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INTERMEDIARY METABOLISM IN *NOCARDIA CORALLINA*:

AN ENZYMIC STUDY

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CHAPTER I

INTRODUCTION

The purpose of this investigation was an examination of the biochemical controls of microbial cellular differentiation. Work paralleling this type of examination is currently in progress in a number of laboratories. In this regard the differentiating slime mold, *Dictyostelium discoideum*, has received the greatest attention. A leader in this field, B. E. Wright (58), has summarized the work on this organism with the hypothesis that during the process of differentiation carbohydrate is used, amino acids are degraded, and finally protein is catabolized. Resulting substrate limitations then determine not only the biosynthetic pathways which will be used but also the morphological state of the organism. The effect of changing metabolic patterns is reflected in the variation in the activities of the enzymes in these cycles.

This alteration in enzyme activity was best shown in several enzymes involved in polysaccharide production.

Pannbacker (39), Roth and Sussman (44), and Loomis and Sussman (29) have established that polysaccharide biosynthesis varies with the morphological state of D. discoideum. At a very late stage in morphogenesis a specific polysaccharide is formed. Trehalose production is low during early stages and increases during the later stages. Uridine diphosphoglucose and uridine diphosphogalactose followed similar patterns. The enzymes involved in the biosynthesis of the polysaccharides were also reported to increase in activity concomitant with the increased need for polysaccharide. Evidence for de novo synthesis of enzyme was examined by Sussman, et al. (50) and by Wright and others (60, 40), using actinomycin D. This antibiotic, which is a powerful inhibitor of protein synthesis, blocked the rise in enzyme activity and thus prevented the production of polysaccharide, therefore indicating de novo synthesis of the enzyme.

The process of spore formation and germination is accompanied by changes in the levels of respiratory enzymes in both fungi and bacteria. M. Sussman and H. O. Halvorson, in a book on sporulation, have summarized the results of many of these investigations. (51) Differences are more noticeable than similarities in the enzyme levels. The most noticeable similarity is the low rate of respiration of the spores. Furthermore, cytochromes appear to be absent from bacterial spores but present in some fungal

spores.

The actinomycete, Nocardia corallina, was chosen as a system to investigate. On the surface this organism seems to be a more simple system than the spore forming fungi and bacteria, which have been used almost solely in the study of the biochemistry of differentiation in the lower organisms. There is a basic assumption that the energy control mechanisms underlying morphogenesis should be somewhat similar in all lower organisms. There is a basic assumption that the energy control mechanisms underlying morphogenesis should be somewhat similar in all lower organisms; consequently, Nocardia corallina should provide data and information with fewer variables.

The concept of the control of metabolic processes by metabolites was first conceived by Yates and Pardee and by Umbarger. With their simultaneous discovery of biosynthetic negative feedback, Yates and Pardee (62) and Umbarger (53) formulated the basic concept that regulation of metabolism is controlled through effector-enzyme interaction. Since then enough cases have been documented in which a product acts on an enzyme, which catalyzes an early step in its biosynthesis, to indicate that negative feedback must be of fundamental importance in biosynthesis. Recent review articles by Atkinson (1), and by Stadtman (49), should be consulted for a more complete analysis of this type of regulation.

Furthermore Nocardia corallina seemed ideal for the study not only of the energy aspects of morphogenesis, but also of the causes and effects of morphological changes since it undergoes a cyclic series of rather simple transformations during its life cycle. It is a nonexacting bacterium which grows well on nutrient agar and posses a life cycle characterized by distinct morphological changes (Fig. 1). The events including germination, hyphal development, and fragmentation describe the primary growth cycle. A secondary cycle, which is less well defined, is the process by which the bacillary cells divide to form the coccoid. Morphological and cytogenetic changes have been characterized by R. B. Webb, J. B. Clark, and H. L. Chance, (56) by R. B. Webb and J. B. Clark, (55) and by O. R. Brown. (6) Prior to germination, nuclear division occurs, the cell becomes oblong or elliptical and outgrowth occurs at one or both ends. Nuclear division apparently accompanies hyphal development, resulting in a multi-nucleated cell. The process of fragmentation begins with cross wall formation. Fragmentation then occurs at the site of this formation and results in the production of rod shaped cells containing one or more nuclei. These bacillary cells again divide by cross wall formation to develop into uninucleated coccoidal cells. Growth then takes place by nuclear division and cell division of the coccoidal cell.

In order to accomplish the stated purpose of this

investigation it became necessary to further characterize the principal metabolic pathways of this organism. Preliminary studies had been conducted by several investigators. Baugh, Claus and Werkman (4) describe an enzyme system capable of reversibly fixing CO_2 if the appropriate nucleotide were present. Midwinter and Batt (35) used oxygen uptake to study respiration and oxidative assimilation in the intact organism. Martin and Batt (30, 31) defined the growth requirements of the bacteria and also studied the induction of enzymes for pyrimidine catabolism. Brown (5, 6) found evidence for the pentose cycle in the organism. Using oxygen uptake curves and observing the increase in the utilization of oxygen in the presence of succinate, malate, α-ketoglutarate, and citrate, he indicated the presence of a Krebs cycle. He also found evidence for glucose-6-phosphate dehydrogenase and isocitrate lyase. None of the enzymes have been characterized. Brown and Reda (8) reported hyphae of the organism possess enzymes capable of converting glucose to fructose-6-phosphate. Brown and Reda also reported a two fold change in the activity of glucose-6-phosphate dehydrogenase of the hyphae.

It has been necessary to devote a large amount of effort to develop techniques to obtain more information and to extend the information already available on the intermediary metabolism of this organism. In this way

new insights might be obtained both into carbohydrate usage and control of metabolism. Techniques have had to be perfected for synchronous growth and disintegration of the cell wall to obtain active preparations of the various enzymes prior to actual assay. Once such preparations were obtained it was possible to characterize the enzymes and to measure their specific activity as a function of growth. The nature of the kinetics of the enzymes and their relative level at the various stages of growth can be used to suggest possible mechanisms for control in this organism.

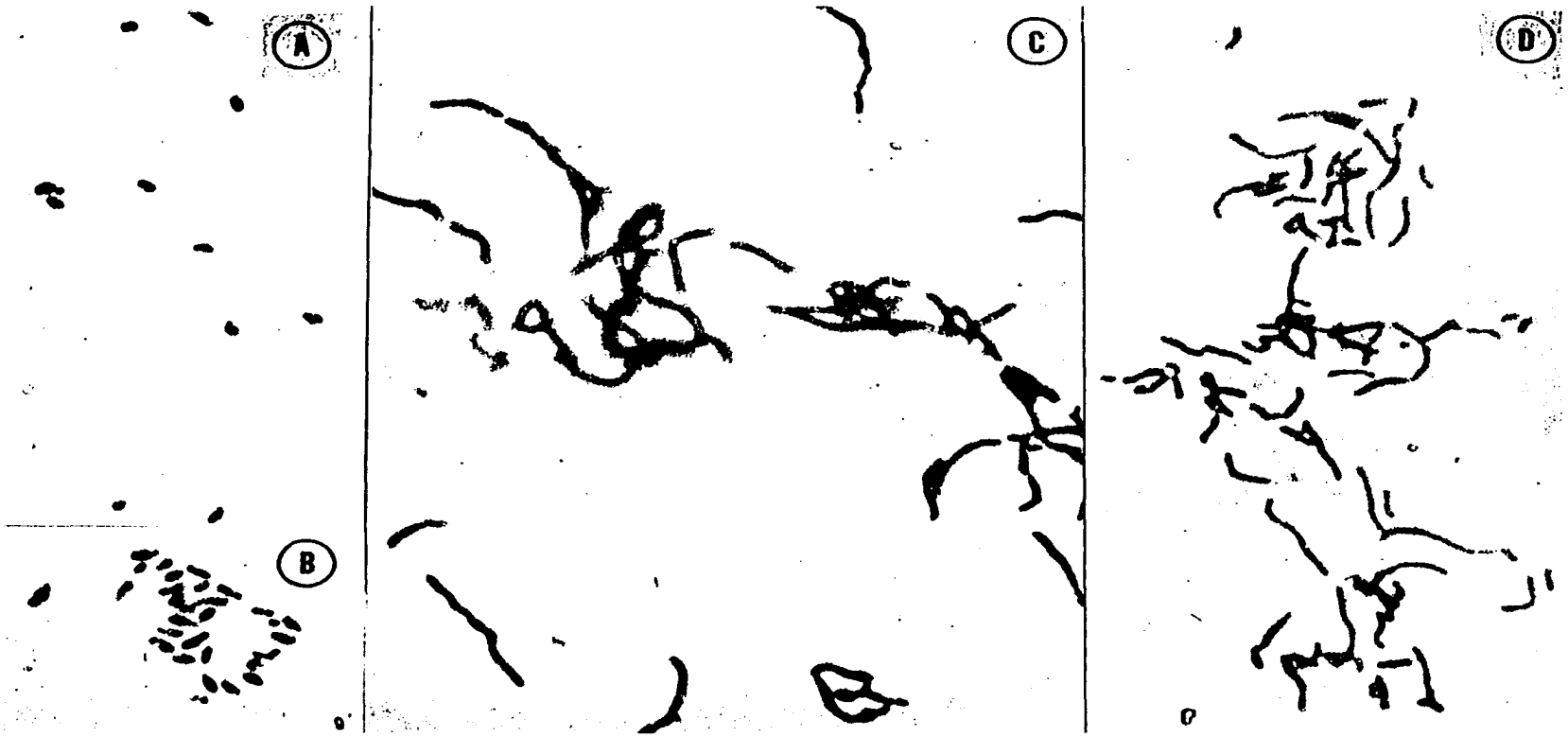


Figure 1. Morphological growth stages of N. corallina

A. Coccoid, B. Germination, C. Full hyphal, D. Early fragmentation

CHAPTER II

EXPERIMENTAL

Growth of the Organism

Cells were routinely obtained from cultures grown at 30°C on 1% fructose supplemented nutrient agar (agar 3% by weight). Both Petri dishes (15 x 150 cm^2) and large Roux bottles were poured and stored at a slant 24 hours at 30°C prior to inoculation. Cultures which had been growing 24 to 48 hours were used as a source of inocula. The bacteria were spread smoothly and uniformly over the surface of the agar using a heavy inoculum. In determining enzyme parameters all cells were grown 24 to 48 hours before harvesting. After 48 hours very little additional growth occurred. The bacteria were washed from the surface of the agar using 0.01 M $NaHCO_3$, centrifuged at 2000 x g and either used directly or stored at -20°C. Under these conditions each Petri dish yielded between 1 and 2 g of bacteria (wet weight) after 48 hours.

Growth of the organism in liquid media was also accomplished. 1% fructose supplemented nutrient broth was used to culture the bacteria. Bacteria were transferred

from a 24 hour slant to form a heavy inoculum in the broth. The media containing bacteria were shaken at 30°C for 48 hours. Serial transfers each time using a 1/10 dilution and flasks having a 2/3 void volume were used to grow increasingly large volumes of bacteria. Finally cells were harvested from 1 l. of medium in a 2.5 l. culture flask after growth for 48 hours at 30°C with shaking. Cells were harvested by centrifugation and frozen. 1 l. of medium yielded ca. 10 g of cells (wet weight).

Synchronous growth on agar plates was obtained by plating cells from 48 hour cultures. The source of cells was a rapidly growing culture which had been transferred several times at 48 hour intervals. Random samples were withdrawn at the various time intervals and examined microscopically to determine the morphological state of the organism. At the desired growth stage cells were harvested, broken, and assayed. All enzymic measurements were performed within 10 hours after breakage of the cells on an unfrozen preparation.

Using the growth conditions mentioned above and a heavy inoculum of a 48 hour culture, synchronous growth in liquid media was observed to the stage of full hyphal growth.

The bacteria were also observed to grow on nutrient agar alone as well as on nutrient agar supplemented with citrate, acetate, or glycerol. Growth on glucose, galactose,

lactose, sucrose and maltose supplemented nutrient agar was also observed. The concentration of all the supplements was 1% by weight.

Cells grew slowly on the defined medium of McClung. (29) This medium contains 2.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 mg $Fe_2(SO_4)_3$, 8.0 mg $MnCl_2 \cdot 4H_2O$, and 2 mg $ZnSO_4 \cdot H_2O$ plus a carbon source in 1 l. of medium. Medium was prepared, sterilized, and growth was observed after allowing the organism to adapt by several transfers to fresh medium.

Cell Disruption

Several techniques were used to break the cell wall of this organism. Sonication, blending, cell lysis by lysozyme, detergent, autolysis, lyophilization and fat extraction were all employed.

In blending experiments 20 g of cells were mixed with 20 gms of homogenizing glass beads (Aloe, number 12) and 20 ml of the desired buffer. The slurry was placed in the 50 ml cup of a Sorvall Omnimixer. The cup was submerged in an ice salt water solution and the blender was set at 55 to 60 v for 10 to 20 min. Glass beads were removed by decantation and cellular debris by centrifugation at 50,000 x g (average), for 2 hours. The supernatant was retained for analysis and either stored frozen or used directly.

Several attempts were made using detergent to

solubilize protein from the pellet previously obtained (centrifugation 50,000 x g, average). Sodium lauryl sulfate was used in these attempts. The pellet from 40 ml of cells was mixed with 1.0 ml of buffer and 10 ml of a solution containing 15% sodium lauryl sulfate in 1% NaCl (made using 0.1 M phosphate buffer pH 6.5 as the solvent). This mixture was blended with an equal amount of glass beads for 15 minutes. After centrifugation for 2 hours, 50,000 x g (average), the supernatant was assayed for enzyme activity.

Breakage attempts upon whole cells using detergent alone or detergent plus lysozyme have been examined. Lysozyme (Worthington) in the quantity of 1 mg per g of bacteria was used in all attempts. In a typical attempt 1 g of bacteria was mixed with lysozyme and the mixture stirred 7 hours at $4^{o}C$. Similarly, cells were combined with lysozyme and detergent and stirred 7 hours at $4^{o}C$. The same experiments were carried out on cells which had been lyophilized and defatted by the technique outlined below. 0.75 g of the cells were used and the technique was identical to that reported for untreated cells. This technique is similar to that reported by Herbert and Pinsent. (18)

Attempts were made to correlate breakage times (as seen by release of $NADP^{+}$ isocitric dehydrogenase) and the time of blending using the homogenizer. Times of up

to 45 minutes were used using 25 g of beads, 25 ml of 0.01 M $NaHCO_3$ and 25 g of cells. 5 ml samples were withdrawn and analyzed for activity. A similar series of experiments was run omitting the glass beads.

In a parallel group of experiments release of NAD^+ isocitric dehydrogenase activity was assayed as a function of blending time. 20 g of cells, 20 g of glass beads and 10 ml of 0.1 M Tris buffer (pH 9.0) were blended for periods of time up to 45 minutes under nitrogen. The supernatant from 3 hours centrifugation, 15,000 x g was assayed for activity.

A second major technique used in cell disruption was sonication. Sonication was used before and after defatting the cells. The procedure involved placing the desired weight of cells in buffer into the cup of a Blackstone, Model number SS3, sonicator. The sample was sonicated the prescribed period of time while circulating a stream of ice water through the jacket of the cup. Temperature of the sample varied from 10 to 15°C. The sample was then collected, cooled to ice water temperature and centrifuged to remove cellular debris.

In sonication attempts using untreated cells 1 g of pellet was placed in 10 ml of buffer and the mixture sonicated 15 min. Attempts were made on larger volumes of solution and greater amounts of bacteria. The effect of sonication for longer and shorter periods of time was also noted.

In the case of cells which had been defatted 0.2 g of the bacteria was used and 10 ml of buffer. The solution was sonicated and the parameters checked as mentioned in the previous paragraph. Debris was removed in both cases by centrifugation at 25,000 x g.

In an attempt to utilize autolysis 3 g of bacteria were combined with 3 ml of 0.1 M Tris buffer (pH 9.5) and the slurry was stirred 3 days at 4°C.

Cells which were to be defatted were lyophilized 24 hours. A 10 g pellet obtained from growth on agar usually yielded 2 g of a pale orange powder of lyophilized bacteria. These bacteria were frozen in an acetone dry ice bath prior to extraction with cold ether. All extraction operations were carried out in the acetone dry ice bath. The bacteria were separated from the supernatant solution by low speed centrifugation using a pre-cooled (-40 to -60°C) rotor. Thus the cells were continually in contact with very cold (temperatures lower than -60°C) solvent. 2 to 3 g of bacteria were extracted with 20 ml portions of ether until a decrease was noted in the color of the extracting solvent. Generally for a sample of bacteria of this size 150 to 200 ml of ether was required and the pellet usually had a 10% loss in dry weight. After the final ether extraction the cells were maintained frozen and the ether removed by lyophilization. In this way a fine powder of defatted bacteria was obtained.

The effect of lysozyme on defatted cells was determined by mixing sufficient cells in 0.1 M phosphate buffer (pH 7.0) to give a stable solution when viewed at 450 mμ several minutes. A lysozyme solution (2 mg/3 ml) was then added and changes in absorbance at 450 mμ were recorded. (18)

The buffers used and the parameters which gave the best results are as reported in the sections devoted to each individual enzyme.

Enzyme Assays

All optical measurements were made on a Hitachi Perkin-Elmer UV visible spectrophotometer, (Model 139). Protein concentration was determined either by the biuret method (11) or by the method of Folin and Ciocalteau (42) using bovine serum albumin as the standard. Velocities reported in optical density changes per minute at 340 mμ may be converted to μmoles by multiplying the change by a constant factor of 0.480 for the 3 ml assays and 0.160 for the 1 ml assays. For purposes of graphical analysis units reported are 0.001 of an optical density unit. Due to the stability of the $NADP^+$ enzyme and its occurrence under several breakage conditions it was chosen as an indicator enzyme.

Optimum breakage conditions for the $NADP^+$ dehydrogenases were very similar. The Omnimixer was used at a setting of 55 v and the cells, glass beads, and buffer

(in a 1:1:1 ratio, wt:wt:vol.) were blended 15 minutes in the ice water bath. Cellular debris was then removed by centrifugation after decanting of the glass beads.

Isocitrate Dehydrogenase

N. corallina possesses both a NAD^+ and a $NADP^+$ dependent isocitrate dehydrogenase. Both enzymes were assayed by measuring the reduction of the nucleotide as observed by an increase in absorbance at 340 mμ. NAD^+ isocitrate dehydrogenase (L_s-isocitrate: NAD^+ oxidoreductase (decarboxylating) EC No. 1.1.1.42) was detected in cell free preparations which had been prepared by two different techniques. Blending 20 g of cells in the presence of 10 ml of 0.1 M Tris (obtained through Sigma) buffer (pH 9.0) and 20 g of beads at a setting of 65v for 30 minutes produced a cell free preparation which possessed greatest isocitrate dehydrogenase. A second breakage attempt involved use of the defatted cells and sonicating for 10 minutes. In both cases a high endogenous activity is seen in the absence of added isocitrate. For this reason a 90% $(NH_4)_2SO_4$ precipitation step and dialysis (against 0.01 M Tris, pH 9.0, containing 0.05 M β-mercaptoethanol) was used prior to actual assay of the enzyme. Under these conditions activity in the absence of either substrate is negligible.

Conditions for a typical assay procedure run in a 1 ml cuvette included 10 mM Tris-HCl, pH 9.0; 2 mM D,

L-isocitrate (Sigma Reagent Grade): 10 mM NAD^+ (Sigma Reagent Grade); 5 mM $MgCl_2$; 5 mM β-mercaptoethanol; and sufficient enzyme preparation to give optical density changes of 0.015 to 0.020 units per 15 seconds. The reaction was started by the addition of isocitrate. After a 3 minute incubation at room temperature isocitrate was added and optical density changes were recorded. Under these conditions the reaction proceeds linearly as a function of time.

Activity of the $NADP^+$ isocitrate dehydrogenase (L_s-isocitrate: $NADP^+$ oxidoreductase (decarboxylating) EC No. 1.1.1.42) was very high under several breakage conditions, including blending and sonication. Kinetic measurements and breakage in several buffers yielded identical results. The pH profile was obtained by preparing the buffers, each 33 mM and using several overlapping points. A typical set of assay conditions in a 3 ml cuvette included 33 mM phosphate buffer, pH 7.5; 2.0 mM D, L-isocitrate; 0.20 mM $NADP^+$; 33 mM $MgCl_2$; and enzyme (crude preparation) diluted to give an optical density change of 0.015 to 0.020 units in a 15 second time interval. Assays were run in 3 ml cuvettes and started by the addition of the diluted enzyme preparation.

L-Malic Dehydrogenase

A reaction which is characterized by the reduction of $NADP^+$ in the presence of L-malic acid has been detected

and characterized in cell free preparations of this organism. The enzyme is relatively unstable showing a 25-50% loss in activity upon freezing and thawing. A similar loss in activity is noted on frozen storage (25-50% per day). This loss is not prevented by 0.05 M β-mercaptoethanol. Solutions of L-malic acid are also unstable and must be prepared fresh daily. A pH profile was obtained by preparing the various buffers (33 mM) without overlapping pH ranges.

A standard assay, in a 3 ml cuvette, included 33 mM glycylglycine, pH 8.5; 50 mM L-malate; 0.20 mM $NADP^+$; 33 mM $MgCl_2$; and 0.1 ml of diluted enzyme preparation. The assays were started by the addition of enzyme. The preparation was obtained from cells broken by blending cells and glass beads 15 minutes in 0.01 M sodium bicarbonate.

Several possible metabolic effectors of the enzyme were assayed using the above conditions. The concentration of each of the effectors was 1 mM and the concentration of L-malate was sufficient to yield half the maximal velocity.

The reverse reaction was checked in a 1 ml cuvette by using 10 mM glycylglycine, pH 8.5; 10 mM oxaloacetate; 10 mM NADH; 10 mM $MgCl_2$; and 0.1 ml of the crude preparation. A second possible reverse reaction was checked by placing the following in a 1 ml cuvette; 10 mM sodium

bicarbonate; 1 mM pyruvate; 1 mM NADPH; 10 mM $MgCl_2$; and 0.1 ml of crude preparation. Both possible reverse reactions were started by the addition of enzyme.

