

**PURIFICATION AND CHARACTERIZATION OF MALATE DEHYDROGENASE  
AND 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM HAEMOPHILUS  
INFLUENZAE**

by

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(ABSTRACT)

*Haemophilus influenzae*, the primary causative factor in bacterial meningitis, displays a unique growth requirement for intact NAD. Selective inhibitors of the pyridine nucleotide-requiring enzymes from *H. influenzae* could have a pronounced effect on growth of the organism.

*Haemophilus* malate dehydrogenase was purified 109-fold with a 26% recovery through a 4-step procedure involving salt fractionation, and hydrophobic and dye affinity chromatography. The purified enzyme was demonstrated to be a dimer of  $M_r = 61,000$ . Initial velocity, product, and dead-end inhibition studies were consistent with an ordered bi bi mechanism in which NAD is the first substrate bound to the enzyme. Several NAD analogs structurally altered in either the pyridine or purine moiety functioned as coenzymes in the reaction catalyzed. Selective interactions occurring at the coenzyme binding sites were investigated. Coenzyme-competitive inhibition by adenosine derivatives demonstrated important interactions of the pyrophosphate moiety of the coenzyme. Positive chainlength effects in the coenzyme-competitive inhibition by aliphatic carboxylic acids indicated the presence of a hydrophobic region close to the pyrophosphate region at the coenzyme binding site. Several structural analogs of NAD and malate were evaluated as selective inhibitors of the enzyme. The enzyme was inac-

tivated by incubation with diethylpyrocarbonate whereas no inactivation was observed with sulfhydryl reagents.

*Haemophilus influenzae* 6-phosphogluconate dehydrogenase was purified 308-fold with a 16% recovery through a 4-step chromatographic procedure involving a Phenyl-Sepharose hydrophobic column, and affinity chromatography on Matrex gel Green A, Matrex gel Red A, and 2',5'-ADP-Sepharose resin. The purified enzyme was demonstrated to be a dimer of  $M_r = 70,000$ . Initial velocity studies of 6-phosphogluconate oxidation indicated a sequential reaction mechanism. Although certain product and dead-end inhibition studies were consistent with an ordered mechanism, the direct binding of 6-phosphogluconate in protection experiments did not support a strictly ordered reaction sequence. Inhibition by adenosine derivatives indicated that the 2'-phosphate is important in binding to the coenzyme binding site of the enzyme.

The 3-acetylpyridine analogs of NAD and NADP which support growth of *H. influenzae* were demonstrated to function as coenzymes with the two dehydrogenases studied. The most effective inhibitors of the purified malate dehydrogenase and 6-phosphogluconate dehydrogenase were observed to inhibit the growth of *Haemophilus influenzae*. However, the most potent inhibition of growth by 3-aminopyridine analogs of NAD and NADP could not be explained on the basis of interactions of these analogs with the two dehydrogenases studied.

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## List of Abbreviations

<b>AAD(P)</b>	3-Aminopyridine adenine dinucleotide (phosphate)
<b>ADP-ribose</b>	Adenosine diphosphoribose
<b>AMP, ADP, and ATP</b>	Adenosine 5'-mono-, di-, and triphosphates
<b>3-APAD</b>	3-Acetylpyridine adenine dinucleotide
<b>Bicine</b>	N,N-Bis(2-hydroxyethyl)glycine
<b>DEPC</b>	Diethylpyrocarbonate
<b>DTT</b>	Dithiothreitol
<b>EDTA</b>	Ethylenediamine tetracetic acid
<b>FDP</b>	Fructose 1,6-diphosphate
<b>FMA</b>	Fluorescein mercuric acetate
<b>Hepes</b>	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
<b>HPLC</b>	High performance liquid chromatography
<b>MDH</b>	Malate dehydrogenase
<b>MTT</b>	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
<b>NAD and NADH</b>	Nicotinamide adenine dinucleotide and its reduced form
<b>NADase</b>	NAD glycohydrolase
<b>NADP and NADPH</b>	Nicotinamide adenine dinucleotide phosphate and its reduced form
<b>NBT</b>	Nitro blue tetrazolium
<b>NMN</b>	Nicotinamide mononucleotide
<b>6-PG and 6-PGDH</b>	6-Phosphogluconate and 6-phosphogluconate dehydrogenase
<b>PMS</b>	Phenazine methosulfate
<b>SDS</b>	Sodium dodecyl sulfate
<b>Tris</b>	Tris(hydroxymethyl)aminomethane
<b>2'P-ADP-ribose</b>	2'-Phosphoadenosine diphosphoribose
<b>2',5'-ADP</b>	2',5'-Adenosine diphosphate

# CHAPTER I

## INTRODUCTION

*Haemophilus* species are gram-negative, aerobic or facultatively anaerobic, coccobacilli which are nonmotile and nonspore-forming bacteria. *Haemophilus* organisms are subclassified by their unique growth requirement for one or both of two factors, X-factor (hemin) and V-factor (intact nicotinamide adenine dinucleotide) (1,2). Several of these organisms are pathogenic to man and other animals. *Haemophilus aegyptius* and *Haemophilus ducreyi* cause acute conjunctivitis and venereal disease in humans, respectively. *Haemophilus somnus* causes infectious thromboembolic meningoencephalitis in cattle. *Haemophilus gallinarium* is responsible for acute respiratory infections in chickens. *Haemophilus suis* and *Haemophilus parahaemolyticus* play significant roles in a variety of serious infectious diseases in swine.

*Haemophilus influenzae*, the prototype of the genus, is the most frequently encountered human pathogen and causes bacterial meningitis in children six months to five years of age when their immune system is not fully developed (3). Various diseases like epiglottitis, pneumonia, urinary infections, and cellulitis are also caused by *H. influenzae* (4-6). There are two major colony types of the organism: (a) small and granular (R or rough) colony of the noncapsulated organism, and (b) mucoid and iridescent (S or smooth) colony of the encapsulated organism. The encapsulated strains are virulent and

serotyped with antisera types a to f on the basis of their unique capsular carbohydrates. The capsule of *H. influenzae* type b which is comprised of polyribosylphosphate is regarded as an important virulence factor. Noncapsulated and nonvirulent *H. influenzae* bacteria are found in the normal flora of the nasopharyngeal region.

Treatment of *H. influenzae* has relied upon the use of antibiotics such as ampicillin and chloramphenicol. However, toxic side effects have precluded the extensive use of chloramphenicol. Recent emergence of strains resistant to these antibiotics has been noted (7-9). All of the ampicillin-resistant organisms possess beta-lactamase which catalyzes the hydrolysis of the beta-lactam ring of ampicillin and structurally-related antibiotics. All of these factors have raised serious concerns about the development of alternate therapeutic agents as exemplified by recent studies of semi-synthetic antibiotics and the development of vaccines (10). Another major concern in conjunction with antibiotic resistance is the recent documentation of increased incidence of *H. influenzae* infections in adults (11).

*Haemophilus influenzae* has an absolute growth requirement for X-factor and for V-factor. The X-factor requirement is due to the loss of the enzymatic capacity to synthesize protoporphyrin IX from  $\delta$ -amino levulinic acid (12). The V-factor requirement is caused by the absence of the nicotinamide phosphoribosyltransferase which is required for the formation of the nicotinamide-ribose bond. Thus the organism can not synthesize NAD from the usual low molecular weight precursors utilized by other bacterial systems. *Haemophilus influenzae* must rely on obtaining NAD from the external environment in which it grows. Nicotinamide and nicotinic acid, as well as the typical precursors of the *de novo* NAD biosynthetic pathways, do not serve as V-factor, while nicotinamide riboside, NMN, NAD, NADP, and the reduced pyridine nucleotides are known to satisfy this growth requirement. NAD is the most effective derivative in supporting growth and is most likely the derivative more readily available in growth environments of the organism.

Enzymes involved in satisfying the V-factor growth requirement for externally provided intact NAD in *H. influenzae* have been studied. A periplasmic nucleotide pyrophosphatase (EC 3.6.1.9) responsible for the hydrolysis of extracellular NAD to NMN and AMP, has been characterized with respect to kinetic parameters, substrate specificity, and selective inhibition (13). A correlation between inhibition of this enzyme and inhibition of growth of the organism was demonstrated. In addition to NAD, the 3-acetylpyridine and thionicotinamide analogs of NAD served as substrates for the pyrophosphatase and were observed to support growth of the organism, suggesting the functioning of these analogs as coenzymes for cytoplasmic dehydrogenases. The presence of a cytoplasmic NMN:ATP adenyltransferase and NAD kinase, capable of intracellular synthesis of NAD and NADP from NMN and ATP, has been demonstrated. In addition to those enzymes required for the establishment of intracellular pyridine nucleotide pools, overall pyridine nucleotide metabolism includes the participation of these molecules in essential oxidation-reduction reactions and perhaps systems involving ADP-ribosylation. Considering the atypical processing of NAD in *H. influenzae*, enzymes involved in the overall metabolism of pyridine nucleotides in this organism may be prime targets for controlling growth of the organism. In this respect, very little is known about the intracellular dehydrogenases of this organism. Also very little information is known about various metabolic pathways in *H. influenzae*.

In studies of glucose metabolism in *Haemophilus* organisms, various excreted acid end-products were identified (14). The major acid end-product was succinate in conjunction with small amounts of acetate, oxalacetate, and pyruvate. A number of enzymes involved in glucose oxidation, such as, phosphoenolpyruvate carboxylase, malate dehydrogenase, fumarase, and succinate dehydrogenase were detected and their activities were measured (14). A partial tricarboxylic acid cycle and high malate dehydrogenase activity were indicated by these studies. Malate dehydrogenase was indicated as an important participant in the partial tricarboxylic acid cycle involving re-

actions from phosphoenolpyruvate through oxalacetate to succinate. Preliminary studies of dehydrogenases in sonicates of *H. influenzae* also demonstrated the malate dehydrogenase activity to be the highest among those enzymes assayed. Considering the important role played by this malate dehydrogenase, it was of interest to purify this enzyme and characterize properties of the enzyme such as kinetic mechanism, functioning of coenzymes, and selective inhibition.

The interactions of NAD and NADP with intracellular dehydrogenases of *H. influenzae* represent an important phase in the overall pyridine nucleotide metabolism of this organism. Therefore, the characterization of enzymes utilizing either NAD or NADP should aid in our understanding of how this organism maintains metabolic processes in the face of a limiting supply of pyridine nucleotides. The NADP-dependent 6-phosphogluconate dehydrogenase plays an important role in regulating the pentose phosphate pathway which produces ribose for nucleic acid synthesis and NADPH for anabolic processes. Characterization of this enzyme would give primary information about the control mechanism of the pentose phosphate pathway, and additional information about regulatory properties of the enzyme in RNA and protein biosynthesis. Considering the important role played by 6-phosphogluconate dehydrogenase in the NADPH/NADP ratio and fatty acid biosynthesis in cells, it was of interest to purify and characterize the *Haemophilus* enzyme to obtain more information on the involvement of NADP-dependent dehydrogenases in pyridine nucleotide metabolism in the *Haemophilus* organisms.

Structural analogs of NAD and NADP prepared by chemical and enzymatic methods have been used extensively to determine the involvement of pyridine nucleotides in the binding and catalytic processes of many dehydrogenases. These analogs were used to study selective and competitive interactions with the enzymes at pyridine nucleotide binding sites of dehydrogenases and other pyridine nucleotide-dependent enzyme systems. In the current study, analogs of NAD and NADP will be used to investigate se-

lective interactions with dehydrogenases of *H. influenzae*. The enzymic functioning of these analogs will be correlated with the ability of these compounds to support or inhibit growth of the *H. influenzae* organism. Selective inhibition of *H. influenzae* pyridine nucleotide-dependent dehydrogenases could complement the known inhibition of the periplasmic nucleotide pyrophosphatase and thereby provide a more effective mechanism for controlling growth of the organism.

## CHAPTER II

### LITERATURE REVIEW

The NAD molecule was identified as the first coenzyme in 1904 by Harden and Young when studying the fermentation of glucose to ethanol. A similar molecule, NADP, detected by Warburg and Christian in 1934, was required for glucose 6-phosphate oxidation by erythrocytes (15). Both NAD and NADP functioned as coenzymes, undergoing two-electron reduction to give the 1,4-dihydronicotinamide derivatives, NADH and NADPH. These coenzymes are ubiquitous in all living systems and play an important role in cellular metabolism. As coenzymes they are required for the reactions of more than 370 of the presently described enzymes (16). Figure 1 presents the molecular structure of NAD(P). In addition to the role of these coenzymes in oxidation and reduction reactions, NAD serves as a substrate for a number of different reactions. NAD functions as a substrate for the DNA ligase involved in DNA synthesis, repair, and recombination in certain procaryotes (17,18). DNA ligase catalyzes the formation of phosphodiester bonds between two polydeoxynucleotides by coupling to the cleavage of the pyrophosphate bond of NAD. NAD glycohydrolases also utilize NAD as a substrate, catalyzing the hydrolysis of the nicotinamide-ribose bond. This glycolytic activity has also been associated with mono and poly ADP-ribosylases catalyzing the transfer of ADP-ribose from NAD to various acceptors. An ADP-ribose transferase

involved in mono ADP-ribosylation of specific proteins has been demonstrated in bacterial toxins, bacteria and mammalian tissues. These enzymes catalyze the cleavage of NAD to nicotinamide and ADP-ribose, and covalently attach the latter moiety to protein acceptors. Diphtheria toxin and *Pseudomonas aeruginosa* exotoxin catalyze the transfer of ADP-ribose to a specific amino acid residue of elongation factor II required for protein synthesis, thus leading to cessation of eucaryotic protein synthesis (19-21). Cholera toxin and *Escherichia coli* enterotoxin carry out ADP-ribosylation of a regulatory protein of adenylate cyclase (22,23). Mono ADP-ribosylation was also found in eucaryotes such as turkey erythrocytes (24) and plays a similar regulatory role as in cholera toxin. Poly (ADP-ribose) synthetases involved in poly ADP-ribosylation are located in the nuclei of most eucaryotes and attach poly (ADP-ribose) molecules covalently to nuclear proteins. Poly ADP-ribosylation in eucaryotes plays a central role in cellular metabolism by promoting chromatin structure changes, modulation of various nuclear enzymes, and the depletion of cellular NAD pools (25).

Although most microorganisms can synthesize NAD from various small molecular weight precursors, certain species of *Haemophilus* were observed to be unable to perform these synthetic processes. In 1917, Davis recognized that two growth factors are required by influenza bacilli (26). A few years later Thjotta and Avery were able to confirm Davis' data and named these substances X- and V-factors (27). Lwoff and Lwoff in studies of *H. influenzae* determined that the V-factor requirement could be met by NAD and NADP (28). These pyridine nucleotides were then chemically reduced and growth studies proved that the reduced coenzymes were also functional as V-factor. Further investigations showed that adenylic acid, nicotinic acid, and nicotinamide were unable to function as V-factor. In the 1940's Gingrich and Schlenck provided evidence that in addition to NAD and NADH serving as V-factor, nicotinamide riboside could also serve as V-factor while nicotinamide, ribose and AMP did not (29,30). They sug-

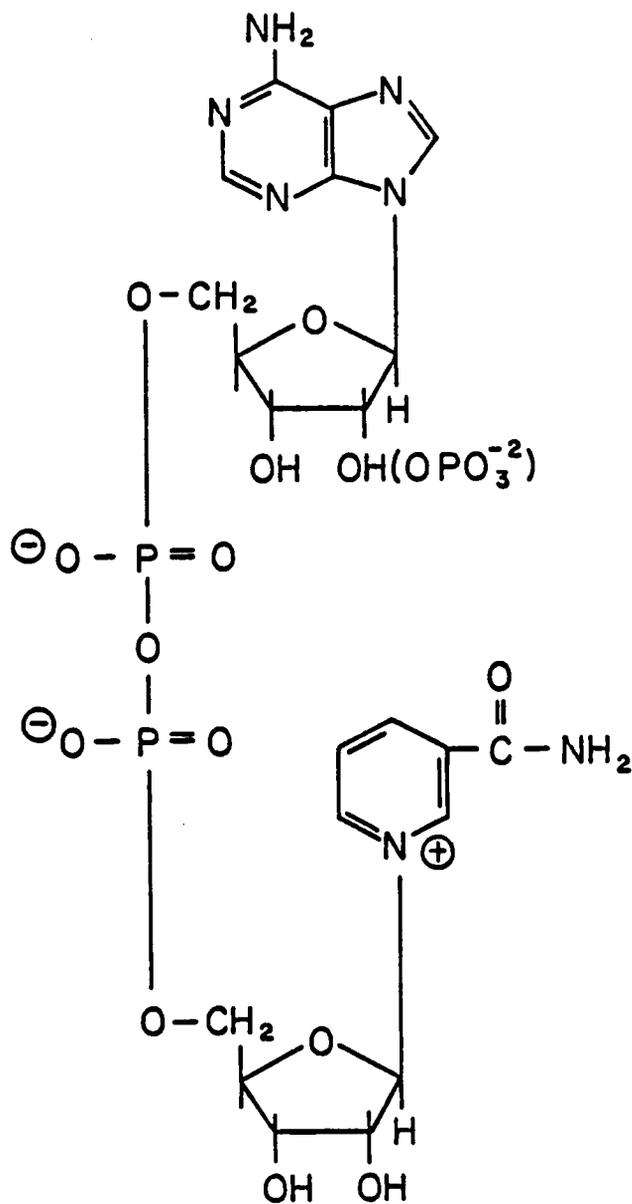


Figure 1. Chemical Structures of Nicotinamide Adenine Dinucleotide (NAD) and Nicotinamide Adenine Dinucleotide Phosphate (NADP): Parentheses indicate the location of phosphate in NADP.

gested that the key biosynthetic step which *Haemophilus influenzae* can not perform is the linkage of nicotinamide to ribose.

Transformation and transduction mediated by *Haemophilus* DNA have been extensively studied (31,32), and enzymology and genetics of their restriction and modification systems have been well-characterized (33,34). In the *H. influenzae* strain, specific restriction endonucleases recognize DNA from other strains. *Haemophilus*-derived restriction enzymes have been used to analyze the structure and function of virus genomes.

Enzymes involved in NAD metabolism in *H. influenzae* have been studied recently by Kahn and Anderson (13). A study of the periplasmic nucleotide pyrophosphatase indicated that external NAD is hydrolyzed to NMN and AMP, prior to the entry of NMN into the cell. In the cytoplasm, NAD is resynthesized from NMN and ATP by NMN:ATP adenylyltransferase, and NAD is converted to NADP by NAD kinase. Subsequently, the intracellular levels of NAD and NADP are established, and the coenzymes are utilized by the various pyridine nucleotide-requiring enzymes.

Most dehydrogenases are highly specific for either NAD or NADP; however, a few enzymes such as glutamate dehydrogenase can function with both NAD and NADP (35,36). NAD-dependent dehydrogenases serve primarily in respiratory processes for the generation of ATP. However, NADP-dependent dehydrogenases are employed to generate reducing power which can be used for the synthesis of pentoses and other essential metabolites.

Most of the NAD-dependent dehydrogenases bind NADH much more tightly than NAD. By contrast most of the NADP-dependent dehydrogenases studied bind the oxidized and reduced coenzyme with similar affinity. This difference in binding may be related to the different redox states of NAD and NADP in the cytoplasm (37). Structural studies of many dehydrogenases revealed the presence of two well-defined regions, one involved in coenzyme binding and the other responsible for catalysis and substrate binding. Comparative studies of the three-dimensional structures of dehydrogenases

have demonstrated that the coenzyme binding regions are highly conserved whereas the catalytic regions are less conserved (38,39). However, cytoplasmic malate dehydrogenase and lactate dehydrogenase are homologous in both the coenzyme binding and the catalytic regions (39). This appears to be due to the fact that the substrates utilized by these two enzymes are closely related, both being 2-hydroxyacids (38).

Many dehydrogenases have been classified as sulfhydryl enzymes on the basis of inactivation by sulfhydryl reagents (40-45). The relative location of essential sulfhydryl groups has been investigated by studies of coenzyme or substrate protection of these inactivation processes (46). A number of pyridine nucleotide analogs have been used to study binding and catalytic processes of many different enzymes using NAD and NADP as substrates or coenzymes. In selective binding studies of octopine dehydrogenase, 21 different NAD analogs were successfully used to probe the environment of the coenzyme binding site on the enzyme (47). Also, by employing a number of NAD analogs structurally altered in either the pyridine or purine moiety, Schuber *et al.* obtained information supporting the oxocarbenium ion mechanism of calf spleen NADase (48). Further applications of different analogs have been reported in recent reviews by Anderson (49), and Woenckhaus and Jeck (50).

The oxidation of malate in most living organisms is catalyzed by two distinct types of pyridine nucleotide-dependent enzymes. In the one case of malate dehydrogenase (MDH), the major product is oxalacetate; whereas, the malic enzyme produces pyruvate and carbon dioxide. The enzymes catalyzing malate to oxalacetate utilize NAD, while the enzymes catalyzing malate to pyruvate use NADP. The purification and characterization of NAD-dependent malate dehydrogenases from eucaryotes have been extensively studied. Malate dehydrogenases occur mostly in two forms, one in the mitochondria, and the other in the cytoplasm. The cytoplasmic MDH isozyme participates in the malate shuttle in order to provide NADH equivalents in the form of malate across the mitochondrial membrane. The mitochondrial enzyme, in addition to its role

in the other half of the malate shuttle, is a major component of the tricarboxylic acid cycle. A third form was identified in the microbody or glyoxysome fraction of some plants and was shown to function in the glyoxalate cycle or in photorespiration (51-54). Molecular properties of NAD-dependent malate dehydrogenases have been studied through gel filtration, X-ray diffraction studies, and amino acid sequence analysis (51-58). In general, both eucaryotic and procaryotic malate dehydrogenases are composed of two identical subunits having dimeric molecular weights of 60,000 to 70,000 (51-67). Fluorescence enhancement studies of porcine heart mitochondrial and cytoplasmic malate dehydrogenases indicated each enzyme contains two equivalent coenzyme binding sites (67). Initial velocity, product inhibition, and isotopic exchange studies with eucaryotic enzymes were consistent with an ordered bi bi mechanism in each case studied (68-71). Dehydrogenases catalyzing the same reaction and isolated from different sources utilize pyridine nucleotide coenzyme analogs quite differently (61,72,73). Coenzyme analogs were used to demonstrate differences in the catalytic properties of the two forms of *Neurospora crassa* malate dehydrogenase (61). The effectiveness of reduction of analogs (analog ratios) has been useful in determining specific properties and heterogeneity of dehydrogenases. Compared to malate dehydrogenases from eucaryotic sources, those from procaryotes have been studied to a lesser degree. Malate dehydrogenases from *Actinomycetes*, *Pseudomonas*, and photosynthetic bacteria have been purified (74-76); however, the mechanisms involved in the hydride transfer reactions are not well understood.

6-Phosphogluconate dehydrogenase (6-PGDH) catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and carbon dioxide. In all cases studied, the enzyme required NADP as the coenzyme, with NAD being completely inactive. This enzyme in conjunction with glucose 6-phosphate dehydrogenase is important in the generation of NADPH for lipid synthesis. 6-Phosphogluconate dehydrogenases have been isolated from bacterial, yeast, and mammalian sources

(102,116-126). In general, these enzymes are dimeric with a monomer molecular weight of 50,000 (116-126). Direct binding studies of sheep liver 6-PGDH with coenzyme and substrate suggested two identical subunits with two independent binding sites for the coenzyme and substrate (128). Sulfhydryl groups were indicated as important in catalysis of many of the 6-phosphogluconate dehydrogenases studied (102,129,130). Kinetic studies of 6-phosphogluconate dehydrogenases from sheep and pig liver were consistent with an ordered bi ter mechanism, with NADP being the first substrate bound, NADPH the last product released, and carbon dioxide the first product released (131).

The main objective of the present study is to establish mechanisms of pyridine nucleotide-dependent dehydrogenases in *H. influenzae*. The enzymes, malate dehydrogenase and 6-phosphogluconate dehydrogenase, were purified and characterized with respect to kinetic parameters, substrate specificity, and selective inhibition.

## CHAPTER III

# EXPERIMENTAL PROCEDURES

### Materials

*Haemophilus influenzae* strain Rd was obtained from Dr. William L. Albritton of the University of Saskatchewan, Saskatoon. Brain Heart Infusion was obtained from Fisher Scientific. All mono- and dinucleotides were purchased from Sigma except thionicotinamide adenine dinucleotide, 3-pyridinealdehyde adenine dinucleotide, nicotinamide 1,N<sup>6</sup>-ethenoadenine dinucleotide, 3-aminopyridine adenine dinucleotide, pyridine adenine dinucleotide, 3-pyridylcarbinol adenine dinucleotide, 3-pyridylacetonitrile adenine dinucleotide, 3-methylpyridine adenine dinucleotide, 3-pyridylacryloamide adenine dinucleotide, and thionicotinamide adenine dinucleotide phosphate which were prepared by published procedures (77-79). Protamine sulfate, streptomycin sulfate, hemin, and histidine were obtained from Sigma. Phenyl-Sepharose CL-4B, 2',5'ADP-Sepharose 4B, and Sephadex G-100 were purchased from Pharmacia. Matrex Red Gel A, Matrex Green Gel A, and Matrex Blue Gel A were obtained from Amicon. Acrylamide, N,N'-methylenebisacrylamide, and Coomassie Brilliant Blue G-250 were purchased from Eastman Kodak. Coomassie Brilliant Blue R-250 was pur-

chased from Bio-Rad. Phenazine methosulfate (PMS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nitro blue tetrazolium (NBT), fluorescein mercuric acetate (FMA), protein molecular weight standards, pyridoxal 5'-phosphate, dithiothreitol (DTT), and Sephacryl S-200 were purchased from Sigma. N<sup>1</sup>-Alkylnicotinamide chlorides were synthesized as previously reported (80). Propionic acid, butyric acid, pentanoic acid, hexanoic acid, octanoic acid, nonanoic acid, and decanoic acid were purchased from Eastman Kodak. Procion Blue HB and heptanoic acid were purchased from Aldrich. 6-Phosphogluconate, D-glucose 6-phosphate, L- $\alpha$ -glycerophosphate, glutathione (oxidized), 3-phosphoglycerate, and fructose 1,6-diphosphate were obtained from Sigma. Succinate was purchased from Baker. Other hydroxy and keto acids, dicarboxylic acids, and buffer salts were purchased from Sigma. All other chemicals were of reagent grade.

