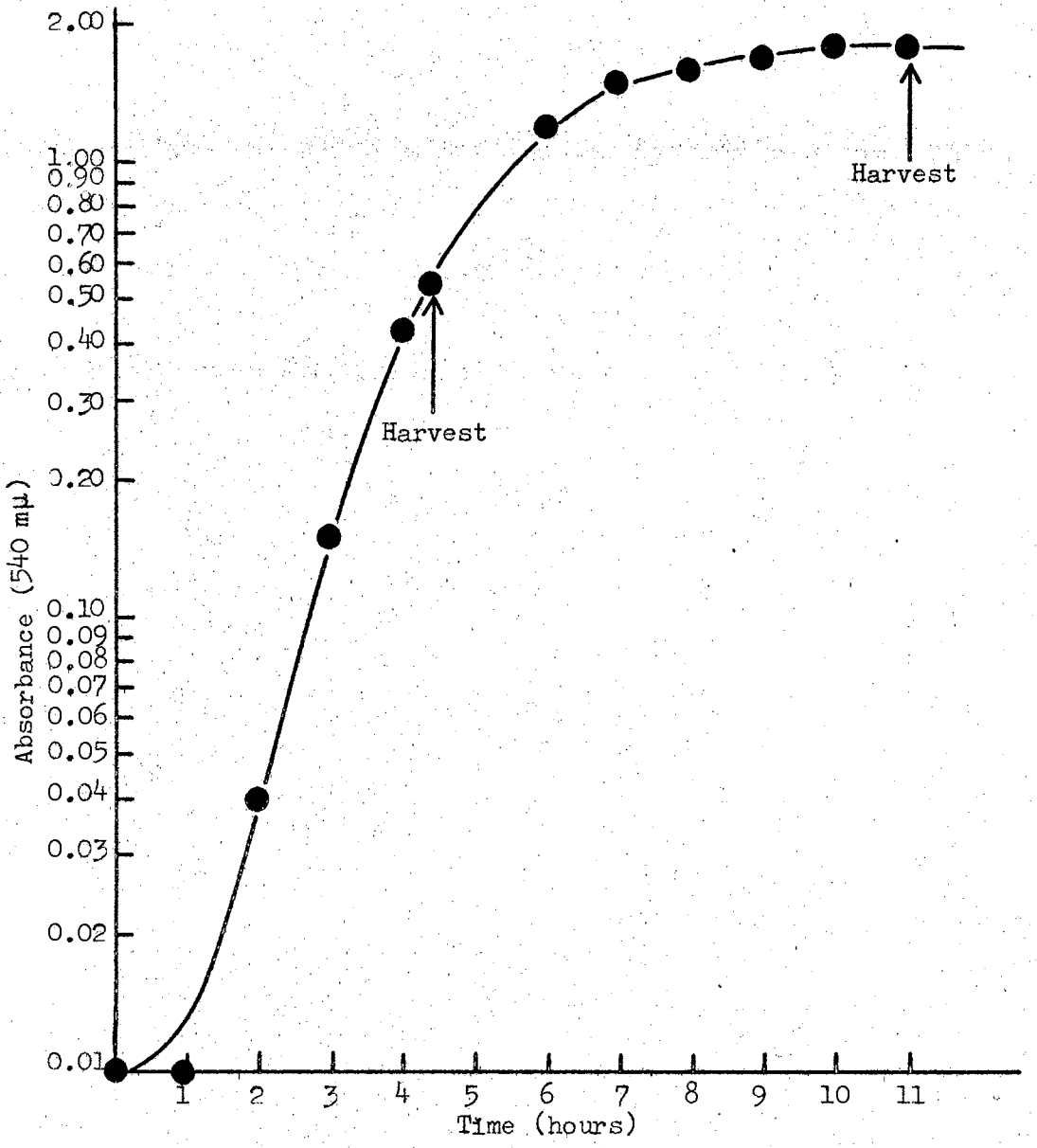

2.00
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Absorbance (540 mμ)
Harvest
Harvest
1
2
3
4
5
6
7
8
9
10
11
Time (hours)

TABLE II

INFLUENCE OF EDTA AND TRIS ON LOG PHASE NUTRIENT BROTH GROWN *FLAVOBACTERIUM* CELLS

Additive and Temperature	Vancomycin Adsorbed (μg)	Per Cent Adsorbed
Control (2 C)	30	3.3
EDTA (2 C)	60	6.6
Tris (2 C)	36	4.0
EDTA plus Tris (2 C)	60	6.6
Control (37 C)	30	3.3
EDTA (37 C)	78	8.7
Tris (37 C)	36	4.0
EDTA plus Tris (37 C)	78	8.7

TABLE III

INFLUENCE OF EDTA ON STATIONARY PHASE NUTRIENT BROTH GROWN *FLAVOBACTERIUM* CELLS

Treatment	Vancomycin Adsorbed (μg)	Per Cent Adsorbed
Control (2 C)	12	1.3
EDTA (2 C)	30	3.3
Control (37 C)	30	3.3
EDTA (37 C)	36	4.0

same low adsorption pattern as demonstrated by the nutrient broth grown cells (Table IV). Thus, the adsorption studies suggest that the cell wall of succinate agar grown cells may more closely resemble the wall of nutrient broth grown cells than the wall of nutrient agar grown cells. Since cells grown on the synthetic and complete agar media differ, it may be that the divalent metal ions are less available for chelation in the succinate grown cells. These experiments suggest that the growth substrate as well as age have a profound effect upon the composition and structure of the cell wall as measured by vancomycin adsorption.