A NAD^+ malic dehydrogenase was also detected. Assay conditions for this enzyme included 10 mM Tris-HCl buffer, pH 8.5; 10 mM L-malate; 1.0 mM NAD^+; 10.0 mM $MgCl_2$; and enzyme preparation in a 1 ml cuvette. The preparation contained 0.05 M β-mercaptoethanol and was obtained from a cell-free preparation produced by blending cells, beads, and buffer (1:1:1) 30 minutes at 65 v. The supernatant (high in NAD^+ endogenous activity) was assayed after being precipitated by 90% $(NH_4)_2SO_4$. Endogenous activity of the pellet (taken up in buffer) was negligible.

Glucose 6-phosphate Dehydrogenase

The presence of this very stable enzyme (glucose-6-phosphate: $NADP^+$ oxidoreductase EC No. 1.1.1.49) was detected by several very different breakage conditions including blending and sonication. Under the assay conditions a linear response with respect to substrate concentration is noted for intervals up to 10 minutes when measuring velocity at V_m. Typical conditions for a 3 ml assay included 33 mM glycylglycine, pH 8.5; 2 mM glucose-6-phosphate; 0.2 mM $NADP^+$; 3.3 mM $MgCl_2$ and 0.1 ml of enzyme preparation (diluted to give optical density changes of 0.010 to 0.015 per 15 seconds). Assays were started by the addition of enzyme. Several possible effectors of

glucose-6-phosphate dehydrogenase were examined by assaying the enzyme at ½ V_m in the presence and absence of these substances at mM concentrations.

Isocitrate Lyase

This enzyme (L_s-isocitrate glyoxylate-lyase EC No. 4.1.31) was detected in cell free preparations obtained by blending 24 to 48 hour cultures with glass beads and 0.01 M sodium bicarbonate (1:1:1) for 15 minutes at a setting of 55 v. The enzyme was assayed in the direction of isocitrate cleavage using phenylhydrazine as a trapping reagent for glyoxylate. Assay conditions used were those of Dixon and Kornberg (15). All reagents were prepared fresh daily in deoxygenated water and the assays were run under anaerobic conditions. Following the addition of enzyme the cuvettes were capped and the increase in absorbance at 324 was recorded. Optical density increases were linear with time but endogenous activities at times were as high at 10% V_m.

A typical set of assay conditions, in a 3 ml cuvette, contained 6.6 mM phosphate buffer, pH 7.0; 2 mM D, L-isocitrate; 2 mM cysteine HCl (neutralized); 3.3 mM $MgCl_2$; 3.3 mM phenylhydrazine; and enzyme sufficient to yield optical density changes of 0.020 to 0.030 units per minute.

Several attempts were made to assay this enzyme by coupling it with $NADP^+$ isocitrate dehydrogenase. Assay

conditions, in a 3 ml cuvette, included 33 mM Tris HCl, pH 7.5; 1 mM glyoxylate (Sigma Reagent Grade); 1 mM succinate; 3.3 mM $MgCl_2$; 1 mM $NADP^+$; isocitrate dehydrogenase 1 unit (Sigma, pig heart) and 0.1 ml enzyme preparation. No activity was seen.

A second attempt to couple the enzyme involved the use of glyoxylate reductase. In this attempt 10 mM phosphate buffer, pH 7.0; 6 mM D, L-isocitrate; 10 mM $MgCl_2$; 1 mM NADH; 1 unit of glyoxylate reductase (Calbiochem, spinach leaf) and 0.1 ml of enzyme were placed in a 1 ml cuvette. No activity was noted.

Aldolase

Aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphatelyase EC No. 4.1.2.6) was detected in preparations obtained from sonication of 0.2 gms of defatted cells in 10 ml of 0.1 M Tris buffer (pH 9.0). The enzyme was assayed by following the oxidation of NADH using the couple of triose phosphate isomerase and L-glycerol phosphate dehydrogenase. Coupled enzymes were diluted to contain approximately 1.1 units of the isomerase and 0.6 units of the dehydrogenase in a 1 ml cuvette which was the reaction vessel. Dilutions were made in such a way that additional coupling enzymes had only a negligible effect on optical density changes. Assay conditions included 10 mM Tris-HCl buffer, pH 7.5; 1.0 mM fructose-1,

6-diphosphate; 1 mM NADH; an excess of the coupling enzymes, and enzyme preparation sufficient to give optical density changes of 0.010 to 0.020 units per 15 seconds. Assays were started by the addition of the enzyme preparation. No activity was seen in the absence of the full components of the assay system. Contamination with NADH oxidase was removed by centrifugation of the preparation at 100,000 x g for 2 hours. Metal ion requirements were investigated as was the effect of other possible substrates for the reaction.

NADH Oxidase

This enzyme was detected in the same preparation as listed for aldolase. It was assayed by the decrease in absorption caused by the oxidation of NADH occurring at 340 mu. The assay contained 10 mM Tris HCl, pH 7.5 1 mM NADH; and enzyme preparation sufficient to give an optical density change of 0.010 to 0.015 units in 15 seconds when assayed in 1 ml cuvettes. The enzyme was found to be very stable stored under 90% $(NH_4)_2SO_4$ at 4°C. The preparation contained 15 to 20 mg per ml of protein (Obtained by 90% $(NH_4)_2SO_4$ precipitation) and usually 0.05 to 0.1 ml of preparation was used in the assays. Assays were started by the addition of NADH after a 5 minute pre-equilibration at room temperature. The pH profile was obtained under similar conditions by varying the buffer and the pH.

Fructose-1,6-Diphosphate Phosphatase and Phosphofructokinase

Several attempts were made to detect the presence of both of these enzymes using different breakage conditions (see results section). Assay conditions for both enzymes involved using the coupling assay enzymes which had the advantage of both rapidity of measurement and detectability of very small amounts of activity for either enzyme.

The coupling system for fructose-1,6-diphosphate phosphatase involves use of hexosephosphate isomerase (1 unit) and glucose-6-phosphate dehydrogenase (0.5 unit). Assays included 10 mM Tris-HCl, pH 8.5, 1 mM fructose-1,6-diphosphate; 1 mM $NADP^+$; phosphohexose isomerase (Sigma, rabbit muscle); glucose-6-phosphate dehydrogenase (Sigma, yeast); and enzyme, in a 1 ml cuvette. The effect of varying pH was checked by running the reaction in the presence of 10 mM Tris pH 7.5 or pH 8.5.

The coupling system for phosphofructokinase involves aldolase, 1 unit (Sigma, rabbit muscle), triose phosphate isomerase, 1 unit (Sigma, rabbit muscle), and L-glycerol phosphate dehydrogenase, 0.5 unit (Sigma, rabbit muscle). 10 mM buffer, pH 7.5 or 8.5; 5 mM fructose-6-phosphate (obtained by mixing glucose-6-phosphate with phosphohexose isomerase and incubating 1 hour at room temperature); 1 mM NADH; coupled enzymes and enzyme sufficient to give optical density changes of 0.010 to 0.015 per

15 seconds were placed in a 1 ml cuvette. Optical density changes at 340 mμ were recorded.

Hexose Phosphate

This enzyme (D-glucose-6-phosphate ketol-isomerase EC No. 5.3.1.9) was assayed by following the reduction of $NADP^+$ at 340 mμ in the presence of glucose-6-phosphate dehydrogenase. No activity was noted in the absence of fructose-6-phosphate. Source of the enzyme was the 15 minute blending using 1:1:1 cells:beads:0.01 M bicarbonate buffer (wt:wt:vol). The assay included 33 mM Tris HCl buffer, pH 7.5; 3 mM fructose 6-phosphate; 1 mM $NADP^+$; 10 mM $MgCl_2$; 0.5 units glucose-6-phosphate dehydrogenase; and enzyme in a 1 ml cuvette.

Hexitol Phosphate Dehydrogenase

The presence of a hexitol phosphate dehydrogenase was detected in defatted cells of this organism. Activity was difficult to maintain, however, and could not be reproduced from preparation to preparation. An additional encountered was the coprecipitation of the enzyme system with that of NADH oxidase. Typical assay conditions in a 1 ml cuvette included 10 mM Tris buffer, pH 7.5; 3 mM glucose-6-phosphate (or fructose-6-phosphate); 1 mM NADH; and enzyme preparation. The reaction showed no metal ion requirement.

Purification Attempts

Purification attempts were made on several of the enzymes and the results are listed under the enzymes in the appropriate section. The great problem not yet overcome in all purification attempts was the inability to precipitate nucleoproteins. In one attempt, 0.5 ml of a 1% protamine sulfate was added to 10 ml of preparation containing 10 to 15 mg of protein per ml. The solution was stirred several hours and centrifuged. Only a very small amount of precipitate formed and the ratio OD_{280}/OD_{260} did not change appreciably. Adjusting the pH to 6.5 or lower prior to addition of protamine sulfate resulted in a loss in activity and no concomitant change in the ratio. $MnSO_4$ in concentrations up to 0.01 M was similarly without effect. The use of DEAE cellulose (prepared as instructed) did serve to accomplish a separation of the nucleoprotein from the other protein.

CHAPTER III

RESULTS

Growth of the Organism

Table 1 summarizes the results of growing the organism on nutrient agar under different conditions. It is obvious that the organism grows well on supplemented nutrient agar and that growth on disaccharides is slower than on monosaccharides.

Table 2 summarizes a similar series of growth experiments on the defined medium of McClung (32, 33). It was noted that growth was very slow on this medium regardless of the source of carbon. The bacteria grew slowly on acetate, citrate, fructose, and glucose, but not at all on glycerol. No attempt was made to detect growth on the medium of Martin and Batt although it is expected growth would be much better in this medium. (30, 31)

Growth of this organism on agar has two very characteristic lag periods (Fig. 2). The first period in which very little increase in wet weight is observed occurs after inoculation and lasts 3 to 4 hours. This is the time interval usually devoted to germination. After an increase in growth rate usually lasting from 1 to 2 hours there

TABLE 1

Growth of *N. corallina* on Supplemented Nutrient Agar after 4 Days

Supplement	Growth	Color of Bacteria
None	+	———
Citrate	+ + +	yellow orange
Acetate	+ + +	yellow
Glycerol	+ + +	orange
Glucose	+ + + +	orange
Galactose	+ + + +	orange
Lactose	+ +	light orange
Sucrose	0	very light orange
Maltose	+ +	light orange

TABLE 2

Growth of *N. corallina* on McClung's Media after 2 Weeks

Carbon Source	Growth	Color of Bacteria
Acetate	+	yellow
Citrate	+	yellow
Fructose	+ +	light yellow
Glucose	+ +	light yellow
Glycerol	0	———

+ Indicates amount of growth after the period of time as shown.

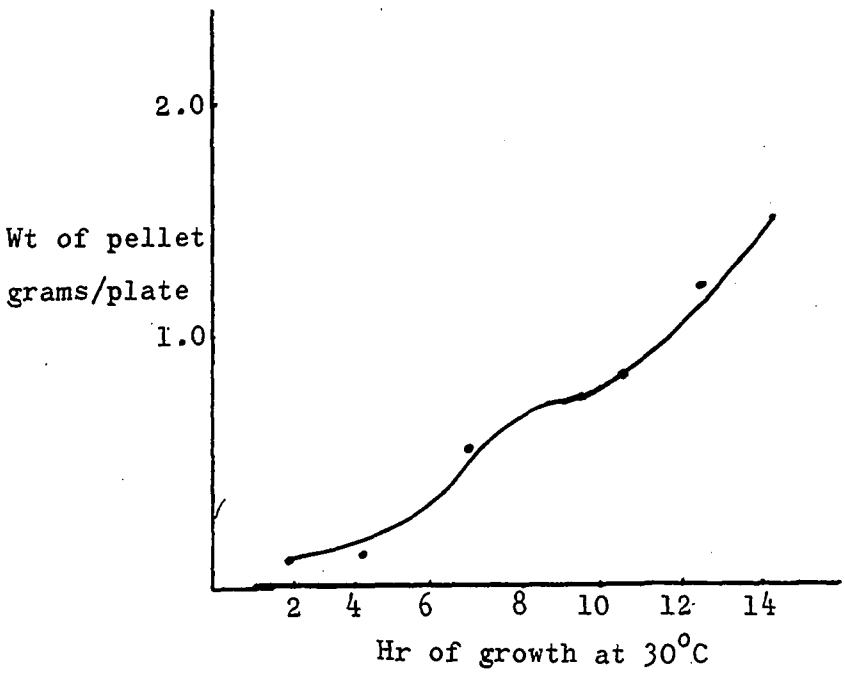


Figure 2. Growth of N. corallina on 1% fructose supplemented nutrient agar. The growth is shown by increase in wet weight of the bacteria harvested from petri dishes (30 cm x 150 cm).

appears to be a second interval of slow growth, lasting from 3 to 4 hours. During this second lag period, synchronous cultures are forming hyphae and preparing to fragment. After fragmentation growth occurs quite rapidly to 24 hours and then growth is much more gradual.

Cell Disruption

Table 3, Part 1 summarizes the results of attempts to break the cells using the Sorvall omnimixer. For these experiments cells, glass beads, and buffer were maintained at a 1:1:1 ratio and blending time was varied (using 20 g of cells).

From these results it may be surmised that the amount of time required to solubilize an enzyme depends upon the nature of that enzyme. Similar results can be reported for $NADP^+$ glucose-6-phosphate dehydrogenase and for $NADP^+$ malic dehydrogenase. The NAD^+ cnzymes were only detected in preparations in which blending was either carried out for longer periods of time or the volume of buffer had been decreased. This is shown again in Table 3, Part 2. For these breakage attempts the ratio of cells to glass beads to buffer was 1:1:0.5 (20 grams of cells, 20 grams of glass beads and 10 ml of Tris buffer).

Results in Table 3 indicate that the NAD^+ enzymes are difficult to solubilize. The $NADP^+$ enzymes appear to be easier to solubilize.

Sonication was the second major technique used in

TABLE 3. PART 1

Breakage Attempts Using Blender

Time of Blending (min)	Protein Yield (mg/ml)	Enzyme Assays Dehydrogenase Activity isocitrate $NADP^+$	NAD^+
5	5-7	+	0
10	10-13	+ + +	0
15	15-20	+ + +	+
30	15-20	0	+ + +
45	15-20	0	+ + +

TABLE 3. PART 2

Breakage Attempts Using Blender on Concentrated Suspensions

Blending Time (min)	Protein Yield (mg/ml)	Breakage Attempts Dehydrogenase Activity
15	15-20	+
30	15-20	+ + +
45	15-20	+ + +

Greatest activity	+ + +
Some activity	+ +
Little activity	+
No activity	0

breakage experiments. In the case of sonication on undried cells the cells were placed in 10 ml of buffer and the mixture was sonicated for the time intervals shown in Table 4, Part 1. Little increase in the yield of protein is noted with more prolonged periods of sonication.

In the case of cells which had been dried and defatted prior to breakage the amount of time required to break cells decreased as is noted in Table 4, Part 2. For such attempts 0.2 g of cells was combined with 10 ml of buffer and sonication carried out for the time intervals as shown. It is apparent that increasing the weight of cells has a very small effect on protein yield. It also suggests that the process of lyophilization and fat extraction renders the cell much more susceptible to lytic action by sound than untreated cells.

The procedure used to extract lipids from the cell wall of this bacterium is very temperature dependent. The amount of time elapsing between lyophilization, lipid extraction, and sonication is critical. Greatest success has been obtained when the entire operation was concluded within 48 hours. Considerable enzyme deactivation occurs if a longer time interval elapses between lyophilization and extraction or between extraction and sonication even if the cell powder is stored at -20°C.

The effect of various buffers and reagents to stabilize the enzyme preparation in the breakage attempts

TABLE 4. PART 1

The Effect of Sonication on Breakage of *N. corallina* Cells

Wt. of Cells Used (g)	Time (min)	Protein Yield (mg/ml)
1	5	1
1	10	1-2
1	15	3.5-5
1	20	3.5-5
5	15	3.5-5
5	15	3.5-5

TABLE 4. PART 2

Sonication of Pretreated Cells

Wt. of Cells (g)	Time (min)	Protein Yield (mg/ml)	Description of Cells
0.2	5	2-3	Lyophilized/Defatted
0.2	10	3.5-4.5	Lyophilized/Defatted
0.2	15	3.5-4.5	Lyophilized/Defatted
0.5	15	5-6	Lyophilized/Defatted
0.2	15	2-3	Lyophilized

have been examined and are included under the appropriate enzyme assay results.

Of the techniques thus far investigated to break the cell of this organism fat extraction will apparently solubilize NAD^+ specific enzymes. Apparently either blending for long periods of time (greater than 30 minutes) or blending in the presence of smaller volumes of buffer will also accomplish this. Both conditions result in the production of a supernatant fluid very high in NAD^+ reduction activity. This activity is noted by the increase in absorption at 340 mμ. Such activity in the presence of NAD^+, but in the absence of any additional substrate is largely lost after one $(NH_4)_2SO_4$ precipitation step. The exact nature of the "endogenous" activity has not been determined.

Several attempts were made to use detergent to solubilize enzymes from this organism. Attempts in which the pellet (isolated by centrifuging the broken cells at 25,000 x g for 3 hours) was mixed with detergent and blended resulted in the production of a gelatinous supernatant. The supernatant obtained after centrifugation at 50,000 x g was clear but solidified upon standing. Thin film dialysis for an hour did not improve the consistency of the preparation. Protein analysis of the supernatant indicated the presence of less than a mg of protein.

Similar results were obtained when whole cells were blended with detergent or with detergent in the presence of lysozyme. It was found that the supernatant became

gelatinous upon refrigeration and that this material was devoid of enzymic activity. When lyophilized, defatted cells were used nearly identical results were seen.

Lysozyme appears to be without effect on this organism. Attempts to lyse the cells using lysozyme either alone or in the presence of detergents led to similar results. Stirring a suspension of cells in lysozyme or in lysozyme plus detergent for several hours led to very little breakage of the cells. When cells were dried, or dried and defatted and then treated with lysozyme very similar results were obtained. In attempts to note lytic activity by a decrease in absorption (in the presence of lysozyme), no such activity was observed.

Finally, stirring the cells in the presence of 0.1 M Tris buffer (pH 9.5) led to very little autolysis of the cells. The protein that is obtained is without activity for any of the enzymes tested.

Purification Attempts

Enzyme purification attempts on this organism for the most part have been unsuccessful. The organism produces great amounts of lipid material particularly if harvested after 24 hours growth. This lipid material appears to interfere with $(NH_4)_2SO_4$ precipitation. 90% $(NH_4)_2SO_4$ will precipitate only 50-75% of the protein from a fresh preparation after a 15 minute centrifugation at 25,000 x g. If the solution is frozen and thawed before precipitation,

90% $(NH_4)_2SO_4$ will precipitate most of the protein after 20 minute centrifugation at 25,000 x g.

Similar attempts to rid the preparation of nucleoprotein have proven unsuccessful as shown in Table 5.

TABLE 5

Attempts to Free Preparations of Nucleoproteins

Material Used	$(\frac{280}{260})$ Before	$(\frac{280}{260})$ After	Amount of Protein Precipitating
$MnCl_2$	0.50	0.50	less than 5%
Protamine sulfate pH 5.5	0.50	0.50	25 to 50%
Protamine sulfate	0.50	0.50	10 to 20%

A chromatography attempt (on DEAE cellulose) did accomplish this separation. Other purification attempts are discussed under the appropriate enzymes.

Enzyme Studies

Nomenclature

Michaelis and Menten (34) have derived an equation which relates initial velocities to substrate concentrations. This equation is:

$$v_o = \frac{V_m}{1 + K_m/s}$$

where v_o is the initial velocity, V_m is the maximum velocity, s is the substrate concentration and K_m is the

Michaelis constant. The constant K_m is defined as that concentration of substrate giving $V_m/2$. Several methods exist to transform the above equation to a linear form. The double reciprical plot of Lineweaver and Burke (25) was used in this study:

$$\frac{1}{v} = \frac{K_m}{V_m}\left(\frac{1}{s} - \frac{1}{V_m}\right)$$

According to this equation a plot of $\frac{1}{v}$ against $\frac{1}{s}$ yields intercepts which can be used to calculate V_m and K_m. These constants are sufficient to define the complete saturation pattern for those enzymes which obey simple Michaelis-Menten kinetics.

For those enzymes which are characterized by sigmoid substrate velocity curves the above parameters do not suffice. While V_m is still appropriate the kinetic constant K_m is not. Koshland (26) has introduced the constant $S_{(0.5)}$ and defined it as the half saturating concentration of substrate.

An equation based on equilibrium considerations very similar to those used in deriving the Michaelis equation was first proposed by Hill (19) to describe the binding of oxygen to hemoglobin. Hill established that the sigmoid shape of the oxygen hemoglobin saturation curve required cooperative binding (entry of each molecule of oxygen into the protein increases the affinity of the protein for the next molecule). This equation was studied by Atkinson, Hathaway, and Smith (2) in the form:

$$\log \frac{v}{V_m - v} = n \log s + \log K$$

The Hill equation supposes an ideal case of infinite cooperativity. In such a case a plot of $\log \frac{v}{V_m\ v}$ against log s would be linear with a slope of n. Changeux (9) referred to this value as an interaction constant. Atkinson demonstrated that the value is dependent not only on the number of binding sites but also on the strength of the interactions. When such interactions are strong the value of n is a true measure of the number of binding sites. In all plots used to measure values for n the entire saturation range from 10 to 90% V_m has been used.

Specific activity (S.A.) is defined as μ moles of substrate transformed per minute per mg of protein.

Isocitrate Dehydrogenase

The NAD^+ specific enzyme was detected in the kinds of preparations that indicate it is particulate bound or protected. It has only been found in cell free extracts after long periods of blending, blending with smaller volumes of buffer, or sonication after fat extraction. These are the same conditions used to solubilize other NAD^+ enzymes which are also found to be membrane associated. This enzyme loses 50% or more of its activity upon freezing and thawing. This loss in activity is prevented to some extent by the addition of 5 mM β-mercaptoethanol. The assay conditions reported are the most favorable that

have been observed (Fig. 3). Assays were performed on preparations obtained from 90% $(NH_4)_2SO_4$ precipitation followed by 1 hour thin film dialysis against 0.01 M Tris HCl pH 8.5 containing 5 mM β-mercaptoethanol. A small decrease in optical density is seen in the absence of isocitrate (Fig. 3, line marked A) indicating the absence of endogenous activity. The metal ion requirement for the enzyme is fulfilled by Mg^{++} or Mn^{++} (Fig. 3, lines B, D, and E). Apparently Mn^{++} will fill this requirement better than Mg^{++}. This could be due to increased activity in the presence of Mn^{++} or to a higher K_m for Mg^{++} than Mn^{++}. Further evidence for the metal ion requirement is the total loss in activity in the presence of 50 mM EDTA. β-mercaptoethanol has a stabilizing effect on the enzyme (Fig. 3, line C). The nonlinear response becomes linear in the presence of this reagent. Sigmoid substrate saturation curves were seen for the enzyme when sufficiently active preparations could be obtained. However, due to the problems encountered in the reproduction of active enzyme preparations, this data is not reported.