## Methods

*Growth of the Organism - Haemophilus influenzae* was grown in 750 ml of Brain Heart Infusion medium in 2.8-l Fernbach flasks at 37°C. The medium containing 28 g of Brain Heart Infusion in 750 ml of distilled water was autoclaved for 15 min at 120°C. A 7.5-ml NAD solution (300  $\mu$ g/ml) and 7.5 ml of a hemin-histidine solution were then added to the cooled broth. The hemin-histidine solution was prepared by adding 7.5 mg each of hemin and histidine to 3.5 ml of distilled water and 0.3 ml of triethanolamine. This mixture was incubated in a water bath at 55°C for 10 min and then an additional 3.8 ml of water were added. Both NAD and hemin-histidine solutions were filter-sterilized using a 0.2-micron Gelman Acrodisc disposable filter assembly while adding to the media. Streptomycin sulfate (450 mg) was added directly to the autoclaved media. Growth was initiated by inoculation with an inoculum that had been stored frozen. Inocula were prepared by mixing 2.5-ml aliquots of the medium containing the bacteria grown to late log phase with 0.5 ml of glycerol. The prepared inocula were frozen rap-

idly in an ethanol - dry ice bath and stored at  $-70^{\circ}\text{C}$  until time of use. Cells were grown in a New Brunswick G-25R incubator with shaking at 120 cycles/min for nine hours. The cells were harvested by centrifugation at  $18,000 \times g$  for 12 min, washed in 50 mM potassium phosphate buffer, pH 7.0, resuspended with a minimal amount of the same buffer, and stored at  $-15^{\circ}\text{C}$  until used.

*Polyacrylamide Gel Electrophoresis* - Polyacrylamide disc gel electrophoresis was performed by the method of Davis (81), and Weber and Osborn (82). A 7.5% separating gel with a 2.5% stacking gel was used. Electrophoresis was carried out at 2.5 mA per gel at  $4^{\circ}\text{C}$ . Gels were stained overnight in 0.25% Coomassie Brilliant Blue R-250, 40% ethanol, and 15% acetic acid. Destaining was followed with a solution containing 40% ethanol and 15% acetic acid for 4 hours. Stained gels were stored in 10% acetic acid. Activity staining for MDH was performed using a redox dye coupling method with PMS and NBT. A freshly-prepared solution containing 50 mM malate, 0.5 mM NAD, 1 mM NBT, 0.1 mM PMS, and 20 mM Hepes, pH 8.5 was used for the staining. Gels were placed in this solution for 3 min in the dark until the purple bands appeared. Stained gels were transferred to 5% trichloroacetic acid for 1 min, rinsed and stored in aqueous solution.

*SDS-Polyacrylamide Gel Electrophoresis* - The subunit molecular weight of the enzyme was determined using 10% SDS gels according to the method of Laemmli (83). Slab gel electrophoresis was carried out using a BRL vertical gel electrophoresis system. Gels were stained by the silver staining method. Gels were fixed in 50% methanol overnight, and then stained in the staining solution for 25 min with constant gentle agitation (40-60 rpm). The staining solution contained 1.0 ml of 7.6% sodium hydroxide, 1.4 ml of 14.8 M ammonium hydroxide, and 1.0 ml of 4.7 M silver nitrate in 100 ml distilled water. The gels were washed with agitation in 2 changes of distilled water for 2 min each. Gels were placed in a developing solution until the band(s) appeared. The developing solution contained 1 ml of 1% citric acid and 0.1 ml 37% formaldehyde in

200 ml deionized water. Once the band(s) became visible, the reaction was stopped by adding 5% acetic acid for 1 min. Gels were rinsed and stored in 20% methanol. Molecular weight of the protein was determined relative to the molecular weight standards of  $\beta$ -galactosidase, 116,250; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400.

*Fluorescence Titration of MDH* - Fluorescein mercuric acetate (FMA) titration of MDH was performed as reported by Dubler and Anderson (84). Stock solutions of 11.9  $\mu$ M FMA were freshly prepared in 50 mM potassium phosphate buffer, pH 7.5. A standard curve for FMA was obtained by adding successive 5- $\mu$ l increments of the stock FMA solution to 2.9 ml of 50 mM potassium phosphate buffer, pH 7.5, either in the presence or absence of 8 M urea. Relative fluorescence intensity in the presence of indicated amount of enzyme in conjunction with the absence of enzyme was measured with emission at 525 nm and excitation at 495 nm. Fluorescence measurements were made on a Perkin-Elmer 650-40 spectrofluorometer.

*Chemical Modification of MDH and 6-PGDH* - Inactivation of MDH by sulfhydryl group or lysine modifying reagents were carried out at 25°C in reaction mixtures (0.1 ml) containing 10 mM potassium phosphate, pH 7.0, 500 ng enzyme, and the indicated amount of reagents. DEPC inactivation reactions were performed at 15°C in reaction mixtures (0.1 ml) containing 10 mM potassium phosphate, pH 7.0, 800 ng enzyme, and the indicated amount of DEPC which was kept at 4°C. Inactivation of 6-PGDH by N-ethylmaleimide was performed at 25°C in reaction mixtures (0.1 ml) containing 10 mM potassium phosphate, pH 7.0, 0.25 ng enzyme, 5% ethanol (to solubilize the maleimide), and the indicated amount of N-ethylmaleimide. At timed intervals, aliquots (10  $\mu$ l) were removed from these reaction mixtures and the remaining enzyme activities were measured spectrophotometrically and plotted on the semilog scale.

*Assays of Selected Enzyme Activities* - The various dehydrogenases studied were assayed at 23°C in 1-ml reaction mixtures containing the following components: (a)

malate dehydrogenase in 45 mM glycine-NaOH buffer, pH 9.6, 3 mM malate, and 0.54 mM NAD; (b) 6-phosphogluconate dehydrogenase in 10 mM potassium phosphate buffer, pH 7.0, 1 mM 6-phosphogluconate, and 0.1 mM NADP; (c) glutamate dehydrogenase in 100 mM potassium phosphate buffer, pH 8.0, 50 mM glutamate, and 0.15 mM NADP; (d) glucose 6-phosphate dehydrogenase in 60 mM glycine-NaOH buffer, pH 9.6, 4 mM glucose 6-phosphate, and 0.1 mM NADP; (e) alcohol dehydrogenase in 25 mM sodium phosphate buffer, pH 8.0, 100 mM ethanol, and 0.54 mM NAD; (f) lactate dehydrogenase in 50 mM Tris-HCl buffer, pH 8.0, 50 mM L-lithium lactate, and 0.54 mM NAD; (g) L- $\alpha$ -glycerophosphate dehydrogenase in 50 mM Tris-HCl buffer, pH 8.0, 4 mM L- $\alpha$ -glycerophosphate, and 0.54 mM NAD; and (h) 3-phosphoglycerate dehydrogenase in 30 mM Tris-HCl buffer, pH 8.5, 2 mM 3-phosphoglycerate, and 0.54 mM NAD. The initial velocities for each enzyme were determined by measuring the increase in absorbance at 340 nm resulting from the reduction of NAD or NADP. The reverse reaction of malate dehydrogenase was assayed in 1-ml reaction mixtures containing 50 mM Tris-HCl buffer, pH 8.5, 40  $\mu$ M oxalacetate, and 15  $\mu$ M NADH. The initial velocity of oxalacetate reduction was determined by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH.

Glutathione reductase activity was measured in a reaction mixture containing 125 mM sodium phosphate buffer, pH 7.5, 1.25 mM EDTA, 4.5 mM oxidized glutathione, and 0.3 mM NADPH in a final volume of 3.0 ml. The initial velocity was determined by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADPH. A unit of enzyme activity was defined as 1  $\mu$ mol of coenzyme oxidized or reduced per min.

Succinate dehydrogenase activity was measured in a reaction mixture containing 42 mM sodium phosphate buffer, pH 7.5, 0.42 mM EDTA, 10 mM succinate, 0.35 mM MTT, and 0.12 mM PMS in a final volume of 1.0 ml. Lactate oxidase activity was measured in a mixture containing 50 mM Tris-HCl buffer, pH 8.0, 50 mM L-lithium

lactate, 0.35 mM MTT, and 0.12 mM PMS in a final volume of 1 ml. Enzyme activities were followed by the reduction of MTT at 578 nm.

Fumarase was assayed in a reaction mixture containing 7.5 mM sodium fumarate, and 30 mM potassium phosphate buffer, pH 7.3 in a final volume of 1 ml. The initial rate of decrease in absorbance at 300 nm was measured.

Ultraviolet and visible absorbance measurements were performed on a Beckman Acta MVI recording spectrophotometer. All measurements of pH were made on a Radiometer digital PHM 52 pH meter equipped with a GK 2321 C combination electrode.

Protein concentrations were determined by the microprotein assay of the Coomassie blue method (85) using bovine serum albumin as a standard. Ultrafiltration and protein concentration were carried out using Amicon PM-10 membranes.

## CHAPTER IV

### RESULTS

Selected enzyme activities were measured in supernatants of 50% *H. influenzae* cell sonicates in 50 mM potassium phosphate buffer, pH 7.0. The presence of MDH, 6-PGDH, glucose 6-phosphate dehydrogenase, glutamate dehydrogenase, succinate dehydrogenase, glutathione reductase, fumarase, and lactate oxidase was demonstrated. Relative activities of these enzymes are listed in Table I. MDH and 6-PGDH activities were observed to be higher than the other enzymes studied.

#### *Purification of H. influenzae MDH*

Fourteen grams of frozen *H. influenzae* cells were dispersed with 14 ml of 50 mM potassium phosphate buffer, pH 7.0 at 4°C using a glass homogenizer. This 50% (w/v) homogenate was sonicated in an ice/salt bath using the microprobe tip of an Ultrasonics Sonifer Cell Disruptor and centrifuged at 17,000 x g for 20 min. The sonication was repeated twice using the pellets of the centrifugations and the supernatants were combined for the protamine sulfate step.

A protamine sulfate solution was added to the sonicate supernatant to remove nucleic acids and the resulting 0.2% protamine sulfate solution was stirred at 4°C for

**Table I. Selected Enzymes in *H. influenzae* Cell Sonicate Supernatant**

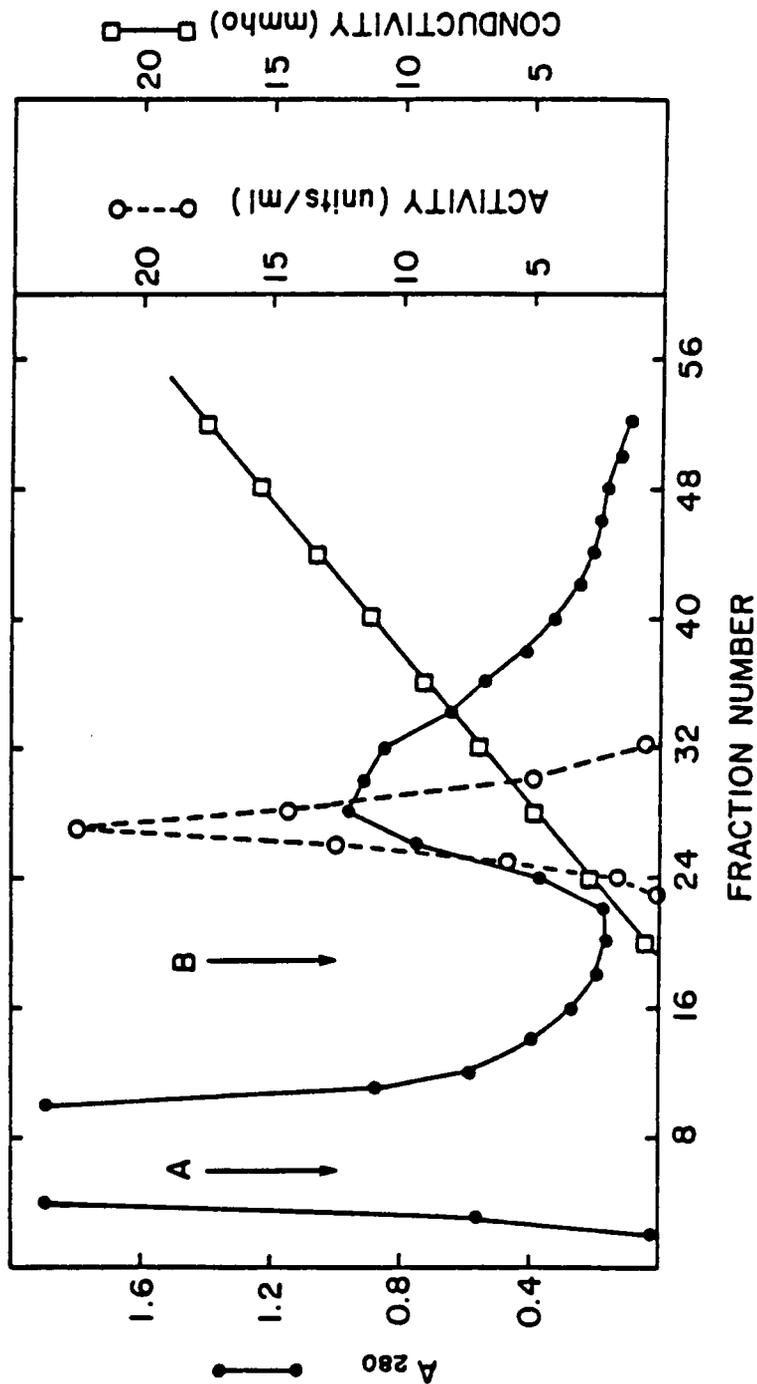
Enzyme	Activity (units/ml)
Malate dehydrogenase	9.5
6-Phosphogluconate dehydrogenase	2.4
Succinate dehydrogenase	1.1
Glucose 6-phosphate dehydrogenase	0.66
Lactate oxidase	0.56
Glutamate dehydrogenase	0.38
Glutathione reductase	0.35
Fumarase	0.32
Alcohol dehydrogenase	— <sup>a</sup>
L- $\alpha$ -Glycerophosphate dehydrogenase	— <sup>a</sup>
Lactate dehydrogenase	— <sup>a</sup>
3-Phosphoglycerate dehydrogenase	— <sup>a</sup>

a = not detected

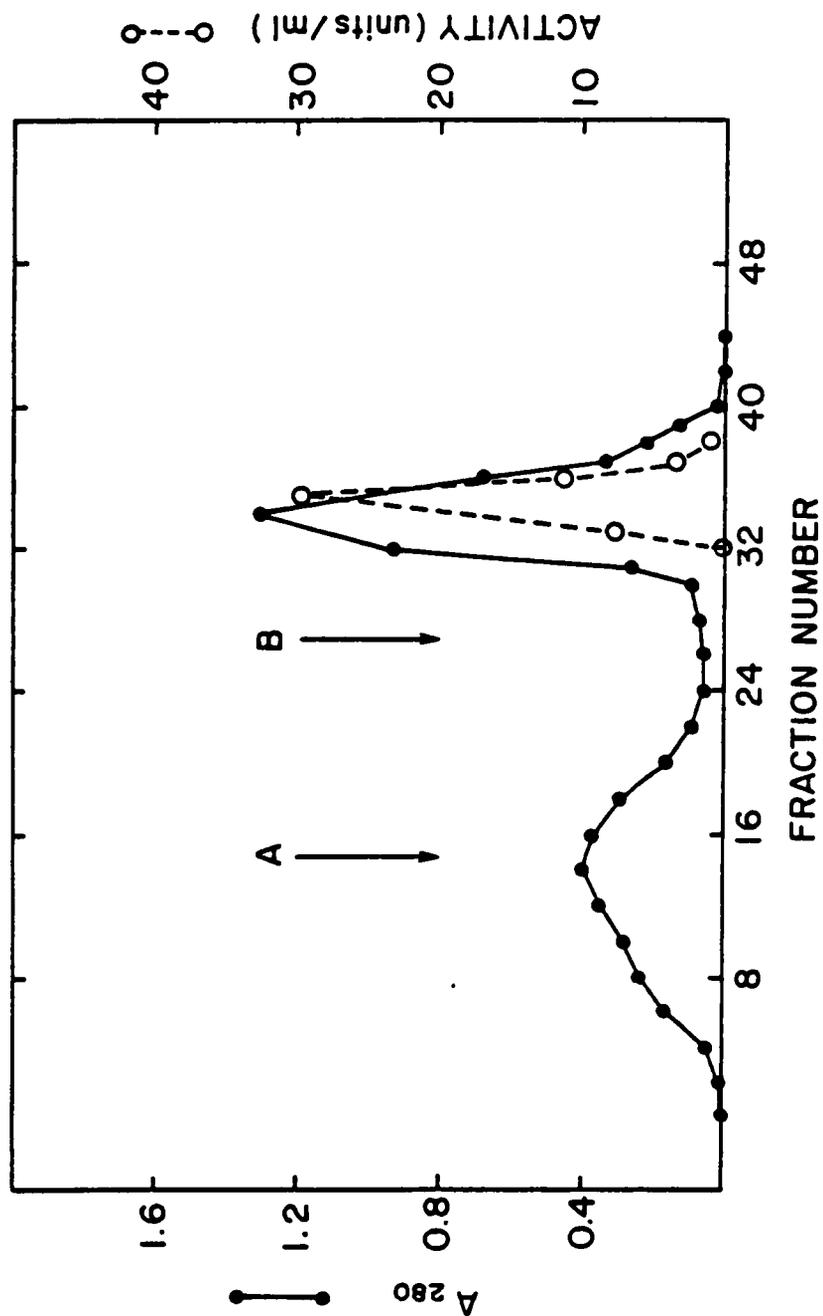
30 min in an ice-bath. After centrifugation at 39,000 x g for 20 min, the pellet from this protamine sulfate step was discarded. The supernatant was adjusted to 40% ammonium sulfate saturation by slowly adding finely-powdered ammonium sulfate with continuous stirring at 4°C. The suspension was centrifuged at 11,000 x g for 30 min and the resulting supernatant was adjusted to 60% ammonium sulfate saturation. The suspension was centrifuged at 11,000 x g for 30 min, the supernate was discarded, and the pellet was resuspended in 10 ml or more of 10 mM potassium phosphate buffer, pH 7.0 to adjust the protein concentration to 8 mg/ml.

At this step in the purification procedure, the MDH would not bind to affinity chromatography resins at protein concentrations higher than 8 mg/ml. All column purification steps were carried out at 4°C. The diluted protein solution was applied to a Matrex gel Green A column (1.0 x 6 cm) equilibrated in 10 mM potassium phosphate buffer, pH 7.0. The column was then washed with the equilibration buffer to remove unbound protein. The malate dehydrogenase was eluted using a linear salt gradient, 0.05 to 0.8 M KCl in 10 mM potassium phosphate buffer, pH 7.0 (2 x 100 ml). The elution profile is shown in Figure 2. Fractions (2.4 ml) were collected and assayed for enzyme activity, and the protein concentration was monitored by 280-nm absorbance. Those fractions containing enzyme activity were pooled and used in the next purification step.

The pooled sample from the Matrex gel Green A column was adjusted to a final concentration of 1 M KCl by adding solid KCl. This solution was applied to a Phenyl-Sepharose CL-4B hydrophobic column (1.0 x 9.5 cm) that was previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0, 1 M KCl. Unbound proteins were washed from the column with the equilibration buffer. The enzyme was eluted with the lower ionic strength 10 mM potassium phosphate buffer, pH 7.0. The elution profile is shown in Figure 3. Fractions (2.4 ml) containing enzyme activity were pooled and used for further fractionation.



**Figure 2.** Affinity Chromatography on Matrex Gel Green A Column: The 40-60% ammonium sulfate fractionated-protein was resuspended in 10 mM potassium phosphate buffer, pH 7.0 (8 mg/ml), and applied to a Matrex gel Green A column (1.0 x 6 cm) that had been equilibrated with 10 mM potassium phosphate buffer, pH 7.0. Arrow A indicates the point of initiation of washing with the equilibration buffer. Arrow B indicates the initiation of a linear salt gradient of 0.05 to 0.8 M KCl in the equilibration buffer (2 x 100 ml).

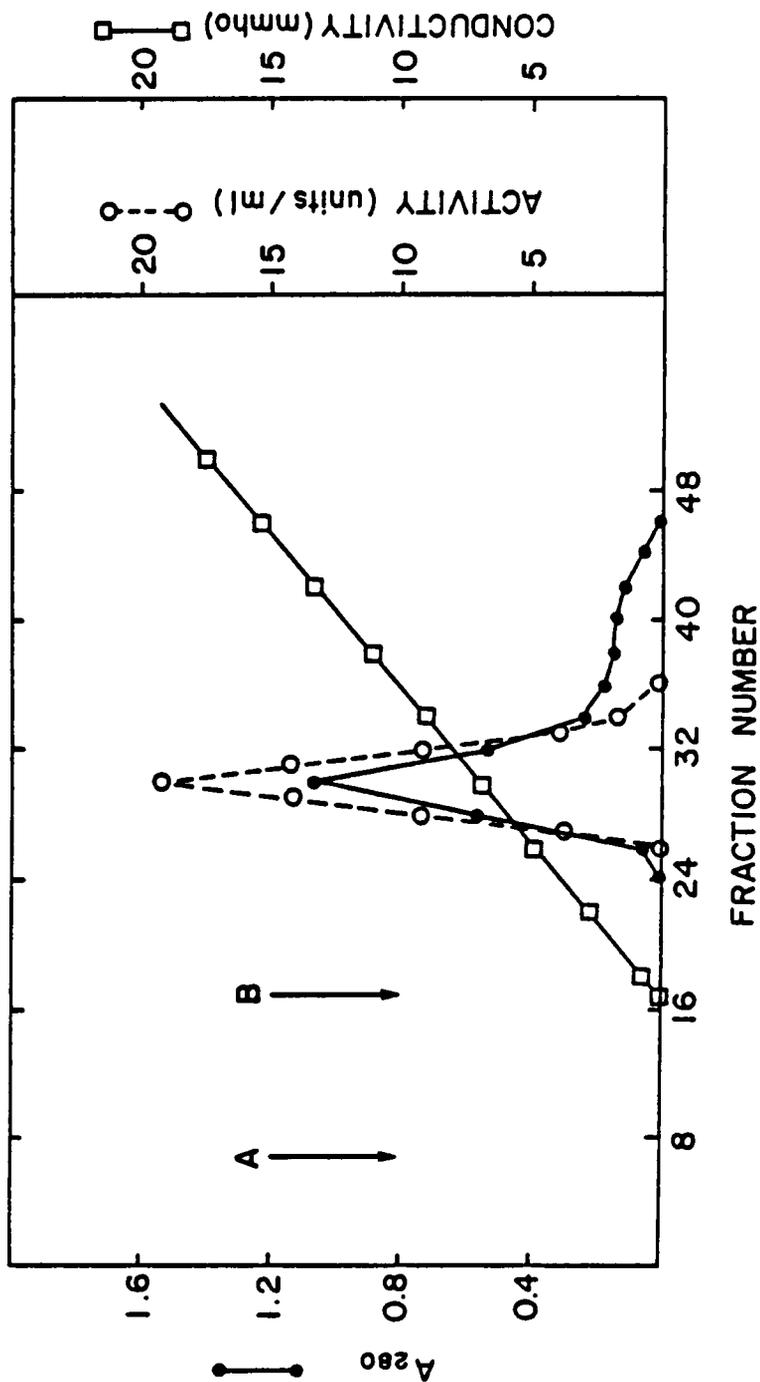


**Figure 3. Phenyl-Sepharose CL-4B Column Chromatography:** The pooled Green A column sample, adjusted to 1 M KCl, was applied to a Phenyl-Sepharose column (1.0 x 9.5 cm) that had been equilibrated with 10 mM potassium phosphate buffer, pH 7.0, 1 M KCl. Arrow A indicates the point of initiation of washing with the equilibration buffer. Arrow B indicates the starting point of elution with the lower ionic strength 10 mM potassium phosphate buffer, pH 7.0.

The sample from the Phenyl-Sepharose column was applied to a Matrex gel Blue A column (1.0 x 5 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. Unbound proteins were removed by washing with the equilibration buffer. The malate dehydrogenase was then eluted from the column using a linear salt gradient, 0.05 to 0.8 M KCl in the same phosphate buffer (2 x 100 ml). The elution profile is shown in Figure 4. Fractions (2.4 ml) were collected and those fractions containing MDH activity were pooled and concentrated by ultrafiltration. A summary of the *H. influenzae* MDH purification procedure is shown in Table II. The enzyme was purified 109-fold with a 26% yield and a final specific activity of 104  $\mu\text{mol}/\text{min}$  per mg protein.

### *Properties of the Purified Enzyme*

The purified enzyme was analyzed by polyacrylamide gel electrophoresis at pH 8.3. The enzyme migrated as a single protein band which corresponded to a malate dehydrogenase determined by activity staining techniques. The molecular weight of the native purified MDH was determined by Sephadex G-100 molecular exclusion column chromatography at 4°C. The column (1.5 x 80 cm) was equilibrated with 50 mM Tris-HCl buffer, pH 8.0, 200 mM KCl. Elution volumes of MDH and standard proteins were determined by measuring the enzyme activity and the protein absorbance at 280 nm, respectively. The calculated  $V_e/V_0$  value of the purified enzyme was 1.69 corresponding to an apparent  $M_r$  of 62,000 as shown in Figure 5 (line A). The purified enzyme exhibited an apparent molecular weight of 60,000 when analyzed by a Bio-Sil TSK-250 gel filtration column using HPLC. SDS-Polyacrylamide gel electrophoresis was employed to determine the molecular weight and the number of subunits. Electrophoretic conditions were essentially those reported by Dreyfuss *et al.* (86), and Thomas and Kornberg (87). In comparison to relative mobilities with standard proteins, an apparent molecular weight of 31,000 was obtained as illustrated in Figure 5 (line B).



**Figure 4. Affinity Chromatography on Matrex Gel Blue A:** The pooled Phenyl-Sepharose column sample was applied to a Matrex gel Blue A column (1.0 x 5 cm) that had been equilibrated with 10 mM potassium phosphate buffer, pH 7.0. Arrow A indicates the point of initiation of washing with the equilibration buffer. Arrow B indicates the point of initiation of a linear salt gradient of 0.05 to 0.8 M KCl in the equilibration buffer (2 x 100 ml).