Influence of pH on EDTA Lysis and Growth Inhibition

Experiments were conducted to determine if the pH of the experimental system was a factor in determining the lytic ability of EDTA. EDTA was adjusted to various pH values, from the normal pH of 4.4 of the disodium salt form, using 0.1 M NaOH. P. fluorescens cells were treated with the different EDTA solutions at 2 C and 37 C for 2 minutes and 15 minutes, 5 ml samples were collected and read immediately at 540 mμ to determine the degree of lysis.

The cellular damage, as measured by lysis, increased as the pH was raised from 4.4. Similar lytic trends were observed for treatment at both 2 minutes and 15 minutes although the longer treatment was more effective at the higher pH values (Figure 5). Thus, EDTA is a much better chelator at higher pH values with its lytic capability increasing concommitantly.

A decreased lytic response was observed with the cells treated at 2 C. At both temperatures, incubation for 15 minutes produced

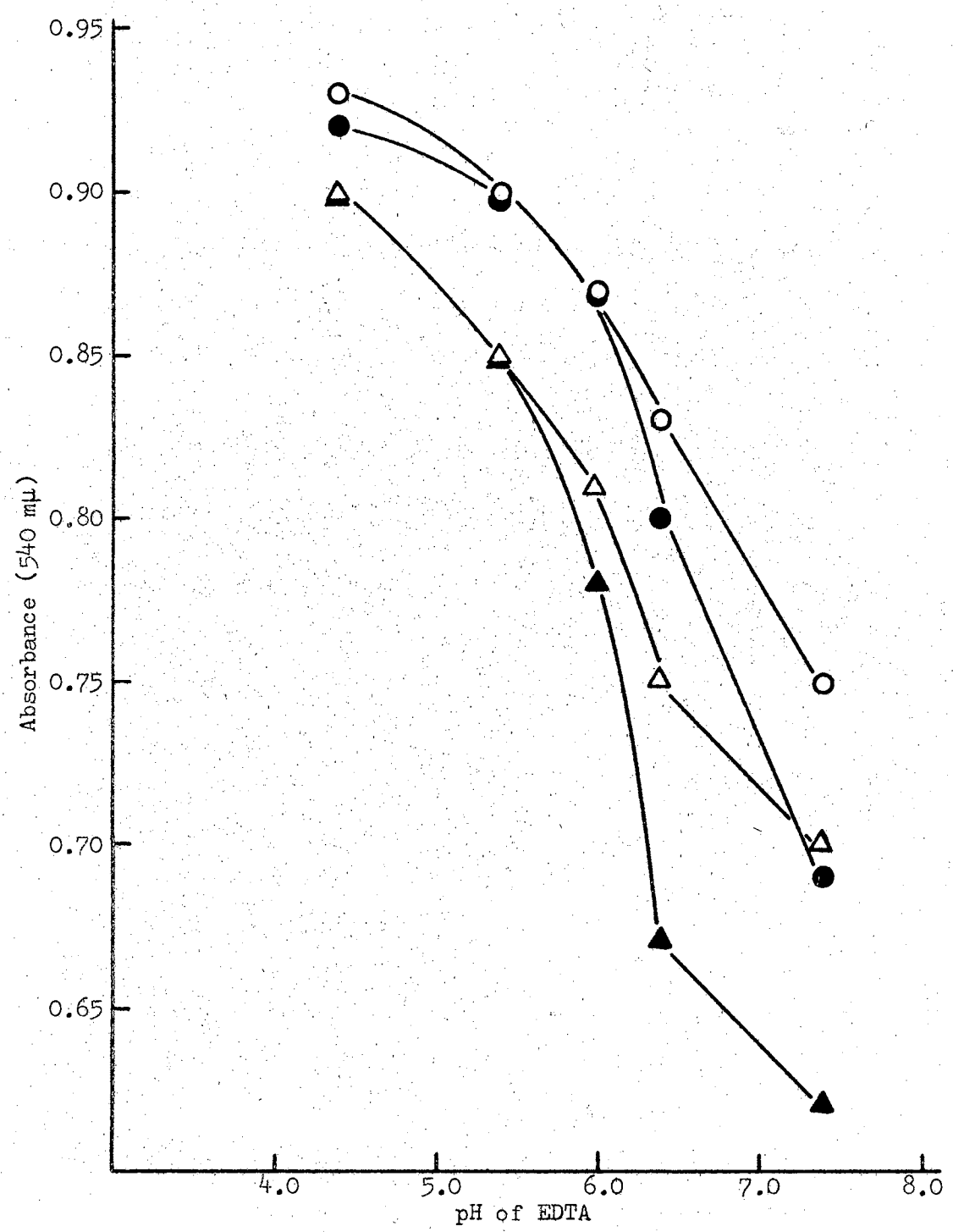
TABLE IV

INFLUENCE OF EDTA AND TRIS ON VANCOMYCIN ADSORPTION OF SUCCINATE AGAR GROWN *FLAVOBACTERIUM* CELLS