The $NADP^+$ specific enzyme has been detected under conditions which indicate that it is a soluble enzyme. It is very stable and was detected under a variety of breakage conditions. No activity is seen in the presence of 50 mM EDTA thus strongly indicating a metal ion

requirement. This requirement is fulfilled by either Mg^{++} or Mn^{++}.

The saturation curves for isocitrate (Fig. 4) and for $NADP^{+}$ (Fig. 5) are hyperbolic. The Michaelis constants are given in Table 7. The Hill plots (Fig. 6) for both substrates yield values whose slopes are very close to one. This indicates the absence of multiple binding sites or interacting binding sites on the enzyme for either substrate.

The pH profile for the enzyme (Fig. 7) was obtained by plotting the maximum activities as a function of the pH of the buffers. In Tris the enzyme has the same activity at pH values from 7.0 to 9.0. There is a rise in the activity of the enzyme in phosphate buffer as the pH of the buffer increases to a pH of 7.5. The activity of the enzyme shows an increase throughout this range in glycylglycine.

Several possible metabolic effectors of this enzyme have been examined. ATP, ADP, and AMP are without effect on the enzyme. Each adenylate was assayed at 3 mM concentration throughout the saturation range for isocitrate. Fructose-1,6-diphosphate, phosphoenol pyruvate, pyruvate, malate, oxaloacetate and glyoxylate at the same concentrations were similarly without effect.

Assay Conditions for Figure 3

10 mM Tris/HCl, pH 8.5

2 mM D, L-isocitrate

1 mM NAD^+

5 mM $MgCl_2/MnCl_2$

5 mM β-mercaptoethanol

Enzyme preparation, 0.05 ml. Assays, in a 1 ml cuvette, were started after 3 min. pre-equilibration by the addition of isocitrate.

A No isocitrate

B No metal ion

C No β-mercaptoethanol

D 5 mM $MgCl_2$

E 5 mM $MnCl_2$

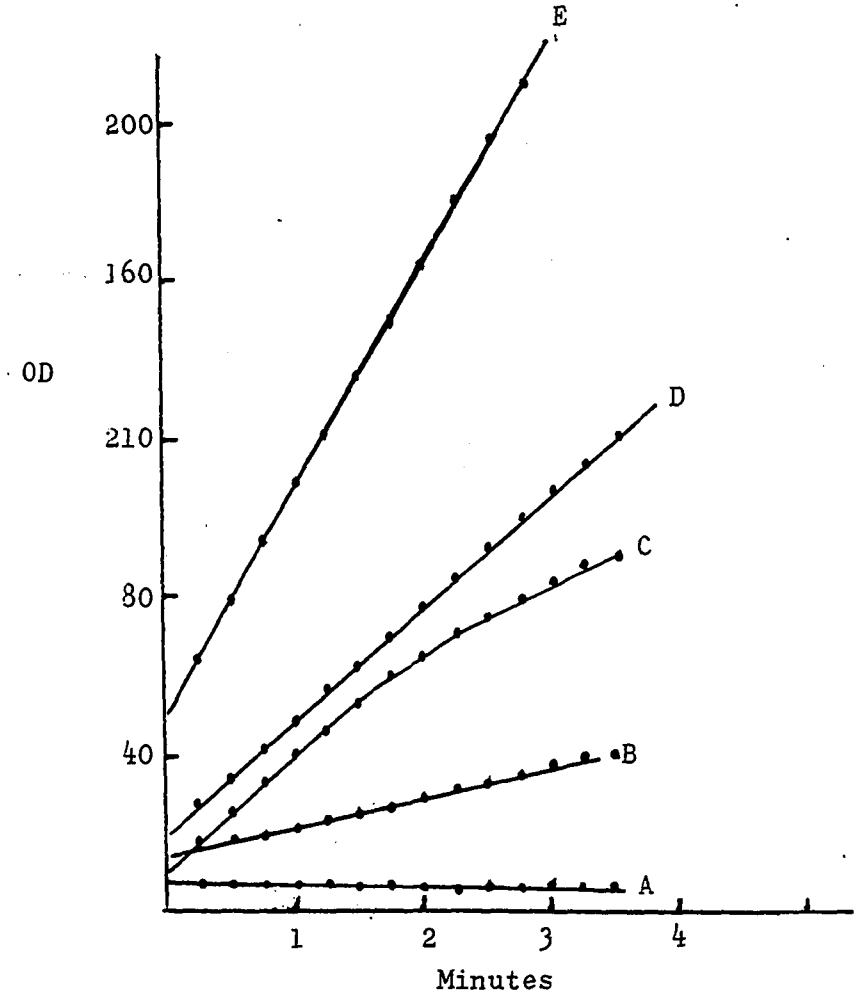


Figure 3. Rate progress curves of NAD^+ isocitrate dehydrogenase from 24 to 48 hr. cultures.

Assay Conditions for Figure 4

200 mM Phosphate buffer pH 7.5

4 mM $NADP^+$

10 mM $MgCl_2$

0.1 ml of preparation and isocitrate as indicated. Reaction was started by the addition of enzyme to assays in a 3 ml cuvette.

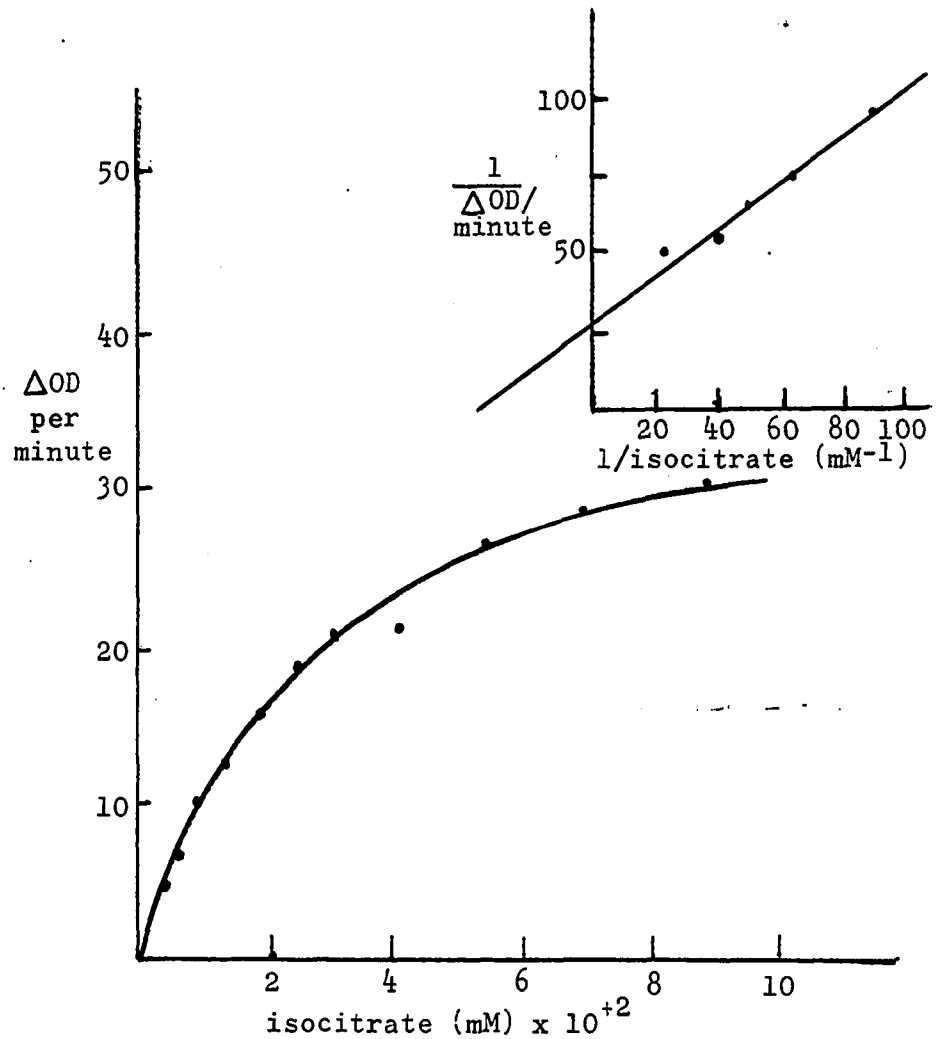


Figure 4. Isocitrate saturation of $NADP^+$ specific isocitrate dehydrogenase from 24 to 48 hr. cultures.

Assay Conditions for Figure 5

200 mM Phosphate buffer

10 mM $MgCl_2$

0.1 ml enzyme preparation and $NADP^+$ as indicated. Reactions were started by the addition of enzyme to assays in a 3 ml cuvette.

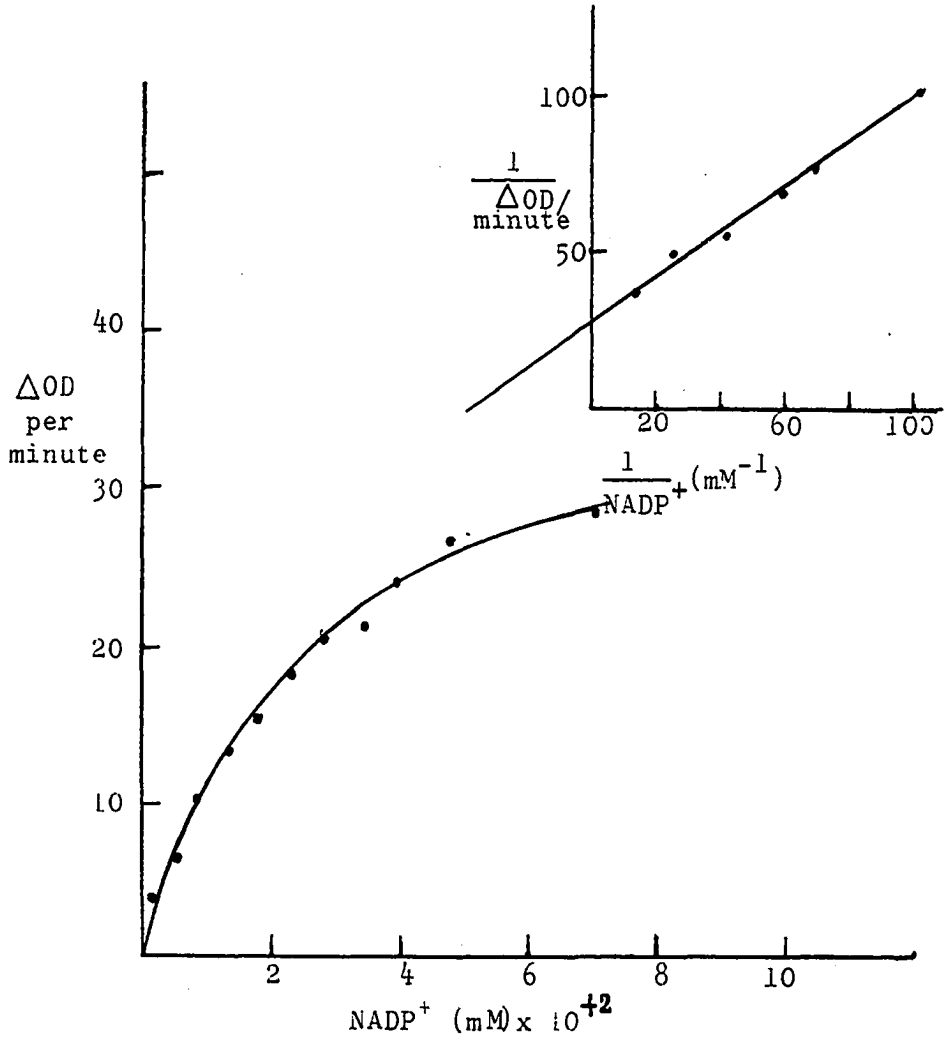
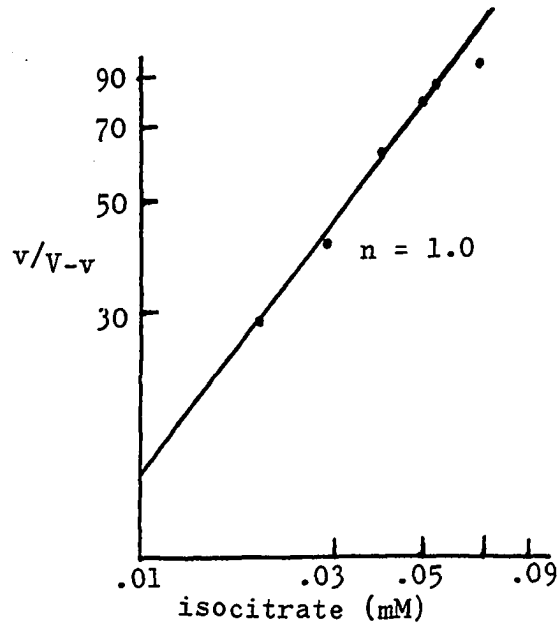


Figure 5. $NADP^+$ saturation of $NADP^+$ specific isocitrate dehydrogenase from 24 to 48 hr. cultures.



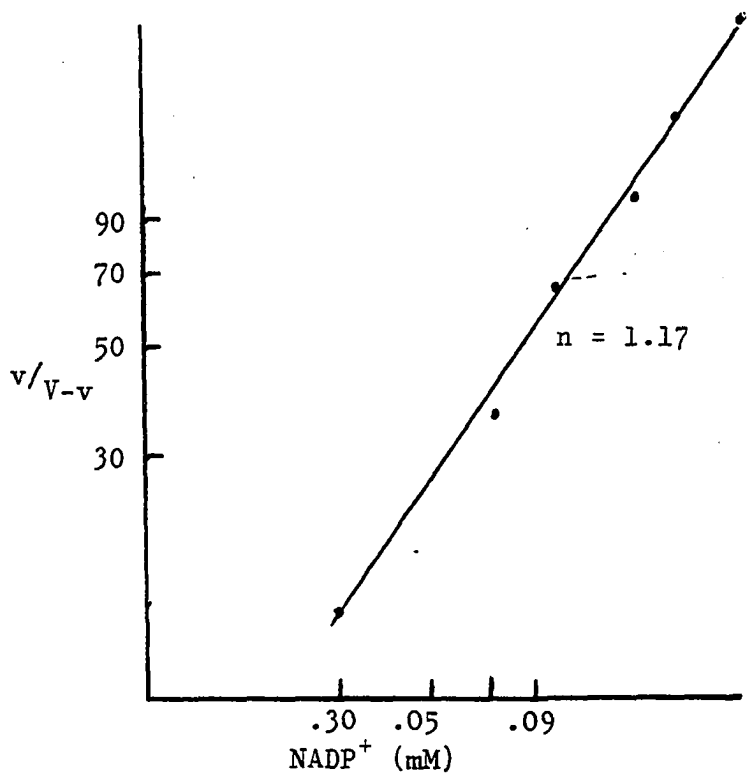


Figure 6. Empirical Hill plots for isocitrate dehydrogenase.

pH profile for $NADP^+$ isocitrate dehydrogenase

Assays were performed using the buffers shown and substrates at saturating concentrations.

A 0.1 M Phthatate

B 0.1 M Cacodylate

C 0.1 M Phosphate

D 0.1 Tris HCl

E 0.1 M Glycylglycine

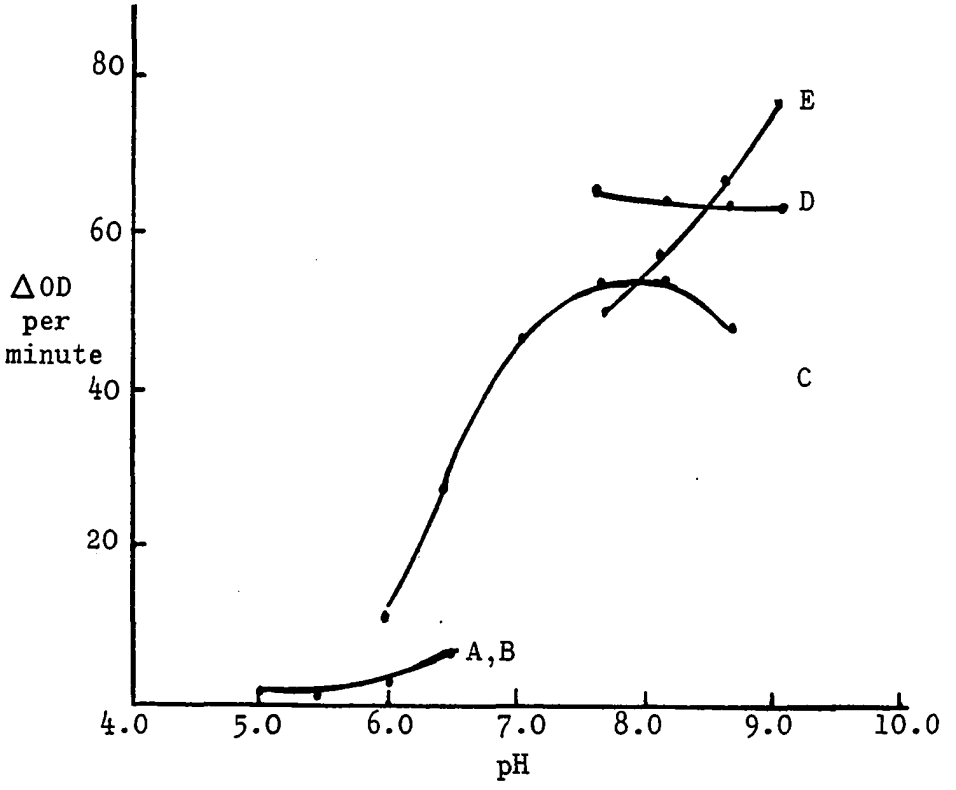


Figure 7. pH profile of $NADP^+$ specific isocitrate dehydrogenase 24-48 hr. cultures.

L-Malic Dehydrogenase

Several characteristics of the NAD^+ enzyme suggest that it, like the NAD^+ isocitric dehydrogenase, is particulate or at least membrane bound. It is a very difficult enzyme to solubilize, having been detected only in 30 minute anaerobic blending experiments in which cells, glass beads, and Tris buffer (0.1 M, pH 9.0) are combined in ratios of 1:1:0.5 (wt:wt:vol, respectively). Results of the type shown in Figure 8 indicate several conditions required to attain maximum enzyme activity. This enzyme has a metal ion requirement which is satisfied by a di-valent metal ion. The activity is greater in the presence of Mn^{++}, 33 mM (not shown), than of 33 mM Mg^{++} (shown by line B). Additional evidence of a metal ion requirement is the complete lack of activity in the presence of 0.1 ml of 0.5 M EDTA.

The $NADP^+$ malic dehydrogenase appears to be a soluble enzyme very similar to $NADP^+$ isocitric dehydrogenase; however, it is not as stable as the isocitric enzyme. Both enzymes can be detected in preparations solubilized by 15 minute blendings at a setting of 55 volts on the Omnimixer. Figure 9 is a substrate saturation curve for $NADP^+$. Double reciprocal plots, which are concave upward, indicate interacting sites for $NADP^+$. This is established in the Hill plot (Figure 10, Part A) which has an interaction constant of 2.5. Figure 10,

Part B demonstrates the effect of plotting 1/v against $1/(NADP^+)^2$, strongly indicating the presence of interacting sites. $(S)_{0.5}$ is given in Table 7.

The sigmoid nature of the saturation curve for L-malic acid (Fig. 11) also suggests the presence of interacting sites. This is further verified by the Lineweaver-Burk plot (see Fig. 11, insert), which is concave upward, by the Hill plot (Fig. 12, Part A) and the plot of $\frac{1}{v}$ against $1/(\text{L-malate})^2$ (Fig. 12, Part B). The values for $(S)_{0.5}$ and the Hill constant are given in Table 7 (Discussion section).

The pH profile (Fig. 13) indicates the enzyme has a peak in activity at 8.5 in either Tris or glycine buffer.

Several possible effectors of this enzyme have been examined. Results are indicated in Figures 14 and 15. Phosphoenolpyruvate, glyoxylic acid, and pyruvate (all 1 mM) are without effect on enzyme activity throughout the saturation range of L-malate (results not shown). Oxaloacetate and L-aspartate are, however, noncompetitive inhibitors of the enzyme (Fig. 14). 7 mM oxaloacetate inhibits the enzyme 50% (Fig. 15, Curve B), while the same concentration of L-aspartate inhibits the enzyme 40% (Fig. 15, Curve A). The physiological significance of these results will be discussed in the appropriate portion of the discussion section.

Due to the instability of the enzyme preparation the action of the effectors is best noted on a freshly prepared enzyme solution. Very little effect is seen if the preparation is allowed to age a week or more at $-20^{\circ}C$. This is apparently true with respect to saturation curves for L-malate and $NADP^{+}$. The degree of interaction between sites on the molecule also decreases with age of the preparation (shown by a decrease in the value for the Hill constant).