**Table II. Purification of *H. influenzae* MDH**

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Cell sonicate	411.4	391	1	100	1
Ammonium sulfate pellet	159.1	371	2	95	2.4
Matrex gel Green A	15.1	246	16	63	17
Phenyl-Sepharose	2.1	153	73	39	77
Matrex gel Blue A	1.0	104	104	26	109

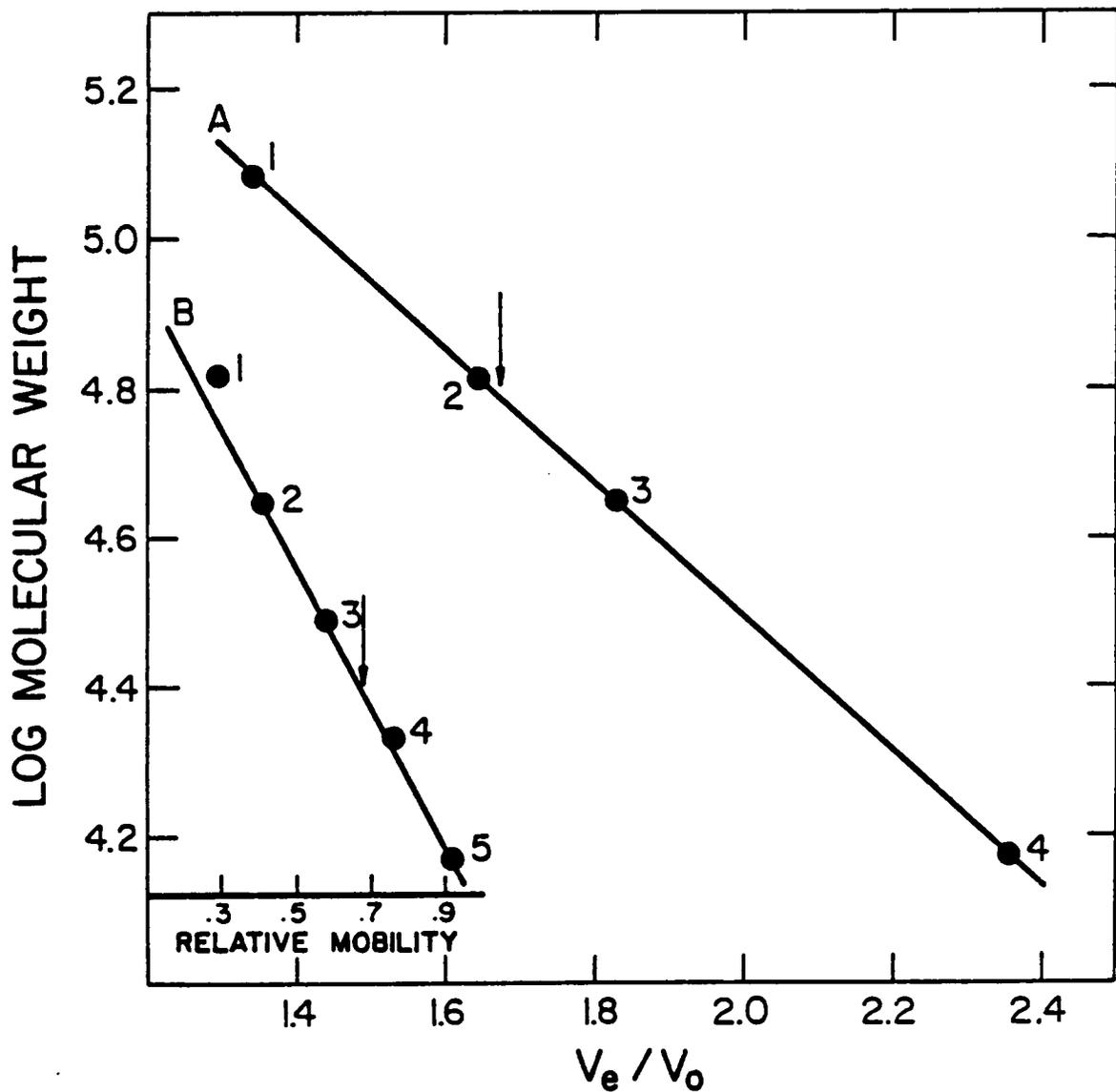


Figure 5. Molecular Weight Determination of *H. influenzae* MDH: A. Gel filtration on Sephadex G-100 column. Molecular weight standards were 1)  $\beta$ -galactosidase, 2) bovine serum albumin, 3) ovalbumin, and 4) lysozyme. B. SDS-Polyacrylamide gel electrophoresis. Molecular weight standards were 1) bovine serum albumin, 2) ovalbumin, 3) carbonic anhydrase, 4) soybean trypsin inhibitor, and 5) lysozyme. The arrows indicate positions determined for *H. influenzae* MDH.

The purified enzyme produced a typical ultraviolet-visible absorption spectrum with a single absorbance maximum at 275 nm. The fluorescence spectrum of the purified enzyme is shown in Figure 6. The enzyme had an excitation maximum at 286 nm and an emission maximum at 337 nm. This is characteristic of the presence of tryptophan residues in the protein.

The purified enzyme was routinely stored at  $-15^{\circ}\text{C}$  at a protein concentration of 200  $\mu\text{g}/\text{ml}$  in a 50% propylene glycol solution containing 100 mM KCl and 10 mM potassium phosphate buffer, pH 7.0. Under these conditions, the enzyme was stable for at least three months with no apparent loss of activity. The enzyme was only slightly less stable in the absence of propylene glycol. The purified enzyme, however, was denatured in more dilute solutions at moderate temperatures. When the enzyme activity was measured at timed intervals during incubation in 10 mM potassium phosphate buffer, pH 7.0, at different temperatures, the rate of thermal denaturation followed first-order kinetics as shown in Figure 7. At  $50^{\circ}\text{C}$ , the rate constant for thermal denaturation was  $0.051 \text{ min}^{-1}$ . The presence of 0.3 mM NAD protected against thermal denaturation resulting in a 46% decrease in the rate constant to  $0.028 \text{ min}^{-1}$ . Concentrations of malate as high as 4 mM did not provide any protection against thermal denaturation.

The effect of pH on enzyme activity was investigated using three different buffers having overlapping  $pK_a$  ranges, such as 45 mM Tris-acetate, 45 mM Hepes-NaOH, and 45 mM glycine-NaOH. Initial velocities of NAD reduction were measured as a function of pH between pH 7.0 and 10.0. With the above buffers and under the conditions of varying NAD concentration at saturating malate, the values of  $V_{\text{max}}/K_m$  exhibited an optimal pH range from pH 9.0 to 10.0 for MDH. At pH 9.6 with glycine-NaOH buffer, the ionic strength of the incubation mixture was increased by the addition of KCl from 10 to 100 mM. The initial velocity values of NAD reduction were not affected by this treatment.

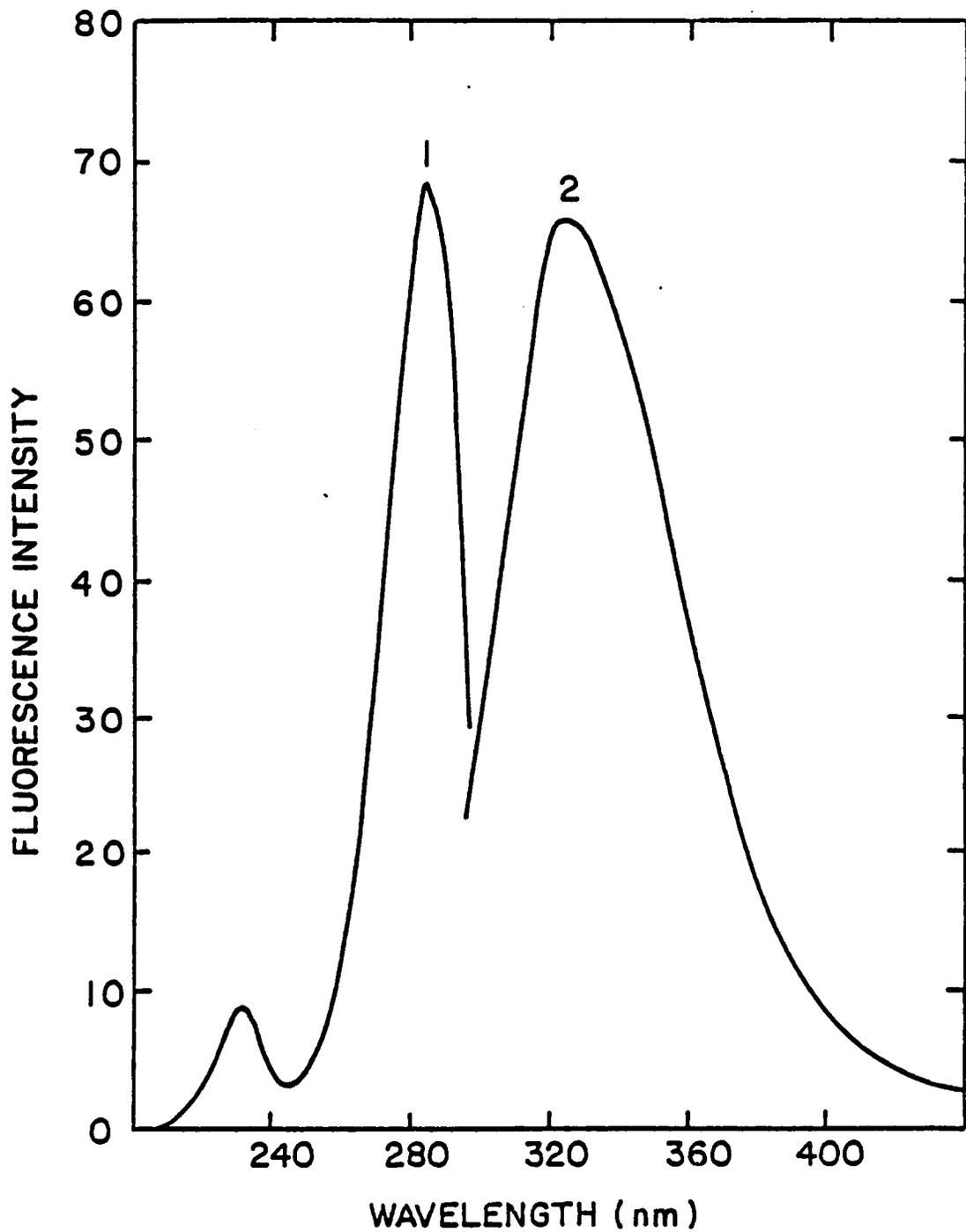


Figure 6. The Fluorescence Spectrum of *H. influenzae* MDH: The excitation spectrum (1) was determined with an emission wavelength at 337 nm and the emission spectrum (2) was determined at an excitation wavelength at 286 nm.

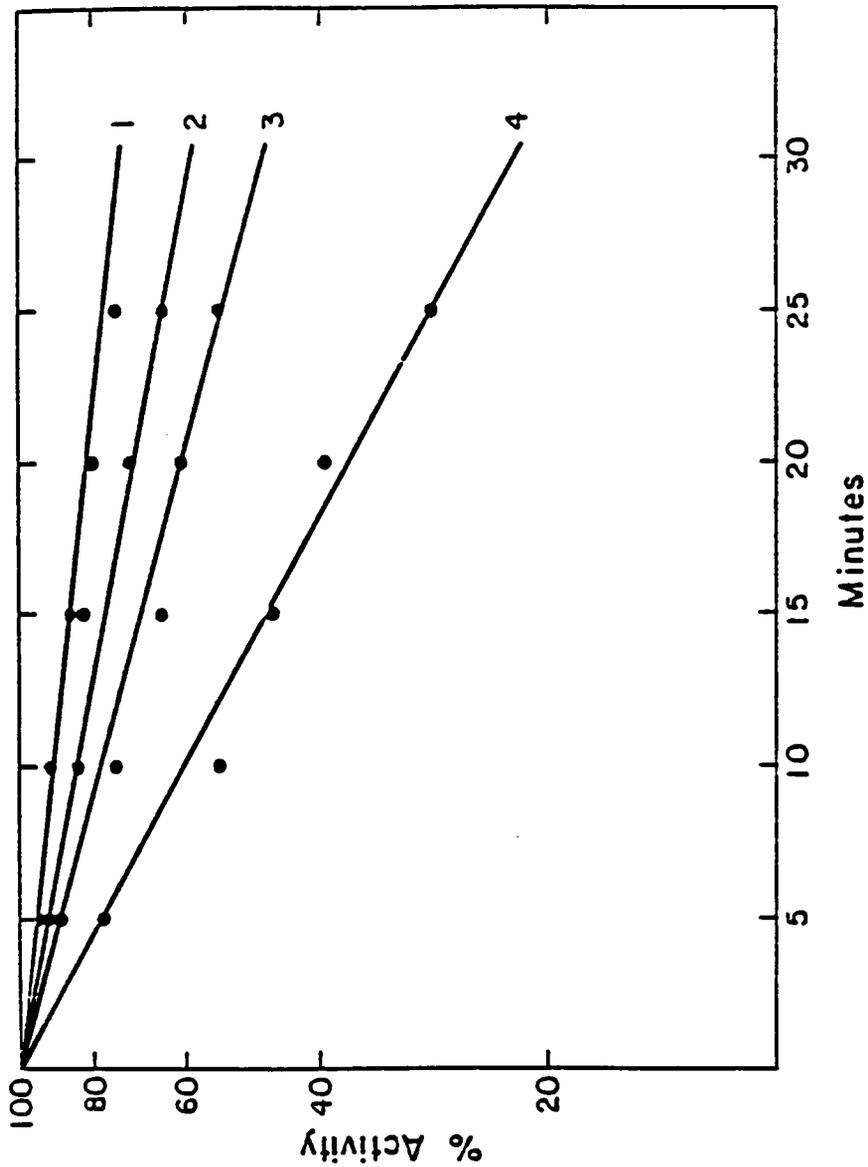


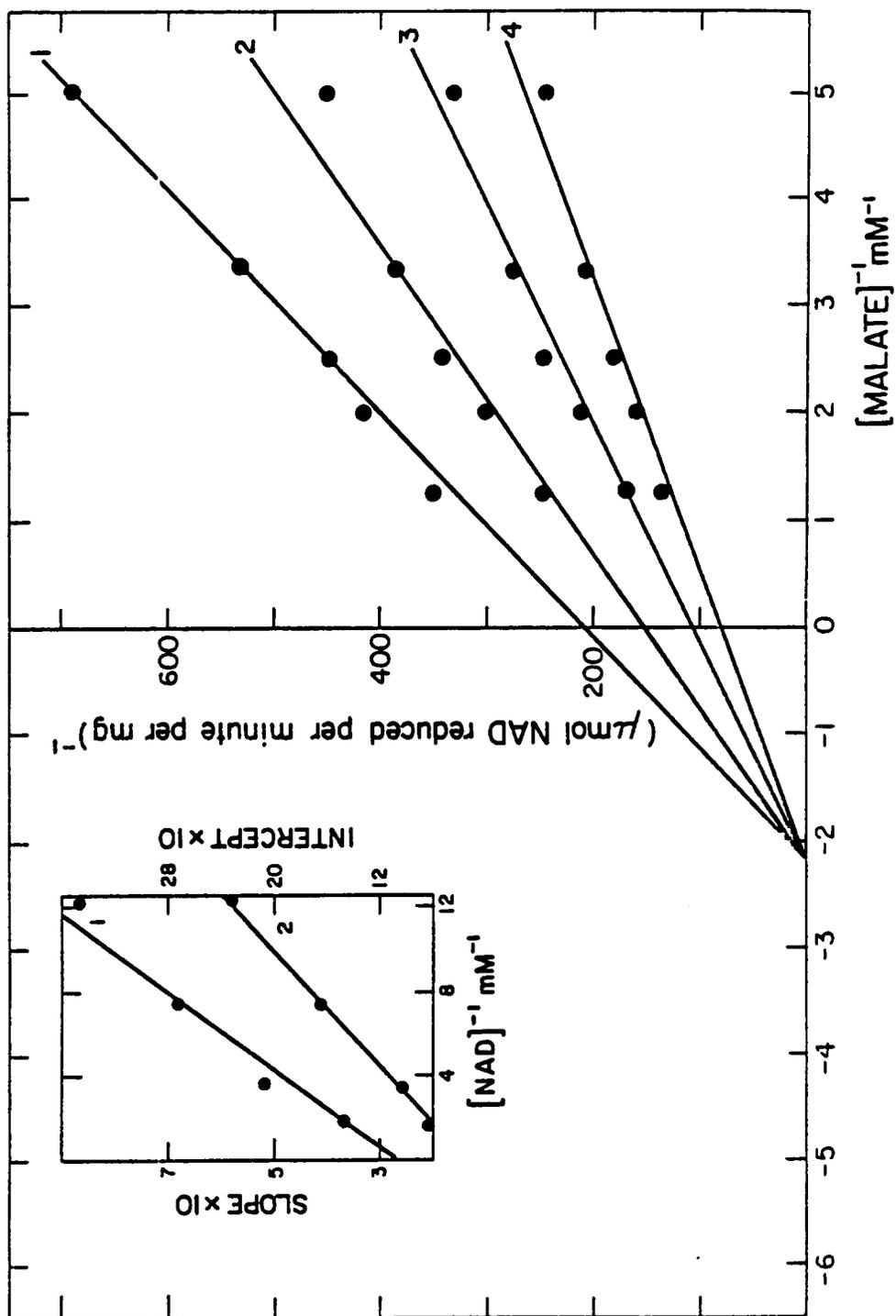
Figure 7. Thermal Denaturation of *H. influenzae* MDH: Incubation mixtures (1 ml) contained 10 mM potassium phosphate buffer, pH 7.0, and 146 ng of enzyme. Enzyme samples were withdrawn at timed intervals, assayed for activity as described in Experimental Procedures, and plotted on the semilog scale. The temperatures of the incubation mixtures were: line 1, 35°C; line 2, 40°C; line 3, 45°C; line 4, 50°C.

## Kinetic Mechanism of Malate Dehydrogenase

Michaelis constants and maximum velocities for both substrates in the forward and reverse directions were determined through initial velocity studies by varying one substrate at several fixed concentrations of the second substrate. NAD reduction was carried out in 45 mM glycine-NaOH buffer, pH 9.6; whereas, NADH oxidation was performed in 50 mM Tris-HCl buffer, pH 8.5. The initial velocities obtained by varying malate concentration at four fixed concentrations of NAD are shown in Figure 8. Slope and intercept replots were used to determine the values for  $K_m$  and  $V_{max}$ . In a separate experiment using saturating concentrations of malate and NAD,  $V_{max}$  values were proportional to enzyme concentration over a 6-fold concentration range. The kinetic parameters for all four substrates in the forward and reverse reactions are listed in Table III. The results obtained by varying each substrate at different fixed concentrations of the appropriate second substrate gave converging line relationships similar to that depicted in Figure 8, indicating a sequential reaction mechanism.

In order to determine whether the sequential reaction mechanism was of an ordered or random type, product and dead-end inhibition (89) were investigated. In the product inhibition studies, inhibition patterns were determined from double reciprocal plots using three fixed product concentrations and varying one substrate at unsaturating or saturating concentrations of the second substrate. When using NAD as the variable substrate, inhibition by one product, NADH, at both unsaturating and saturating concentrations of malate was competitive as shown in Figure 9. When inhibition by NADH was examined with malate as the variable substrate at an unsaturating concentration of NAD, noncompetitive inhibition was observed (Figure 10). No inhibition by NADH was observed when a saturating concentration of NAD was employed.

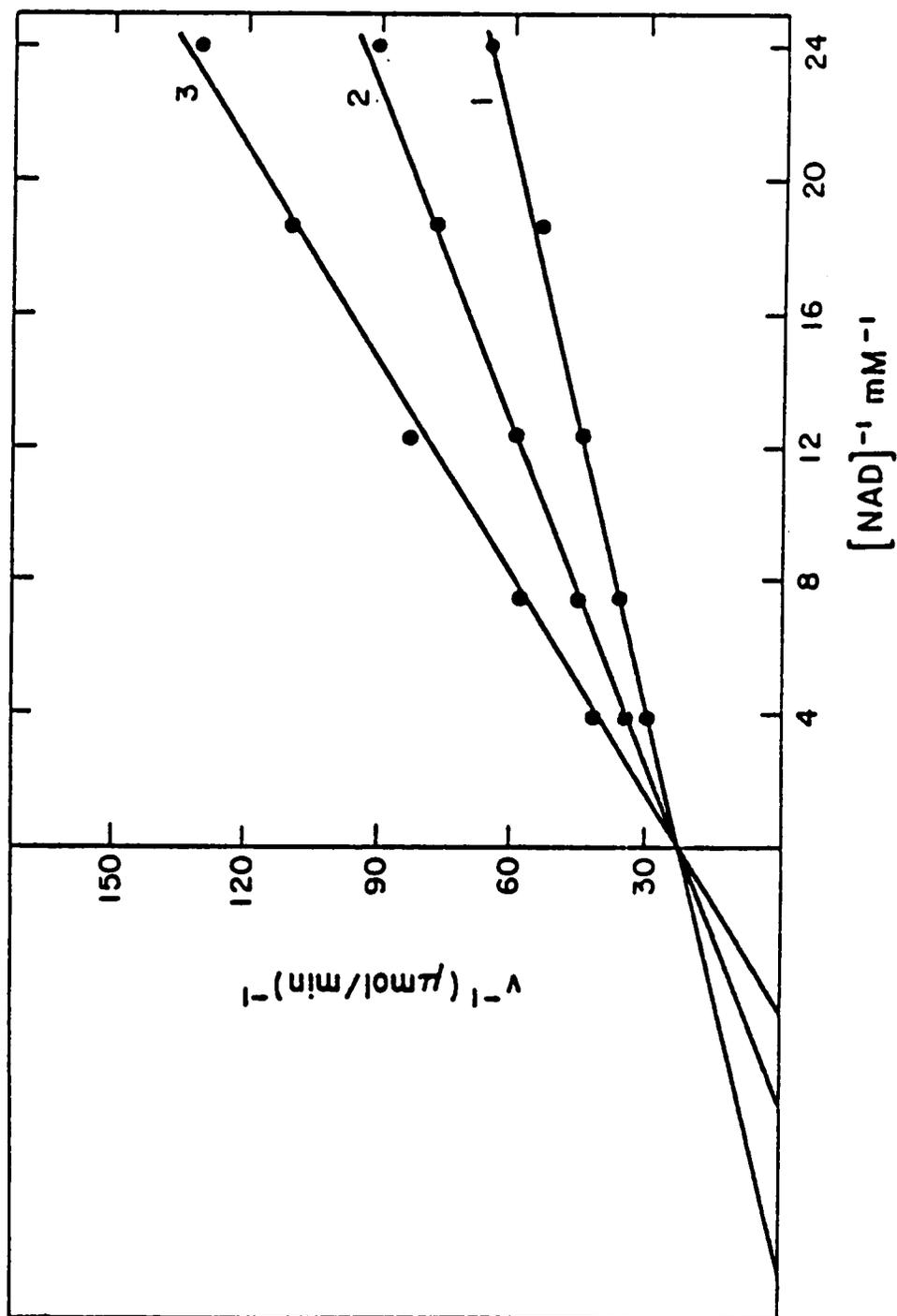
When using NAD as the variable substrate, product inhibition by oxalacetate was evaluated. Noncompetitive inhibition at an unsaturating concentration of malate (Fig-



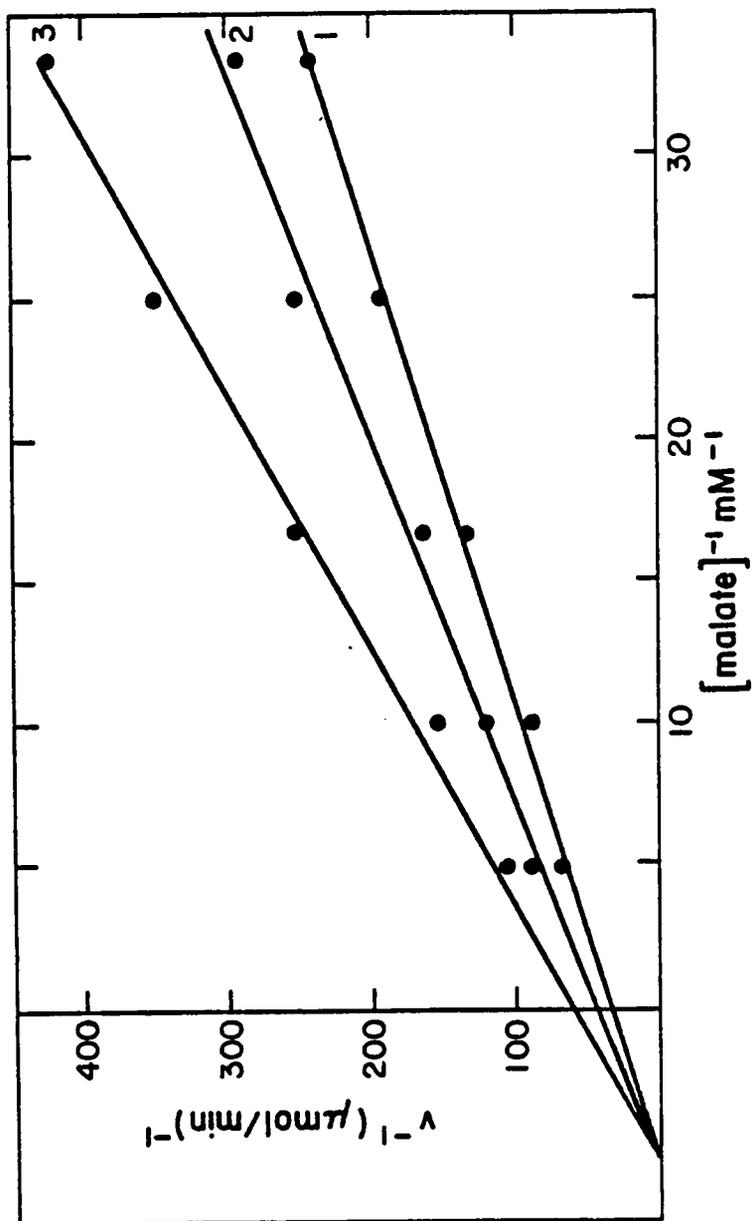
**Figure 8. Effect of NAD and Malate Concentrations on Initial Velocities:** The assay mixtures contained concentrations of malate varying from 0.2 to 0.8 mM at four fixed concentrations of NAD in 45 mM glycine-NaOH buffer, pH 9.6. Reactions were initiated by the addition of 110 ng of MDH. The concentrations of NAD used ( $\mu\text{M}$ ) were: line 1, 81; line 2, 135; line 3, 270; line 4, 540. Inset is a replot of slopes (line 1) and intercepts (line 2).

**Table III.  $K_m$  and  $V_{max}$  Values for Substrates of *H. influenzae* MDH**

Substrate	$K_m$ ( $\mu\text{M}$ )	$V_{max}$ ( $\mu\text{mol}/\text{min}/\text{mg}$ )
Malate	290	23
NAD	57	23
Oxalacetate	13	0.6
NADH	2	0.6



**Figure 9. Product Inhibition of MDH by NADH as a Function of NAD Concentration at a Saturating Concentration of Malate:** Reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 41 to 270  $\mu$ M NAD, 3 mM malate, and 50 ng of enzyme. NADH concentrations were: line 1, none; line 2, 2  $\mu$ M; line 3, 2.5  $\mu$ M.