Treatment	Vancomycin Adsorbed (μg)	Per Cent Adsorbed
Control (2 C)	36	4.0
EDTA (2 C)	42	4.7
Tris (2 C)	48	5.3
EDTA plus Tris (2 C)	48	5.3
Control (37 C)	36	4.0
EDTA (37 C)	42	4.7
Tris (37 C)	36	4.0
EDTA plus Tris (37 C)	60	6.6

Figure 5. Lysis of P. fluorescens Cells Following Treatment With EDTA at Various pH Values

Lysis of P. Fluorescens cells following EDTA treatment over several pH values: ○, 2 C treatment for 2 minutes; ●, 2 C treatment for 15 minutes; △, 37 C treatment for 2 minutes; ▲, 37 C treatment for 15 minutes.


0.95
0.90
0.85
0.80
0.75
0.70
0.65
Absorbance (540 mμ)
4.0
5.0
6.0
7.0
8.0
pH of EDTA

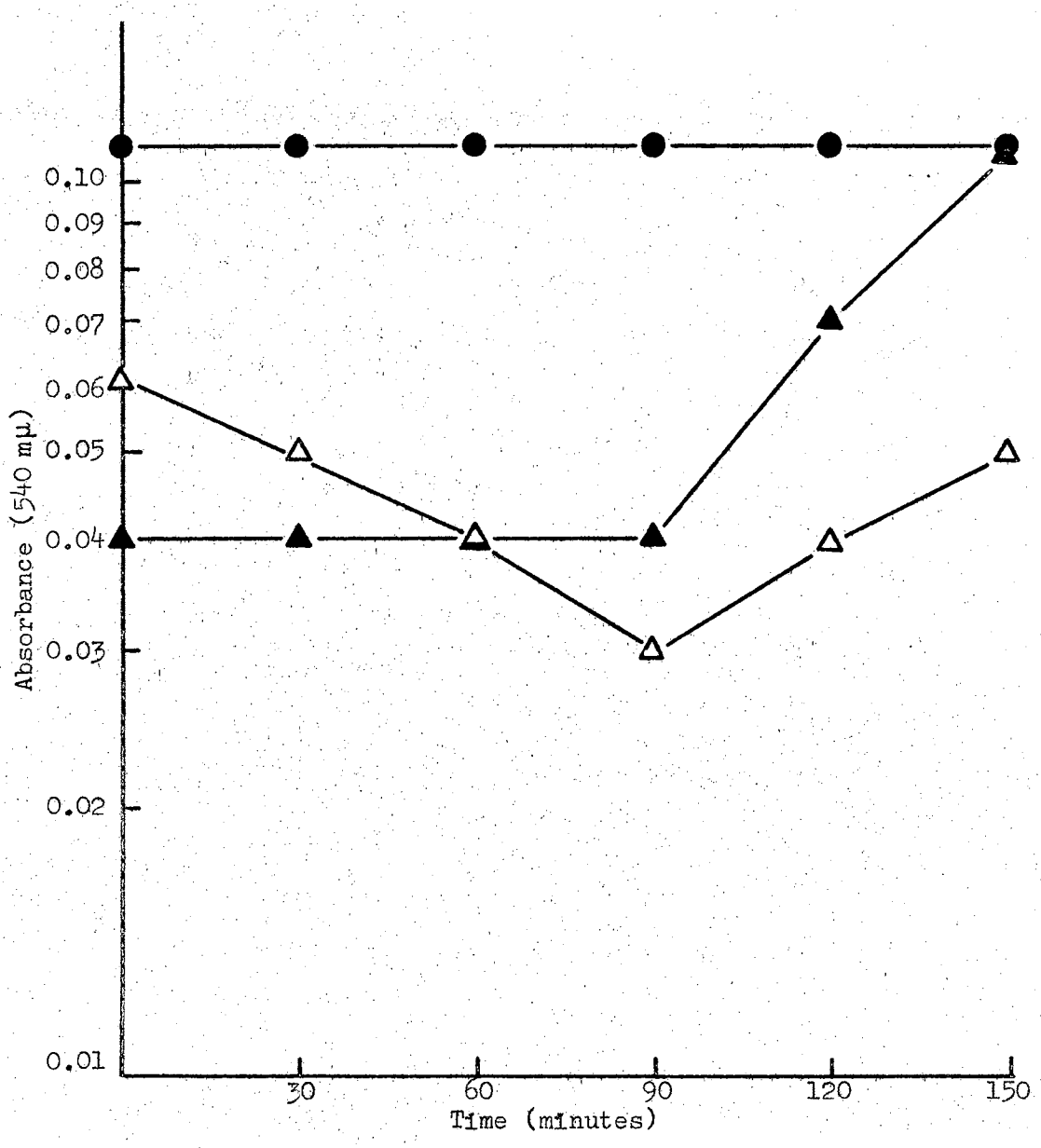
additional lysis when compared to the 2 minute treatment. This would be expected if an enzymic process were partially responsible for cell lysis since the chelating effect of EDTA is rapid reaching a maximum in 2 minutes (Gray and Wilkinson, 1965). Since the effect of treatment at 37 C was consistently greater than treatment at 2 C, it would appear that some enzymic activity may be evident in the process and the rate is slower at the low temperature.