Assay conditions for NAD^+ linked L-malate dehydrogenase in a 1 ml cuvette

33 mM Tris buffer, pH 7.5

10 mM L-malate

1 mM NAD^+

33 mM $MgCl_2$

enzyme sufficient to display the activity shown

A No L-Malate

B No $MgCl_2$

C Full components

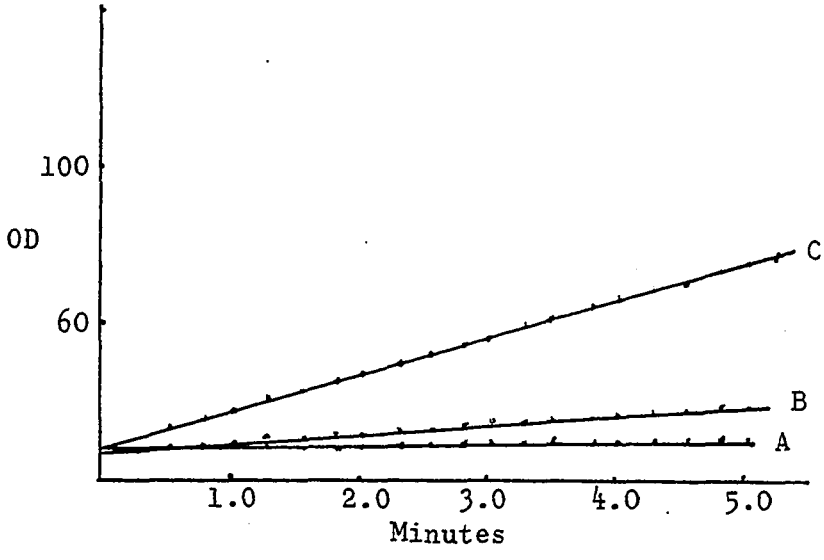


Figure 8. Rate progress curves from NAD^+ L-malic dehydrogenase 24-48 hr. cultures.

Assay conditions for $NADP^+$ saturation of $NADP^+$ linked L-malate dehydrogenase

Assays were begun by the addition of enzyme to a 3 ml cuvette.

100 mM Glycylglycine buffer, pH 8.5

150 mM L-Malate, pH 7.0

10 mM $MgCl_2$

0.1 ml enzyme and $NADP^+$ concentration as shown

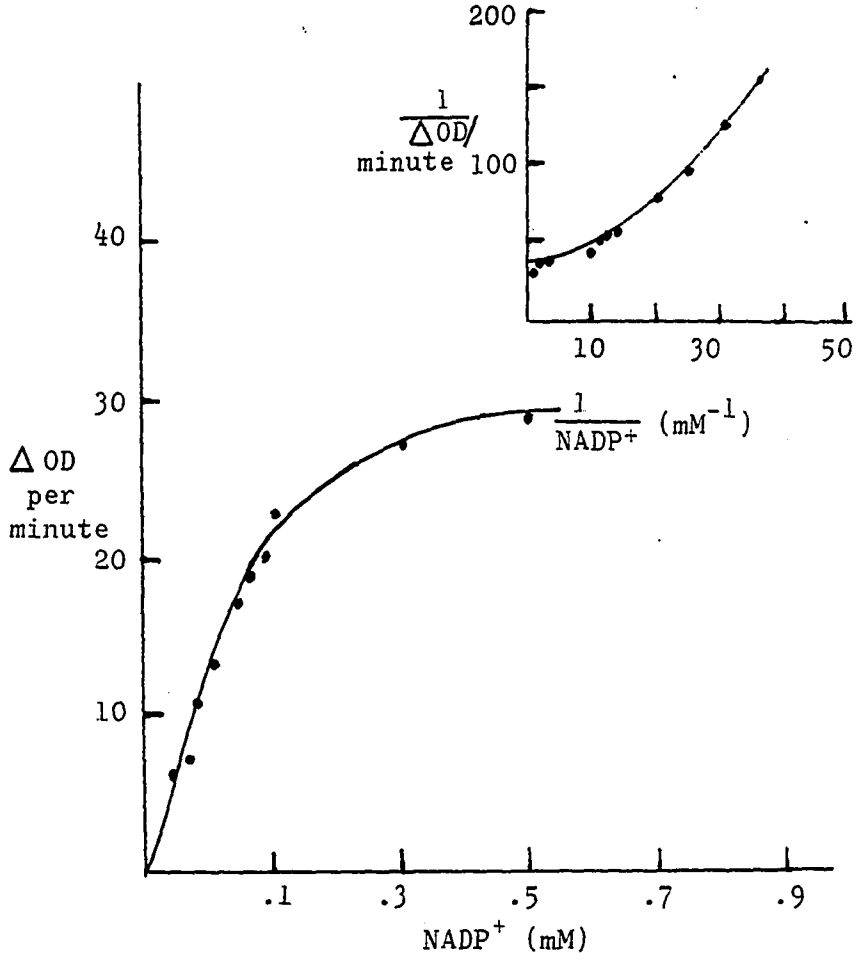


Figure 9. $NADP^+$ saturation of $NADP^+$ L-Malic dehydrogenase 24-48 hr. cultures.

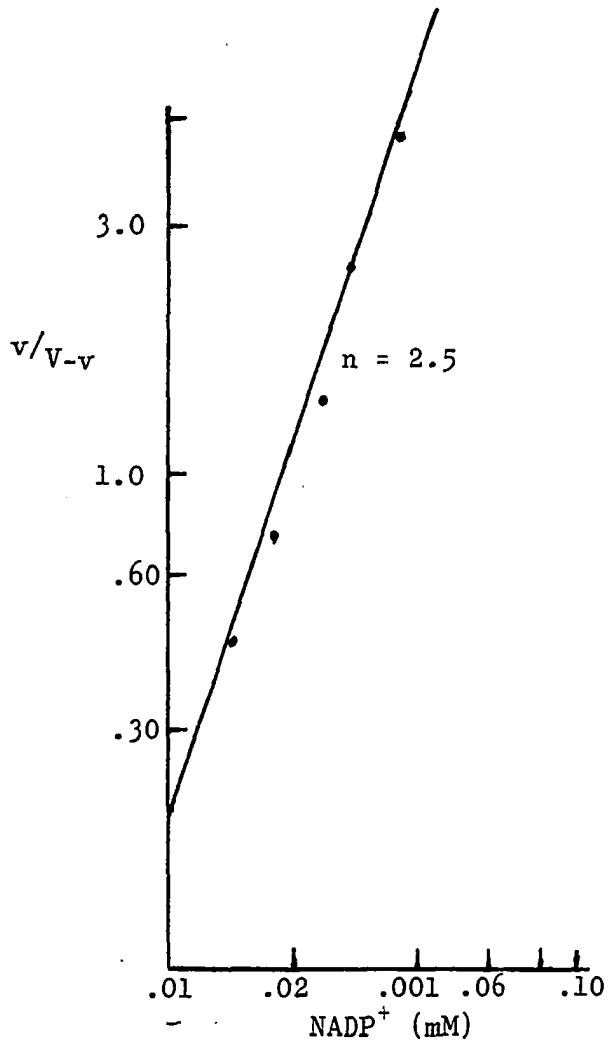


Figure 10. Part A Hill plot of $NADP^+$ saturation

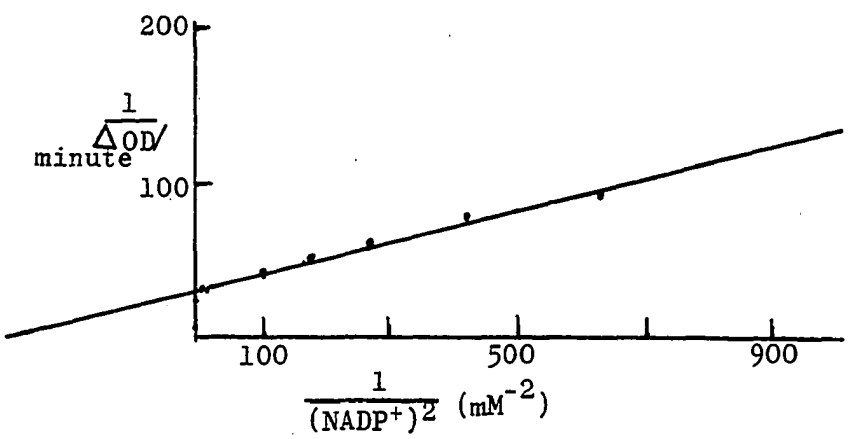


Figure 10. Part B 1/v. vs. $1/(NADP^+)^2$ for L-malate dehydrogenase.

Conditions for L-malate saturation of malic dehydrogenase

100 mM Glycylglycine buffer pH 7.5

0.5 mM $NADP^+$

10 mM $MgCl_2$

0.1 ml enzyme and L-malate concentration as shown

Assays were started by the addition of enzyme to a 3 ml cuvette.

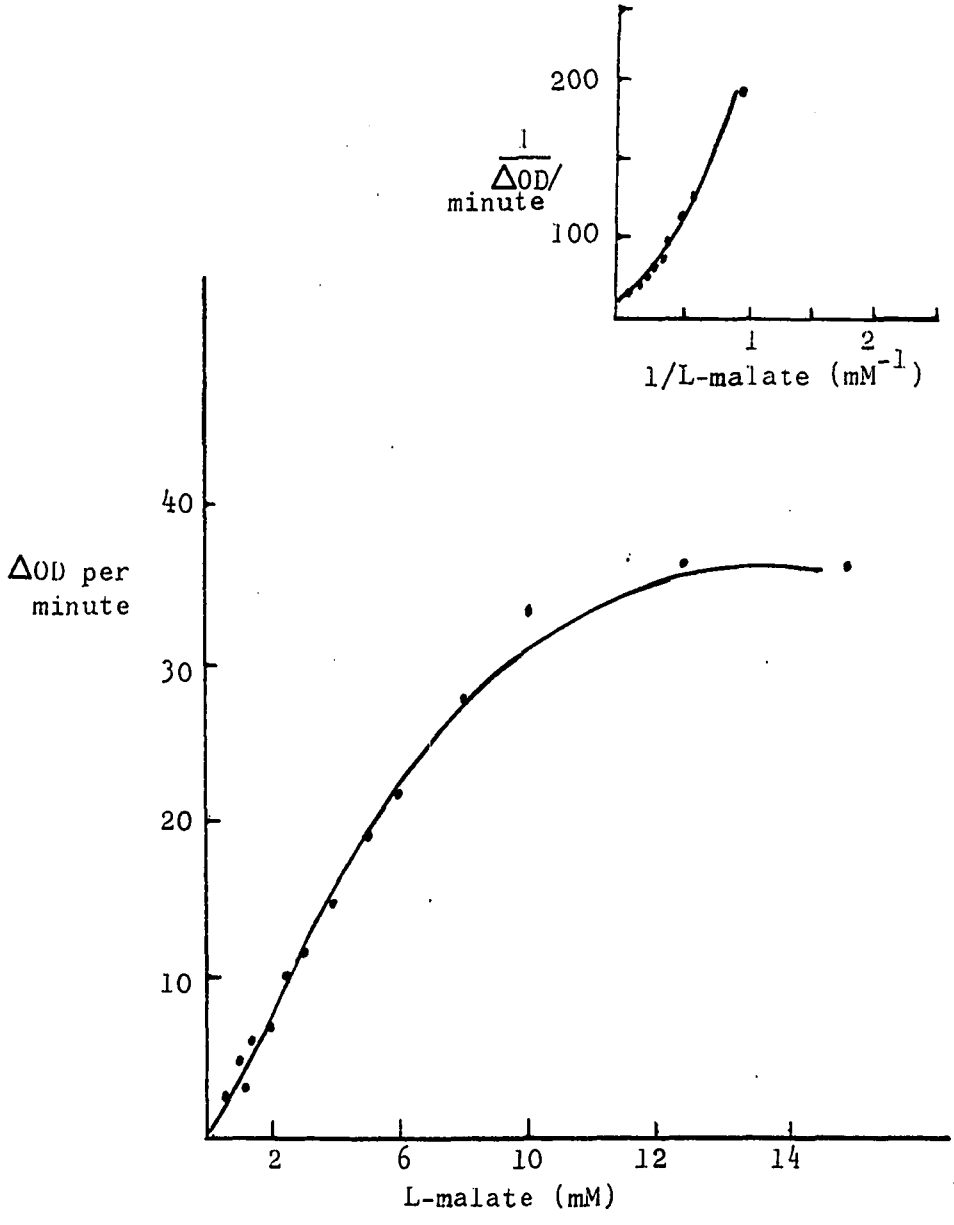


Figure 11. L-malate saturation of $NADP^+$ specific L-malate dehydrogenase from 24-48 hr. cultures.

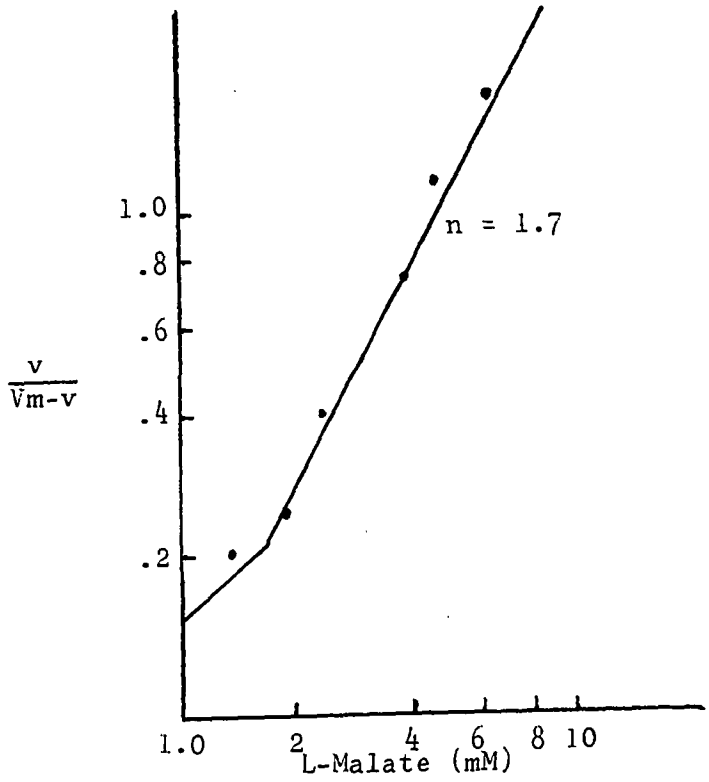


Figure 12. Part A. Hill plot of L-malate saturation.

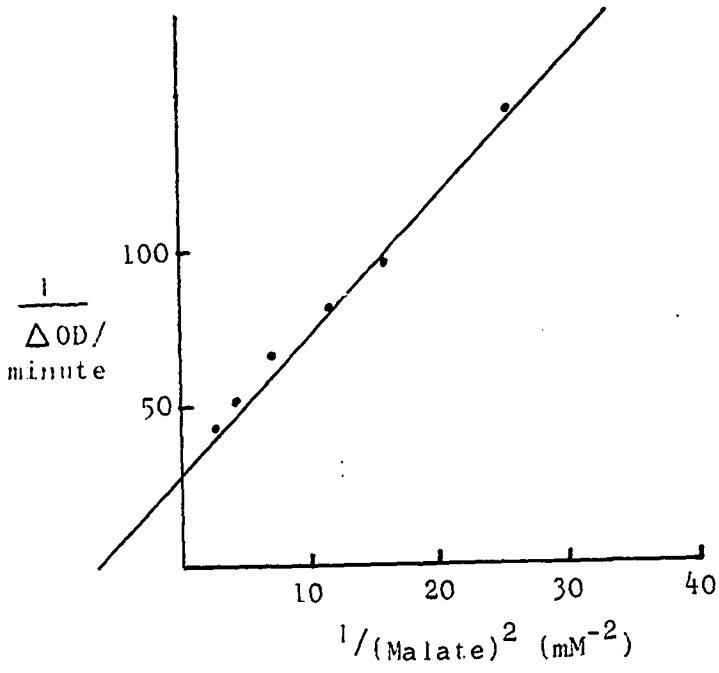


Figure 12. Part B. $1/v$ vs $1/(\text{malate})^2$ for L-malate saturation of $NADP^+$ specific L-malate dehydrogenase.

Assay conditions for pH profile

Buffer as shown and substrates at saturating concentrations

Buffers:

0.1 M Tris HCl pH 7.5-8.5 +

0.1 M phosphate 6.5-8.0 o

0.1 M glycine 8.5-9.5 x

0.1 M carbonate/bicarbonate 9.0-10 e

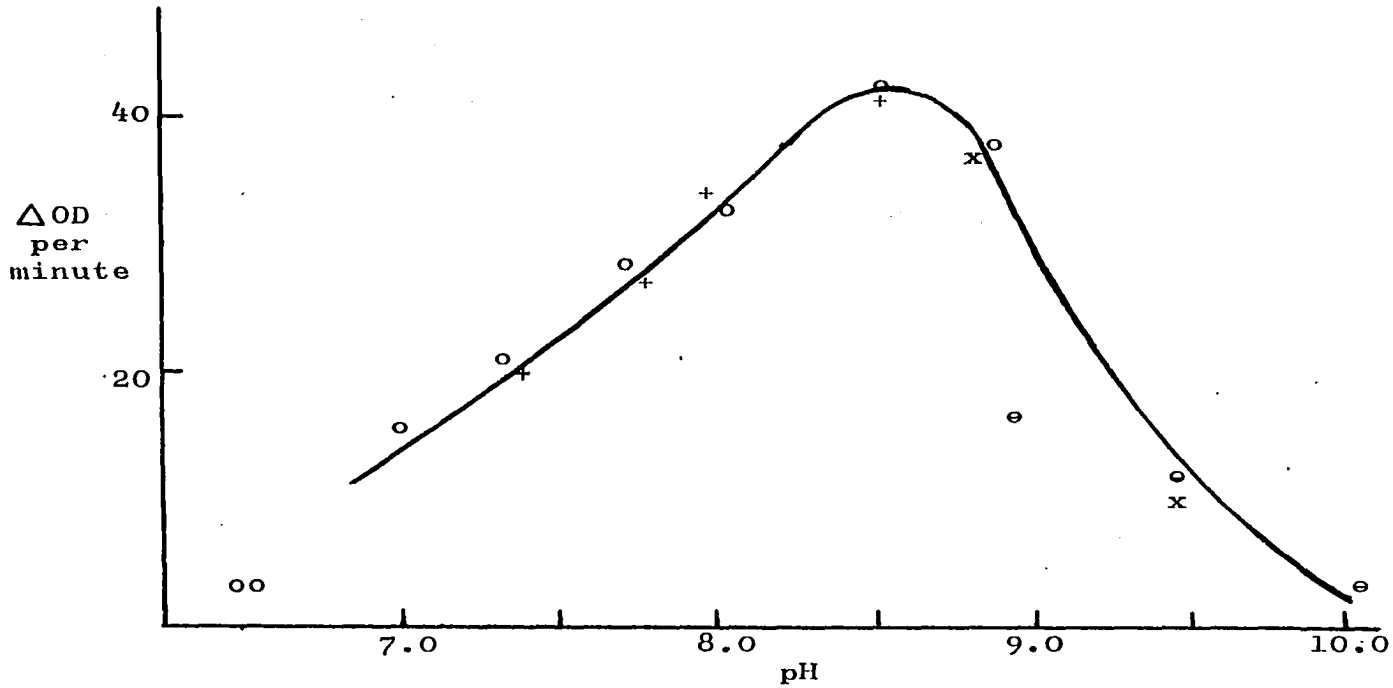


Figure 13. pH profile of $NADP^+$ L-malate dehydrogenase

Assay conditions to detect the presence of inhibitors of NADP linked L-malate dehydrogenase

100 mM Glycylglycine buffer (pH 8.5)

0.6 mM $NADP^+$

10 mM $MgCl_2$

3 mM effector

enzyme and concentration of L-malate as shown in a 3 ml cuvette

A No effector

B L-aspartate

C Oxaloacetate

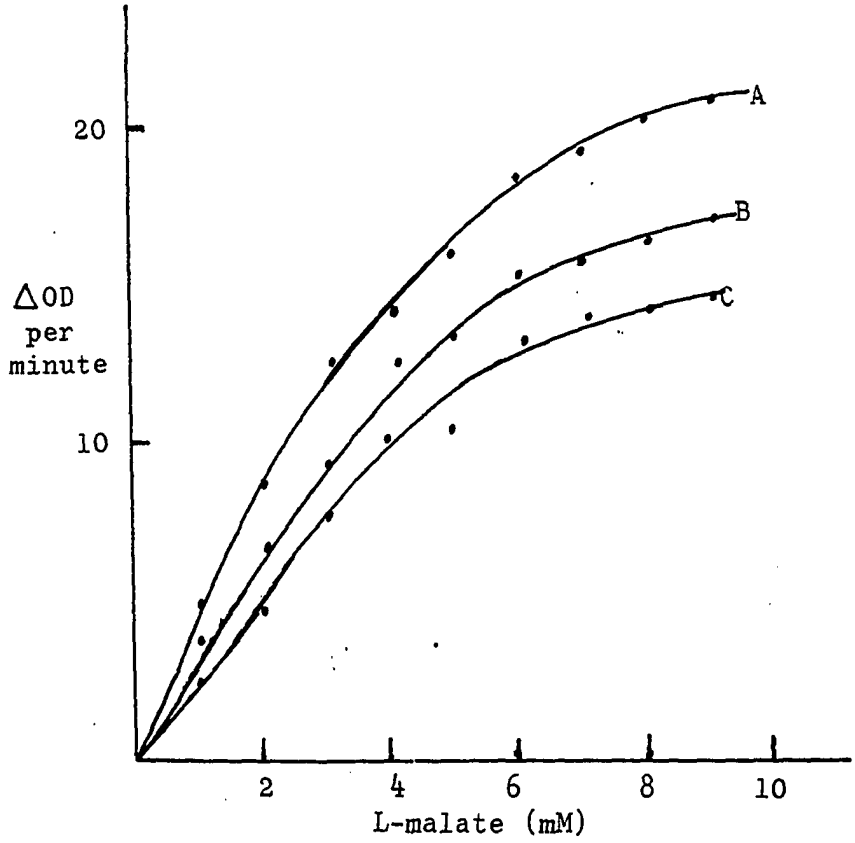


Figure 14. Effectors of $NADP^+$ L-malate dehydrogenase

Assay conditions to demonstrate effect of inhibitors on $NADP^+$ L-malate dehydrogenase

100 mM Glycylglycine buffer, pH 8.5

10.5 mM L-malate, pH 7.5

0.6 mM $NADP^+$

10 mM $MgCl_2$

0.1 ml enzyme preparation and inhibitor concentration as shown

A L-aspartate

B Oxaloacetate

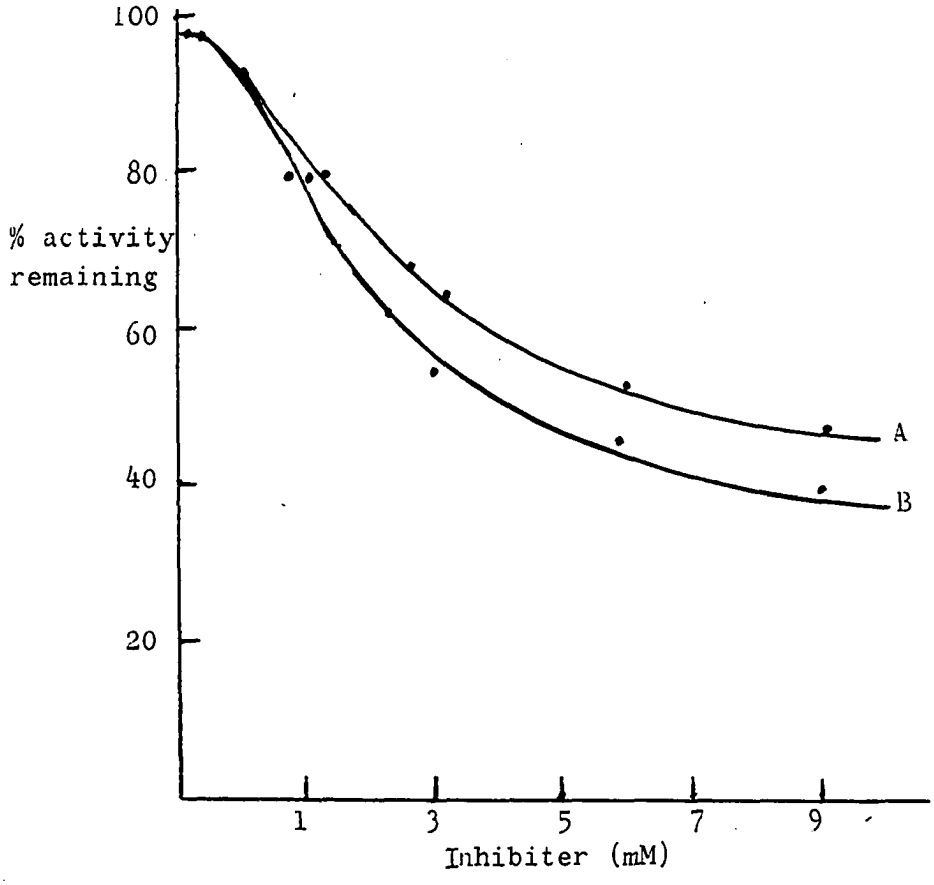


Figure 15. Inhibitors of $NADP^+$ L-malate dehydrogenase at 3.5 mM L-malate.