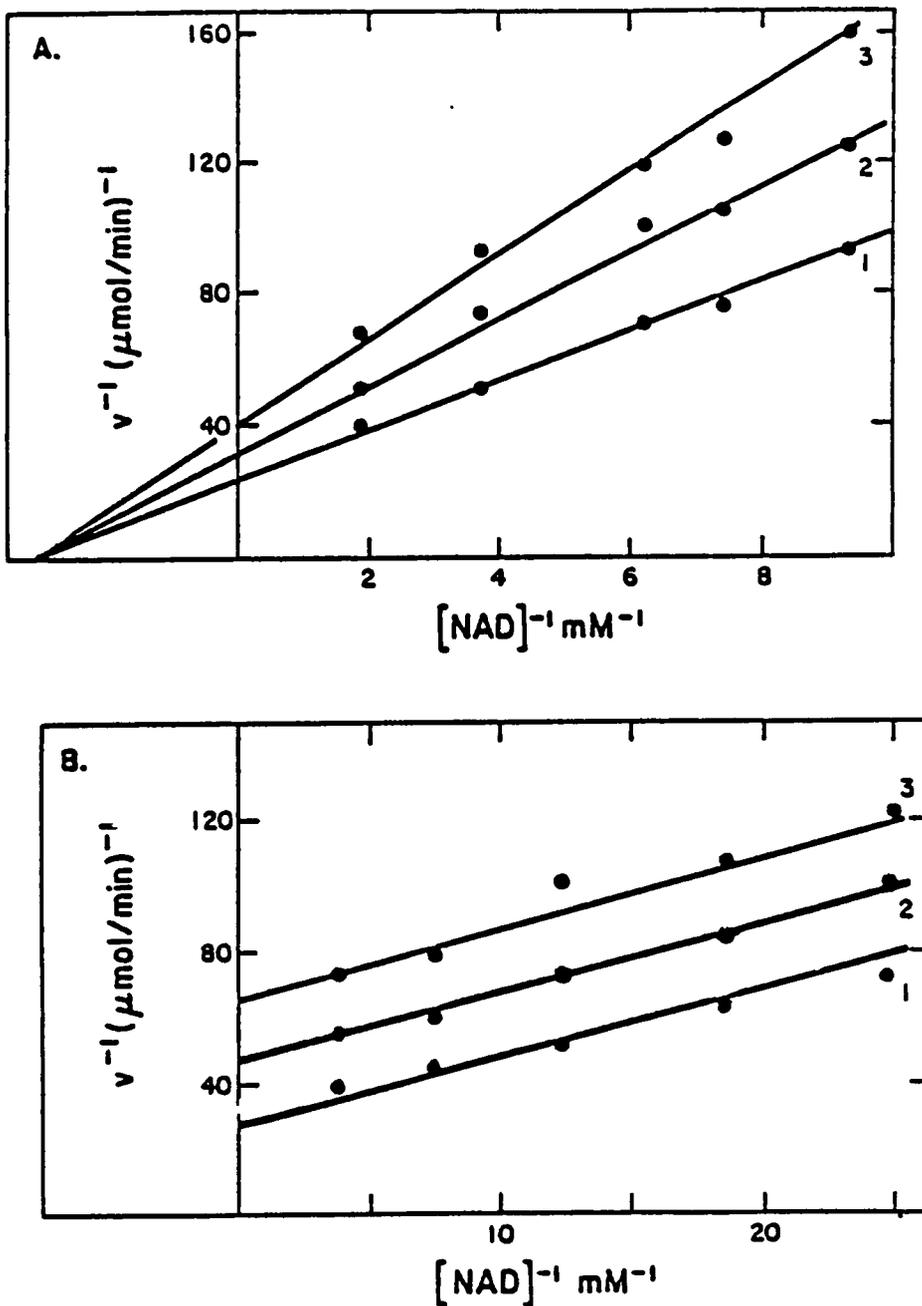
**Figure 10. Product Inhibition of MDH by NADH as a Function of Malate Concentration at an Unsaturating Concentration of NAD:** Reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 54  $\mu\text{M}$  NAD, 30 to 200  $\mu\text{M}$  malate, and 50 ng of enzyme. NADH concentrations were: line 1, none; line 2, 0.5  $\mu\text{M}$ ; line 3, 1  $\mu\text{M}$ .

ure 11, panel A) and uncompetitive inhibition at a saturating concentration of malate (Figure 11, panel B) were observed. When inhibition by oxalacetate was studied with malate as the variable substrate, noncompetitive inhibition was observed at both unsaturating and saturating concentrations of NAD (Figure 12).

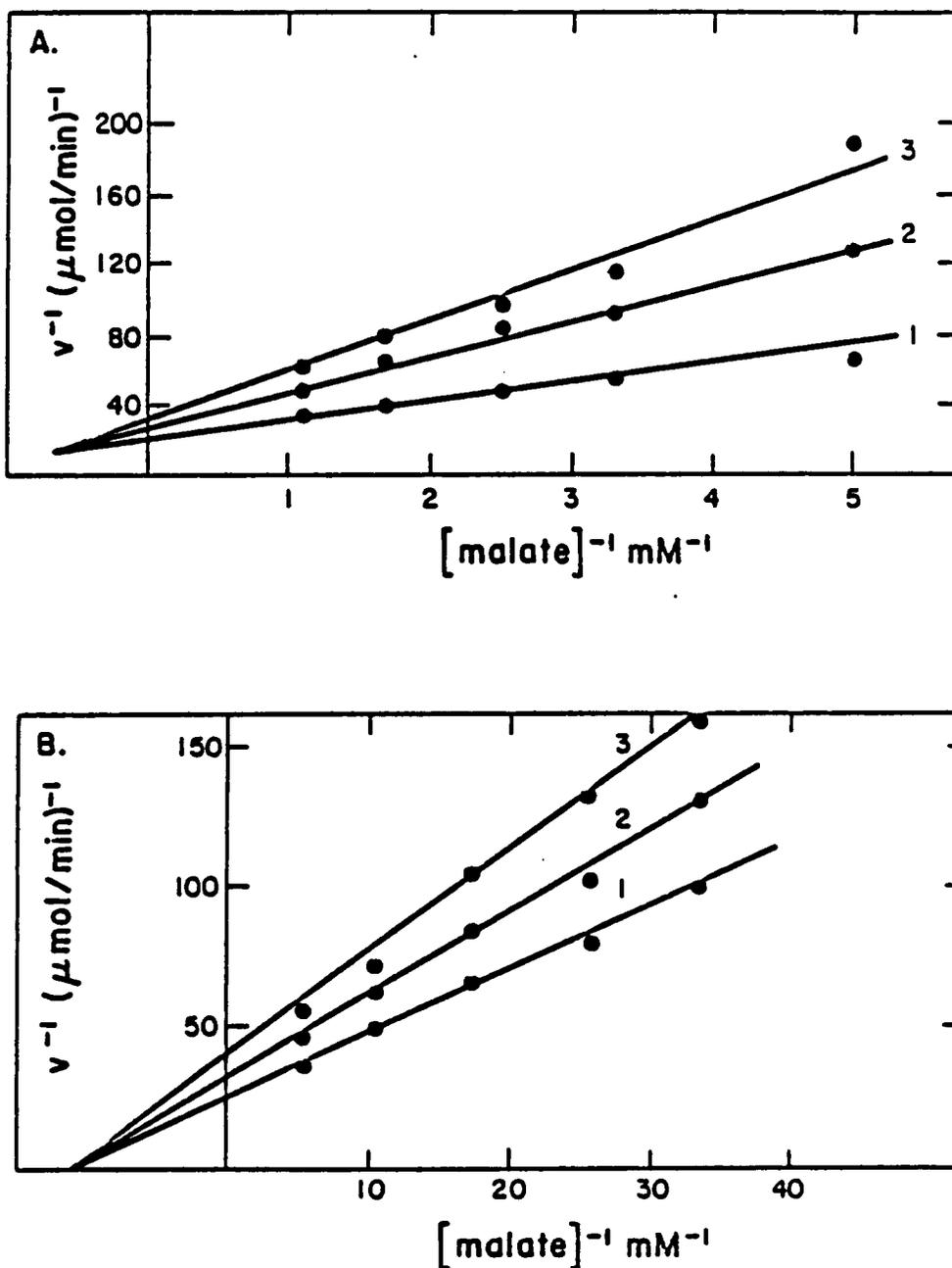
Structural analogs of NAD and malate, adenosine diphosphoribose (ADP-ribose) and hydroxymalonate, respectively, were studied as dead-end inhibitors. Inhibition by ADP-ribose was competitive when NAD was used as the variable substrate (Figure 13, panel A) and noncompetitive when malate was used as the variable substrate (Figure 13, panel B). Inhibition by hydroxymalonate was uncompetitive when NAD was used as the variable substrate (Figure 14, panel A) and competitive when malate was used as the variable substrate (Figure 14, panel B). The  $K_i$  values determined for ADP-ribose and hydroxymalonate were 50  $\mu\text{M}$  and 1.6  $\mu\text{M}$ , respectively. The results of both product and dead-end inhibition studies are summarized in Table IV. The results of these studies were consistent with an ordered bi bi mechanism in which NAD is the first substrate binding to the enzyme and NADH is the second product dissociating from the enzyme.

### Coenzyme Specificity

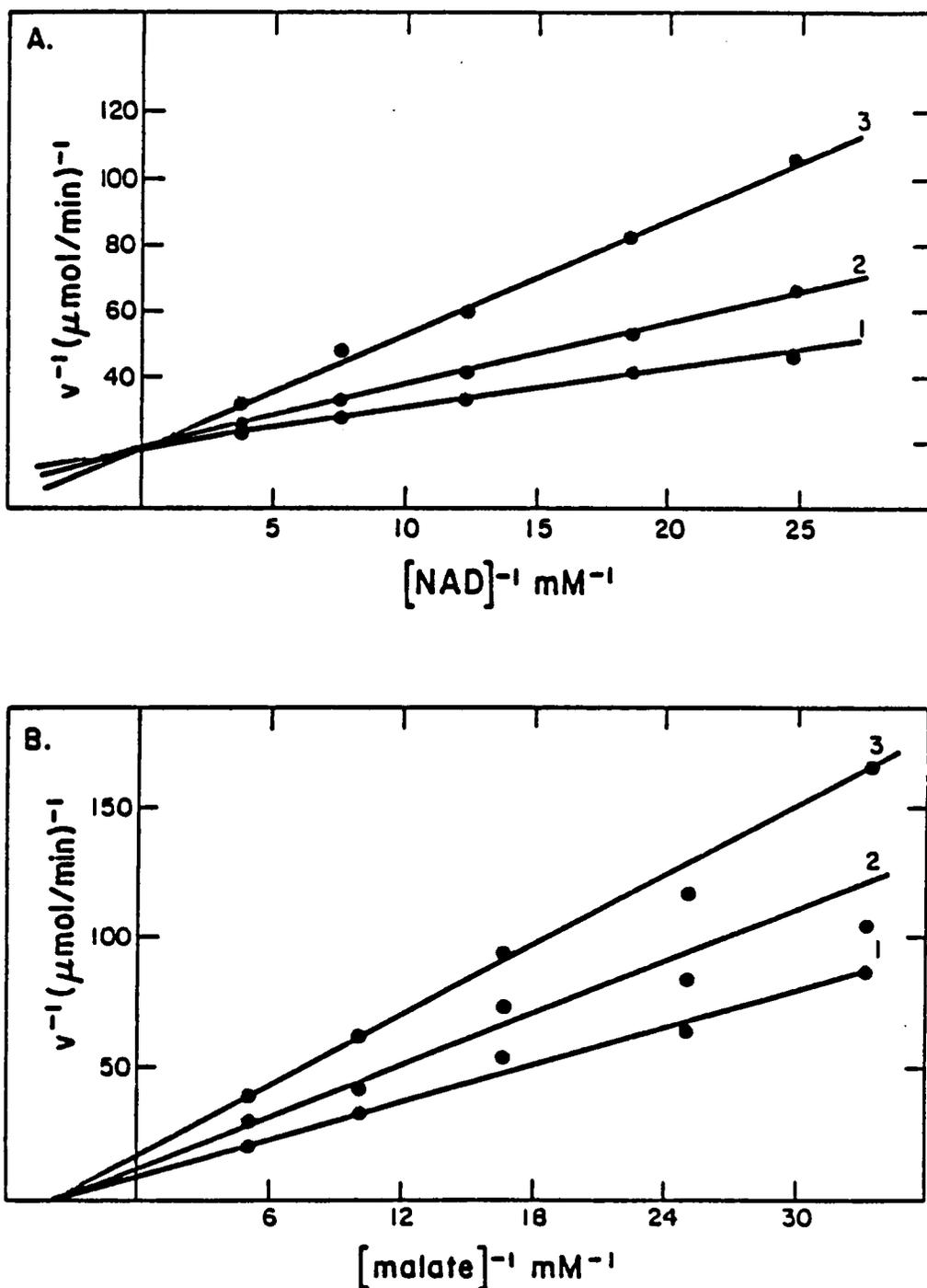
Several pyridine nucleotide analogs structurally altered in either the pyridine or purine moiety were studied to determine their ability to function as coenzymes for the purified *H. influenzae* MDH. Initial velocity studies described in Figure 8 were conducted by replacing NAD with various NAD analogs as coenzymes for MDH. Kinetic constants obtained by extrapolation to x-axis and y-axis intercepts from double reciprocal plots are shown in Table V. The first six analogs listed exhibited coenzyme activity in the MDH-catalyzed reaction. Reactions involving 3-acetylpyridine adenine dinucleotide exhibited kinetic parameters very similar to those observed with NAD. Reactions of the remaining functional analogs varied with respect to  $K_m$  and  $V_{max}$  values. Reductions were not observed with NMN, NADP, nicotinic acid adenine dinucleotide,



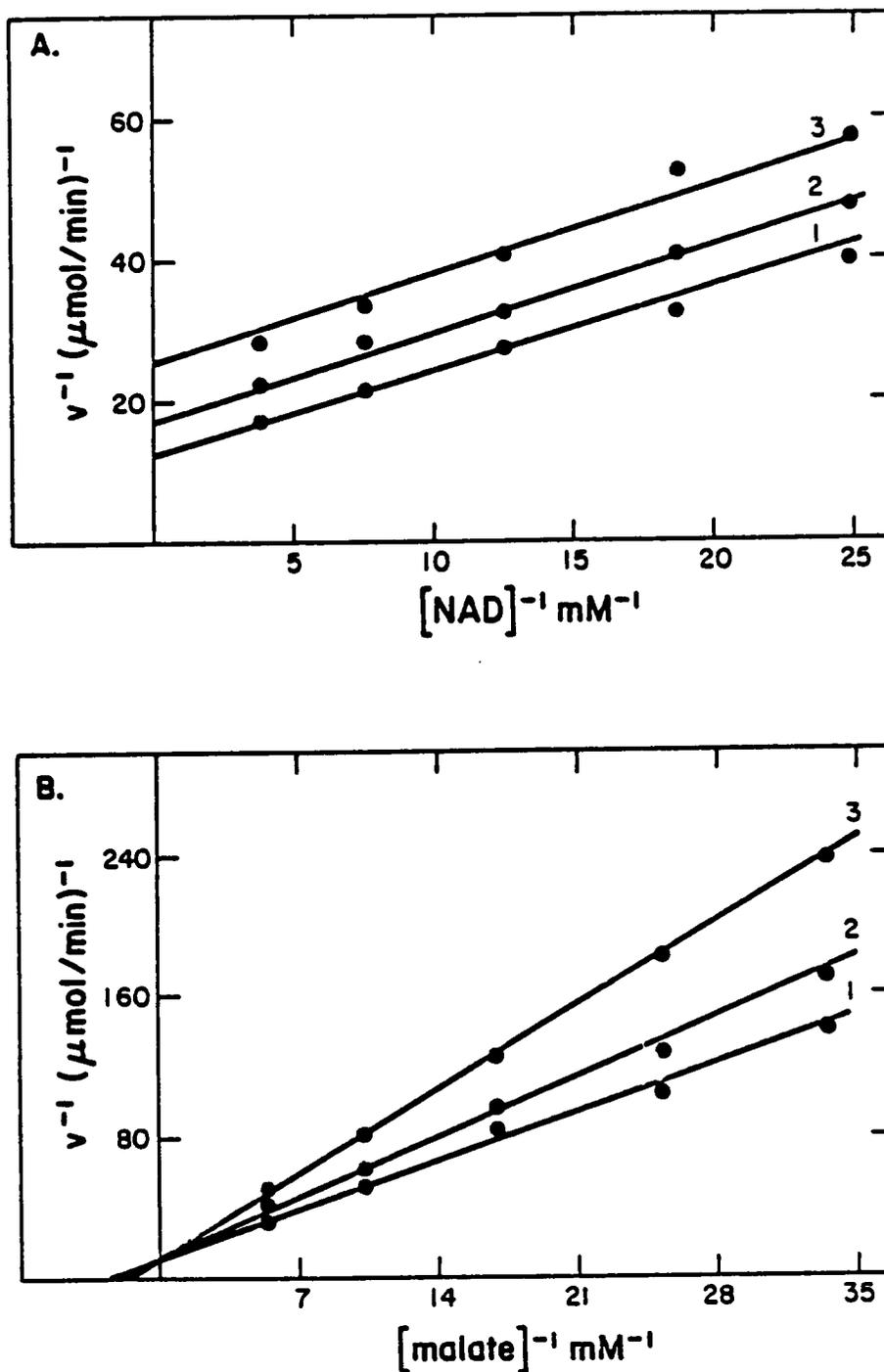
**Figure 11. Product Inhibition of MDH by Oxalacetate as a Function of NAD Concentration:** A. At an unsaturating concentration of malate, reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 110 to 540  $\mu$ M NAD, 300  $\mu$ M malate, and 80 ng of enzyme. Oxalacetate concentrations were: line 1, none; line 2, 5  $\mu$ M; line 3, 10  $\mu$ M. B. At a saturating concentration of malate, reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 40 to 270  $\mu$ M NAD, 3 mM malate, and 40 ng of enzyme. Oxalacetate concentrations were: line 1, none; line 2, 20  $\mu$ M; line 3, 50  $\mu$ M.



**Figure 12. Product Inhibition of MDH by Oxalacetate as a Function of Malate Concentration:** A. At an unsaturating concentration of NAD, reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 54  $\mu\text{M}$  NAD, 0.2 to 0.9 mM malate, and 80 ng of enzyme. Oxalacetate concentrations were: line 1, none; line 2, 10  $\mu\text{M}$ ; line 3, 20  $\mu\text{M}$ . B. At a saturating concentration of NAD, reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 540  $\mu\text{M}$  NAD, 30 to 200  $\mu\text{M}$  malate, and 80 ng purified enzyme. Oxalacetate concentrations were: line 1, none; line 2, 10  $\mu\text{M}$ ; line 3, 15  $\mu\text{M}$ .



**Figure 13. Dead-end Inhibition of MDH by ADP-ribose:** A. As a function of NAD concentration, reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 41 to 270  $\mu$ M NAD, 6 mM malate, and 68 ng of enzyme. ADP-ribose concentrations were: line 1, none; line 2, 20  $\mu$ M; line 3, 50  $\mu$ M. B. As a function of malate concentration, reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 2 mM NAD, 30 to 200  $\mu$ M malate, and 170 ng of enzyme. ADP-ribose concentrations were: line 1, none; line 2, 50  $\mu$ M; line 3, 100  $\mu$ M.



**Figure 14. Dead-end Inhibition of MDH by Hydroxymalonate:** A. As a function of NAD concentration, reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 41 to 270  $\mu$ M NAD, 6 mM malate, and 100 ng of enzyme. Hydroxymalonate concentrations were: line 1, none; line 2, 1.25 mM; line 3, 2.5 mM. B. As a function of malate concentration, reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 2 mM NAD, 30 to 200  $\mu$ M malate, and 32 ng of enzyme. Hydroxymalonate concentrations were: line 1, none; line 2, 0.5 mM; line 3, 1 mM.

**Table IV. Studies of the MDH Kinetic Mechanism**

A) Product inhibition studies				
Inhibitor	Variable Substrate			
	NAD (A)		Malate (B)	
	Unsaturated B	Saturated B	Unsaturated A	Saturated A
NADH	C	C	NC	None
Oxalacetate	NC	UC	NC	NC

B) Dead-end inhibition studies	
Inhibitor	$1/v$ vs. $1/[NAD]$
Adenosine diphosphoribose	C
Hydroxymalonnate	UC

Inhibitor	$1/v$ vs. $1/[malate]$
Adenosine diphosphoribose	NC
Hydroxymalonnate	C

C = Competitive inhibition  
 NC = Noncompetitive inhibition  
 UC = Uncompetitive inhibition

Table V.  $K_m$  and  $V_{max}$  Values for NAD Analogs

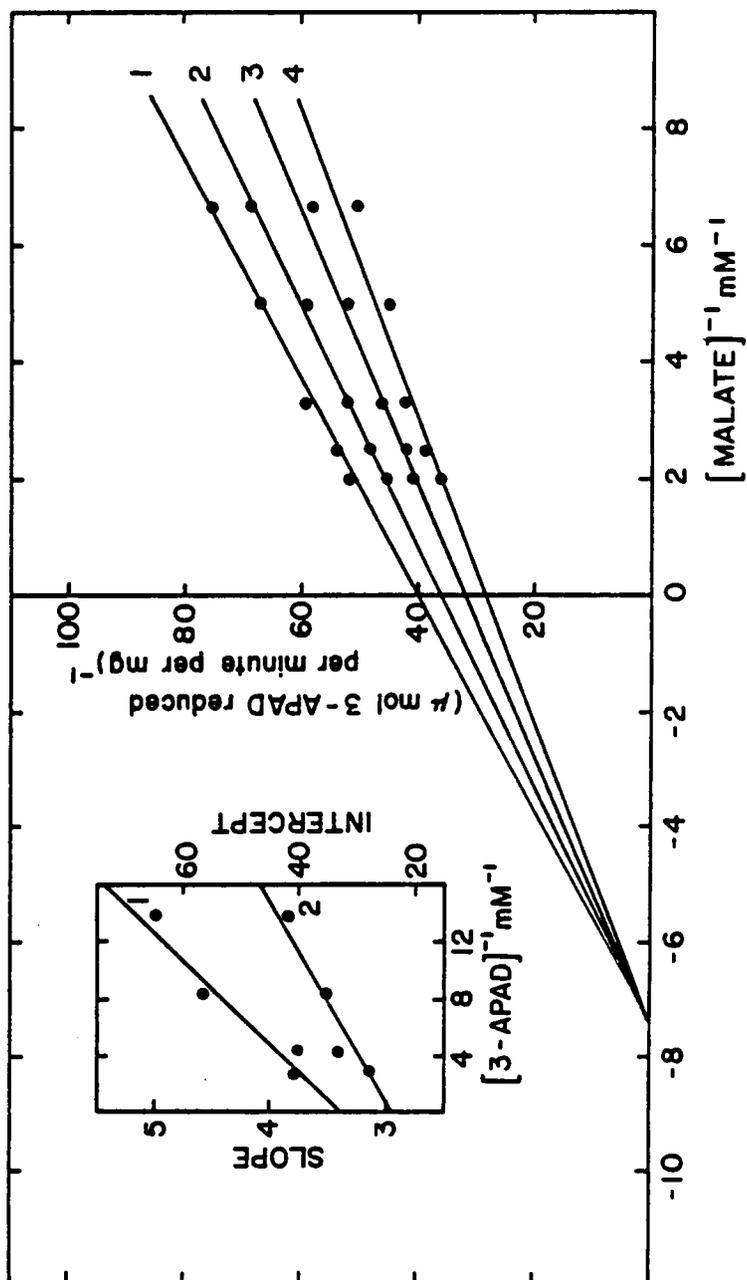
NAD analog	$K_m$ ( $\mu\text{M}$ )	$V_{max}$ ( $\mu\text{mol}/\text{min}/\text{mg}$ )
Nicotinamide adenine dinucleotide	57	23
3-Acetylpyridine adenine dinucleotide	79	45
Thionicotinamide adenine dinucleotide	86	5
3-Pyridinealdehyde adenine dinucleotide	220	16
Nicotinamide hypoxanthine dinucleotide	230	15
Nicotinamide guanine dinucleotide	4000	3.2
Nicotinamide 1, <i>N</i> <sup>6</sup> -etheno-adenine dinucleotide	1540	4.4
Nicotinamide mononucleotide	— <sup>a</sup>	— <sup>a</sup>
Nicotinic acid adenine dinucleotide	— <sup>a</sup>	— <sup>a</sup>
Nicotinamide adenine dinucleotide phosphate	— <sup>a</sup>	— <sup>a</sup>
3-Pyridylacryloamide adenine dinucleotide	— <sup>a</sup>	— <sup>a</sup>
3-Aminopyridine adenine dinucleotide	— <sup>a</sup>	— <sup>a</sup>

a = no reaction detected

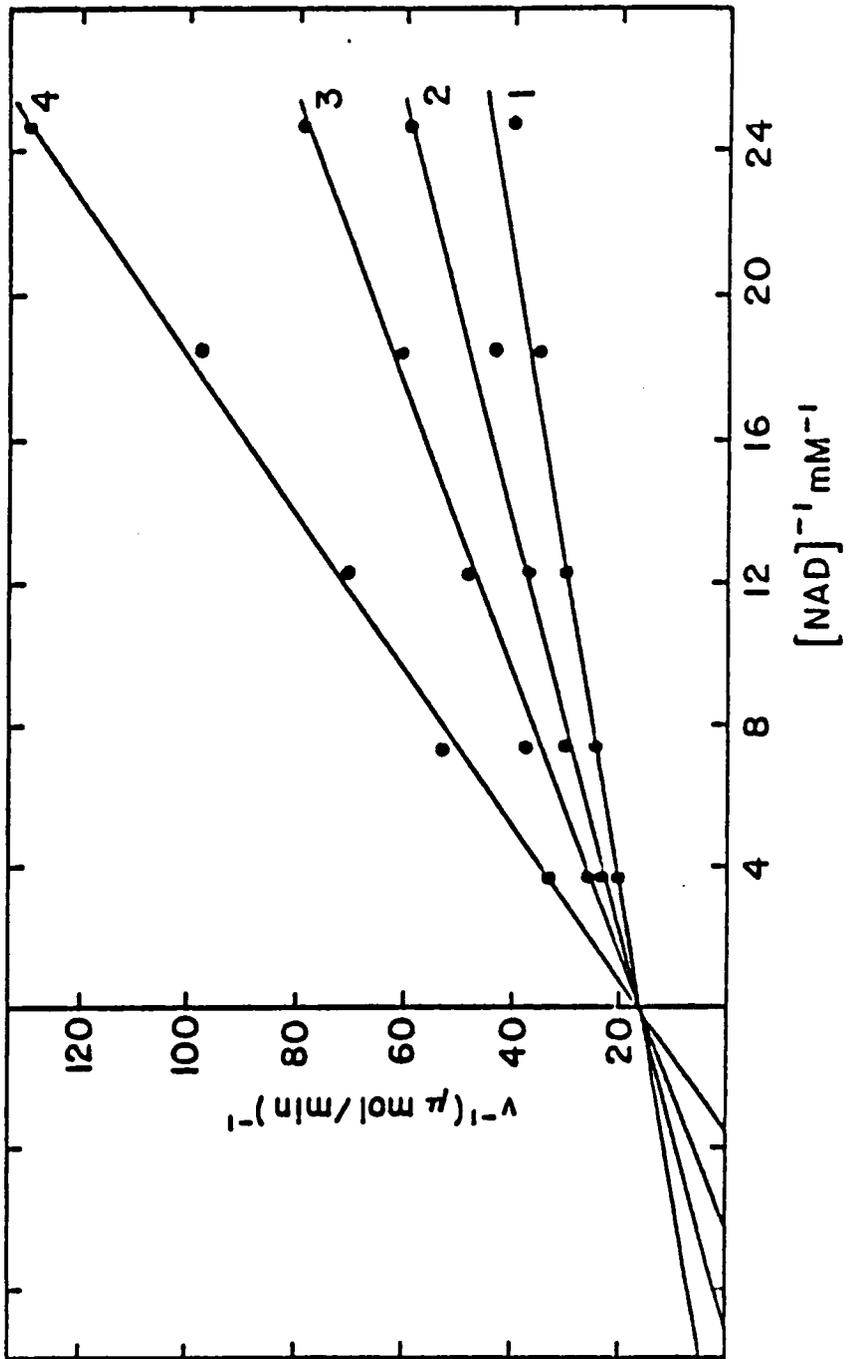
3-aminopyridine adenine dinucleotide, and 3-pyridylacryloamide adenine dinucleotide. The addition of millimolar concentrations of AMP did not promote the reduction of nicotinamide mononucleotide. Initial velocity studies of the 3-acetylpyridine and thionicotinamide analogs gave converging line relationships, confirming the sequential reaction mechanism determined with NAD as a coenzyme. The results obtained with 3-acetylpyridine adenine dinucleotide (3-APAD) are shown in Figure 15.