Repaske (1958) reported that the lytic ability of the EDTA-Tris buffer system was much more efficient as the pH was raised from 7.2 to 8.6. These results (Figure 5) support the concept that the pH is an important variable and that EDTA functions as a very strong chelating agent at acidic pH values with increased chelating abilities in the basic pH range. The chelating ability of EDTA in the acidic range indicates that hydrogen ions are lost from the EDTA molecule at low pH values suggesting that the pKa values of some of the ionizable groups are quite low.

The lytic response of cells treated with EDTA at a pH near neutrality is further demonstrated by growth studies performed by suspending cells in nutrient broth after EDTA treatment. Cells treated for 15 minutes at 2 C with EDTA (pH 7.4) show some evidence of recovery after incubation in nutrient broth for 90 minutes at 37 C (Figure 6), whereas complete recovery is observed in nutrient broth with cells treated at 2 C for 15 minutes with pH 4.4 EDTA (Figure 2). Cells treated with EDTA at pH 7.4 do not show further lysis when held at 2 C in nutrient broth (Figure 6).

Figure 6. Growth of P. fluorescens Following Treatment With EDTA at pH 7.4

The effect of EDTA treatment (15 minutes) at pH 7.4 was measured by following growth in nutrient broth after EDTA treatment: ●, treatment at 2 C or 37 C, grown at 2 C; ▲, treatment at 2 C, grown at 37 C; △, treatment at 37 C, grown at 37 C.


0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Absorbance (540 mμ)
30
60
90
120
150
Time (minutes)

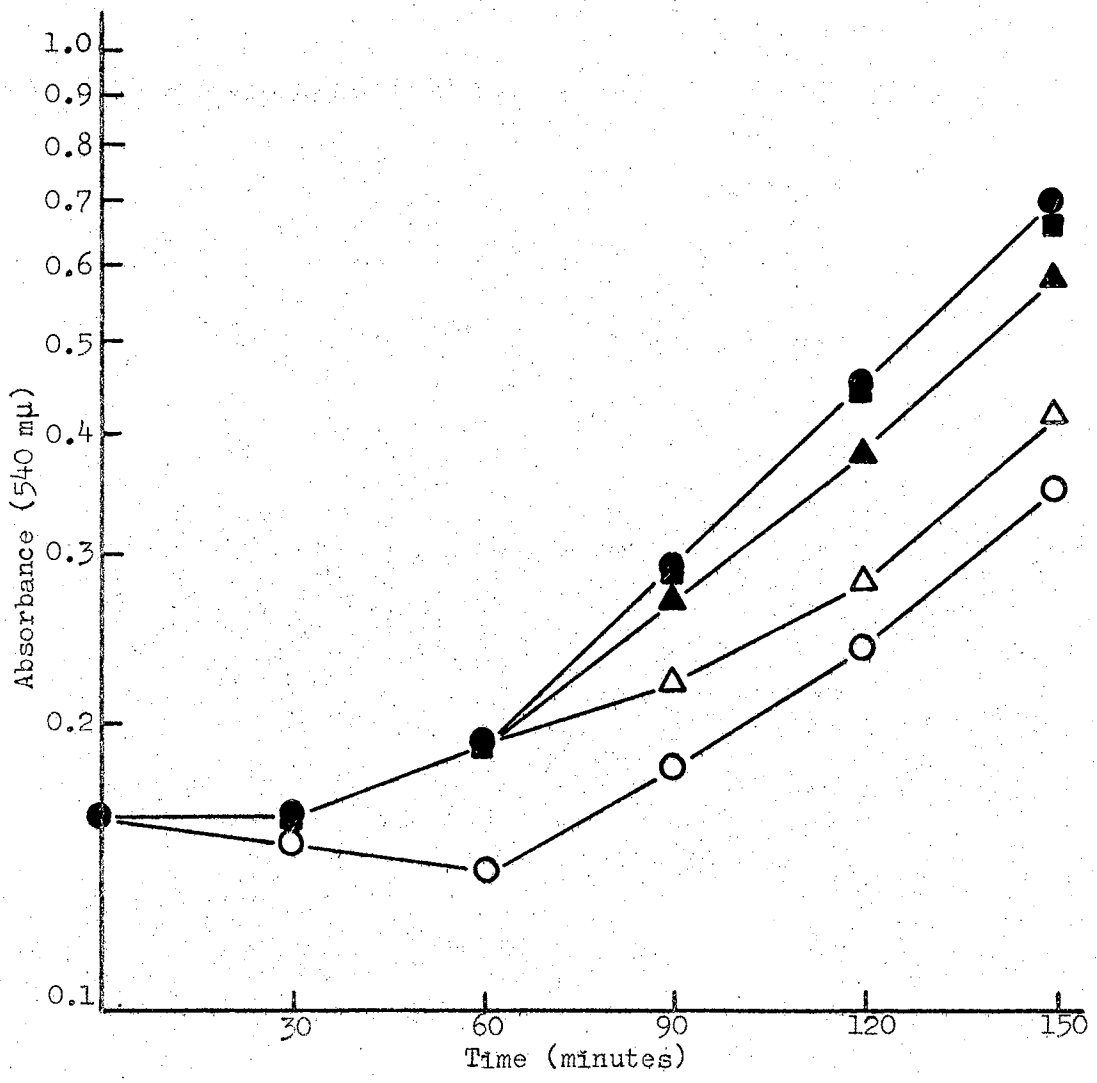
Influence of Magnesium on Growth Following EDTA Treatment