Glucose-6-phosphate Dehydrogenase

This enzyme shows an absolute specifity for $NADP^+$. Breakage conditions are identical to those already listed for other $NADP^+$ enzymes thus indicating that this is also a soluble enzyme. The metal ion requirement for this enzyme is satisfied by Mg^{++}. There is no activity in the presence of 50 mM EDTA.

The saturation curve for glucose-6-phosphate (Fig. 16) is hyperbolic. Lineweaver-Burk plots (see Fig. 16, insert) are linear and the slope of the Hill plot for this substrate (Fig. 18, Part A) is nearly unity. All of these indicate the absence of either multiple binding sites or interacting sites on the enzyme for this substrate. For the Michaelis constant, see Table 7 in the Discussion section. The saturation curve for $NADP^+$ is shown in Figure 17. The probable presence of strong interaction (or multiple binding sites) on the enzyme is indicated by the double reciprical plot (Fig. 17, insert) and the Hill plot (Fig. 18, Part B). Values for the kinetic constants are again reported in Table 7.

The pH profile for this enzyme (Fig. 19) has a peak at 8.5 in all buffers listed. The enzyme is more active in glycylglycine than in any other buffer tested. Figure 19 portrays the action of several potential effectors on this enzyme. The enzyme is active only toward glucose-6-phosphate. Glucose-1-phosphate and orthophosphate are

apparently not bound by the enzyme (Fig. 19, line A). The phosphorylated sugars, fructose-1,6-diphosphate and fructose-6-phosphate, do, however, have an effect on the enzyme (Fig. 19, lines B and C). Glyoxylate was the only other effector shown to have significant inhibitory power. 3 mM fructose 1,6-diphosphate inhibited the enzyme 20% at 0.7 mM glucose-6-phosphate concentrations. Possible metabolic aspects of these effectors will be further discussed in the appropriate discussion section. Pyruvate, phosphoenolpyruvate, ATP, ADP, AMP, oxaloacetate and fumarate all at 3 mM concentrations have no effect on this enzyme at 0.7 mM glucose-6-phosphate concentrations.

Assay conditions for glucose-6-phosphate saturation of glucose-6-phosphate dehydrogenase

100 mM Glycylglycine, pH 8.5

0.4 mM $NADP^+$

10 mM $MgCl_2$

0.1 ml enzyme preparation and glucose-6-phosphate concentration as shown in a 3 ml cuvette

Assays were started by addition of enzyme preparation.

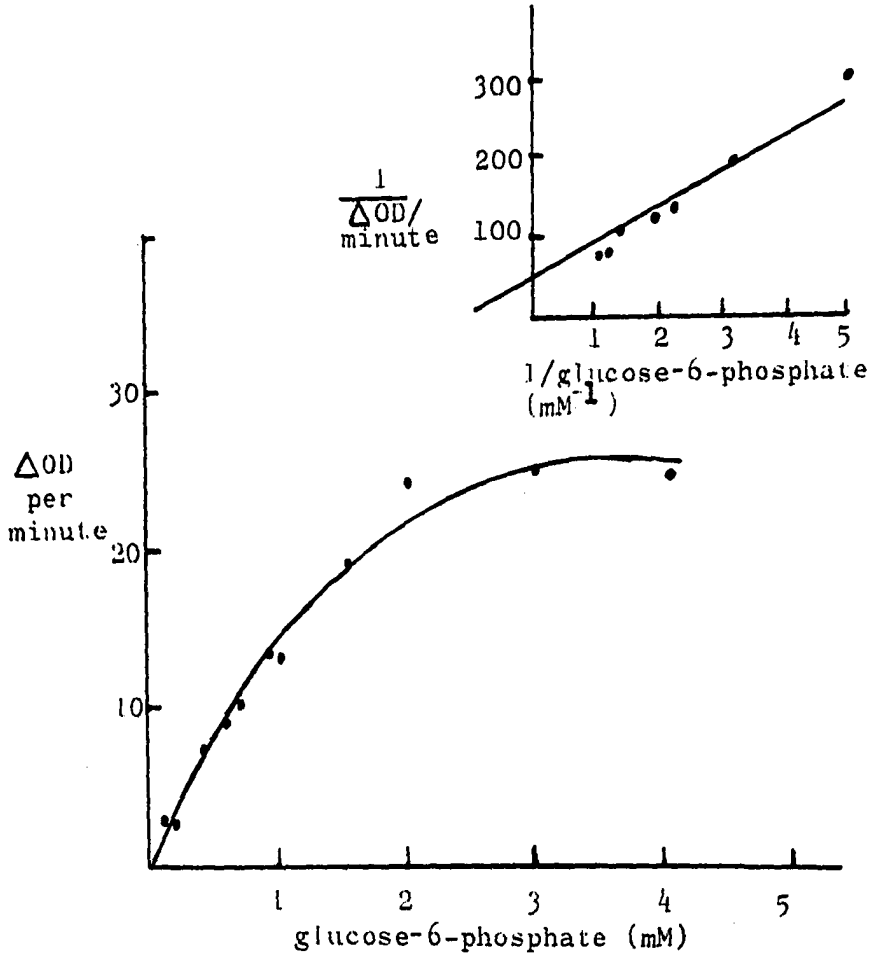


Figure 16. Glucose-6-phosphate saturation of glucose-6-phosphate dehydrogenase from 24 to 48 hr. cultures.

Assay conditions for $NADP^+$ saturation of glucose-6-phosphate dehydrogenase

100 mM Glycylglycine, pH 8.5

6 mM Glucose-6-phosphate

10 mM $MgCl_2$

0.1 ml enzyme and the concentration of $NADP^+$ as shown, in a 3 ml cuvette. Assays were started by the addition of enzyme.

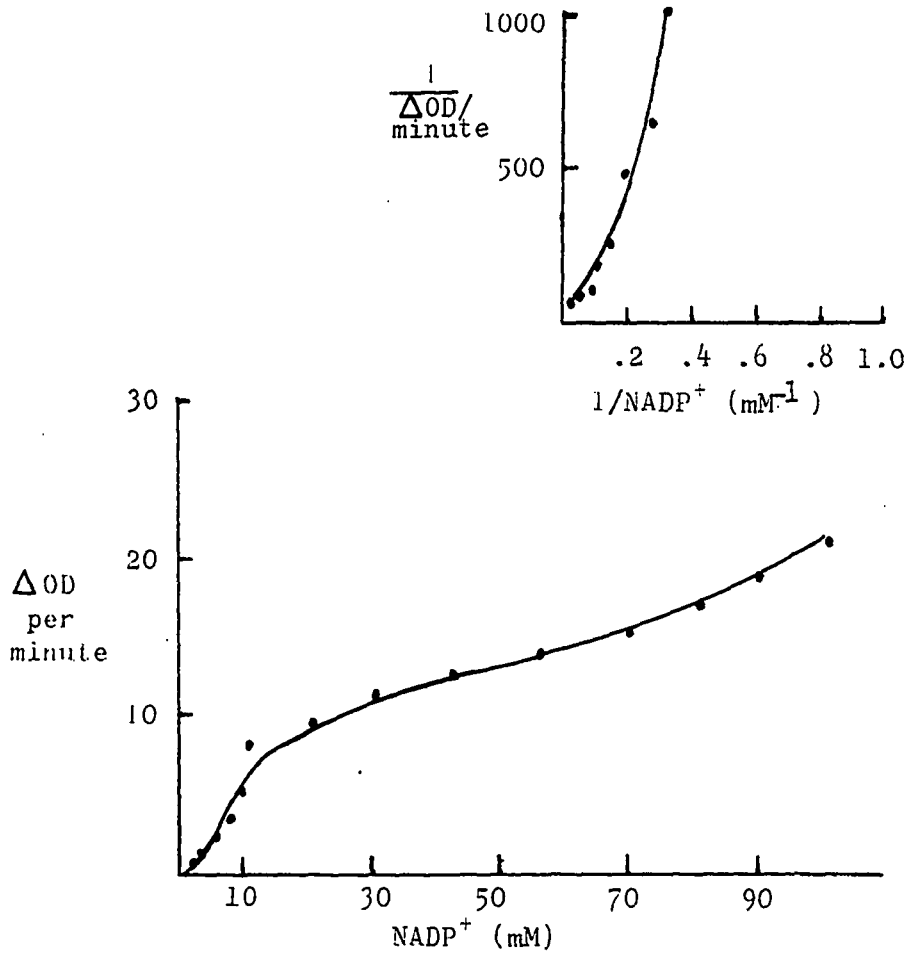


Figure 17. $NADP^+$ saturation of glucose-6-phosphate dehydrogenase.

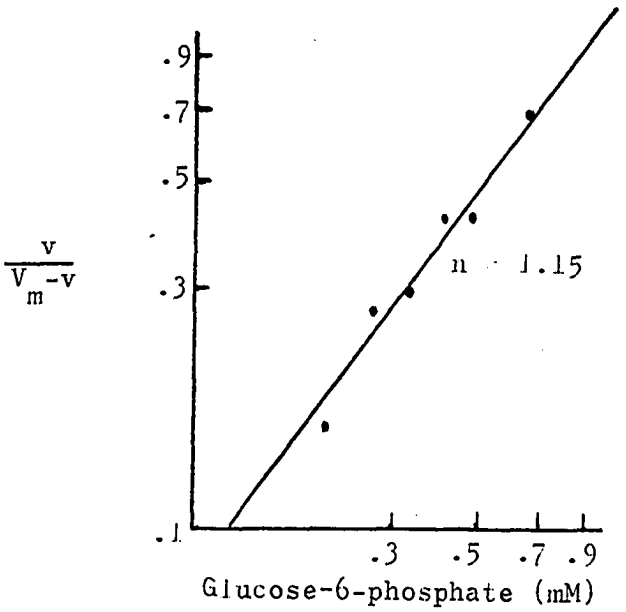


Figure 18. Part A. Hill plot of glucose-6-phosphate saturation.

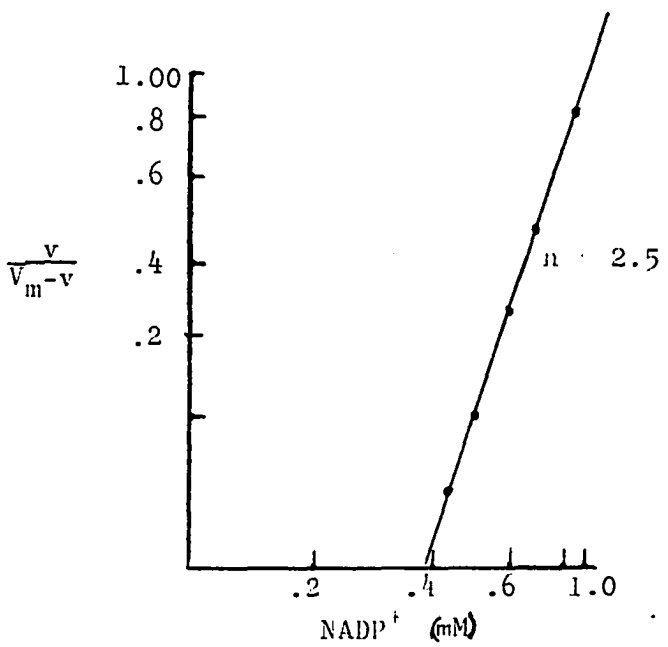


Figure 18. Part B. Hill plot of $NADP^+$ saturation of glucose-6-phosphate dehydrogenase.

Assay conditions for pH profile of glucose-6-phosphate dehydrogenase

Substrates used were at saturating concentrations and buffers were as shown

Buffers

A 0.10 M Phosphate

B 0.10 M Tris

C 0.10 M Glycylglycine

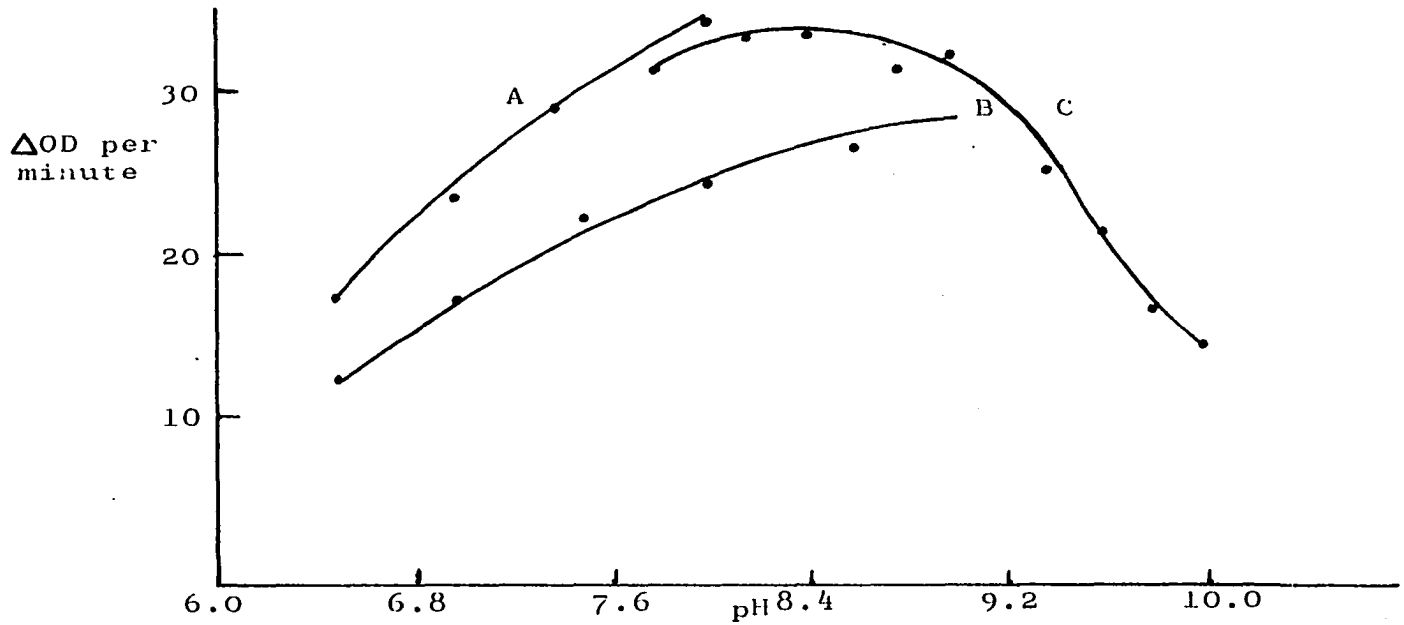


Figure 19. pH profile of glucose-6-phosphate dehydrogenase.

Assay conditions for inhibitor curves

100 mM Glycylglycine

6 mM Glucose-6-phosphate

0.6 mM $NADP^+$

10 mM $MgCl_2$

3 mM Inhibitor

0.1 ml enzyme in a 3 ml cuvette

A Glucose-6-phosphate, glucose-1-phosphate, orthophosphate

B Fructose 1,6-diphosphate ⊕

C Glyoxylate, Fructose-6-phosphate ⊖

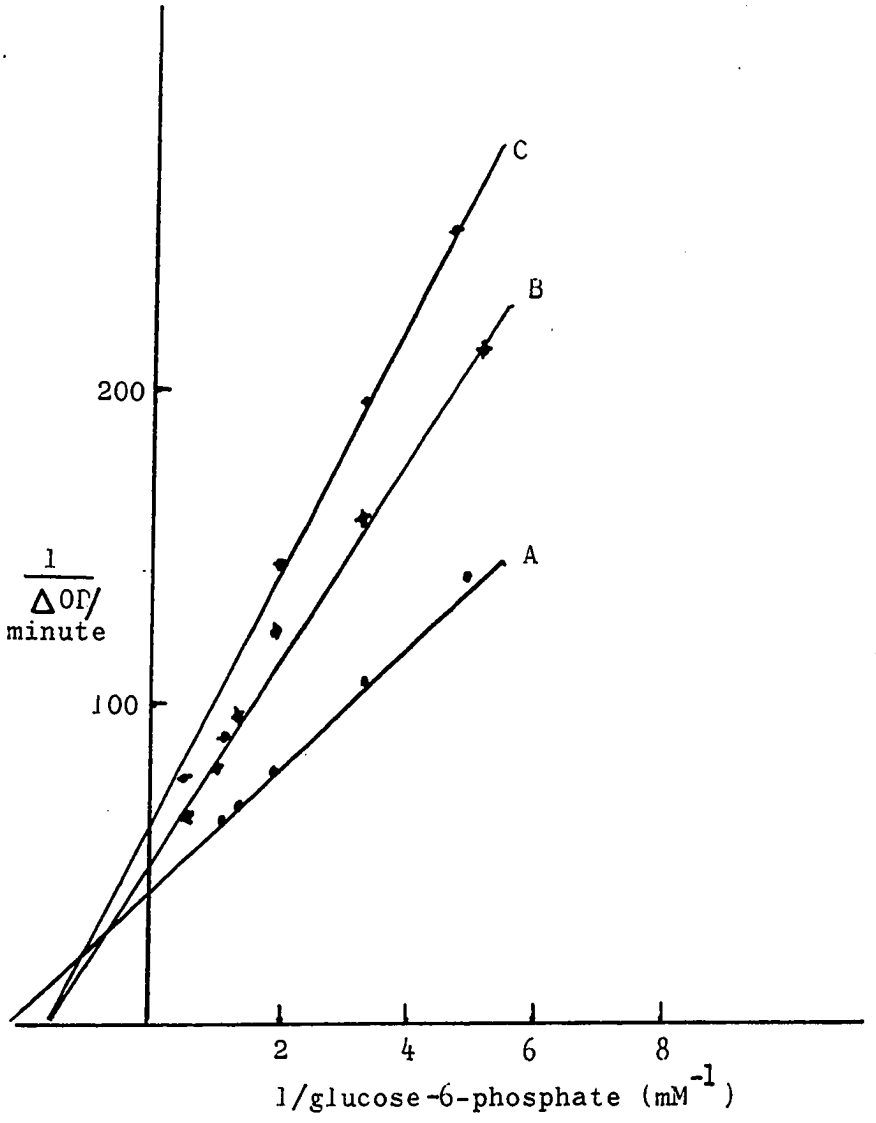


Figure 20. Inhibition of glucose-6-phosphate saturation of glucose-6-phosphate dehydrogenase.

NADH Oxidase

This enzyme, like the NAD^+ enzymes already discussed, also appears to be membrane bound. It has been detected in cells which were disrupted either by sonication of lyophilized defatted cells or by blending cells for long periods of time (30 minutes with 1:1:0.5 ratio of cells: beads:0.1 M Tris buffer, pH 9.5). Additional evidence of the particulate nature of this enzyme is that it can be effectively removed from preparations by centrifugation at 100,000 x g for 2 hours.

The saturation curve (Fig. 21) indicates the presence of interacting sites on the enzyme for NADH. This is further established by the double reciprocal plot (Fig. 21, insert), which is concave upward, the Hill plot (Fig. 22) and the plot of $\frac{1}{v}$ against $\frac{1}{s^2}$ (Fig. 22, insert). The saturation curve (Fig. 21) also indicates very strong inhibition of the enzyme by the substrate, NADH. The range of optimum activity for the enzyme is 0.05 to 0.09 mM NADH. As can be seen by the curve, 0.2, 0.3, and 0.5 mM inhibit the enzyme 50, 75 and 85% respectively. The pH profile (Fig. 23) shows a broad plateau in activity starting at pH 7.5 in all three buffers tested. The enzyme was very stable to freezing and thawing conditions. It was assayed after 90% $(NH_4)_2SO_4$ precipitation and thin film dialysis against 0.1 M Tris, pH 7.5. NADH oxidase displays no metal ion requirement.

The presence of possible effectors for this enzyme has not been determined. Kinetic constants are summarized in Table 7.

Assay conditions for saturation of NADH oxidase

100 mM Tris, pH 7.5

Enzyme preparation and NADH as shown, in a 1 ml cuvette

Assays were started by addition of NADH after 5 min. pre-equilibration at room temperature.