### Studies of Enzyme Inhibition

The binding of a substrate or coenzyme to an enzyme can result from the interactions of different portions of these ligands with different regions of the enzyme binding site. Properties of the binding sites on the enzyme can be investigated by the study of competitive inhibitors of the enzyme. In studies of coenzyme-competitive inhibition of MDH, initial velocities were determined by varying the concentration of NAD at several fixed concentrations of various inhibitors. Several adenosine derivatives such as adenosine, AMP, ADP, and ADP-ribose were demonstrated to be effective coenzyme-competitive inhibitors. Inhibition of *H. influenzae* MDH by ADP is representative of the inhibition observed with all of the coenzyme-competitive inhibitors studied and is shown in Figure 16. The  $K_i$  values decreasing in the order of adenosine > AMP > ADP > ADP-ribose were observed (Table VI). N<sup>1</sup>-Methyl- and N<sup>1</sup>-octylnicotinamide chloride did not inhibit the malate dehydrogenase at 100 mM. The addition of ADP to reaction mixtures containing 100 mM N<sup>1</sup>-methyl- or N<sup>1</sup>-octylnicotinamide chloride did not produce inhibition beyond that observed with ADP alone. Seven structural analogs of NAD which did not serve as coenzymes were evaluated as coenzyme-competitive inhibitors. The  $K_i$  values obtained for these compounds are listed in the Table VII. *Haemophilus influenzae* MDH was shown to bind effectively to the blue affinity column, Matrex gel Blue A. This result led to the study of inhibition by the free ligand, Procion



**Figure 15. Effect of 3-Acetylpyridine Adenine Dinucleotide (3-APAD) and Malate Concentrations on Initial Velocities:** Reaction mixtures contained concentrations of malate varying from 0.15 to 0.5 mM at four fixed concentrations of 3-APAD in 45 mM glycine-NaOH buffer, pH 9.6. Reactions were initiated by the addition of 17 ng of MDH and initial velocities were measured at 365 nm spectrophotometrically. The concentrations of 3-APAD used ( $\mu\text{M}$ ) were: line 1, 72; line 2, 121; line 3, 241; line 4, 362. Inset is a replot of slopes (line 1) and intercepts (line 2).



**Figure 16. Coenzyme-competitive Inhibition of MDH by ADP:** Reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 6 mM malate, 50 ng of enzyme, and 41 to 270  $\mu\text{M}$  NAD at four different concentrations of ADP. The concentrations of ADP used were: line 1, none; line 2, 0.5 mM; line 3, 1.5 mM; line 4, 3.0 mM.

**Table VI. Inhibition of *H. influenzae* MDH by Adenosine Derivatives**

Inhibitor	$K_i$ (mM)
Adenosine	12.7
AMP	1.1
ADP	0.85
ADP-ribose	0.05

**Table VII. Inhibition of *H. influenzae* MDH by NAD Analogs**

NAD analog	$K_i$ (mM)
Nicotinamide 1, <i>N</i> <sup>6</sup> -ethenoadenine dinucleotide	5.50
Pyridylacetonitrile adenine dinucleotide	3.48
3-Aminopyridine adenine dinucleotide	2.47
Pyridine adenine dinucleotide	0.79
Nicotinic acid adenine dinucleotide	0.58
3-Methylpyridine adenine dinucleotide	0.14
3-Pyridylcarbinol adenine dinucleotide	0.02

Blue HB. As anticipated, the dye displayed a coenzyme-competitive inhibition with a  $K_i$  value of  $0.19 \mu\text{M}$  which was by far the most effective inhibition of the enzyme.

Since a pyrophosphate interaction was indicated by the binding of adenosine compounds, other negatively-charged molecules, such as aliphatic carboxylic acids of varying chainlength were tested for coenzyme-competitive inhibition. The results of the analysis as to type of inhibition and  $K_i$  values are shown in Table VIII. Shorter chain carboxylic acids displayed competitive inhibition whereas longer chain derivatives showed a mixed-type inhibition. The  $K_i$  values decreased with increasing chainlength of the alkyl groups of the carboxylic acids. A plot of the logarithm of the reciprocals of the  $K_i$  values versus the chainlength of the alkyl substituents of carboxylic acids is shown in Figure 17. The importance of the negatively-charged carboxylate anion in binding was supported by the absence of coenzyme-competitive inhibition by butanol. At a concentration ( $200 \text{ mM}$ ) higher than the  $K_i$  for butyric acid, butanol did not inhibit the enzyme.

Multiple inhibition analysis described by Yonetani and Theorell (90) was used to determine which regions of the coenzyme binding site of MDH interact with different coenzyme-competitive inhibitors. Initial velocities were measured for each inhibitor pair by varying one inhibitor at three fixed concentrations of the second inhibitor. A plot of the ratio of the initial velocity in the absence of inhibitors ( $V_o$ ) to the initial velocity in the presence of inhibitors ( $V_i$ ) against the concentration of the first inhibitor,  $I_1$ , results in a series of straight lines that intersect at an abscissa value of  $-\alpha K_i$ .  $\alpha$  is defined as an inhibitor interaction constant which is a measure of the interactions existing between  $I_1$  and  $I_2$  in the  $E I_1 I_2$  complex. The interaction constant,  $\alpha$ , can be calculated from the value  $-\alpha K_i$  by substituting the known  $K_i$  value determined from previous inhibition studies of the inhibitor,  $I_1$ . Multiple inhibition studies with the inhibitor pair, AMP and ADP-ribose, were performed. The data were plotted as  $V_o/V_i$  against the concentration of AMP. A parallel line relationship was obtained as shown in Figure 18, indicating an  $\alpha$  value of infinity. The following coenzyme-competitive inhibitor pairs

**Table VIII. Inhibition of *H. influenzae* MDH by Carboxylic Acids**

Carboxylic acid	Type of inhibition	$K_i$ (mM)
Acetic acid	Competitive	258
Propionic acid	"	174
Butyric acid	"	146
Pentanoic acid	"	87
Hexanoic acid	"	60
Heptanoic acid	"	29
Octanoic acid	"	21
Nonanoic acid	Mixed-type	15
Decanoic acid	"	8

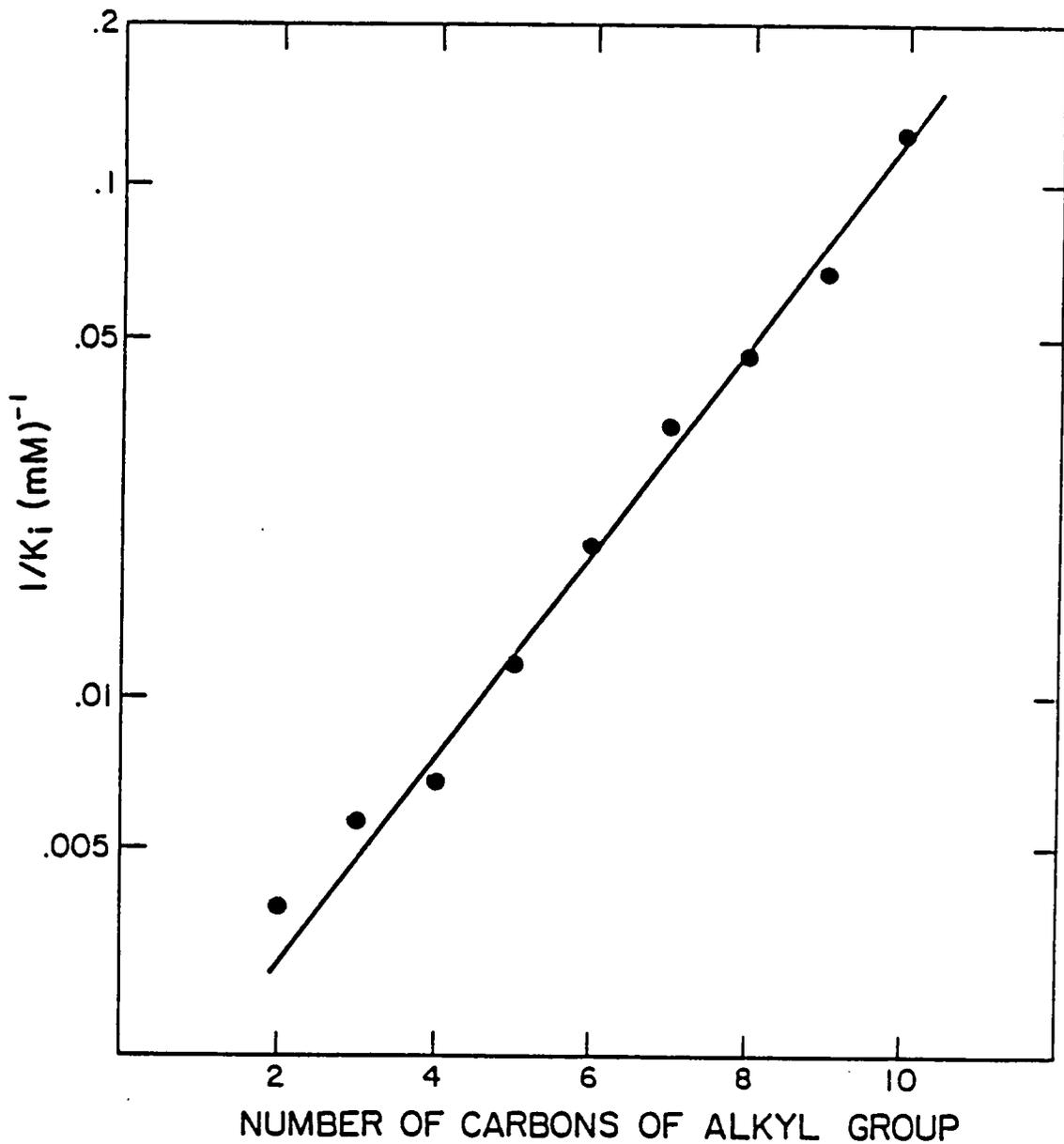
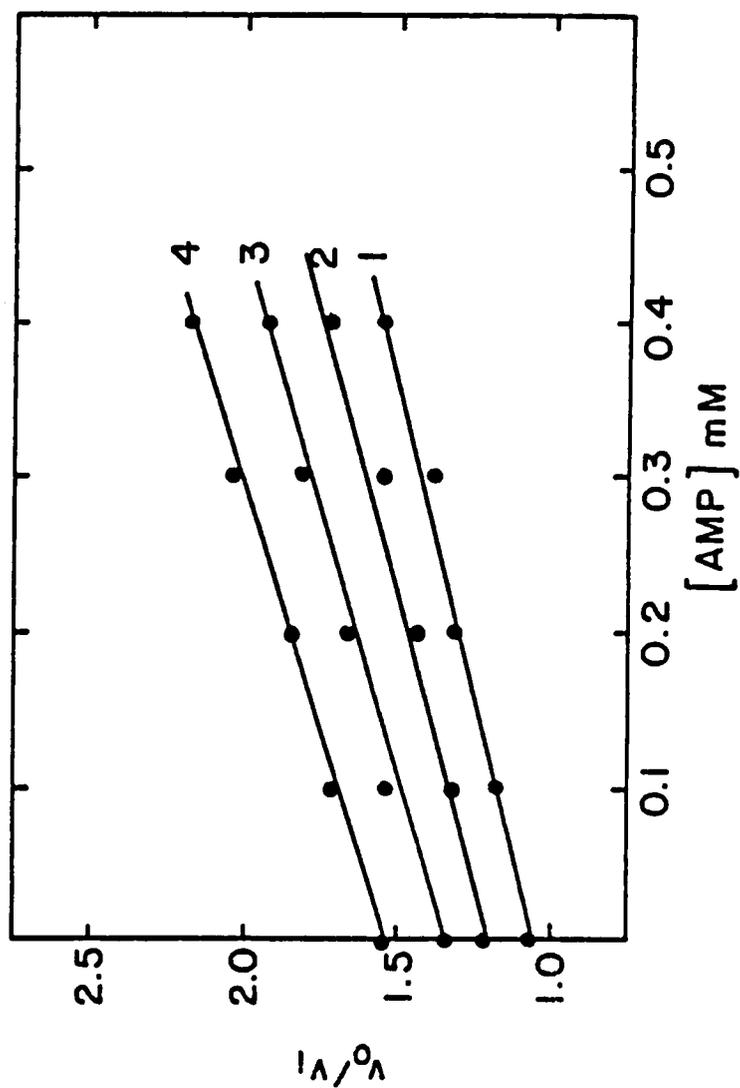


Figure 17. Chainlength Effects on Carboxylic Acid Inhibition: The relationship of the logarithm of the reciprocals of the inhibitor constants to the chainlength of the carboxylic acid inhibitor.



**Figure 18. Multiple Inhibition of MDH by AMP and ADP-ribose:** Reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 54  $\mu$ M NAD, and 6 mM malate in a total volume of 1 ml. The concentration of AMP was varied from 0 to 14 mM. The concentrations of ADP-ribose used were: line 1, 0.02 mM; line 2, 0.04 mM; line 3, 0.06 mM; line 4, 0.08 mM.

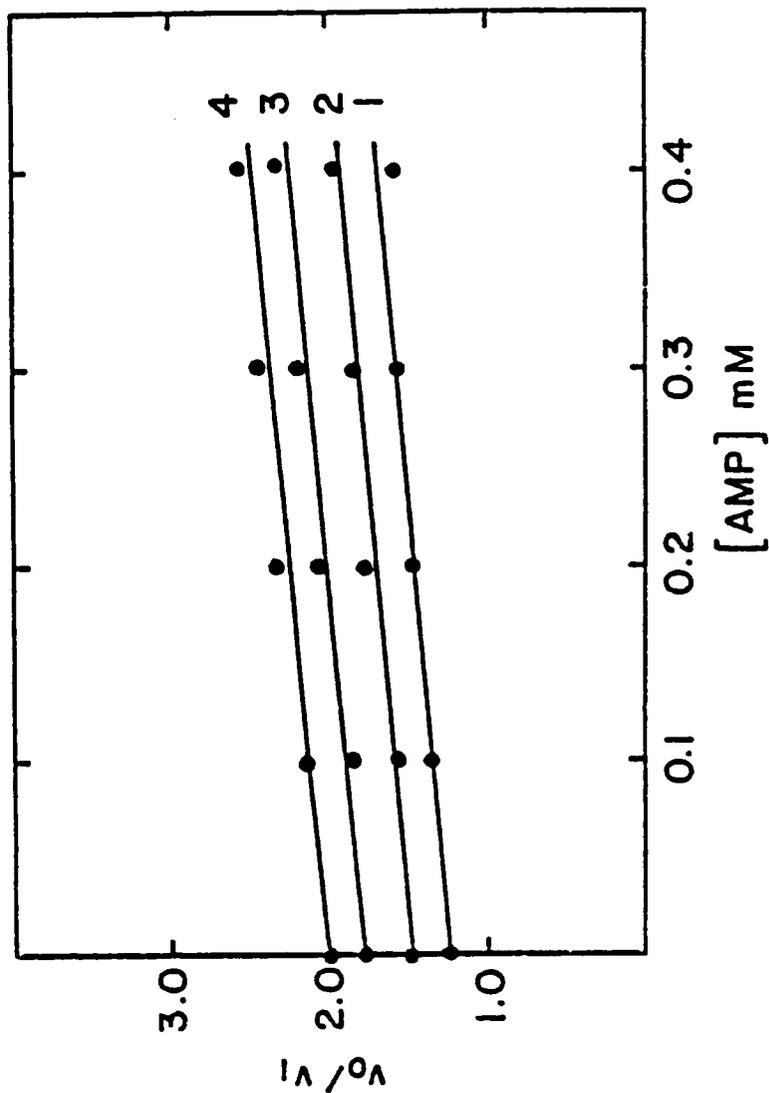
gave inhibitor interaction constants of infinity: AMP and hexanoic acid, ADP and hexanoic acid, ADP-ribose and hexanoic acid, AMP and ADP-ribose, and AMP and ADP. The results obtained with AMP and hexanoic acid are shown in Figure 19.

Structural analogs of malate were also studied for selective inhibition of the enzyme. Substrate-competitive inhibition was determined in 1.0-ml reaction mixtures containing 45 mM glycine-NaOH buffer, pH 9.6, 0.54 mM NAD, and malate varied from 60 to 400  $\mu$ M. Both mono- and dicarboxylic acids were employed as substrate analogs in the study of inhibition of MDH. The  $K_i$  values are listed in Table IX. Of all the substrate analogs studied,  $\alpha$ -ketoglutarate was the most effective inhibitor with an inhibitor dissociation constant of 25  $\mu$ M.

### Chemical Modification of the Enzyme

The chemical modification of *H. influenzae* MDH was investigated to identify possible amino acid residues involved in catalysis. Many dehydrogenases require the presence of a free sulfhydryl group for catalytic activity. Nucleophilic group modifying reagents, relatively selective for the sulfhydryl group of cysteine residues, such as iodoacetamide, iodoacetic acid, N-ethylmaleimide, and fluorescein mercuric acetate (FMA), were incubated with purified MDH. They did not affect the activity of the enzyme over a 60-min period when incubated at a concentration of 5 mM.

The importance of a pyrophosphate region of the NAD binding site of MDH, determined in inhibition studies with adenosine compounds, suggested the presence of positively-charged amino acid residues of the enzyme that interact with the negatively-charged pyrophosphate group of the coenzyme molecule. The lysine modifying reagent, pyridoxal 5'-phosphate, caused no inactivation of the enzyme at a concentration of 1 mM. Diethylpyrocarbonate (DEPC), which reacts predominantly with histidyl residues at pH 7.0 (91), inactivated the MDH enzyme. The DEPC inactivation was time-dependent and followed pseudo-first-order kinetics as shown in Figure 20. Pseudo-



**Figure 19. Multiple Inhibition of MDH by AMP and Hexanoic Acid:** Reaction mixtures contained 45 mM glycine-NaOH buffer, pH 9.6, 54  $\mu$ M NAD, and 6 mM malate in a total volume of 1 ml. The concentrations of AMP was varied from 0 to 14 mM. The concentrations of hexanoic acid used were: line 1, 20 mM; line 2, 40 mM; line 3, 60 mM; line 4, 80 mM.

**Table IX. Substrate-competitive Inhibition of *H. influenzae* MDH**

Inhibitor	$K_i$ (mM)
Fumaric acid	18.0
Succinic acid	15.4
$\alpha$ -Hydroxyglutaric acid	14.9
Malonic acid	12.7
Ketomalonic acid	2.7
Hydroxymalonic acid	1.6
$\alpha$ -Ketoglutaric acid	0.025
Aspartic acid	— <sup>a</sup>
$\alpha$ -Hydroxybutyric acid	— <sup>a</sup>
Pyruvic acid	— <sup>a</sup>
D-Lactic acid	— <sup>a</sup>
L-Lactic acid	— <sup>a</sup>

a = no inhibition detected

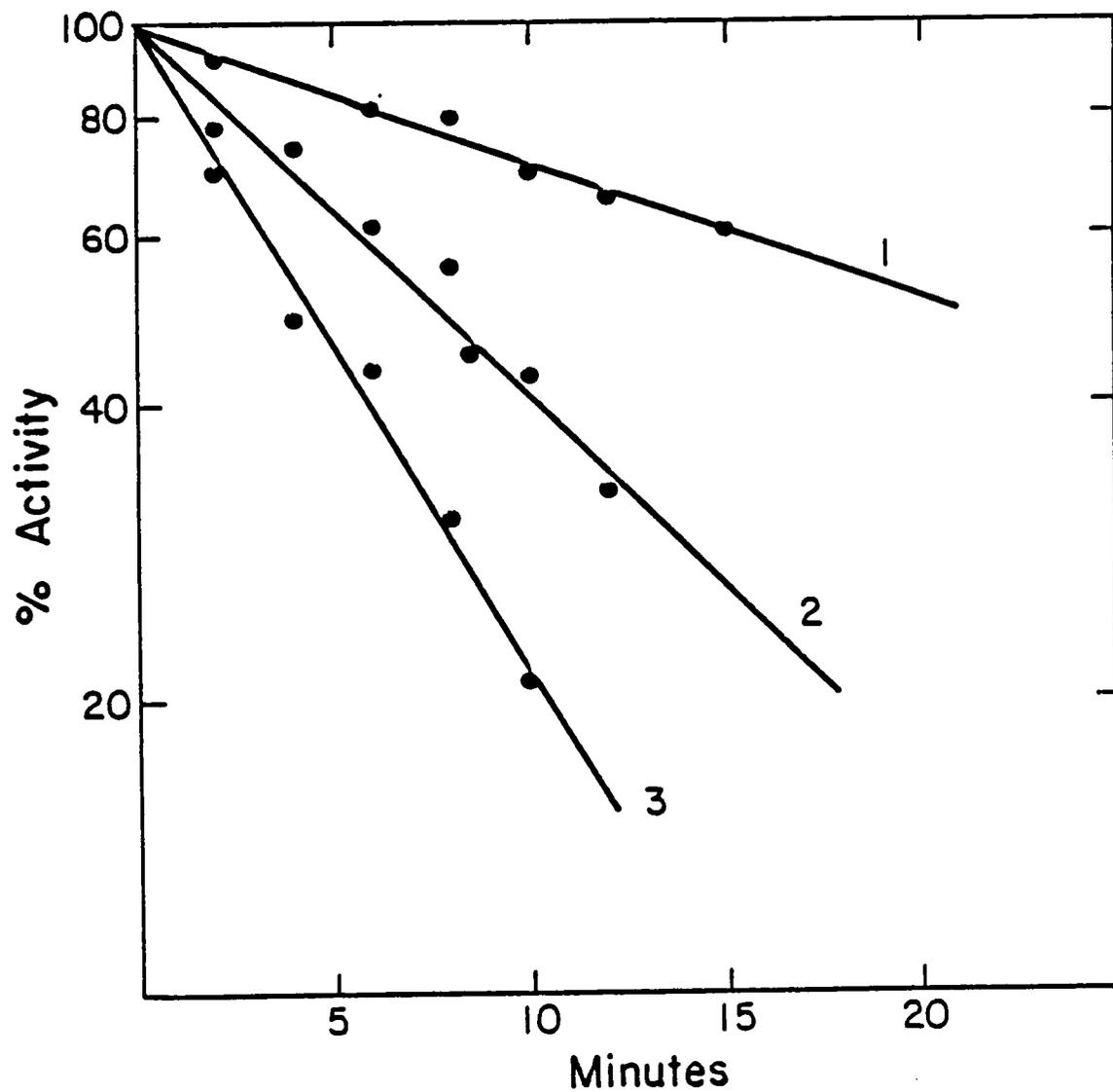
first-order rate constants for the inactivation reactions calculated using the equation  $k_1 = 0.693/t_{1/2}$  were  $0.082 \text{ min}^{-1}$  and  $0.143 \text{ min}^{-1}$  for 2.5 mM and 4 mM DEPC, respectively.

The purified MDH was titrated with FMA in the presence and absence of 8 M urea as described in Experimental Procedures. Fluorescence intensity versus FMA concentration was measured according to Hsu and Lardy (88) and is shown in Figure 21. The fluorescence titration demonstrated the presence of 2 sulfhydryl groups per 61,000 molecular weight enzyme. In the absence of urea the titratable sulfhydryls were not observed, suggesting the unavailability of sulfhydryl groups in the native enzyme.

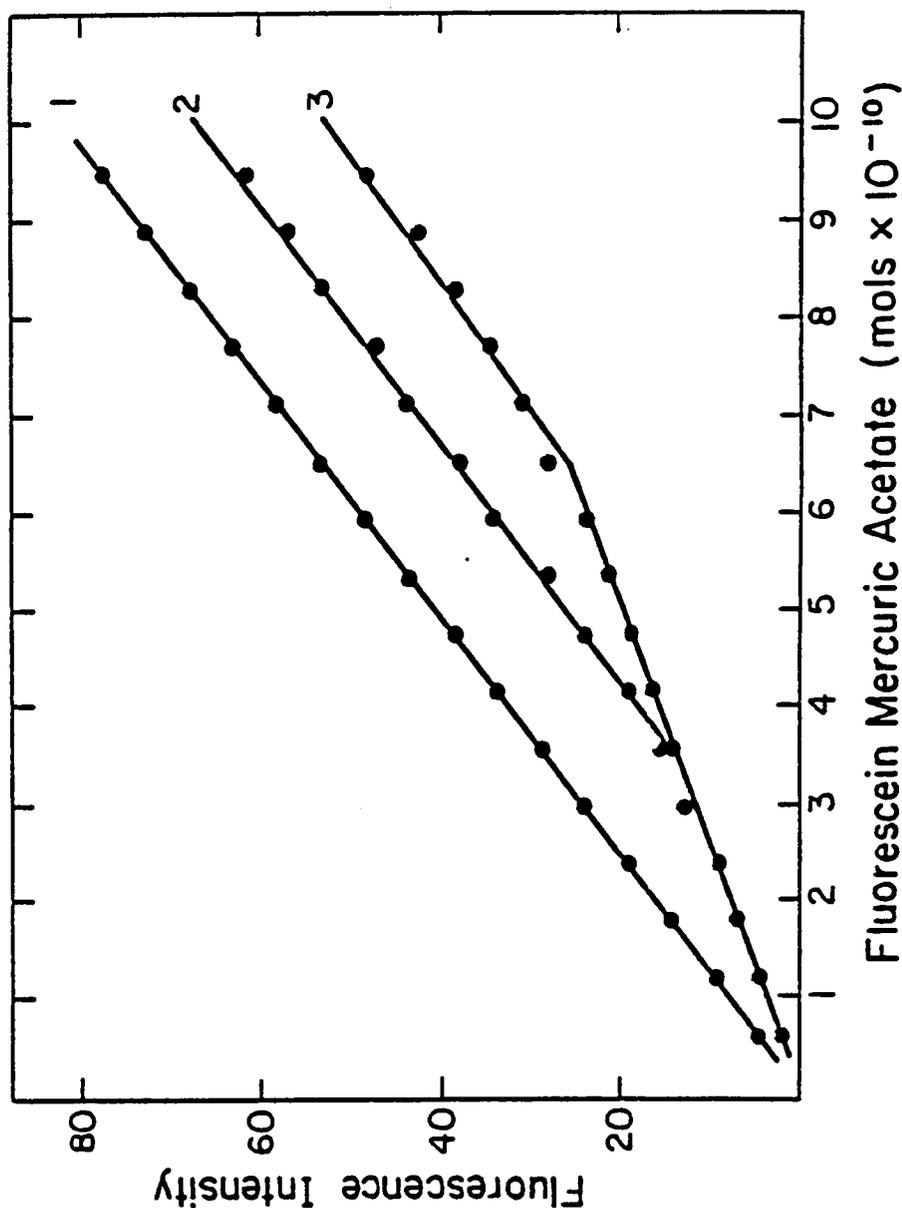
### ***Purification of 6-Phosphogluconate Dehydrogenase***

The initial purification steps for 6-phosphogluconate dehydrogenase, including sonication, protamine sulfate and salt fractionation, were carried out in essentially the same manner described above for purification of the *H. influenzae* MDH. The first column chromatography using Matrex gel Green A affinity column was effective in separating the two enzymes since 6-PGDH did not bind to this column whereas the *H. influenzae* MDH did. All column purification steps were carried out at 4°C.