Experiments were conducted to determine if the action of EDTA against P. fluorescens involves only chelation and was influenced by the presence of cations. Cells were treated with 0.001 M EDTA (pH 4.4) in the presence and absence of an equimolar concentration of Mg^{++} in the sulfate salt form. The cells were washed, suspended in nutrient broth in the presence and absence of Mg^{++}, and growth was followed (Figure 7). Cells treated with EDTA in the presence of an equimolar concentration of Mg^{++} showed a growth response in nutrient broth only slightly slower than the control indicating the presence of Mg^{++} during EDTA treatment protects the cells. Cells treated with EDTA in the presence of Mg^{++} and grown in nutrient broth containing Mg^{++} showed a response which coincided with growth of the control. Cells treated with EDTA and grown in nutrient broth containing Mg^{++} showed a much greater response than cells grown in nutrient broth without Mg^{++}. The presence of Mg^{++} in the growth medium enhanced recovery of the cells regardless of whether Mg^{++} was or was not present during EDTA treatment.

Cells treated with EDTA in the presence of an equimolar amount of Mg^{++} and grown in nutrient broth exhibited slightly slower growth than the control; however, divalent metal cations in the wall no doubt compete with the free Mg^{++} ions for EDTA binding to some extent which would explain the slightly retarded growth curve. Thus, it would appear that EDTA action involves primarily the mechanism of chelation. These results augment the study of Repaske (1958) who reported that the action of EDTA on Escherichia coli and Azotobacter vinelandii was transient and did not permanently condition the cell for lysis. Since cells treated with EDTA and grown in the presence of Mg^{++} exhibited a reversal

Figure 7. Reversal and Protection by Mg^{++} of Lysis Initiated by EDTA

The influence of 0.001 M Mg^{++} on the EDTA (0.001 M) effect determined by following growth in nutrient broth: ●, control; ■, EDTA plus Mg^{++} treated, grown in nutrient broth plus Mg^{++}; ▲, EDTA plus Mg^{++} treated, grown in nutrient broth; △, EDTA treated, grown in nutrient broth plus Mg^{++}; ○, EDTA treated, grown in nutrient broth.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance (540 mμ)
30
60
90
120
150
Time (minutes)

of the EDTA effect, EDTA appears to have a transient effect on P. fluorescens also. In addition, EDTA treatment in the presence of Mg^{++} showed little if any harmful effect on the cells which suggests that the primary mechanism by which EDTA affects P. fluorescens is by chelation.

Autolysis of P. fluorescens in Buffer

The results obtained from the growth and adsorption studies have offered some evidence for the presence of an autolytic enzyme. Experiments were conducted to determine if lysis occurred in untreated cells using buffer solutions (0.05 M) to cover a wide range of pH values. Although these cells were not treated with EDTA, it is assumed that sufficient change occurred in the integrity of the cell wall to activate the enzyme. The cells were suspended in water using a Vortex mixer, placed in the various buffer solutions and incubated at 37 C for 15 minutes prior to reading the absorbance at 540 mμ. Lysis was measured and two peaks were evident. One peak occurred in the acidic pH range along with a corresponding peak in the basic range (Figure 8). These results would lend further evidence for the presence of an autolytic enzyme(s). Since lytic peaks were observed in both acidic and basic pH ranges, then more than one enzyme may be responsible for the degradation of the cell wall of P. fluorescens.