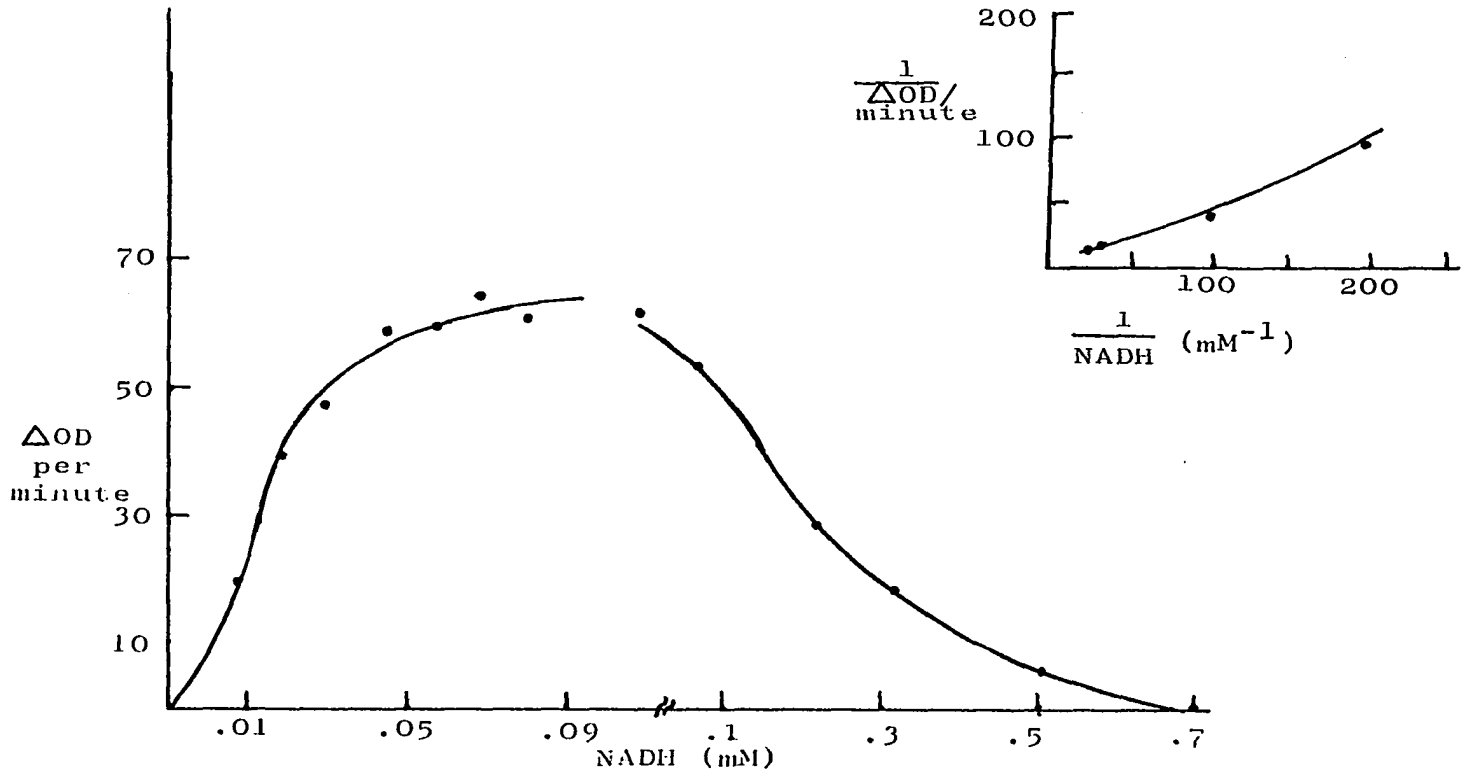
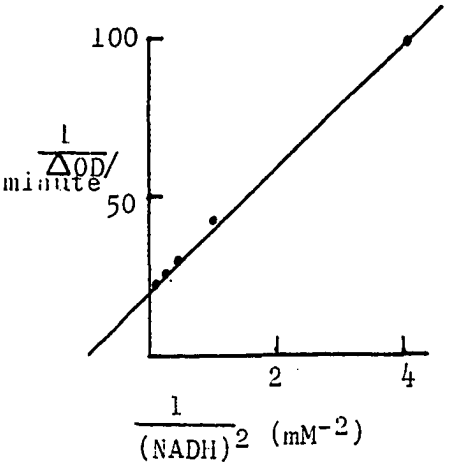


Figure 21. NADH saturation of NADH oxidase.



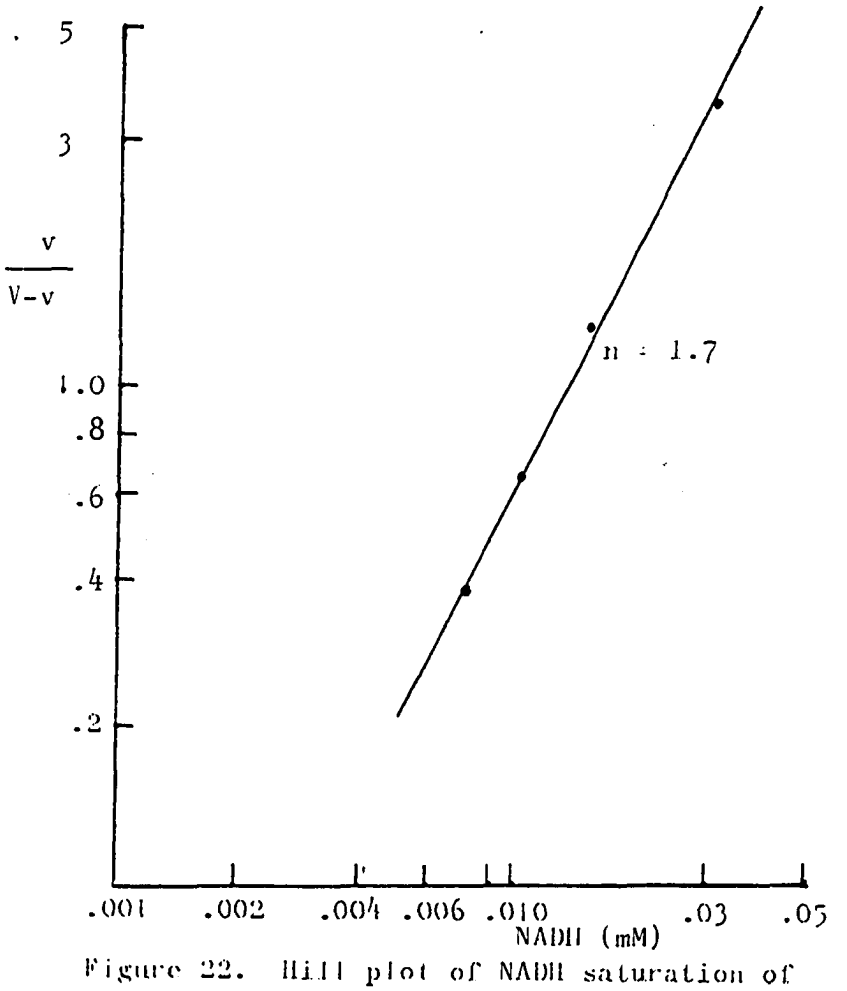


Figure 22. Hill plot of NADH saturation of NADH oxidase.

Assay conditions for the pH profile of NADH oxidase

100 mM buffer

0.07 mM NADH

0.1 ml enzyme in a 1 ml cuvette

A 100 mM Tris Δ

B 100 mM Phosphate o

C 100 mM Glycylglycine ⊖

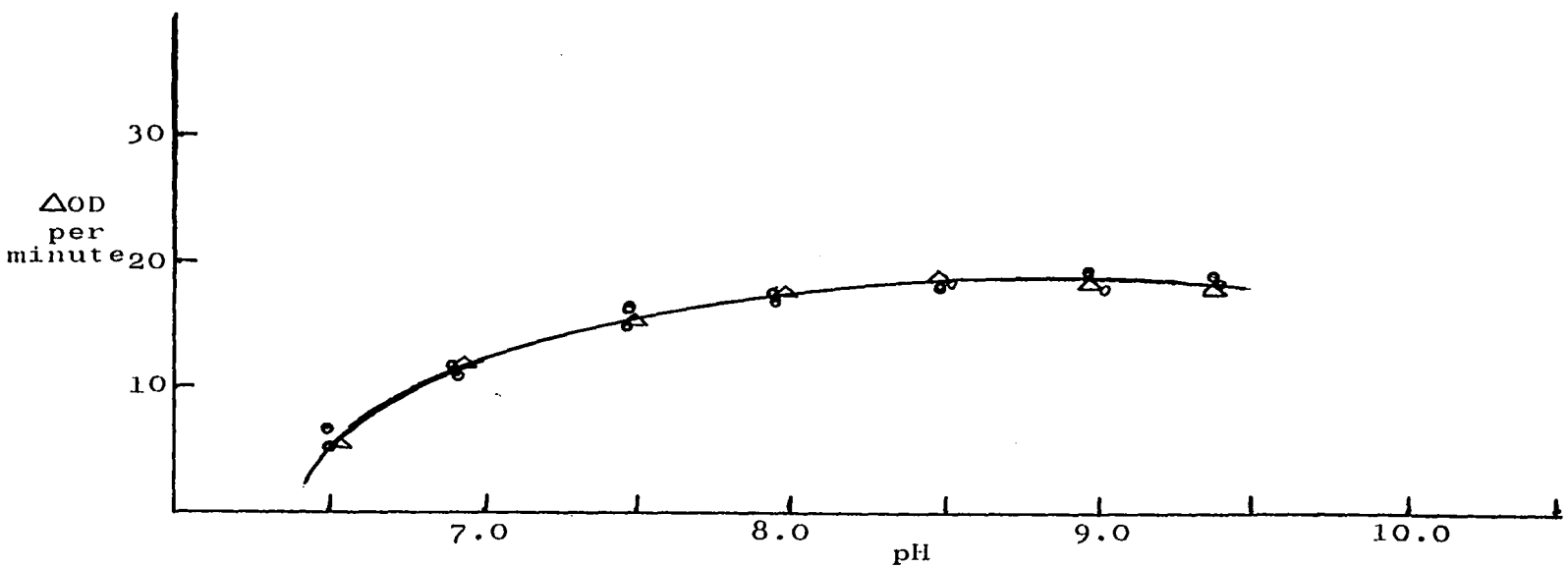


Figure 23. pH profile of NADH oxidase.

Fructose 1,6-Diphosphate Aldolase

This enzyme displays the kinetics shown in Fig. 24. It shows typical Michaelis Menten kinetics, no metal ion requirement and a specifity for fructose-1,6-diphosphate. Fructose-1-phosphate (3.0 mM), glucose-1-phosphate (30 mM), fructose-6-phosphate (30 mM) and glucose 6-phosphate (30 mM) are all without activity, when assayed under maximum conditions for cleavage of fructose-1,6-diphosphate. See Table 7 for the Michaelis constant.

Assay conditions

100 mM Tris, pH 7.5

2 mM NADH

1 unit Triosephosphate isomerase

0.5 unit L-Glycerol phosphate dehydrogenase

Enzyme preparation and fructose-1,6-diphosphate as shown.

Reaction was started by addition of fructose-1,6-diphosphate to a 1 ml cuvette.

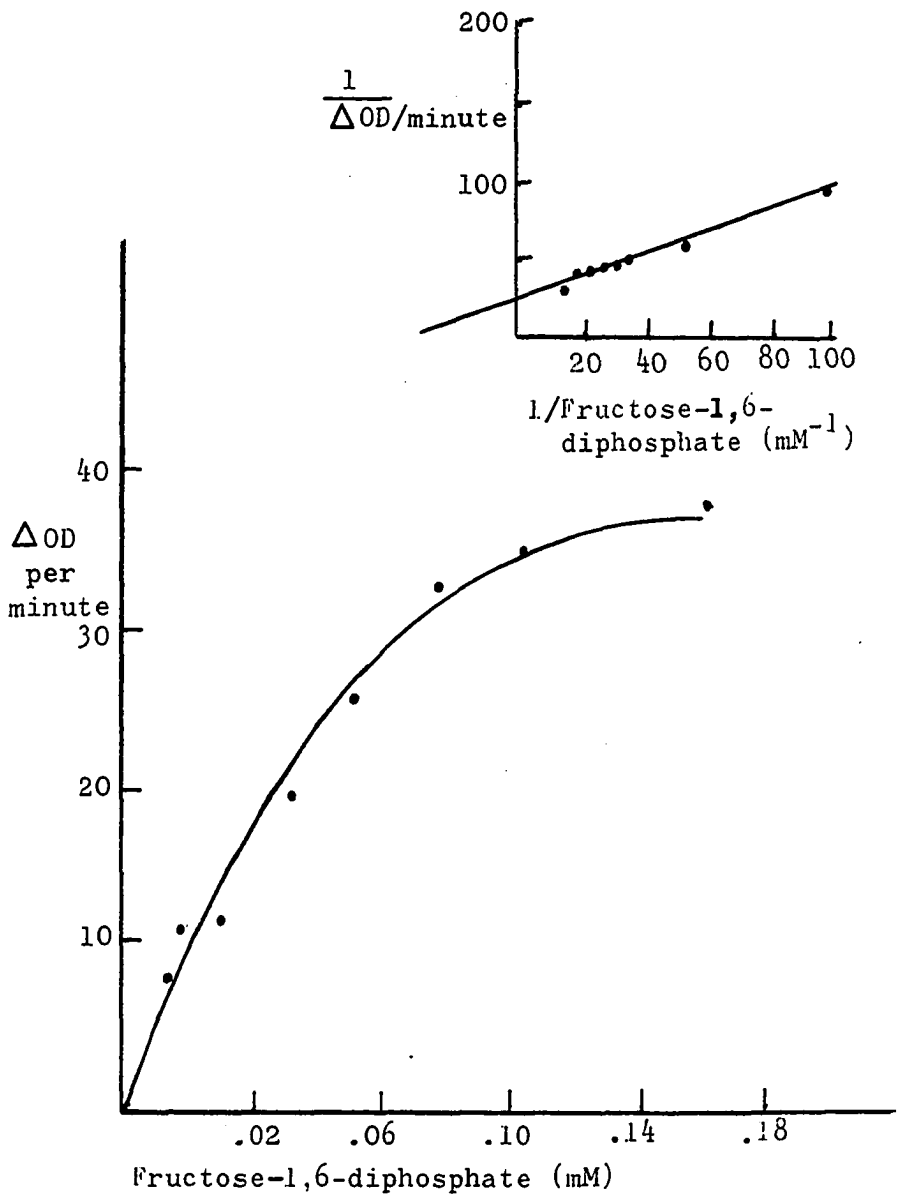


Figure 24. Fructose-1,6-diphosphate saturation of Fructose-1,6-diphosphate aldolase

Hexose Phosphate Isomerase

A typical saturation curve for hexose phosphate isomerase is reported in Figure 25. Km for fructose-6-phosphate is 0.075 mM at pH 7.5 in Tris buffer (Table 7). No inhibition plots were run. The curve is hyperbolic, displaying normal Michaelis Menten kinetics.

Assay conditions for hexose phosphate isomerase

100 mM Tris, pH 7.5

1 mM $NADP^+$

1 unit of Glucose-6-phosphate dehydrogenase

0.1 ml enzyme and fructose-6-phosphate as shown

Assay was started by the addition of fructose-6-phosphate to a 1 ml cuvette.

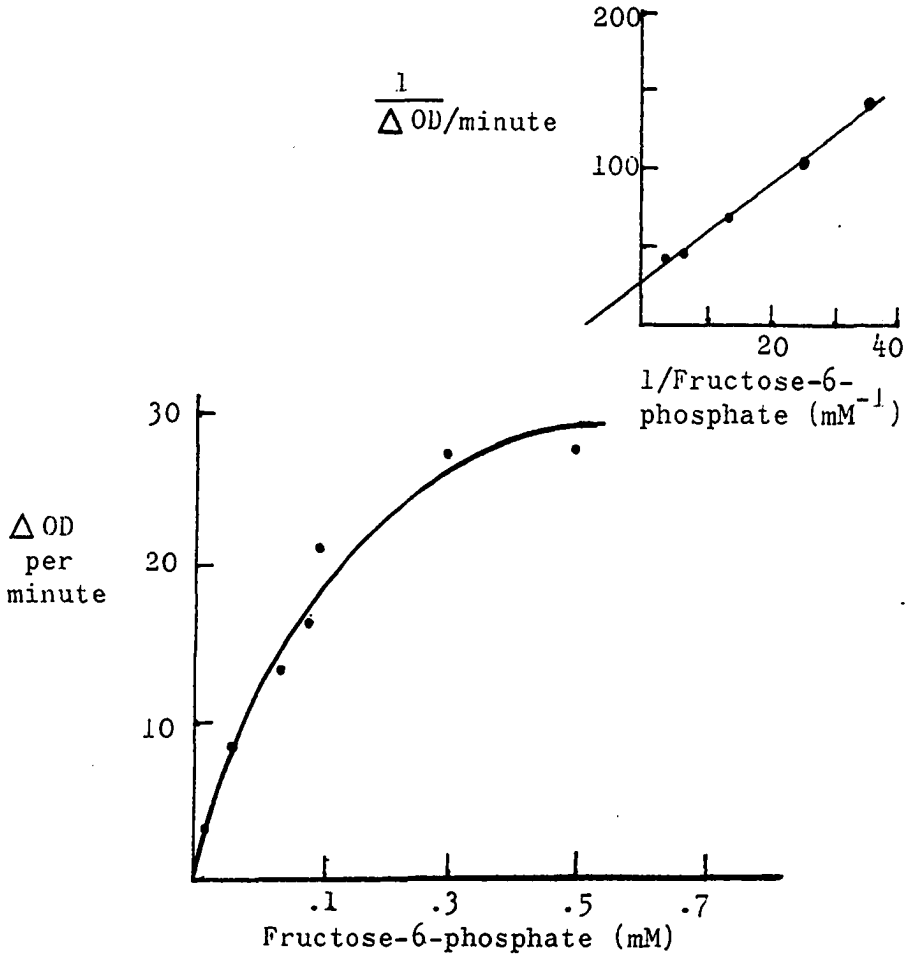


Figure 25. Fructose-6-phosphate saturation of hexose phosphate isomerase

Isocitrate Lyase

This enzyme was assayed in the direction of cleavage using phenylhydrazine as a trapping agent for glyoxylate. The saturation curve (Fig. 26) indicates the presence of interacting sites which is further indicated by the Hill plot (Fig. 27). No possible effectors other than the adenylates have been assayed and these (at mM concentrations) were without effect. Due to these assay conditions it was not possible to check inhibitory characteristics with Krebs cycle or glycolytic intermediates that possess aldehyde or ketone functional groups. The pH profile (Fig. 28) indicates a peak at 7.0 with maximum activity in phosphate buffer. There is less activity in Tris although the peak of activity is between 7 and 7.5. Kinetic constants are summarized in Table 7.

Assay conditions for isocitrate saturation of isocitrate lyase

100 mM Phosphate buffer, pH 7.0

6 mM Cysteine HCl, pH 7.0

10 mM $MgCl_2$

10 mM Phenylhydrazine

0.1 ml enzyme and isocitrate as shown.

Assay was started by addition of isocitrate to deoxygenated samples containing full components in 3 ml cuvettes.

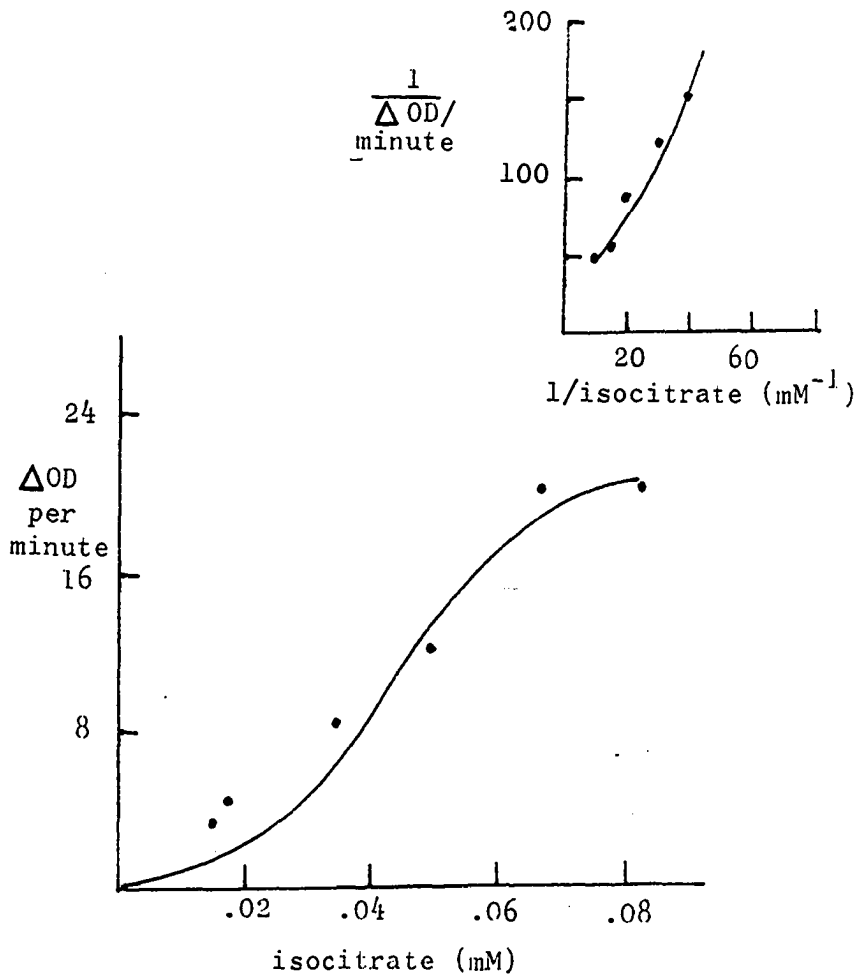


Figure 26. Substrate saturation curve for isocitrate lyase

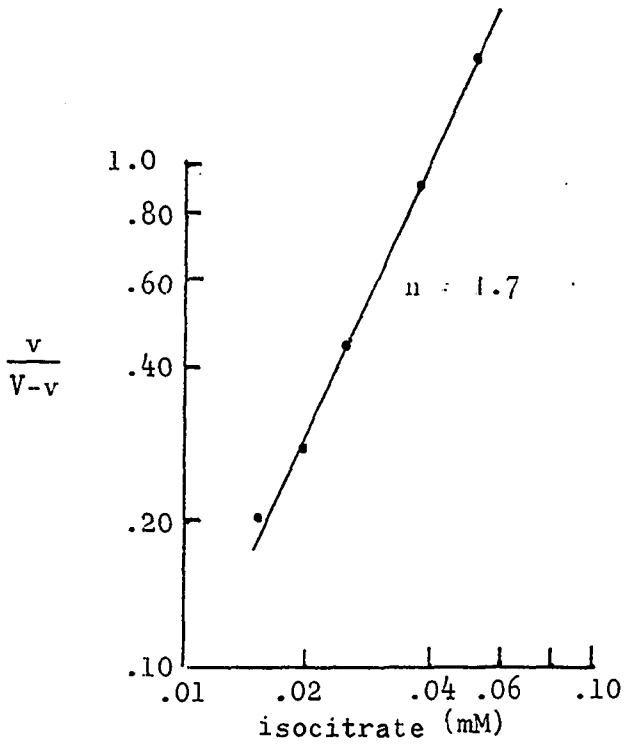


Figure 27. Part A. Hill plot of isocitrate saturation

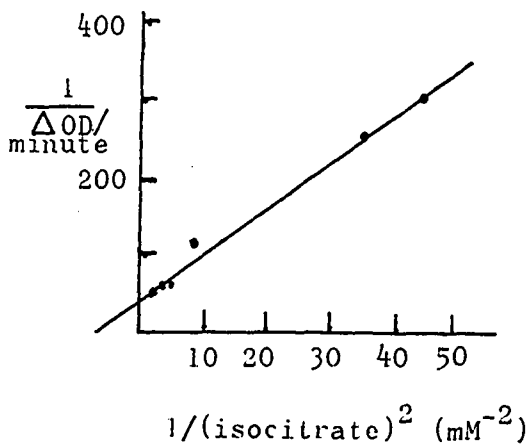


Figure 27. Part B. $1/v$ vs. $1/S^2$ plot for the saturation of isocitrate lyase

Assay conditions for the pH profile of isocitratase included 100 mM buffer and a saturating concentration of isocitrate using conditions as shown in Figure 27.