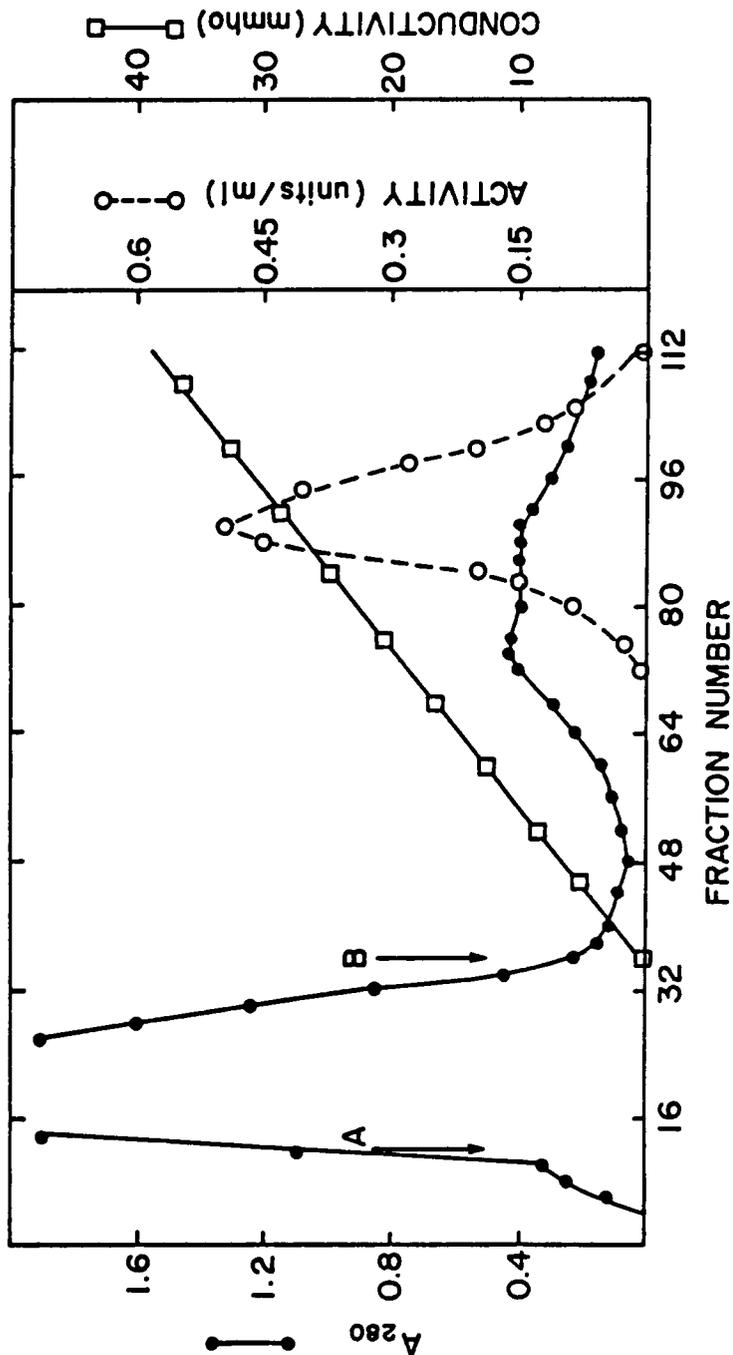
The eluate of the Matrex gel Green A column in 10 mM potassium phosphate buffer, pH 7.0, was adjusted to a final concentration of 1 mM 6-phosphogluconate. This protein solution was applied to a Matrex gel Red A column (1.4 x 14 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM disodium ethylenediamine tetracetic acid (EDTA) and 0.5 mM DTT. Unbound proteins were washed from the column with equilibration buffer. The enzyme was eluted by a linear salt gradient, 0 to 1 M KCl in the same buffer (2 x 100 ml). The elution profile is shown in Figure 22. Fractions (2.4 ml) containing enzyme activity were pooled and concentrated by ultrafiltration.



**Figure 20. Inactivation of MDH with Diethylpyrocarbonate (DEPC):** Enzyme inactivation and reaction mixtures were described in Experimental Procedures. The concentrations of DEPC used were: line 1, 1.5 mM; line 2, 2.5 mM; line 3, 4 mM.



**Figure 21. Fluorescence Titration of MDH by Fluorescein Mercuric Acetate:** Reaction mixtures contained 8 M urea, 50 mM potassium phosphate buffer, pH 7.5, and fluorescein mercuric acetate as indicated. Enzyme additions were: line 1, none; line 2,  $1.28 \times 10^{-11}$  moles; line 3,  $3.2 \times 10^{-11}$  moles. Fluorescence intensity was measured at 525 nm with excitation at 495 nm.



**Figure 22. Affinity Chromatography on Matrex Gel Red A:** The pooled Green A column sample adjusted to 1 mM 6-phosphogluconate was applied to a Matrex gel Red A column (1.4 x 14 cm) that had been equilibrated with 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 0.5 mM DTT. Arrow A indicates the point of initiation of washing with the equilibration buffer. Arrow B indicates the starting point of a linear salt gradient of 0 to 1 M KCl in the equilibration buffer (2 x 100 ml).

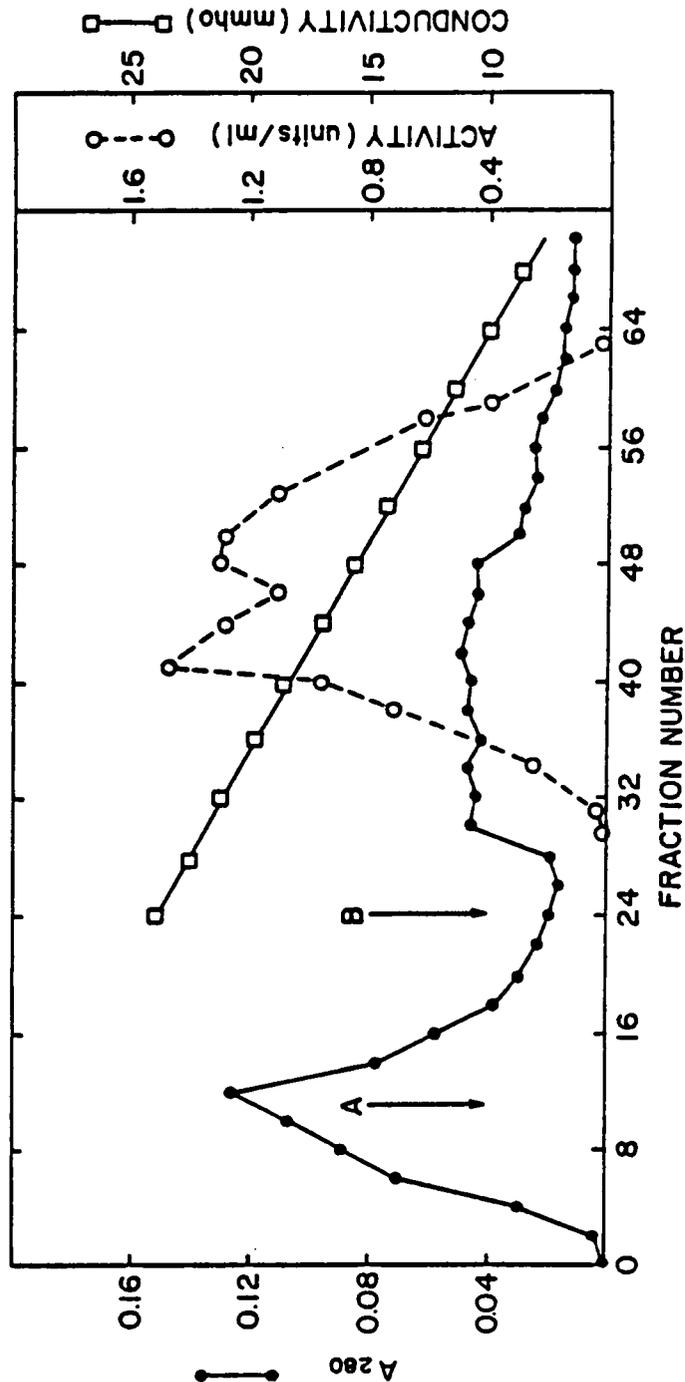
The pooled, concentrated sample from the Matrex gel Red A column was adjusted to a final concentration of 1 M KCl by addition of solid KCl. This solution was applied to a Phenyl-Sepharose CL-4B column (1.0 x 9 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0 containing 1 M KCl, 1 mM EDTA and 0.5 mM DTT. Unbound proteins were washed from the column with equilibration buffer. The enzyme was eluted by a reversed linear salt gradient, 0.5 M to 0 M KCl in the same buffer (2 x 100 ml). The elution profile is shown in Figure 23. Fractions (2.4 ml) containing enzyme activity were pooled and concentrated by ultrafiltration.

The sample from the Phenyl-Sepharose column was applied to a 2',5'ADP-Sepharose 4B column (1.0 x 7 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0 containing 1 mM EDTA and 0.5 mM DTT. After washing the column with the equilibration buffer, the enzyme was eluted by a linear salt gradient, 0 to 1 M KCl in the same buffer (2 x 100 ml). The elution profile is shown in Figure 24. Fractions (2.4 ml) containing enzyme activity were pooled and concentrated by ultrafiltration. A summary of the *H. influenzae* 6-PGDH purification procedure is shown in Table X. The enzyme was purified 308-fold with a 16% yield and a final specific activity of 40  $\mu\text{mol}/\text{min}$  per mg protein.

### ***Properties of the Purified 6-PGDH Enzyme***

The purified 6-PGDH enzyme was analyzed by polyacrylamide gel electrophoresis as described above for *H. influenzae* MDH. The enzyme migrated as a single protein band indicating electrophoretic homogeneity.

The molecular weight of the 6-phosphogluconate dehydrogenase was determined under native conditions of gel filtration on a Sephacryl S-200 column (1.6 x 75 cm) at 4°C. The column was equilibrated with 50 mM Tris-HCl buffer, pH 8.0, 200 mM KCl. Elution volumes of the purified enzyme and standard proteins were determined by measuring the enzyme activity, and the protein absorbance at 280 nm, respectively



**Figure 23. Phenyl-Sepharose CL-4B Column Chromatography:** The pooled Red A column sample adjusted to 1 M KCl was applied to a Phenyl-Sepharose column (1.0 x 9 cm) that had been equilibrated with 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 0.5 mM DTT, and 1 M KCl. Arrow A indicates the point of initiation of washing with the equilibration buffer. Arrow B indicates the starting point of a reversed linear salt gradient of 0.5 to 0 M KCl in the 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 0.5 mM DTT (2 x 100 ml).

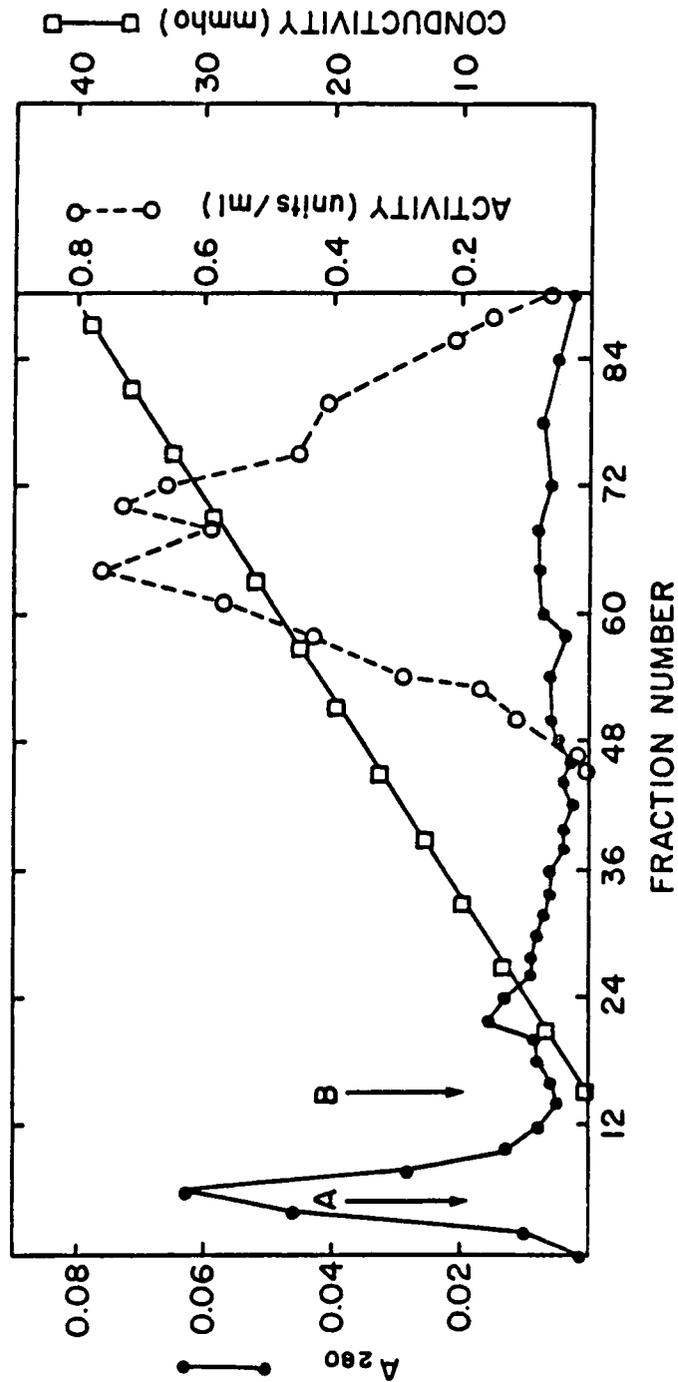


Figure 24. 2',5'ADP-Sepharose Column Chromatography: The pooled Phenyl-Sepharose column sample was applied to a 2',5'ADP-Sepharose column (1.0 x 7 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 0.5 mM DTT. Arrow A indicates the point of initiation of washing with the equilibration buffer. Arrow B indicates the point of initiation of a linear salt gradient of 0 to 1 M KCl in the equilibration buffer (2 x 100 ml).

**Table X. Purification of *H. influenzae* 6-PGDH**

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Cell sonicate	1185	154	0.13	100	1
Ammonium sulfate pellet	400	124	0.31	81	2.4
Matrex gel Red A	18.9	68	3.6	44	28
Phenyl-Sepharose	2.8	55	19.4	36	149
2',5'ADP-Sepharose	0.63	25	40	16	308

(EDTA and DTT were included in the elution buffer). The calculated  $V_e/V_0$  value of the enzyme was 1.38 corresponding to an apparent  $M_r$  of 70,000 as shown in Figure 25 (line A). The number of subunits and the subunit molecular weight of the enzyme was determined under denaturing SDS-polyacrylamide gel electrophoresis. In comparison to relative mobilities with standard proteins, an apparent molecular weight of 34,000 was obtained as shown in Figure 25 (line B).

The purified *H. influenzae* 6-PGDH was stored at 4°C at a protein concentration of 10 µg/ml in 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 0.5 mM DTT. Under these conditions, the enzyme was stable for a few months without apparent loss of activity. Thermal denaturation was conducted for the purified 6-PGDH over the measurable range of 48 to 56.5°C. The rate of thermal denaturation at different temperatures was shown to follow first-order kinetics (Figure 26) when the enzyme activity was assayed at timed intervals during incubation in 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 0.5 mM DTT. At 55°C the rate constant for thermal denaturation was 0.060 min<sup>-1</sup>. The presence of 1 mM 6-phosphogluconate protected against thermal denaturation as shown in Figure 26, whereas 0.1 mM NADP did not show any protection against thermal denaturation.

The effect of pH on the purified 6-PGDH was studied. Initial velocities of NADP reduction were determined as a function of pH between pH 6.0 and 9.6 using the buffers, 10 mM potassium phosphate, 10 mM Bicine-NaOH, 10 mM Tris-HCl, and 10 mM glycine-NaOH. Initial velocities were measured at each pH by varying the concentration of NADP at a saturating concentration of 6-phosphogluconate.  $K_m$  and  $V_{max}$  values over the pH range studied, were determined from double reciprocal plots and the value of  $V_{max}/K_m$  exhibited an optimum pH of 8.0.

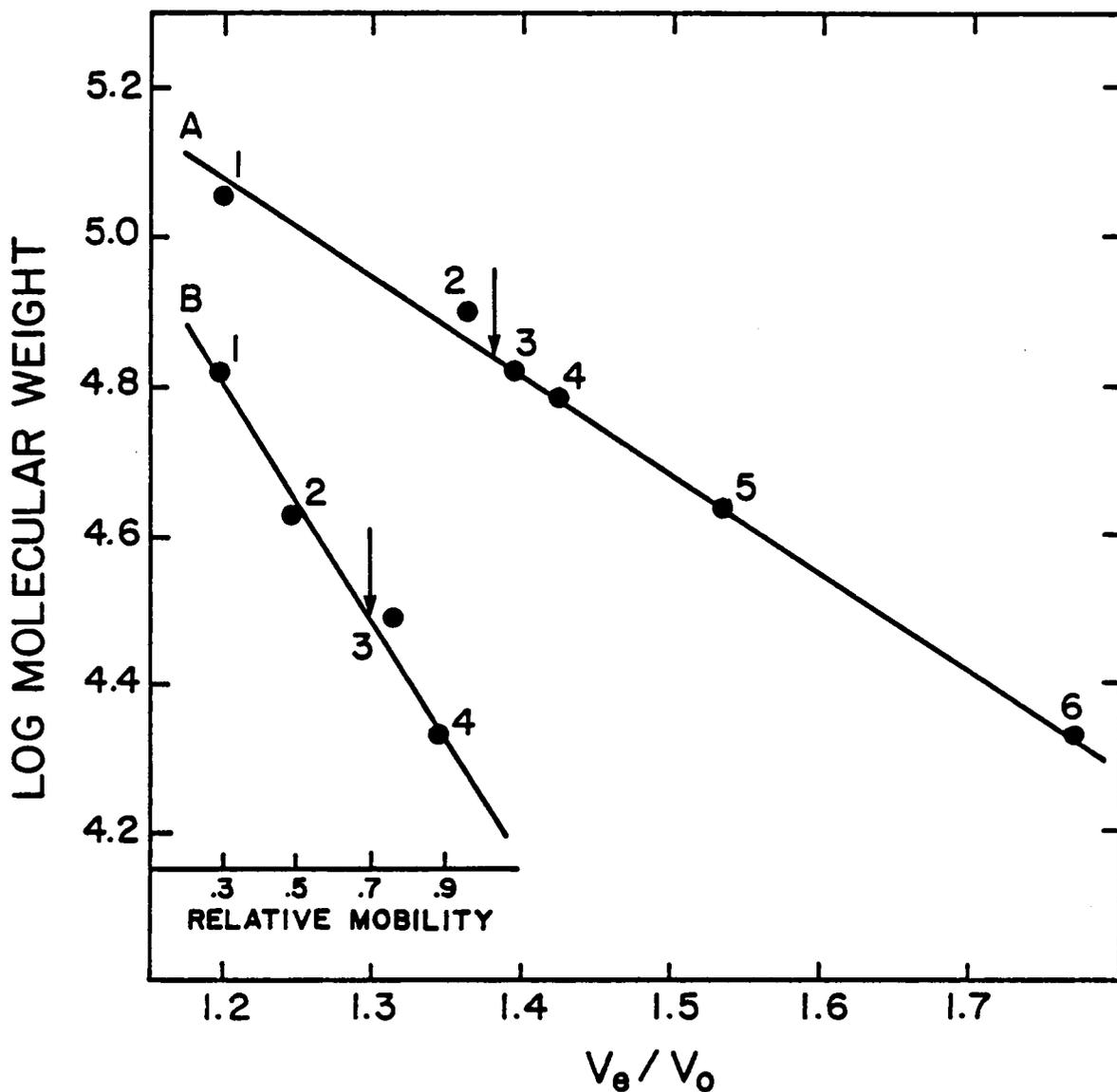


Figure 25. Molecular Weight Determination of 6-PGDH: A. Gel filtration on Sephacryl S-200 column. Molecular weight standards were 1)  $\beta$ -galactosidase, 2) horse liver alcohol dehydrogenase, 3) bovine serum albumin, 4) *H. influenzae* MDH, 5) ovalbumin, and 6) soybean trypsin inhibitor. B. SDS-Polyacrylamide gel electrophoresis. Molecular weight standards were 1) bovine serum albumin, 2) ovalbumin, 3) carbonic anhydrase, and 4) soybean trypsin inhibitor. The arrows in A and B denote positions determined for *H. influenzae* 6-PGDH.

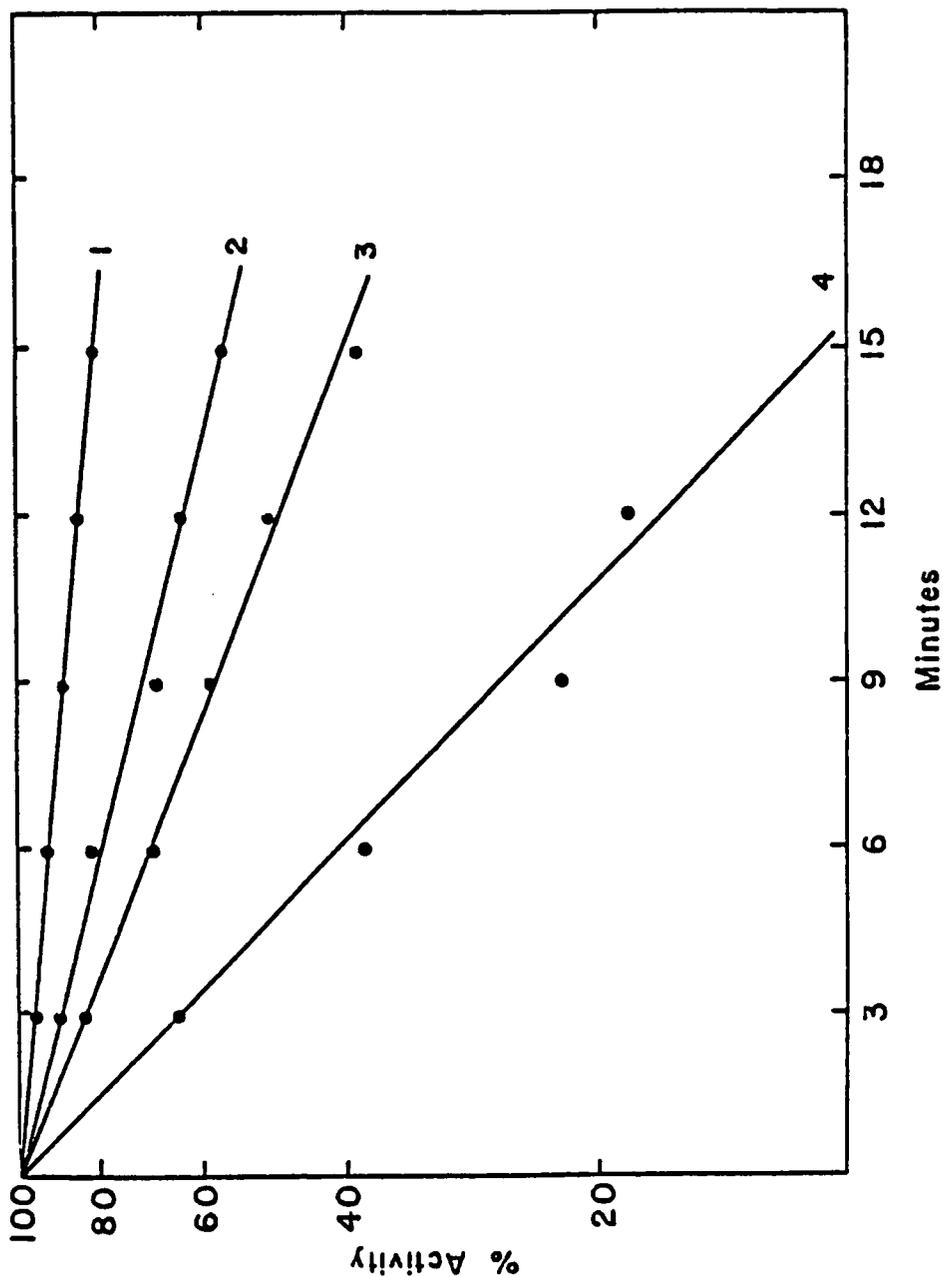


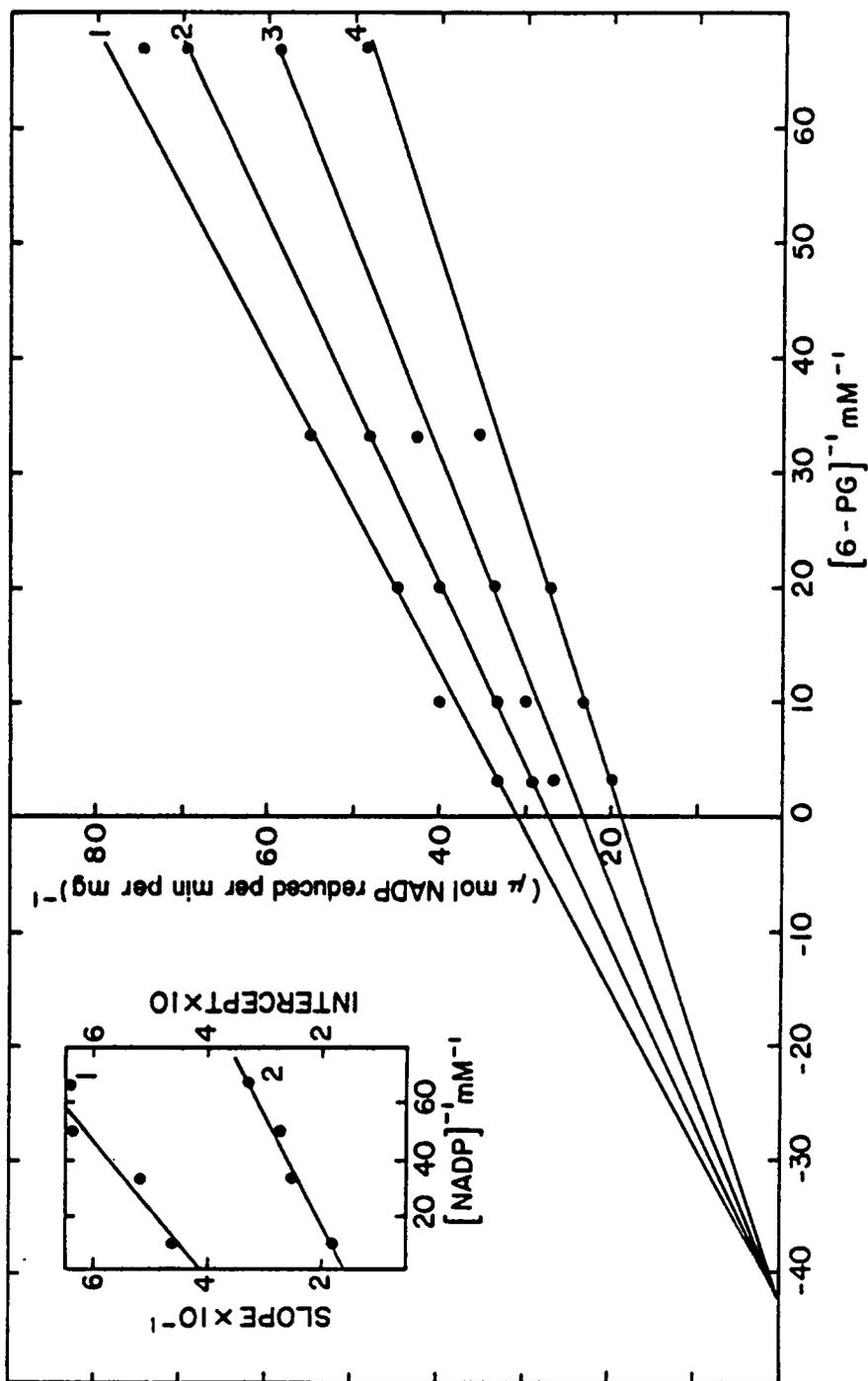
Figure 26. Thermal Denaturation of *H. influenzae* 6-PGDH: Incubation mixtures (1 ml) contained 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 0.5 mM DTT, and 105 ng of enzyme, assayed for activity as described in Experimental Procedures, and plotted on the semilog scale. The temperatures of the incubation mixtures were: line 1, 48°C; line 3, 55°C; line 4, 56.5°C. Line 2 indicates the inclusion of 1 mM 6-phosphogluconate in the incubation mixture at 55°C.