Since Mg^{++} ions were effective in reversing and protecting P. fluorescens from the EDTA effect, various divalent cations were added to the citrate-phosphate buffer system to ascertain if normal autolysis could be arrested by addition of Mg^{++}, Mn^{++}, Fe^{++}, Ca^{++}, Zn^{++}, or Na^{+}. Neither mono- or divalent cations appear to have any effect on arresting autolysis at pH 5.4 (Table V) which suggests that natural autolysis

Figure 8. Autolysis of P. fluorescens in Buffer

Cells were suspended in the appropriate buffer solutions (0.05 M) ranging from pH 4.4 to 10.4. Autolysis was observed by reading the absorbance at 540 mμ after a 15 minute incubation period. Initial absorbance was 0.50.

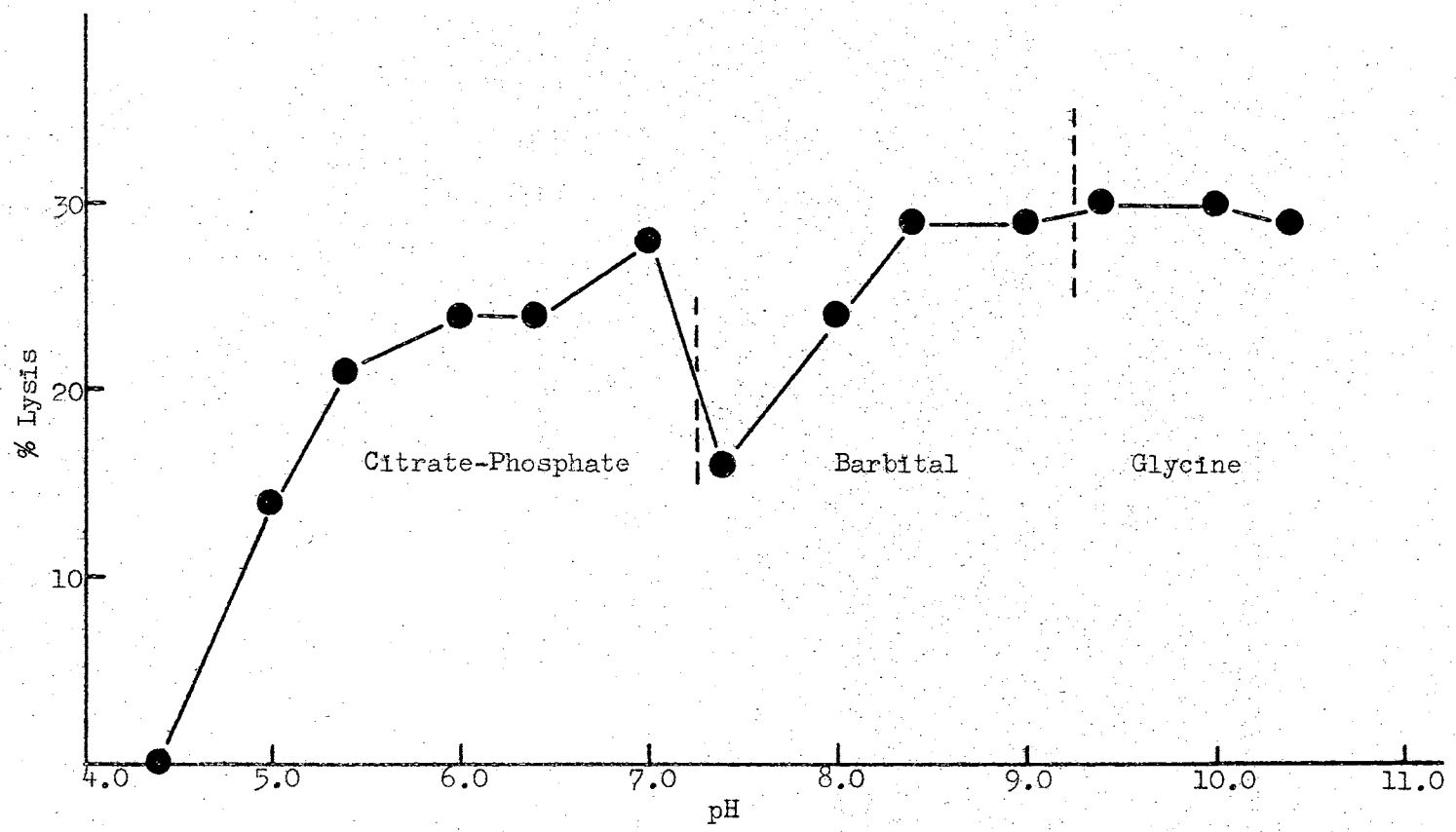

% Lysis
30
20
10
Citrate-Phosphate
Barbital
Glycine
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
pH

TABLE V

INFLUENCE OF MONO- AND DIVALENT CATIONS ON AUTOLYSIS OF P. FLUORESCENS SUSPENDED IN CITRATE-PHOSPHATE BUFFER (pH 5.4) AT 37 C

Cations	Per Cent Lysis
Control	73
Mg^{++}	73
Mn^{++}	77
Fe^{++}	82
Zn^{++}	65
Ca^{++}	65
Na^{+}	65

Final cation concentration was 0.00001 M for the divalent cations and 0.00002 M for Na^{+}. The sulfate salt form was used in all cases. One ml of water was added to the buffer suspension to serve as a control. The initial absorbance at 540 mμ was 0.11. Samples (5 ml) were read after a 15 minute incubation period.

may differ from the autolytic phenomenon observed following EDTA treatment, although enzyme(s) appear to be involved in both cases.