A Tris buffer

B Phosphate buffer

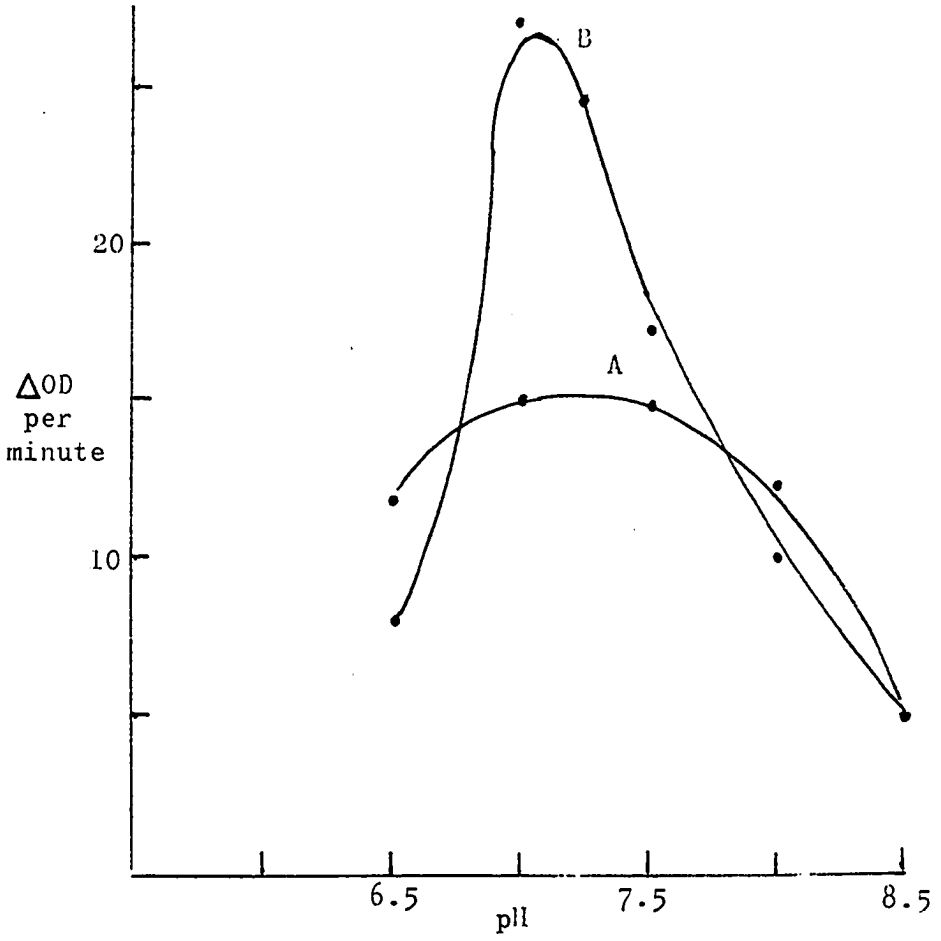


Figure 28. pH profile of isocitrate lyase

Phosphofructokinase and Fructose-1,6-diphosphatase

Table 6 summarizes the breakage attempts used to obtain active preparation of both of these enzymes. It can be seen that a variety of conditions were used to solubilize the enzymes but in all cases no activity was found. The assay conditions are suitably chosen to provide a very sensitive test for very small amounts of activity. It is felt the reason for the lack of activity probably resides in the breakage conditions used and some more effective means will be required to solubilize these enzymes.

Assay conditions used for phosphofructokinase coupled enzymes

33 mM Tris, pH 7.5

5 mM Fructose-6-phosphate

1 mM ATP

1 mM NADH

1 unit Aldolase

1 unit Triosephosphate isomerase

0.5 unit L-glycerol phosphate dehydrogenase

0.1 ml enzyme

Reactions were started by the addition of fructose 6-phosphate to a 1 ml cuvette.

Assay conditions used for fructose-1,6-phosphatase

33 mM Tris, pH 8.5

1 mM Fructose-1,6-diphosphate

1 mM NADP

1 unit H exose phosphate isomerase

0.9 unit Glucose-6-phosphate dehydrogenase

0.1 ml enzyme preparation

Reactions were started by addition of fructose-1,6-diphosphate.

TABLE 6. Breakage Attempts for Phosphofructokinase and Fructose 1,6-Diphosphatase Using Sonication

PART A. In 0.1 M Tris Buffer pH 9.0
(All attempts in 10 ml of solution)

Addition to Buffer	Bovine serum Albumin mg/ml	Sonication Time (min)
0.01 M EDTA	0.0	15
0.01 M β-Mercaptoethanol	0.0	15
0.01 M EDTA and 0.01 M β-Mercaptoethanol	0.0	15
0.01 M EDTA and 0.10 M $MgCl_2$	0.0	15
0.01 M EDTA	10	15
0.01 M β-Mercaptoethanol	10	15
0.01 M EDTA and 0.01 M β-Mercaptoethanol	10	15
0.01 M EDTA and 0.10 M $MgCl_2$	10	15

PART B. In 0.1 M Tris Buffer pH 7.5
(in 10 ml of solution)

Addition to Buffer	Bovine serum Albumin mg/ml	Sonication Time (min)
0.01 M EDTA	-	15
0.01 M β-Mercaptoethanol	-	15
0.10 M $MgCl_2$	-	15
0.01 M EDTA	10	15
0.01 M β-Mercaptoethanol	10	15
0.10 M $MgCl_2$	10	15

Effect of Stage of Growth on Enzyme Activity

As was discussed in the introduction, there is growing experimental evidence that transitions from one growth stage to another are accompanied by both structural changes and changes in enzyme patterns within microorganisms. This could be thought of as a programming by the cell to control metabolic and biosynthetic activities (1) by influencing the production of the enzymes involved in these functions, (2) by controlling the activity of those enzymes which are present, and (3) by selective degradation of existing enzymes. In order to further understand these same controls in Nocardia it was decided to determine the activity of several of the suspected key enzymes as a function of growth. The relative activities of these enzymes could then serve as a guide to the interpretation of the events taking place within the cell.

The first enzymes to be investigated in this manner were the $NADP^+$ reductases. The results (Fig. 29, 30, and 31) indicate there is an alteration in the activity of these enzymes. All three have relatively low activity during the early stage of growth (germination) followed by a rise in activity as the cells develop into full hyphae (usually 7 to 10 hours). All these enzymes display greatest activity at the full hyphal stage of growth (8 to 9 hours), after which time there is a decrease in

activity for all three.

It is of interest to note that isocitrate dehydrogenase shows the greatest increase in activity. This enzyme shows a three-fold increase while the L-malic and glucose-6-phosphate dehydrogenases display an increase of two-fold.

The decrease in activity is also much more gradual after the fragmentation stage for isocitrate dehydrogenase than for the other $NADP^+$ dehydrogenases. The patterns of activity changes as a function of growth have been reproduced a number of times. All of this demonstrates the results shown are real measurements of the relative levels of the enzymes. The circles at the points indicate approximate reproducibility.

The pattern of activity for isocitrate lyase is, however, very different (Fig. 32). This enzyme displays very little activity when the dehydrogenases are the most active, that is during hyphal development (7 to 10 hours). Circles again indicate approximate ranges of reproducibility. The rise in activity occurs at that stage of growth associated with fragmentation of the hyphae (12 hours). After this event the activity of the enzyme remains at relatively the same level throughout the later stages of growth.

The substrate saturation curves at early stages of growth for each enzyme (Fig. 33, 34, 35, and 36) and the

corresponding pH profiles for each enzyme (Fig. 37, 38, 39, and 40) are in agreement with curves reported in the appropriate part of the Results section. All of this is evidence that the kinetic properties of the enzymes investigated remained the same; thus, suggesting that the enzymes studied at early stages of growth and at later stages were identical.

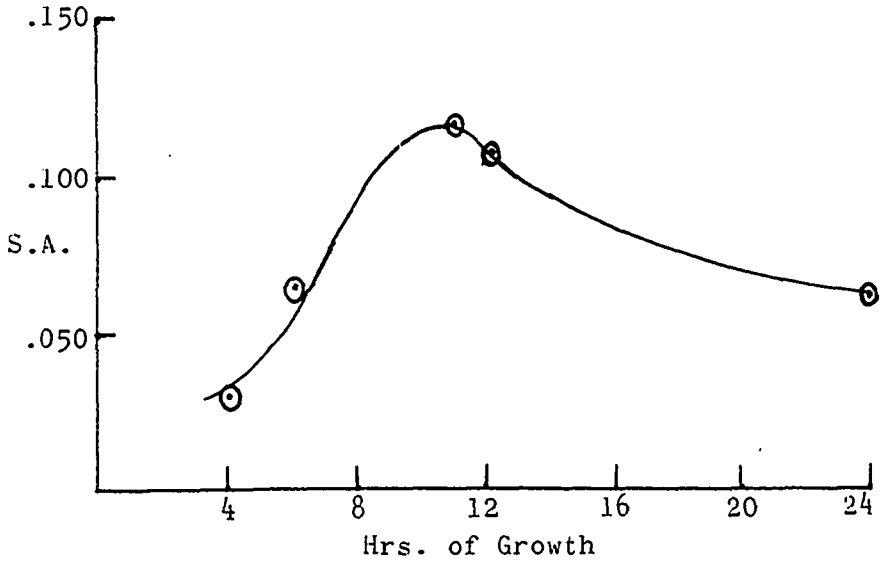


Figure 29. Variation in specific activity of $NADP^+$ isocitrate dehydrogenase as a function of growth of N. corallina.

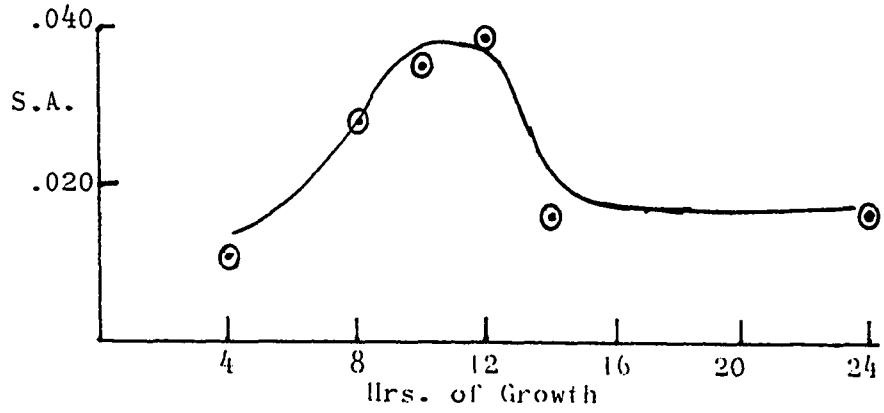


Figure 30. Variation in specific activity of $NADP^+$ L-malate dehydrogenase as a function of growth of N. corallina.

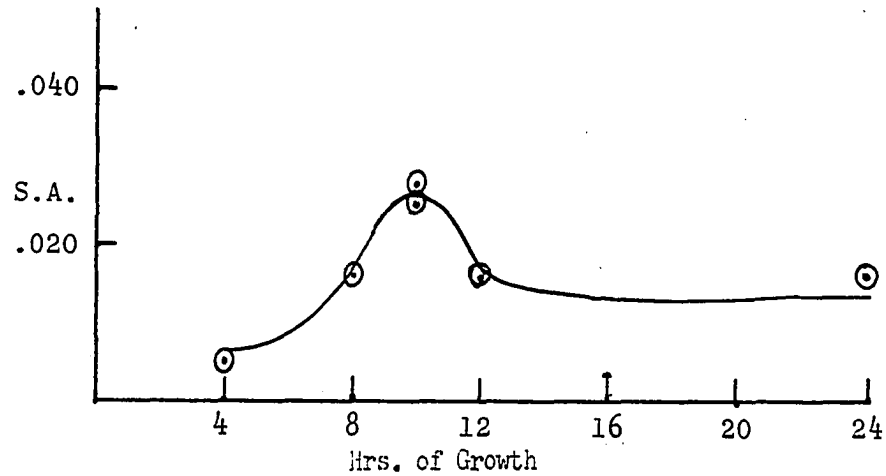


Figure 31. Variation in specific activity of glucose-6-phosphate dehydrogenase as a function of growth of N. corallina.

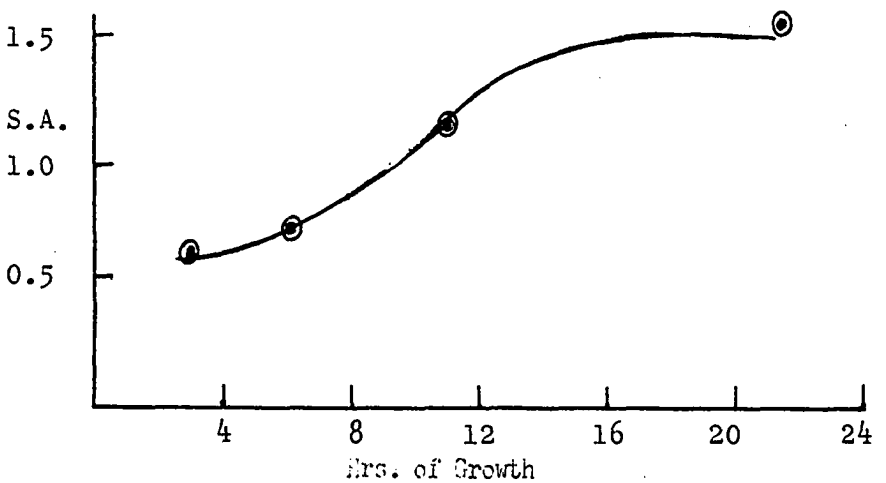


Figure 32. Variation in specific activity of isocitratase as a function of growth of N. corallina.

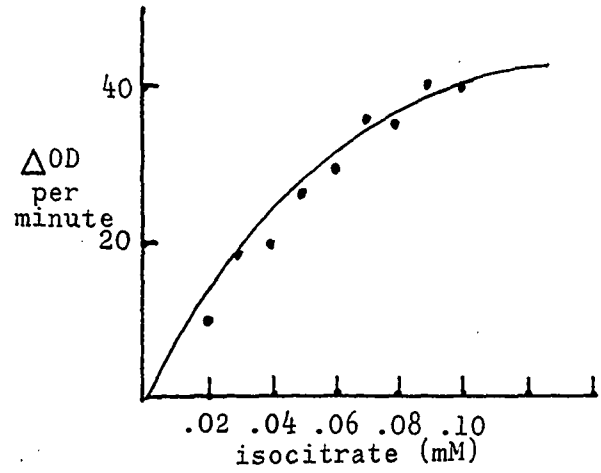


Figure 33. Isocitrate saturation of isocitrate dehydrogenase at the early hyphal stage (4 hr.)

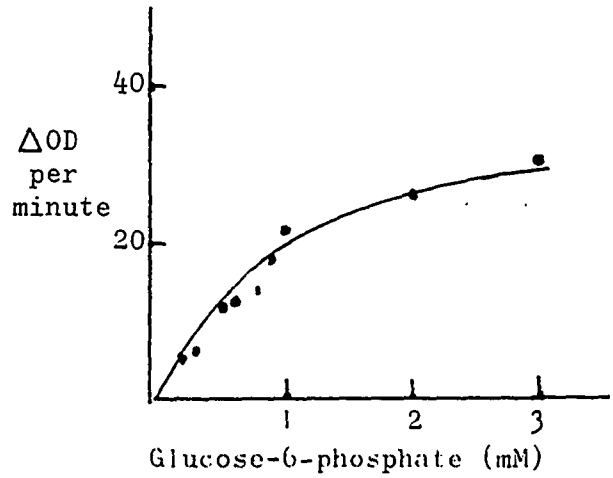


Figure 34. Glucose-6-phosphate saturation of glucose-6-phosphate dehydrogenase at full hyphal stage (11 hr.)

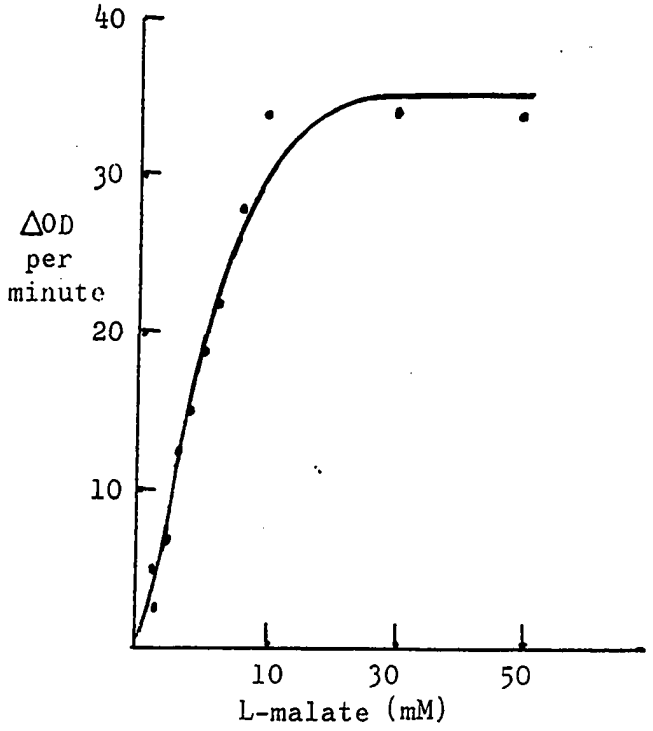


Figure 35. L-malic saturation of $NADP^+$ L-malic dehydrogenase in early hyphal stage (6 hr.)

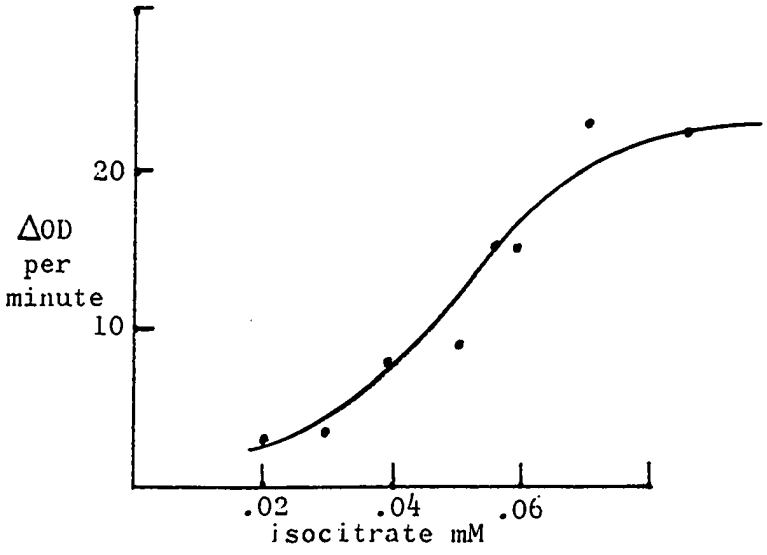


Figure 36. Isocitrate saturation of isocitratase in late hyphal stage (12 hr.)