## Kinetic Mechanism of 6-Phosphogluconate Dehydrogenase

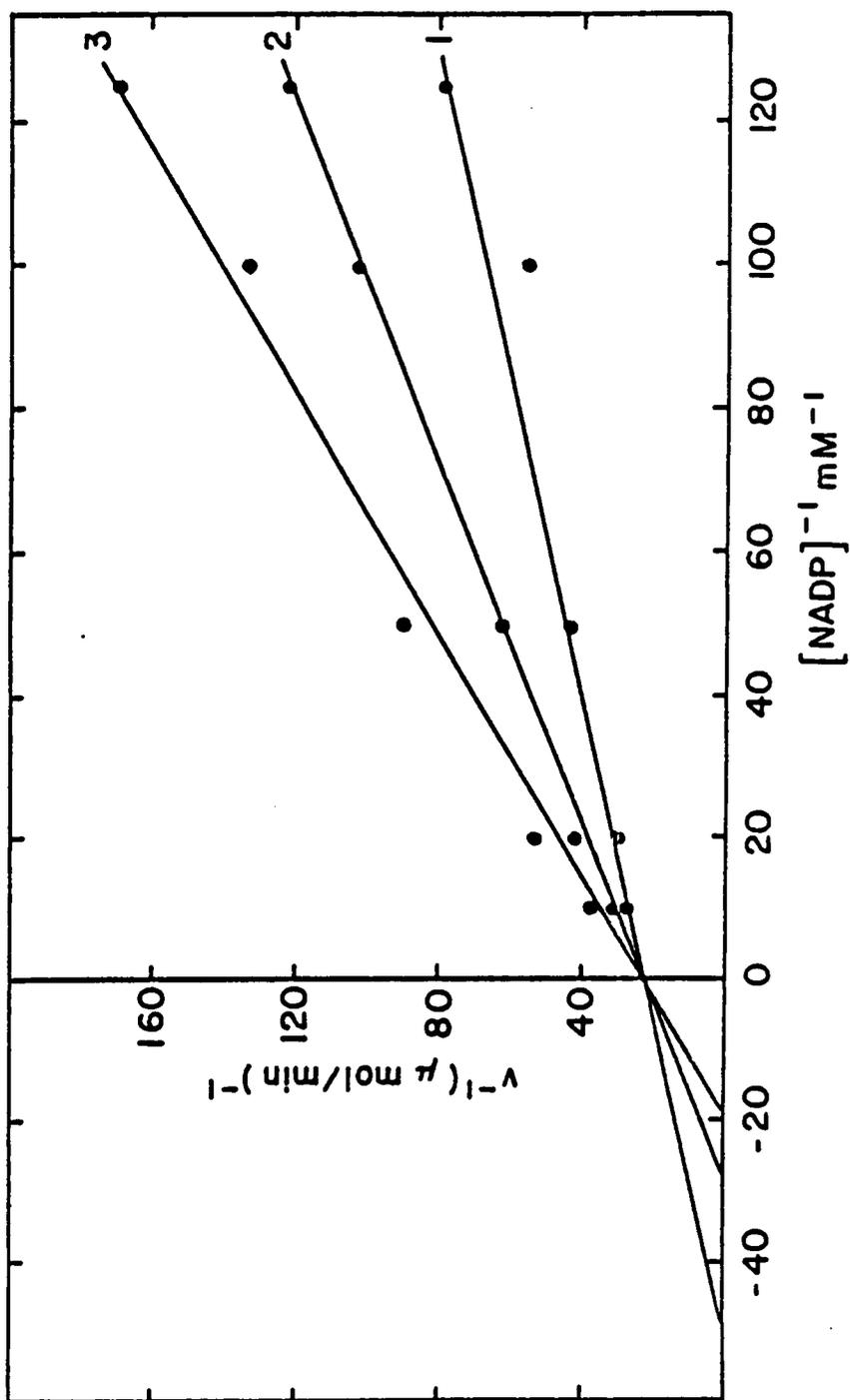
Kinetic parameters of 6-PGDH were measured through initial velocity studies as described above for the malate dehydrogenase. NADP reduction was carried out as described in Experimental Procedures. The initial velocities obtained by varying 6-phosphogluconate concentration at four fixed concentrations of NADP are shown in Figure 27. Slope and intercept replots were used to determine the values for  $K_m$  and  $V_{max}$ . The  $K_m$  and  $V_{max}$  values determined for NADP and 6-phosphogluconate (6-PG) were 16  $\mu$ M and 10  $\mu$ M, respectively. The results obtained by varying NADP at different fixed concentrations of 6-phosphogluconate also gave a converging line relationship similar to that depicted in Figure 27. These results indicated a sequential reaction mechanism.

Product and dead-end inhibition studies were investigated to determine whether the sequential reaction mechanism was of an ordered or random type and were performed in the same manner as with *H. influenzae* MDH. In the product inhibition studies, when NADP was the variable substrate, inhibition by one product, NADPH, at both unsaturating and saturating concentrations of 6-phosphogluconate, was competitive (Figure 28). When inhibition by NADPH was examined with 6-phosphogluconate as the variable substrate at an unsaturating concentration of NADP, noncompetitive inhibition was observed (Figure 29). No inhibition by NADPH was observed when a saturating concentration of NADP was employed.

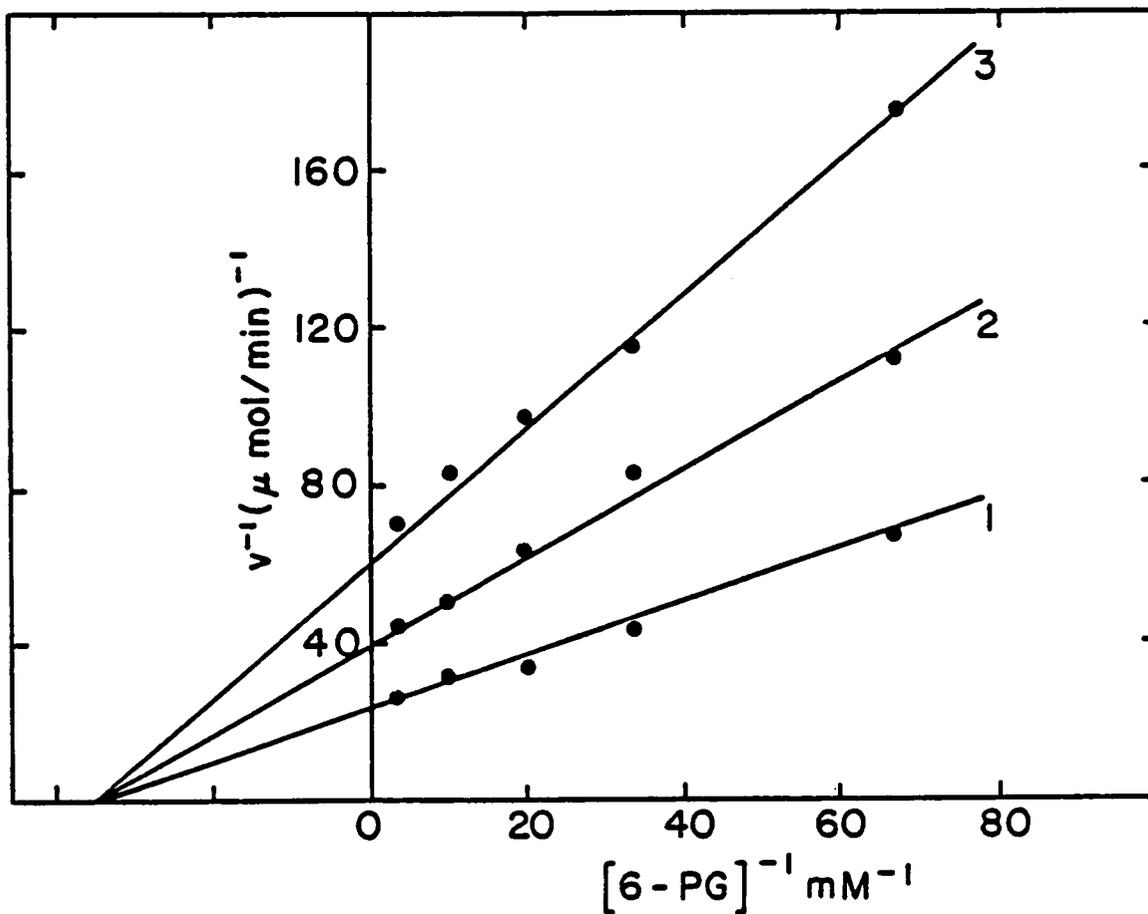
Structural analogs of NADP and 6-phosphogluconate, 2',5'-ADP and fructose 1,6-diphosphate (FDP), respectively, were used as dead-end inhibitors. Inhibition by 2',5'-ADP was competitive when NADP was used as the variable substrate (Figure 30, panel A) and noncompetitive when 6-phosphogluconate was used as the variable substrate (Figure 30, panel B). Inhibition by FDP was noncompetitive when NADP was used as the variable substrate and competitive when 6-phosphogluconate was used



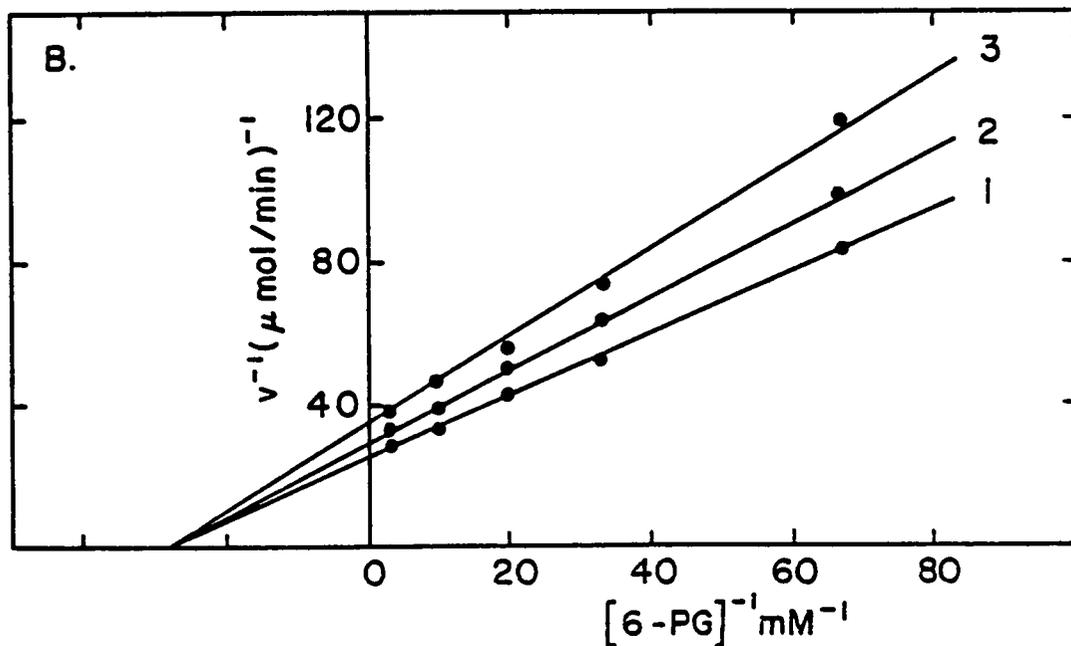
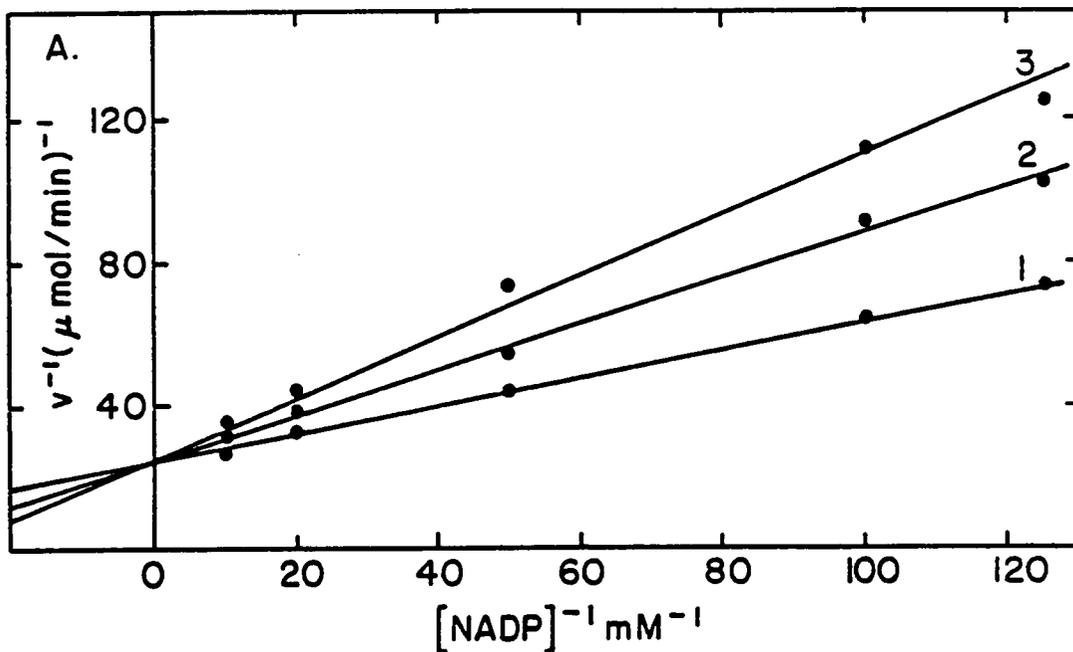
**Figure 27. Effect of NADP and 6-Phosphogluconate Concentrations on Initial Velocities:** The assay mixtures contained concentrations of 6-phosphogluconate varying from 15 to 300  $\mu\text{M}$  at four fixed concentrations of NADP in 10 mM potassium phosphate buffer, pH 7.0. Reactions were initiated by the addition of 225 ng of 6-PGDH. The concentrations of NADP used ( $\mu\text{M}$ ) were: line 1, 15; line 2, 20; line 3, 30 ; line 4, 100. Inset is a replot of slopes (line 1) and intercepts (line 2).



**Figure 28. Product Inhibition of 6-PGDH by NADPH as a Function of NADP Concentration:** At a saturating concentration (1 mM) of 6-phosphogluconate, reaction mixtures contained potassium phosphate buffer, pH 7.0, 8 to 100  $\mu$ M NADP, and 145 ng of enzyme. NADPH concentrations were: line 1, none; line 2, 10  $\mu$ M; line 3, 50  $\mu$ M.



**Figure 29. Product Inhibition of 6-PGDH by NADPH as a Function of 6-Phosphogluconate Concentration:** At an unsaturating concentration (20  $\mu\text{M}$ ) of NADP, reaction mixtures contained 10 mM potassium phosphate buffer, pH 7.0, 15 to 300  $\mu\text{M}$  6-phosphogluconate, and 237 ng of enzyme. NADPH concentrations were: line 1, none; line 2, 10  $\mu\text{M}$ ; line 3, 30  $\mu\text{M}$ .



**Figure 30. Dead-end Inhibition of 6-PGDH by 2',5'-ADP:** A. As a function of NADP concentration, reaction mixtures contained 10 mM potassium phosphate buffer, pH 7.0, 1 mM 6-phosphogluconate, 8 to 100  $\mu\text{M}$  NADP, and 147 ng of enzyme. 2',5'-ADP concentrations were: line 1, none; line 2, 10  $\mu\text{M}$ ; line 3, 20  $\mu\text{M}$ . B. As a function of 6-phosphogluconate concentration, reaction mixtures contained 10 mM potassium phosphate buffer, pH 7.0, 100  $\mu\text{M}$  NADP, 15 to 300  $\mu\text{M}$  6-phosphogluconate, and 147 ng of enzyme. 2',5'-ADP concentrations were: line 1, none; line 2, 10  $\mu\text{M}$ ; line 3, 20  $\mu\text{M}$ .

as the variable substrate. The  $K_i$  values determined for 2',5'-ADP and FDP were 18  $\mu$ M and 0.98 mM, respectively.

### Coenzyme Specificity

Several NADP analogs altered in either the pyridine or purine moiety were examined for their ability to serve as coenzymes for *H. influenzae* 6-PGDH. Initial velocities were determined by replacing NADP with the various NADP analogs as coenzymes at five concentrations between 8 and 400  $\mu$ M.  $K_m$  and  $V_{max}$  values were obtained by extrapolation to x-axis and y-axis intercepts from double reciprocal plots and are listed in Table XI. Reactions involving these NADP analogs varied with respect to  $K_m$  and  $V_{max}$  values. Pyridine-modified analogs had higher  $K_m$  and lower  $V_{max}$  values than those values observed with NADP, whereas purine-modified analogs had higher  $K_m$  and approximately the same  $V_{max}$  values. Reductions were not observed with NAD and 3-aminopyridine adenine dinucleotide phosphate (AADP).

### Studies of Enzyme Inhibition

Binding sites for the coenzyme, NADP, were probed by inhibition studies through the use of coenzyme-competitive inhibitors. Five adenosine derivatives were demonstrated to be effective coenzyme-competitive inhibitors of the purified 6-PGDH. In studies of coenzyme-competitive inhibition of 6-PGDH, initial velocities were determined by varying the concentration of NADP at several fixed concentrations of various inhibitors. The inhibitor dissociation constants obtained for these compounds and AADP are listed in the Table XII. The results show that 2',5'-ADP and 2'-phosphoadenosine diphosphoribose (2'P-ADP-ribose) were the most effective inhibitors of all the adenosine derivatives tested, and the  $K_i$  values were 200 times lower than those of 5'-adenosine diphosphate and adenosine diphosphoribose. 5'-Adenylic

**Table XI.  $K_m$  and  $V_{max}$  Values for NADP Analogs**

NADP analog	$K_m$ ( $\mu M$ )	$V_{max}$ ( $\mu mol/min/mg$ )
Nicotinamide adenine dinucleotide phosphate	16	65
3-Acetylpyridine adenine dinucleotide phosphate	59	46
Thionicotinamide adenine dinucleotide phosphate	67	20
Nicotinamide 1, $N^6$ -etheno-adenine dinucleotide phosphate	39	71
Nicotinamide hypoxanthine dinucleotide phosphate	61	71

**Table XII. Inhibition of *H. influenzae* 6-PGDH**

Inhibitor	$K_i$ (mM)
5'-Adenylic acid	— <sup>a</sup>
2'-Adenylic acid	2.68
5'-Adenosine diphosphate	2.85
2',5'-Adenosine diphosphate	0.018
3',5'-Cyclic adenosine diphosphate	— <sup>a</sup>
Adenosine diphosphoribose	2.82
2'-Phosphoadenosine diphosphoribose	0.016
3-Aminopyridine adenine dinucleotide phosphate	0.096

a = no inhibition detected

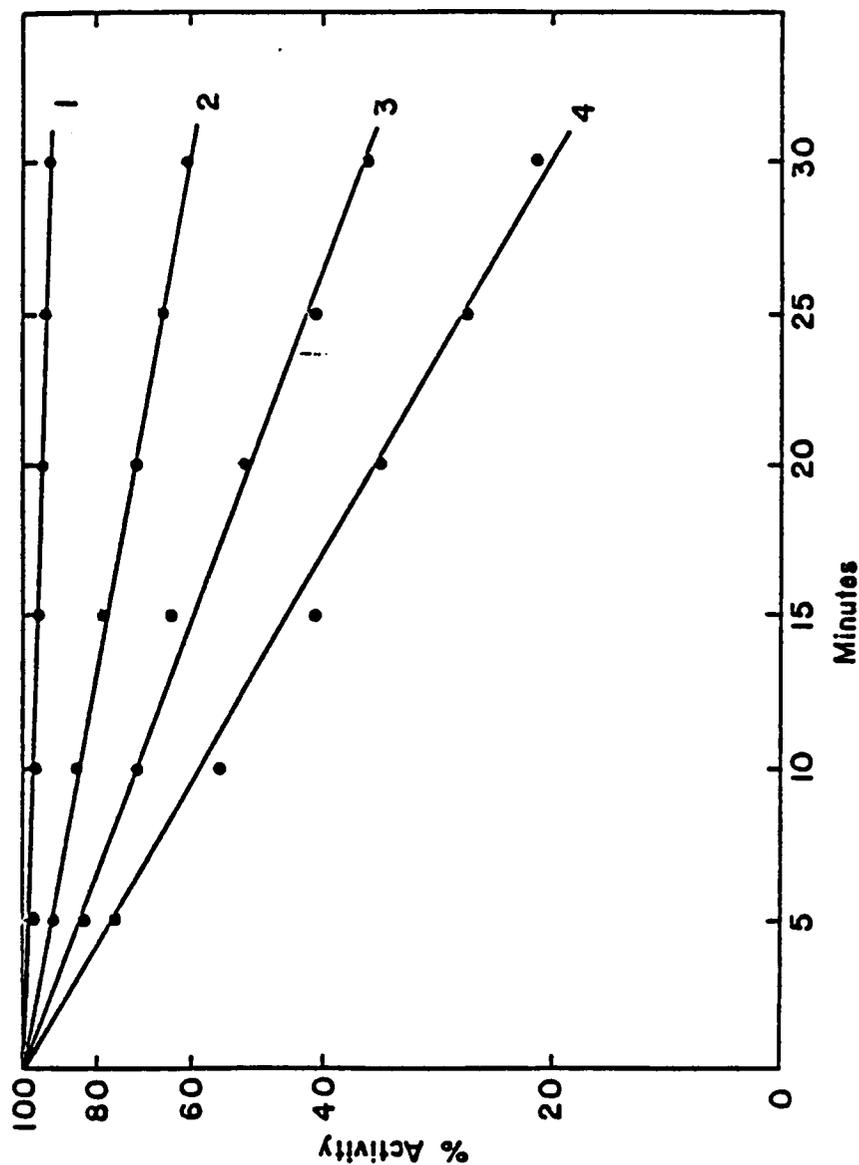
acid and 3',5'-cyclic adenosine diphosphate did not cause inhibition of the enzyme at concentrations as high as 15 mM and 7.5 mM, respectively.

### **Chemical Modification of the Enzyme**

The chemical modification of 6-PGDH was investigated to identify possible amino acid residues essential for catalytic activity. *H. influenzae* 6-PGDH was inactivated by incubation with a sulfhydryl group modifying reagent, N-ethylmaleimide, at 25°C and pH 7.0. Inactivation showed time-dependent pseudo-first-order kinetics. No loss of enzyme activity was observed over the incubation period with 5% ethanol in the absence of N-ethylmaleimide. At 1 mM N-ethylmaleimide the rate constant for inactivation was 0.0354 min<sup>-1</sup>. The second-order rate constant for the N-ethylmaleimide inactivation of the enzyme was 25 M<sup>-1</sup> min<sup>-1</sup>. This was obtained from the slope of the graph which expressed pseudo-first-order rate constants as a function of N-ethylmaleimide concentration. The presence of 1 mM 6-phosphogluconate protected against N-ethylmaleimide inactivation whereas 0.1 mM NADP did not protect the enzyme against inactivation.

### ***Studies of Growth Inhibition of Haemophilus influenzae***

Procion Blue HB, the 3-pyridylcarbinol analog of NAD,  $\alpha$ -ketoglutarate, the 3-aminopyridine analog of NADP (AADP), 2',5'-ADP, and 2'P-ADP-ribose, which represent the most effective inhibitors of the *H. influenzae* MDH and 6-PGDH, were tested for their ability to inhibit the growth of the organism. In studies evaluating the ability of compounds to function as growth inhibitors, growth was initiated by adding 0.5 ml of freshly grown cells (late linear phase), containing 1  $\mu$ g/ml NAD, into the media that contained 0.1  $\mu$ g/ml of NAD and an indicated amount of inhibitor (Table XIII), in a volume of 50 ml. Other growth conditions were essentially the same as described in Experimental Procedures and the growth of the organism was measured by following the



**Figure 31. Inactivation of 6-PGDH with N-Ethylmaleimide:** Enzyme inactivation and reaction mixtures were described in Experimental Procedures. The concentrations of N-ethylmaleimide used were: line 1, none; line 2, 0.5 mM; line 3, 1 mM; line 4, 2.5 mM.

increase of turbidity at 660 nm. These compounds were observed to be active as growth inhibitors in the presence of limiting NAD concentration. The concentrations of each inhibitor used were five times the  $K_i$  value with the exception of AADP where 1  $\mu\text{g/ml}$  of the inhibitor was employed. Growth inhibition was expressed as doubling time and the percent inhibition of growth is listed in Table XIII. The value of the reciprocal of the doubling time ( $1/dt$ ) for the control was set at 100 and the % decrease in the  $1/dt$  in the presence of various inhibitors compared to the  $1/dt$  for the control was equated to % inhibition.