Osmoplast Stabilization

P. fluorescens cells can be converted to osmoplasts by treatment with EDTA in the presence of 0.5 M sucrose. The term osmoplast is used to designate an osmotically shockable rod rather than a true spheroplast. Phase-contrast microscopy of the stabilized cells indicated the presence of rods with no spherical cells evident. To confirm that these cells were true osmoplasts, a 1:5 dilution was made of the cells in distilled water. Dilution of the stabilized cells in water produced lysis which confirmed osmoplast formation.

A slight decrease in optical density was observed with EDTA treated cells suspended in sucrose. This is probably due to the stripping away of some of the cell wall material which produces a decrease in cell refractility (Figure 9). Stabilization was achieved, however, after an initial drop in absorbance at 540 mμ.

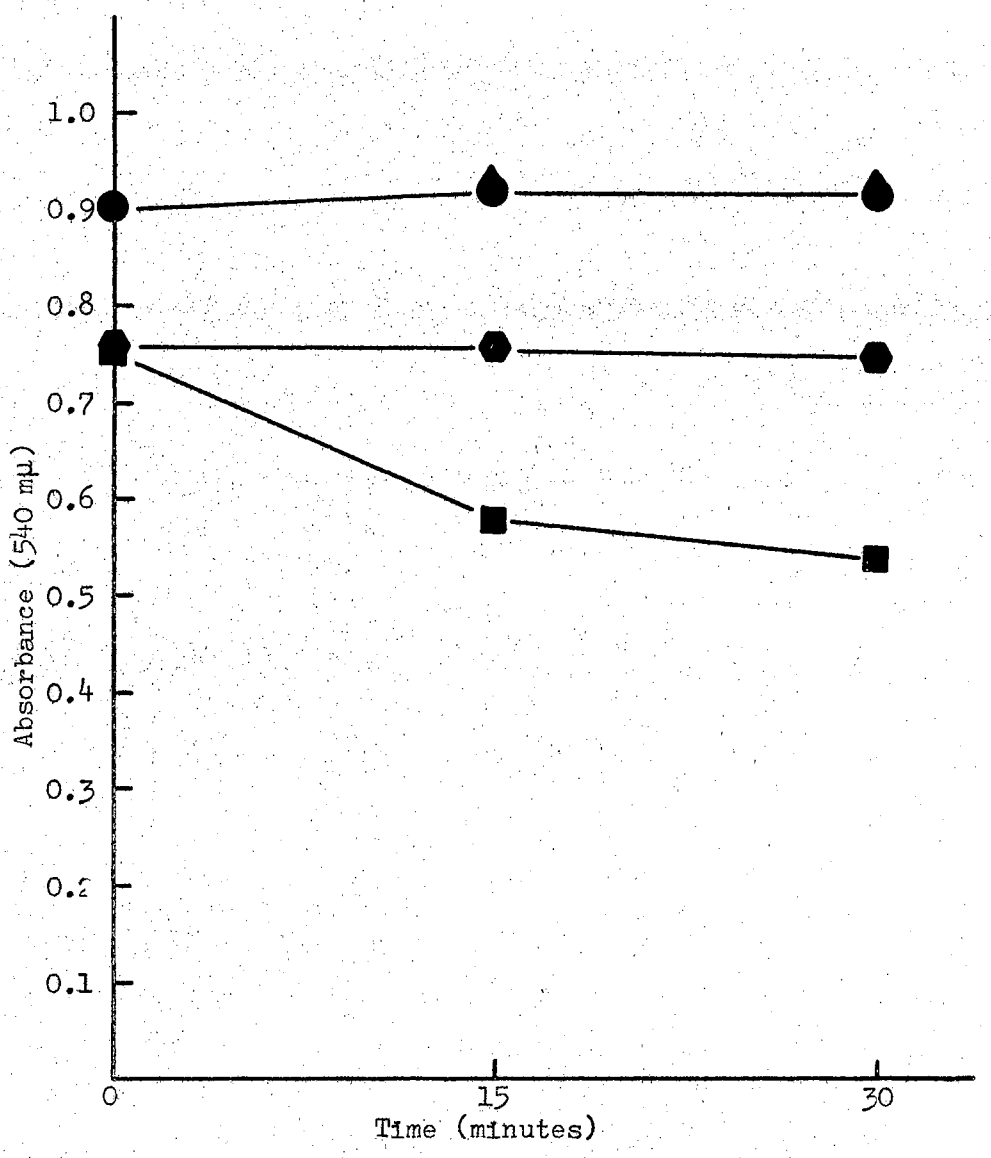
These data support the observation of Voss (1964) that osmotically fragile rods are formed by lysozyme-EDTA-Tris buffer treatment in sucrose. McQuillen (1960) reported the formation of true E. coli spheroplasts by the same procedure.