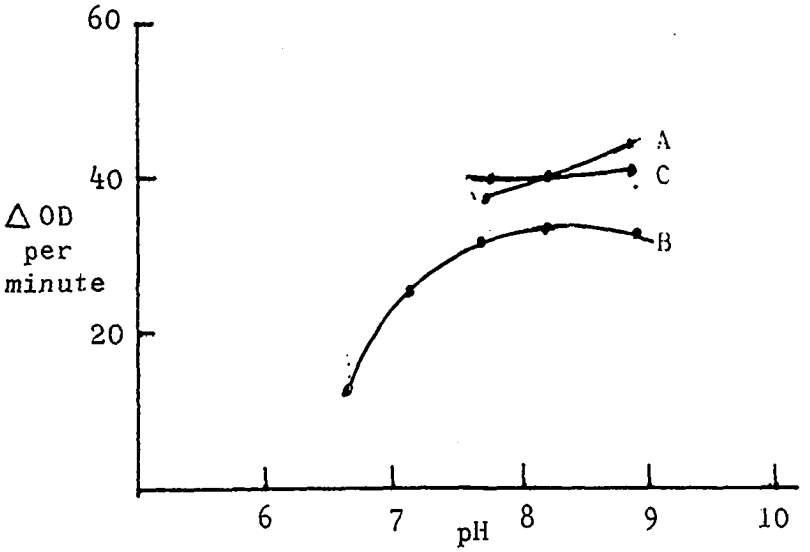


Figure 37. pH profile of NADP$^+$ isocitrate dehydrogenase at late hyphal stage (13 hr.) A) glycylglycine, B) phosphate, C) Tris

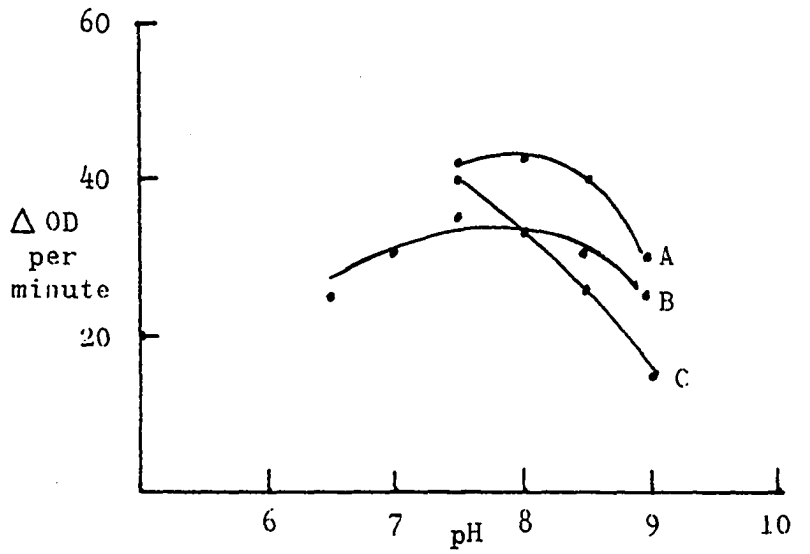


Figure 38. pH profile of glucose-6-phosphate dehydrogenase at late hyphal stage (13 hrs.) A) glycylglycine, B) phosphate, C) Tris

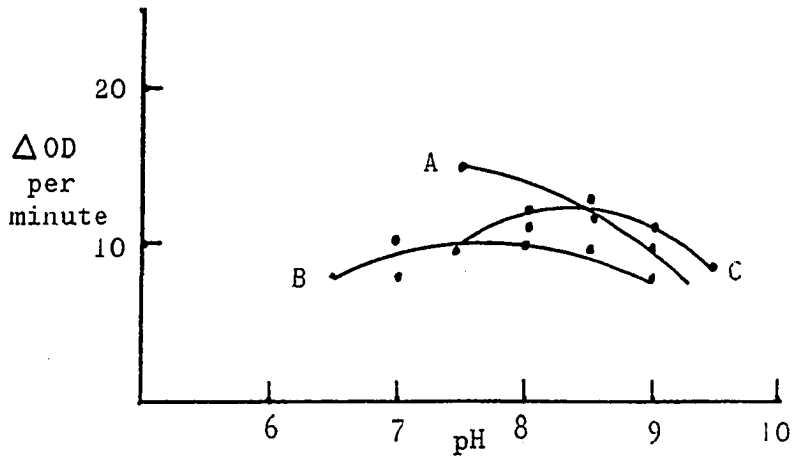


Figure 39. pH profile of $NADP^+$ L-malate dehydrogenase at late hyphal stage (13 hrs.)
A) Tris, B) phosphate, C) glycylglycine

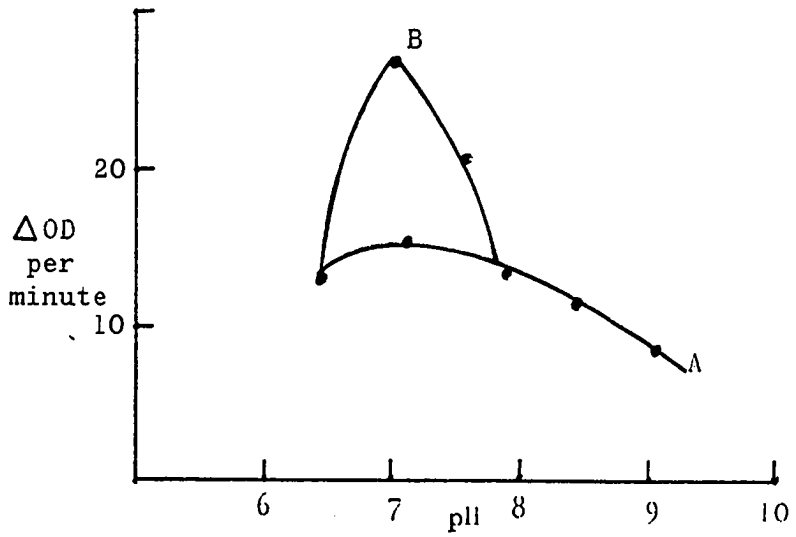


Figure 40. pH profile of isocitrate lyase at late hyphal stage (12 hrs.) A) Tris, B) phosphate

CHAPTER IV

DISCUSSION

The purpose of this investigation was to study control mechanisms of cellular differentiation in Nocardia corallina. In order to accomplish this, it was necessary to examine enzymes functional in the central pathways of carbohydrate metabolism. Since the various morphological states of the bacteria could be distinguished, and a large percentage of population could be maintained in any one stage, it was possible to measure enzyme activity as a function of growth. Information of this type can ultimately be used to establish the molecular basis for control of cellular differentiation both in Nocardia and probably in other organisms.

Isocitrate Dehydrogenase

The occurrence within this organism of a NAD^+ specific isocitrate dehydrogenase may be taken as evidence for the occurrence of a citric acid cycle in Nocardia. The enzyme is very unstable and apparently associated with particulate matter. The presence of a divalent metal ion requirement and the stabilizing influence of

β-mercaptoethanol has also been described. From the large amount of protein used in the assays (2 to 3 mg/ml in a 1 ml cuvette) it would seem that this enzyme is both difficult to solubilize and apparently present in relatively small concentrations. All of these factors, with the exception of the effect of the mercaptan, tend to classify this enzyme with many of the other NAD^+ isocitrate dehydrogenases which have been examined (23). Kinetic parameters for the Nocardia enzyme have not been determined due to the instability of the preparation.

The presence of a very active $NADP^+$ specific isocitrate dehydrogenase in Nocardia has been detected. This enzyme which has been kinetically characterized fits into the general pattern of similar enzymes from other sources (36, 37). The enzyme has a very high affinity for both substrates (see Table 7) and a pH profile which plateaus above 7.5. The absence of interacting sites on the enzyme and the complete absence of regulation by any of the metabolic effectors examined indicates that this enzyme does not respond to control by feedback inhibition by the adenylates, fructose-1,6-diphosphate, phosphoenol pyruvate, pyruvate, malate, oxaloacetate and glyoxylate. This indicates this enzyme probably is not a Krebs cycle enzyme. It likely functions in the production of NADPH.

It is likely the rise in activity, which is seen when activity is plotted as a function of growth, is due

to de novo synthesis of the enzyme. This view seems particularly attractive since there is no change in the kinetic constants or in the pH profile of the enzyme. Clark and Brown (7) have reported the disappearance of fat bodies in this organism during the process of fragmentation. If it can be assumed that fats are being stored throughout the process of hyphal development, the NADPH will be required for the synthesis of these fats. Ball (3) in a study which parallels this investigation, has calculated that the glucose-6-phosphate dehydrogenase in adipose tissue is not present at a level high enough to account for all the NADPH which could be required for fat synthesis. It would, therefore, seem entirely likely that this rise in glucose-6-phosphate dehydrogenase activity could be due to an increased need for the NADPH produced. This reasoning relies upon the basic assumption of a well organized cell in which each enzyme is capable of responding to some type of regulatory signal. This investigation has not established the complete absence of an intracellular modulator which could be responsible for the response. It does, however, establish that the modulator cannot be any of the common metabolites. It would seem likely, then, that this rise in enzyme activity may be due to de novo synthesis of the enzyme.

L-Malate Dehydrogenase

Much of what has been said about the NAD^+ specific isocitrate dehydrogenase could equally apply to this enzyme. It is not stable, it is difficult to solubilize, apparently present in small amounts, and it displays a divalent metal ion requirement. Determination of the kinetic constants and any possible regulation of this enzyme will be dependent upon the discovery of a more effective means of solubilizing the enzyme and maintaining its activity.

The occurrence of a reaction which is characterized by the reduction of $NADP^+$ in the presence of L-malic acid was reported (see Experimental). The exact nature of this reaction has not been completely characterized. The sigmoidal character of the velocity-substrate curve has been established for both substrates through the use of Hill and the Lineweaver-Burk plots. This observation has not been reported, to the best of my knowledge, for any of the other $NADP^+$ malic dehydrogenases. This type of a response of velocity to increasing substrate concentrations indicates that this $NADP^+$ dehydrogenase may be a possible regulatory enzyme.

Evidence for the nature of the reaction was first discovered in an investigation of possible effectors of the enzyme. It was found that of all the modifiers investigated only L-aspartate and oxaloacetate had any

effect on the enzyme. Other possible modifiers investigated included pyruvate, succinate, phosphoenolpyruvate, and glyoxylate. The fact that oxaloacetate inhibits this enzyme in a manner which is characteristic of several very different pyridine linked dehydrogenases is indicative of the reaction:

$$\text{L-Malate} + \text{NADP}^+ \rightleftharpoons \text{Oxaloacetate} + \text{NADPH}$$

For such a reaction, assuming an ordered binding in which $NADP^+$ binds first and L-malate second with oxaloacetate being released first followed by NADPH, an ordered mechanism has been hypothesized. In such a system, with L-malate as the variable substrate and the enzyme unsaturated with $NADP^+$, the product, oxaloacetate, is expected to be a noncompetitive inhibitor (13). The fact that the reverse reaction (oxaloacetate + NADPH $\rightleftharpoons$ L-malate + $NADP^+$) could not be detected may be due to unfavorable assay conditions.

Aspartate is an inhibitor of $NADP^+$ L-malic enzyme in Neurospora crassa. It is possible this effector may be functioning in a similar fashion in Nocardia. (64, 65)

At least one other possibility exists which would also explain these inhibitory responses. It is possible this enzyme could be the $NADP^+$ malic enzyme. This enzyme has been implicated in a regulation of the C_4 acids of the Krebs cycle by causing a decarboxylation of L-malic acid to produce pyruvate. This enzyme in E. coli responds

to induction by malate and it, like the Nocardia enzyme, is inhibited by oxaloacetate. (21)

Certainly this enzyme merits further study. The loss in activity which the enzyme suffers on storage is also reflected in the decrease in the strength of interactions. There is also a decrease in the response of aged preparations to effectors. This decrease could be due to dissociation of the enzyme into subunits, inactivation of effector sites, or irreversible loss of some component of the enzyme which served to stabilize it. One other possibility is that the preparation could consist of more than one enzyme. The decrease in interactions could then be due to a decreased concentration of an unstable enzyme. The inactivation was not prevented by β-mercaptoethanol.

This enzyme like the $NADP^+$ specific isocitrate dehydrogenase displayed a definite increase in activity at the stage of full hyphal growth of the organism. Again due to the calculations of Ball and others (3, 22) it might be predicted this increase in activity occurs at an advantageous time for the cell. The enzyme increases in activity of those times when NADPH is again most required.

This information serves as additional evidence for the nature of the enzyme catalyzed reaction. The rise in activity can be readily understood in the light of

the following discussion. If this enzyme should in fact be the $NADP^+$ malic enzyme a different pattern of activity would be expected. In fact it might be expected that this enzyme which functions in a control of the level of Krebs cycle intermediates might more closely parallel the pattern in activity seen for isocitrate lyase.

A basic question which remains unanswered concerns the exact nature of the reaction. This could most readily be answered by enzyme purification and analysis of the products of the reaction. Such a task will, of course, be dependent upon the development of a method for stabilizing the preparation.

Isocitrate Lyase

The presence of this enzyme in Nocardia may be taken as primary evidence for the occurrence of the glyoxylate cycle. The enzyme is stable to freezing and storage but requires the presence of a sulfhydryl compound in the assay. The assay method depends upon the use of phenylhydrazine to trap the glyoxylate which is formed from the cleavage reaction (Dixon and Kornberg, 15). All of the assays reported for this enzyme use either phenylhydrazine (14), dinitrophenylhydrazine (38), or semicarbazide (14). The differences noted in both inhibitory patterns and kinetic constants by using the direct assay, as compared to the discontinuous or sampling assay, have been summarized by Syrett and John (52). These investigators

noted a ten-fold difference in the kinetic constants for the enzyme by the two different assay methods.

Optimum activity for this enzyme requires the presence of both a sulfhydryl compound and a divalent metal ion. The value reported for the half saturating concentration of substrate indicates that this enzyme, like the $NADP^+$ isocitrate dehydrogenase has a very high affinity for isocitrate. This agrees well with reports from the literature (24, 25, 38).

The question of the purpose of this pathway in the metabolism of the organisms in which it occurs, has been discussed by Kornberg and others (24, 25, 49). It is believed that a principal role for this enzyme is that of maintaining the level of citric acid cycle intermediates. It has been well established that this enzyme is under both feedback and induction-repression control.

From the evidence obtained in this study it appears that the Nocardia enzyme also is responsive to controls of this type. The very best evidence for this is the curve which is obtained when activity is plotted as a function of growth. A very different pattern emerges when this plot is compared to that of the dehydrogenases previously reported. No change occurs either in kinetic parameters or the pH optimum. However, where the dehydrogenases have the greatest activity isocitrate lyase has the least and vice versa.

This can be rationalized by speculating upon what is happening inside the cell. Isocitrate lyase activity is low when most metabolites are being stored as fats (high dehydrogenase activity). At the point that fats are being degraded, isocitrate lyase activity apparently increases to a relatively stable value. In early stages of growth (germination to early hyphal) the organism is growing chiefly on the fructose in the medium. This observation is paralleled by a study conducted in yeast which showed that growth on a carbohydrate repressed the synthesis of the enzyme. The rise in activity that occurs after hyphal growth, during the process of fragmentation, can be explained since during fragmentation fats are being catabolized to furnish energy for the biosynthetic processes concomitant to cell wall biosynthesis. This fat degradation results in the production of acetyl-CoA. It is a gluconeogenic precursor which serves either to activate or to cause de novo synthesis of isocitrate lyase. This leads to an increased production of glyoxylate and succinate. The glyoxylate then condenses with acetyl-CoA in a second reaction to produce malate. Both steps serve to resupply the diminished levels of the Krebs cycle intermediates which are being used for amino acid synthesis and gluconeogenic purposes. Smith and Gunsalus (47), Levy (27), Sjorgen and Romano (46) have reported a stimulation of this enzyme by acetate in the medium in a variety of

microorganisms. It would seem likely that the Nocardia enzyme is also subject to regulation by acetate or some closely related metabolite. The exact nature of the metabolic effector has not been characterized from Nocardia. More detailed analysis will be dependent upon enzyme purification and analysis. It would be also of considerable interest to examine the levels of this enzyme from growth on a defined medium.

Enzymes Utilizing Phosphorylated Hexoses

The presence, in the cell free extract, of several enzymes which are active in the metabolism of hexoses is evidence for the presence of glycolytic and glucogenic pathways in this organism. The enzymes have been detected and characterized with respect to kinetic constants but effector actions have been determined for only one enzyme.

Racker (42) has hypothesized that orthophosphate, which is an inhibitor of glucose-6-phosphate dehydrogenase in mammalian cells, plays an important regulatory role. Phosphate serves as a metabolic regulator because it is required during glycolysis. Inhibition of the pathways for oxidation of hexoses thus serves as an effective control to channel the carbohydrates through the glycolytic pathways. No such inhibitory effect by phosphate is noted on the Nocardia enzyme at any concentration of glucose-6-phosphate (the entire saturation range for glucose-6-phosphate was assayed). The saturation curve for this

enzyme displays an increase in activity at high substrate concentrations (greater than 5 mM). This could be due to high substrate activation of one enzyme or the presence of more than one iso-enzyme. This type of problem could be resolved by enzyme purification and a more detailed kinetic analysis on the purified preparation.

The presence of an inhibitory effect by glyoxylate, fructose-6-phosphate, and fructose-1,6-diphosphate has been observed. It is of some value to consider possible regulatory roles for the compounds. Certainly the fact that fructose-6-phosphate and fructose-1,6-diphosphate inhibit the enzyme could be due to binding at the same site on the enzyme as glucose-6-phosphate. The question of the role of glyoxylate can not be explained this readily. It will be remembered, however, that in this organism there was an inverse relationship between glucose 6-phosphate dehydrogenase and isocitrate lyase. It would seem likely then that the product of the cleavage reaction could work in a direct manner to control the dehydrogenase activity. This could be of possible physiological significance since high levels of isocitrate lyase activity are seen especially at those stages in which polysaccharide synthesis is also high. Thus the effect of glyoxylate inhibition of glucose-6-phosphate dehydrogenase could serve to channel carbohydrates into biosynthetic pathways. These speculations indicate that this enzyme probably is under some type of

regulatory control. The exact nature of this control will be dependent upon a more complete knowledge of other control mechanisms in this organism.

Brown and Reda (8) have reported a two-fold increase in the activity of glucose-6-phosphate dehydrogenase of the hyphae and this agrees well with the results reported in this investigation. The pattern of enzyme activity and the nearly identical pH profiles and kinetic plots parallel the results reported for the other $NADP^+$ dehydrogenases. It is assumed these results serve to strengthen the case for a large supply of NADPH for biosynthetic purposes.

None of the enzymes of the pentose phosphate shunt have been examined in this organism. Knowledge of control mechanisms within this pathway is scant. Racker (42) has pointed out that hexose phosphate isomerase is a likely enzyme on which to expect some type of control to exist. Several investigators have shown the 6-phosphogluconate (41), sedoheptulose-7-phosphate (54), and erythrose-4-phosphate (16) inhibit hexose phosphate isomerase as does ATP and orthophosphate (42). According to Racker this enzyme serves as a branch point to channel carbohydrates either through the pentose cycle or into glycolysis. This enzyme has been characterized from Nocardia but no inhibitor plots were obtained.

The presence in this organism of an aldolase which converts fructose-1,6-diphosphate to C_3 intermediates has

been taken as further evidence for the occurrence of the Embden-Meyerhof-Parnas pathway for carbohydrate utilization. This enzyme, from mammalian sources, has been reported to cleave fructose-1-phosphate at a rate considerably less than fructose-1,6-diphosphate (49). The enzyme from rabbit muscle is interesting because the reaction is inhibited by aldehydes (in particular glyceraldehyde), by ATP, and by fructose-6-phosphate. The enzyme of Nocardia apparently will not cleave fructose-1-phosphate.

The inability to detect phosphofructokinase or fructose-1,6-diphosphatase is probably due to techniques used in the isolation procedure rather than the absence of the enzymes. It would be interesting to reexamine these enzymes from growth on defined media to ascertain whether a repression or induction could account for this observation.

NADH Oxidase

The presence in Nocardia of an enzyme which functions in the oxidation of NADH has been established. This enzyme is very stable and several aspects already discussed indicate the particulate nature of this enzyme. No effectors of this enzyme have been studied but very strong interactions are seen in the saturation curve. Inhibition of the enzyme at high substrate concentrations is also very pronounced. One of the most important aspects of this enzyme

deserving close examination will concern the detection of regulators for the enzyme. The adenylates especially should be examined.

TABLE 7

Kinetic Constants for Enzymes Detected in *Nocardia corallina*

Enzyme	Substrate	Kinetic Constants K_m or $(S)_{0.5}$	Kinetic Constants Hill Constant
$NADP^+$ isocitrate dehydrogenase	$NADP^+$	0.03	1.17
	isocitrate	0.027	1.0
$NADP^+$ L-malate dehydrogenase	$NADP^+$	0.05	2.5
	L-malate	4.5	1.7
Glucose-6-phosphate dehydrogenase	$NADP^+$	0.007	2.5
	Glucose-6-phosphate	0.7	1.15
NADH oxidase	NADH	0.015	1.7
Fructose-1,6-diphosphate aldolase	Fructose-1,6-diphosphate	0.030	____
Hexose phosphate isomerase	Fructose-6-phosphate	0.075	____
Isocitrate lyase	Isocitrate	0.050	1.7

CHAPTER V

SUMMARY

Several enzymes which function in the intermediary metabolism of Nocardia corallina have been characterized.

The enzymes for glucose metabolism (glucose-6-phosphate dehydrogenase, hexose phosphate isomerase, and fructose-1,6-diphosphate aldolase) have been detected and characterized by pH profiles and kinetic constants in N. corallina. Phosphofructokinase and fructose-1,6-diphosphate aldolase have not been detected. The presence of several enzymes involved in the Embden-Meyerhof-Parnas pathway is very good evidence for the occurrence of this central pathway in this organism.

Similar results have been reported for the enzymes of the Krebs cycle. The presence of an NAD^+ isocitrate dehydrogenase and an NAD^+ malic dehydrogenase has been observed.

The presence of an $NADP^+$ specific isocitrate dehydrogenase and of an $NADP^+$ specific L-malate dehydrogenase has been detected and characterized with respect to kinetic constants. The absence of any metabolic effectors for the $NADP^+$ isocitrate dehydrogenase was observed. The presence of two effectors for $NADP^+$ L-malate dehydrogenase has also

been noted. Possible metabolic roles for the effectors of the malic enzyme were discussed as were the unusual substrate saturation curves for $NADP^+$ L-malate dehydrogenase.

Isocitrate lyase has similarly been detected thus indicating the presence of a glyoxylate cycle in this organism. Effectors have not been examined for this enzyme. Kinetic constants and pH profiles were reported for this enzyme.

The activities of the three $NADP^+$ linked dehydrogenases and isocitrate lyase were measured as a function of the stage of growth. A three-fold change in activity was noted for the $NADP^+$ specific isocitrate dehydrogenase. A two-fold change in activity was noted for the other enzymes. No differences were noted either in the pH profiles or in the saturation curves, thus indicating no elaboration of significantly different enzymes. Possible metabolic significance of these enzymes were discussed.

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