Mutagen Treatment of P. Fluorescens

Ultraviolet irradiation was used to determine if an EDTA-resistant mutant could be isolated. Since resistance to EDTA would incur either a drastic change in cell wall composition or the impairment of an autolytic enzyme, the later would be the more probable mutation to occur

Figure 9. The Stabilization of Osmoplasts of P. fluorescens

Cells were converted to osmoplasts by EDTA (pH 7.4) treatment in the presence of 0.5 M sucrose: ●, HOH control; ▲, sucrose control; ⬢, sucrose plus EDTA treated; ■, EDTA treated.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance (540 mμ)
0
15
30
Time (minutes)

in the formation of an EDTA-resistant strain.

The isolated cultures were characterized by normal growth on 0.01 M EDTA nutrient agar as compared to slow growth by the wild type organism. However, additional growth and vancomycin adsorption studies did not conclusively define further differences.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Growth studies using P. fluorescens cells treated with EDTA at 2 C and 37 C suggest that an autolytic enzyme(s) may be activated during EDTA treatment. Since temperature should not significantly affect the chelating ability of EDTA, the lysis observed with cells growing in nutrient broth following EDTA treatment at 37 C, which differs from the lag in growth following EDTA treatment at 2 C, must be attributed to a second parameter involved in cell wall degradation. Since enzyme activity is greatly reduced at 2 C, these results support the hypothesis that this second step in cell wall degradation may involve the activation of a degradative enzyme(s) associated with the cell wall. Autolytic studies involving untreated cells suggest the presence of more than one enzyme since two lytic peaks were observed - one in the acidic and one in the basic pH range.

Vancomycin adsorption studies using nutrient agar grown cells of a species of Flavobacterium indicate that the ease with which the enzyme(s) is activated depends to some extent on the growth substrate. Nutrient broth grown cells showed little if any lysis when placed in buffer solution at 37 C. Similarly, succinate agar grown cells did not readily lyse under the same conditions. These results suggest that the cell wall composition and integrity is dependent on the growth substrate.

Increased adsorption of vancomycin by nutrient agar grown cells

following treatment with EDTA at 37 C as compared to no profound increased adsorption when treated with EDTA at 2 C lends further evidence to the supposition that the complete process involves more than just chelation and that an autolytic enzyme may be functional. Cells treated with EDTA and/or Tris indicated that Tris compounded the effect of EDTA as reported by Eagon and Asbell (1966). It is concluded from the data obtained with vancomycin adsorption studies that these two lytic compounds have different modes of action. The site of Tris action is unknown at this time.

Cells treated with EDTA at 37 C, washed and placed in nutrient broth plus Mg^{++} experienced growth comparable to the control though somewhat retarded. Mg^{++} virtually negates the action of EDTA against P. fluorescens in that the cation either protects or enhances recovery of the cell. The results suggest that the EDTA effect involves only chelation of metal ions associated with the cell wall. No evidence was obtained that suggested that the EDTA molecule affected the cell by any mechanism other than by chelation. Apparently, when Mg^{++} is present, the autolytic enzyme(s) is not activated since the integrity of the cell wall is not altered. Repaske (1958) theorized that the metal is coordinated at a minimum of two sites on the cell surface, one of these being stronger than the other. EDTA chelates the metal by substituting for the weaker of the two bonds and forms an EDTA-metal complex which remains linked to the cell surface through the metal. The dissociation of EDTA from the metal complex must be high since washing (dilution) necessitates the adding of EDTA again to promote lysis. Evidence that the EDTA effect is transitory as reported by Repaske (1958) was substantiated by results obtained from experiments in this study using Mg^{++}.

The results suggest the presence of an autolytic enzyme(s) system which further degrades the cell wall of P. fluorescens and the species of Flavobacterium after initial alteration of wall integrity resulting from chelation of critical cations by EDTA. Growth studies and vancomycin adsorption studies augment this hypothesis. Moreover, results reported by various authors working with P. aeruginosa can be satisfactorily explained by the hypothesis of this study regarding the presence of an autolytic system. Gray and Wilkinson (1965) reported a reduction in the release of cell solutes by EDTA at 0-2 C as compared to leakage of cellular materials following treatment at 20 C. Leive (1965a) reported that actinomycin uptake by EDTA treated cells did not occur at 0 C and suggested a difference in the permeability of the cell surface at 0 C and 37 C. Cox and Eagon (1968) reported that significant quantities of P. aeruginosa cell wall material were released by incubating the cell walls in water with no prior EDTA treatment. An autolytic enzyme(s) activated by partial cell wall damage resulting from EDTA treatment could account for the phenomena observed in the above mentioned papers. More evidence is needed to ascertain the presence of this autolytic system. Further work should be directed toward the isolation and characterization of the enzyme(s) involved in this degradative process.

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VITA

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