**Table XIII. Inhibition of the Growth of *H. influenzae* with NAD as V-factor**

Inhibitor	Concentration ( $\mu$ M)	Inhibition (%)
3-Pyridylcarbinol adenine dinucleotide	100	74
$\alpha$ -Ketoglutarate	125	62
Procion Blue HB	1	84
ADP	1.6	65
2',5'-ADP	90	66
2'P-ADP-ribose	80	66
3-Aminopyridine adenine dinucleotide phosphate	1.2	100

## CHAPTER V

### DISCUSSION

*Haemophilus influenzae* type b is the primary cause of bacterial meningitis in infants and is responsible for 8,000 cases annually in the U.S. (3). Other related *Haemophilus* species have been implicated in numerous diseases in humans and other animals. Treatment of *H. influenzae* infections has relied upon the use of ampicillin and chloramphenicol; however, a continuing increase in strains resistant to these antibiotics has been observed (7-9). *H. influenzae* exhibits an absolute growth requirement for intact NAD or related nicotinamide derivatives. The inability to synthesize NAD from the usual precursors and the likelihood that the availability of intact NAD in the environment could be somewhat limited suggests that the organism must have developed unique mechanisms to satisfy metabolic needs for NAD and NADP. Therefore, enzymes involved in pyridine nucleotide metabolism may also exhibit unique properties. The *H. influenzae* periplasmic nucleotide pyrophosphatase, observed to be responsible for hydrolysis of external NAD which provides the NMN required for intracellular resynthesis of NAD, exhibited negative cooperativity (13). Thus the enzyme catalyzes the hydrolysis of NAD at very low NAD concentrations. The pyrophosphatase showed an unusual response to structural alterations in the purine portion of dinucleotide substrates, such as loss of cooperativity and decreased functioning of substrates. It was

of interest to study other NAD- and NADP-dependent enzymes in *H. influenzae* with respect to investigating unusual processes involved in pyridine nucleotide interactions. In this respect, the malate dehydrogenase, which was previously indicated to be of importance in the partial tricarboxylic acid cycle observed in studies of glucose metabolism (14), and 6-phosphogluconate dehydrogenase, which may play an important role in regulating the pentose phosphate pathway (92,93) were chosen for the present investigation. Relatively little is known about the enzymes involved in pyridine nucleotide metabolism in *H. influenzae*. Knowledge of the properties of NAD- and NADP-requiring dehydrogenases should aid in understanding the effects of pyridine nucleotide derivatives observed previously in studies of support or inhibition of growth of the organism (13). Therefore it was of interest to purify and characterize representative NAD- and NADP-dependent dehydrogenases of *H. influenzae* and compare the properties with corresponding eucaryotic enzymes which have been extensively studied.

In sonicates of *H. influenzae* the MDH activity was the highest among those dehydrogenases found (Table I). The enzyme plays an important role in a reported partial tricarboxylic acid cycle from phosphoenolpyruvate through oxalacetate to succinate. In the present study, *H. influenzae* MDH was purified 109-fold with a 26% recovery through a 4-step procedure involving salt fractionation, and hydrophobic and dye affinity chromatography (Table II). Triazinyl dye affinity chromatography has been used effectively for the purification of malate dehydrogenases from a number of different organisms, which was attributed to the fact that the enzyme can be bound to the affinity resins and eluted selectively by a mixture of NAD and malate. For the elution of *H. influenzae* MDH, 0.25 M KCl was used to avoid interference by substrates in subsequent enzyme characterization studies. The purified enzyme migrated as a single band during native gel electrophoresis and stained positively for both protein and enzyme activity. The molecular weight of the purified enzyme was determined by both gel filtration and gel electrophoresis. Under nondenaturing conditions, the enzyme exhibited a molecular

weight of 61,000. Under denaturing conditions of SDS-polyacrylamide gel electrophoresis, a molecular weight of 31,000 was observed indicating a dimeric structure in the native enzyme. The MDH isozymes from eucaryotes were reported to be dimers having molecular weights of 60,000 to 70,000. Subunits of the cytoplasmic enzymes from both pig and beef heart were determined to be identical by ultracentrifugation under denaturing conditions and tryptic peptide mapping techniques (94). Some bacteria such as *Bacillus* and *Eubacteriales* species have tetrameric forms of MDH (63,64). However, under denaturing conditions these tetrameric enzymes were dissociated into a monomeric form having identical subunit molecular weights as most dehydrogenases (66). The native molecular weight of 61,000 determined in this study for the *H. influenzae* MDH is consistent with the molecular weights reported for most malate dehydrogenases (51-66). Factors concerning the identification of the *Haemophilus* enzyme subunits were not researched; however, in all cases where subunit structure of MDH was studied, identical subunits were observed.

An optimal pH range of 9 to 10 in malate oxidation for procaryotic and eucaryotic malate dehydrogenases has been reported (74-76). In the pH study of *H. influenzae* MDH, an optimal pH range 9 to 10 was also indicated. The effect of ionic strength on this enzyme was determined by adding different concentrations of KCl to the reaction mixture in glycine-NaOH buffer, pH 9.6. Increasing the ionic strength from 10 to 100 mM did not cause a significant effect on initial velocities of NAD reduction. The purified enzyme was observed to be stable when stored at -15°C in a 50% propylene glycol solution containing 100 mM KCl and 10 mM potassium phosphate buffer, pH 7.0. Thermal denaturation was observed at moderate temperatures and protection against thermal denaturation was achieved by the presence of NAD.

Many dehydrogenases have been demonstrated to operate through an ordered binding of substrates. Isotopic exchange studies of malate dehydrogenases from eucaryotes indicated an ordered bi bi mechanism which involved orderly binding of

coenzyme and substrate to form a ternary complex (69,70). Less mechanistic information is available for bacterial malate dehydrogenases. Since the reaction catalyzed by MDH is bireactant in both directions, a number of different kinetic mechanisms could be possible. With the *H. influenzae* enzyme, initial velocities of NAD reduction and NADH oxidation carried out by varying one substrate at several fixed concentrations of the appropriate second substrate led to converging line relationships in double reciprocal plots. With each substrate studied a sequential reaction mechanism was indicated. The  $K_m$  values for the substrates in the forward and reverse reactions were NAD, 57  $\mu\text{M}$ ; NADH, 2  $\mu\text{M}$ ; malate, 290  $\mu\text{M}$ ; and oxalacetate, 13  $\mu\text{M}$  (Table III). The question of whether the sequential reaction mechanism was of an ordered or random type was investigated through product and dead-end inhibition studies. The results of the product and dead-end inhibition studies are summarized in Table IV. The uncompetitive inhibition by the product, oxalacetate, when NAD was varied at a saturating concentration of malate, is characteristic of the ordered bi bi mechanism. The remaining product inhibition profile was likewise consistent with this mechanism. Structural analogs of NAD and malate were used as dead-end inhibitors. In the dead-end inhibition studies the uncompetitive inhibition by hydroxymalonate as a function of NAD concentration was also consistent with the ordered bi bi mechanism, since noncompetitive inhibition would have been expected in a random process. Both product and dead-end inhibition studies indicated an ordered bi bi mechanism (Figure 32) in which NAD is the first substrate bound to the enzyme and NADH is the second product dissociating from the enzyme. Protection of the enzyme against thermal denaturation by NAD but not by malate further supported the ordered mechanism. Knowledge of the order of binding of substrates and release of products can be useful in mapping the binding sites of substrates and in the preparation of selective inhibitors of the enzyme.

Several pyridine nucleotide analogs altered in either the pyridine or purine moiety were studied as coenzymes for *H. influenzae* MDH. Kinetic parameters for six analogs

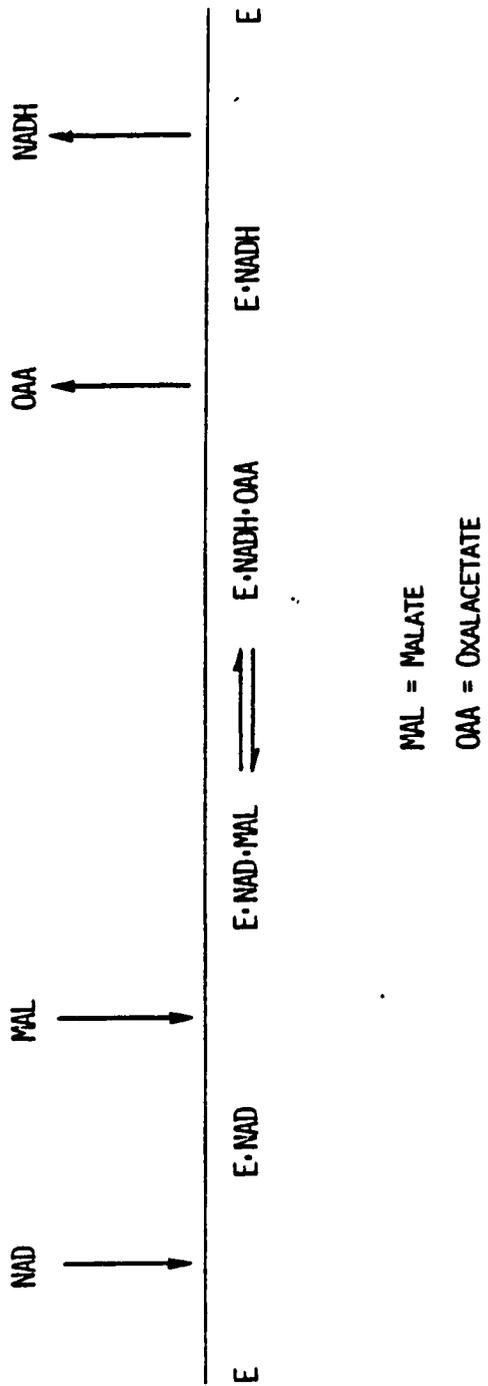


Figure 32. The Pattern of Ordered Bi Bi Mechanism of *H. influenzae* Malate Dehydrogenase

of NAD exhibiting coenzyme activity are listed in Table V. The purified MDH can be classified strictly as an NAD-dependent dehydrogenase since no activity with NADP was observed at high enzyme concentrations. The purified enzyme was observed to function with several NAD analogs structurally altered in either the purine or pyridine portion of the dinucleotide molecule. Of special interest was the functioning of the 3-acetylpyridine and thionicotinamide analogs, both of which were observed previously to support growth of *H. influenzae* in the absence of NAD. The substitution of groups at position 3 of the pyridinium moiety of NAD resulted in somewhat higher  $K_m$  and lower  $V_{max}$  values compared to those values obtained with NAD. In studies of pig heart MDH, hydrogen bonding involving the carboxamide group of the nicotinamide moiety of NAD was considered responsible for providing the correct stereospecificity for the hydride ion transfer reaction to and from carbon 4 of the pyridine ring (95). All of the dinucleotides shown to function as coenzymes with the *H. influenzae* MDH (Table V) have 3-position groups capable of hydrogen bonding. The inability of the vinylogous 3-pyridylacryloamide adenine dinucleotide to serve as a coenzyme could be attributed either to an improper orientation of the hydrogen bonding carboxamide group or to steric hindrance related to the large size of this 3-position functional group. The inability of the negatively-charged nicotinic acid analog is in agreement with the observed ineffectiveness of this derivative with several other dehydrogenases (96). Alteration in the purine moiety of the coenzyme, as in the case of the guanine and ethenoadenine derivatives, resulted in a significant decrease in the functioning of these dinucleotides as coenzymes. Such large effects from purine alterations were also observed in reactions catalyzed by the *H. influenzae* nucleotide pyrophosphatase (13). Thus, the adenine portion of the coenzyme appears to be more important in binding to the enzyme than does the pyridinium portion. NMN was completely inactive as a coenzyme, alone and in the presence of adenosine derivatives, indicating the importance of the adenylyl moiety for nucleotide binding and catalysis. NMN was included in these studies since the re-

duction of NMN has been observed in a few dehydrogenase reactions (97-99). Since NMN is a product of the *H. influenzae* periplasmic nucleotide pyrophosphatase reaction and is known to support growth of the organism, the inability of NMN to function with the MDH confirms the need for the intracellular resynthesis of NAD to meet the coenzymic requirement of at least one metabolically important dehydrogenase.

Selective inhibition of enzymes involved in NAD internalization and utilization in *H. influenzae* could have a pronounced effect on growth of the organism. Specific interactions of the dinucleotide in binding and catalytic processes can be applied to design effective inhibitors of enzymes that could be tested for growth inhibition of the organism. Recent site-mapping studies of the *H. influenzae* periplasmic nucleotide pyrophosphatase (13) led to the recognition of several effective inhibitors of the enzyme. Of special interest was the correlation between ADP inhibition of the nucleotide pyrophosphatase and ADP inhibition of growth of the organism. In the present study, selective inhibition of the purified MDH was investigated in attempts to determine important interactions in the binding of ligands at the coenzyme and substrate binding sites of the enzyme. Derivatives of the adenosine portion of NAD were tested to determine the importance of this functional group in enzyme recognition. The type of inhibition and inhibitor dissociation constants were determined through double-reciprocal plotting methods (99) using several fixed concentrations of the inhibitor. The results demonstrated that all adenosine derivatives studied inhibited the enzyme in a coenzyme-competitive manner. The  $K_i$  values determined reflect the effectiveness of inhibition. The  $K_i$  values for AMP and ADP were 12 times lower than that of adenosine (Table VI). The significant increase in binding of AMP and ADP relative to adenosine indicated an importance for ionic interactions occurring at a pyrophosphate region of the NAD binding site. The enhanced binding of ADP-ribose compared with that of ADP could reflect an importance of a region on the enzyme which interacts with the additional ribose group on the ADP molecule. For pig heart mitochondrial MDH, ATP, ADP, and

AMP were competitive inhibitors with respect to NADH (101). ADP-ribose has been demonstrated as a coenzyme-competitive inhibitor of several malate dehydrogenases (51,52). Similar studies with L- $\alpha$ -glycerophosphate dehydrogenase have demonstrated enzyme recognition of the adenosine and pyrophosphate moieties of the coenzyme molecule. However, in yeast and horse liver alcohol dehydrogenases the pyrophosphate region appeared less important, suggesting no uniform pattern of binding and perhaps a mechanistic difference in the utilization of pyridine nucleotides by different enzymes. Although adenosine derivatives were inhibitory, no inhibition was observed at 100 mM concentrations of nicotinamide derivatives, such as N<sup>1</sup>-methylnicotinamide chloride and N<sup>1</sup>-octylnicotinamide chloride. Although selective interactions with the pyridinium moiety of the coenzyme are to be expected, the lack of effective binding of N<sup>1</sup>-alkylnicotinamide chlorides suggests that such interactions are not available in the absence of the ADP-ribose portion of the coenzyme. Such observations would be consistent with a conformational change induced by the binding of the adenosine half of the coenzyme molecule. The lack of inhibition by N<sup>1</sup>-alkylnicotinamide chlorides was also observed with rabbit muscle lactate dehydrogenase, pig heart and rat liver malate dehydrogenase, yeast glucose 6-phosphate dehydrogenase and 6-PGDH (102). In contrast, the specific binding of N<sup>1</sup>-alkylnicotinamide chlorides was demonstrated with yeast alcohol dehydrogenase, rabbit muscle glycerophosphate dehydrogenase, and rat ovarian 20 $\alpha$ -hydroxysteroid dehydrogenase (46,103,104). In the former cases it is likely that the binding of the purine portion of the coenzyme induces a conformational change, leading to proper orientation of nicotinamide moieties. Coenzyme analogs that are chemically unreactive in dehydrogenase reactions such as pyridine adenine dinucleotide, 3-pyridylcarbinol adenine dinucleotide, 3-pyridylacetonitrile adenine dinucleotide, 3-methylpyridine adenine dinucleotide, nicotinic acid adenine dinucleotide, and 3-aminopyridine adenine dinucleotide were evaluated as coenzyme-competitive inhibitors and the respective  $K_i$  values are listed in Table VII. Of the NAD analogs studied, the

3-pyridylcarbinol adenine dinucleotide was the most effective inhibitor among this class of compounds exhibiting a  $K_i$  value of 20  $\mu\text{M}$ . The hydrogen bonding possibilities for the 3-carbinol substituent may play a role in the better binding of this derivative as in the case of coenzyme specificity studies, which would be consistent with the involvement of hydrogen bonding in the cozymic functioning of dinucleotides. The remaining NAD analogs were much less effective as inhibitors of the enzyme. Selective interactions of the enzyme with substituents in the 3-position of the pyridine ring of NAD analogs were indicated. In earlier studies (49), it was speculated that a 3-position carbon, double bonded to oxygen, sulfur or nitrogen, was an absolute requirement for the functioning of the NAD analogs in dehydrogenase reactions. Also, sufficient electron withdrawing power by the substituent in the 3-position is necessary to promote cozymic function (49). Of special interest was the ineffectiveness of the 3-aminopyridine analog as an inhibitor of *H. influenzae* MDH. This analog served as a substrate for *H. influenzae* nucleotide pyrophosphatase and functioned as a potent growth inhibitor of the organism at 1.0  $\mu\text{M}$  concentration (13). Therefore this exceptional growth inhibition of *H. influenzae* by the 3-aminopyridine analog could not be explained by interactions of the analog with malate dehydrogenase. The mechanism of growth inhibition by the 3-aminopyridine analog of NAD remains to be clarified. Procion Blue HB, the ligand used effectively for affinity chromatography in the purification of MDH, was a very effective coenzyme-competitive inhibitor exhibiting a  $K_i$  value of 0.2  $\mu\text{M}$ .

Since inhibition of the enzyme by adenosine derivatives indicated an importance for interactions of negatively-charged phosphate or pyrophosphate groups, straight chain aliphatic carboxylic acids were studied as coenzyme-competitive inhibitors. Pure coenzyme-competitive inhibition was observed with carboxylic acids varying in chainlength from acetic to octanoic acid (Table VIII). The mixed-type inhibition observed with nonanoic and decanoic acids could indicate secondary binding at the substrate binding site. Positive chainlength effects obtained are shown in Figure 17 and

indicate a facilitation of binding through nonpolar interactions. The change in the logarithm of  $1/K_i$  per methylene group ( $\Delta \log K_i$ ) obtained from this relationship was used to calculate the change in free energy of binding from the following equation  $\Delta \Delta G = 2.3 RT \Delta \log K_i$ . Positive chainlength effects were observed with a change in free energy of binding per methylene group of 0.2 Kcal/mole. This value is slightly below the range (0.3-0.9 Kcal/mole) expected for chain-chain interactions through dispersion forces (132), indicating that some steric hindrance may result in less effective nonpolar interactions. The importance of the negatively-charged carboxylate anion in binding was confirmed by the lack of inhibition with butanol. The binding of the carboxylic acids arises from a combination of interactions involving a pyrophosphate region and a closely-associated hydrophobic region at the coenzyme binding site. This speculation was supported by data obtained in multiple inhibition studies. Pairs of competitive inhibitors were studied for simultaneous binding or mutual exclusion at the coenzyme binding site. The interaction constant of infinity, observed in the multiple inhibition studies of the inhibitor pair, ADP and hexanoic acid, indicates that these two inhibitors utilize a common region of the NAD binding site, presumably the pyrophosphate region. Parallel lines were also obtained in studies of the inhibitor pairs, hexanoic acid and AMP, and hexanoic acid and ADP-ribose. Facilitation of binding of carboxylic acids by nonpolar interactions suggests the presence of a hydrophobic region nearby the pyrophosphate region of the NAD binding site. Carboxylic acids were previously employed as coenzyme-competitive inhibitors for several NAD(P)-requiring dehydrogenases (16,106). In studies of rabbit muscle L- $\alpha$ -glycerophosphate dehydrogenase, inhibition by fatty acids was proposed to be related to endogenous regulation of this enzyme in the cell (106). The coenzyme-competitive inhibition of dehydrogenases by compounds having no obvious structural analogy to the coenzyme molecule can be explained on the basis of interactions of these compounds with different regions existing in the coenzyme binding site. In the case of the *Haemophilus* MDH, the

presence of a hydrophobic region adjacent to the pyrophosphate region of the NAD binding site provides the necessary two-point interactions for the selective binding of aliphatic carboxylic acids. Without prior knowledge of the spatial arrangement of regions at this binding site, coenzyme-competitive inhibition by carboxylic acids would not have been anticipated. The competitive binding of fatty acids and alkylammonium ions to the NAD binding site of rabbit muscle L- $\alpha$ -glycerophosphate dehydrogenase was likewise explained by selective interactions of these compounds with closely associated regions at the coenzyme binding site (106).

*H. influenzae* MDH was also inhibited effectively by structural analogs of malate. The substrate-competitive inhibitors studied are listed in Table IX. Hydroxy- and keto-monocarboxylic acids were not inhibitory at 20 mM concentration. Of the dicarboxylic acids studied,  $\alpha$ -ketoglutarate was the most effective, exhibiting a  $K_i$  value of 25  $\mu$ M. The better inhibition by the keto acid was consistent with the observation that the  $K_m$  for malate is 22-fold higher than that of the keto substrate, oxalacetate. Although effectively bound,  $\alpha$ -ketoglutarate did not serve as a substrate.

A number of amino acid residues involved in catalytic activity can be examined using various chemical reagents. In chemical modification studies of malate dehydrogenases from a variety of sources, only histidine has been clearly implicated as a catalytic active residue. *H. influenzae* MDH lost activity in a first-order process in the presence of diethylpyrocarbonate, indicating a potential involvement of a histidine residue. The sulfhydryl modifying reagents, N-ethylmaleimide, iodoacetamide, and iodoacetic acid did not inactivate the *H. influenzae* MDH enzyme. The reaction of pig cytoplasmic MDH with sulfhydryl reagents likewise indicated no involvement of cysteine residues in the catalytic mechanism of this enzyme. Two cysteine residues reacted with Ellman's reagent, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), with no loss of activity (107). Other cysteine modifying reagents such as *p*-hydroxymercuribenzoate and 4,4'-bisdimethylaminodiphenylcarbinol also did not affect the pig cytoplasmic

dehydrogenase activity. With pig heart mitochondrial MDH, sulfhydryl reagents were effective in modifying two essential thiol groups per enzyme molecule (108). Both modifications were protected by the binding of coenzyme. Therefore the mitochondrial MDH differs from the cytoplasmic enzyme in having an essential thiol group on each subunit located close to the coenzyme binding site. Although the sulfhydryl reagents studied did not inactivate the *Haemophilus* MDH, it was of interest to determine the presence and availability of free sulfhydryl groups in the enzyme. Under denaturing conditions, the fluorescence titration with FMA revealed the presence of two sulfhydryl groups per 61,000 molecular weight which were not available for reaction in the native enzyme. The involvement of a specific histidyl residue in the catalytic mechanism of malate dehydrogenases appears to be well established (109-114). Previous investigation of pig heart cytoplasmic MDH with chemical modifying reagent, diethylpyrocarbonate, suggested that an essential histidine exists at the active site of the enzyme. Inactivation of mitochondrial MDH with iodoacetamide was also related to the modification of a histidine residue (115). The amino acid sequence of the region containing the active site histidine was determined and was analogous to that of lactate dehydrogenase (116), implying that catalytically active residues share extensive homology among dehydrogenases.

*H. influenzae* 6-phosphogluconate dehydrogenase was purified 308-fold with a 16% recovery through a 4-step chromatographic procedure involving a Phenyl-Sepharose hydrophobic column and affinity chromatography on Matrex gel Green A, Matrex gel Red A and 2',5'ADP-Sepharose 4B (Table X). Immobilized triazine affinity dye chromatography was successfully used to purify a number of 6-phosphogluconate dehydrogenases from different sources (116-119). The molecular weight of the *Haemophilus* enzyme was determined by gel filtration on Sephacryl S-200 and SDS gel electrophoresis. The native enzyme exhibited a 70,000 molecular weight. Under denaturing conditions a subunit molecular weight of 34,000 was observed indicating a dimeric

structure in the native enzyme. 6-Phosphogluconate dehydrogenases isolated from different bacterial and mammalian sources have dimer molecular weights of 100,000 (116-126). Pig liver 6-PGDH, however, is a dimer of 83,000 molecular weight (127).

The kinetic mechanism of 6-PGDH was investigated through initial velocity studies. Initial velocity studies of NADP reduction performed with the *H. influenzae* 6-PGDH enzyme by varying one substrate at several fixed concentration of the second substrate, indicated a sequential reaction mechanism. The  $K_m$  values for the substrates in the forward reaction were NADP, 16  $\mu\text{M}$ , and 6-phosphogluconate, 10  $\mu\text{M}$ . Subsequent studies designed to distinguish between an ordered and random mechanism were inconclusive. Although certain product and dead-end inhibition studies were consistent with an ordered mechanism in which NADP is the first substrate bound to the enzyme, the observed protection by 6-phosphogluconate in the thermal denaturation and N-ethylmaleimide inactivation of the enzyme was not consistent with this ordered mechanism. Both ordered and random mechanisms have been reported for sheep liver 6-PGDH (131).

Substrate specificity of *H. influenzae* 6-PGDH was investigated. The importance of the 2'-phosphoryl group of NADP was indicated by the inability of NAD to serve as a coenzyme at high enzyme concentrations. In the present coenzyme specificity studies, several NADP analogs functioned as coenzymes for the purified *H. influenzae* 6-PGDH (Table XI). Alterations in the pyridinium moiety of the coenzyme were shown to affect the kinetic parameters of the reaction. Substitution at position 3 of the pyridinium moiety of NADP, as in the thionicotinamide and 3-acetylpyridine analogs, resulted in higher  $K_m$  and lower  $V_{\text{max}}$  values than those values with NADP. Alterations in the purine moiety of the coenzyme likewise affected kinetic parameters as indicated by the results obtained with the hypoxanthine and ethenoadenine analogs of NADP (Table XI). Changes in the adenine moiety had a lesser effect on  $K_m$  and  $V_{\text{max}}$  values than alterations in the pyridinium moiety.

The results in Table XII demonstrated that those adenosine derivatives which have a phosphate group at the 2'-position were more effectively bound to the enzyme, which is consistent with the specificity of this dehydrogenase for NADP. 2',5'-ADP was bound 160 times better than 5'-ADP (Table XII). This very important interaction of the 2'-phosphate group in enzyme binding has also been demonstrated for other NADP-dependent dehydrogenases, i.e., yeast 6-PGDH (102). This implies that the additional phosphate facilitates the binding of coenzyme, presumably through electrostatic interactions with a positively-charged amino acid residue of the protein. AMP was bound very poorly but the presence of a pyrophosphate group as in the ADP, produced an enhanced binding of the molecule. The  $K_i$  values obtained for 2',5'-ADP and 2'-P-ADP-ribose were similar, indicating that the additional ribose on the 2'-P-ADP-ribose does not enhance the binding of the nucleotide.

The 6-PGDH was effectively inactivated by N-ethylmaleimide, indicating sulfhydryl involvement in catalysis. N-Ethylmaleimide inactivation was protected by 6-phosphogluconate, but not with NADP. The second-order rate constant for N-ethylmaleimide inactivation of the enzyme ( $25 M^{-1}min^{-1}$ ) was similar to that of yeast 6-PGDH ( $18.5 M^{-1}min^{-1}$ ). Protection by 6-PG but not by NADP was also observed with yeast 6-PGDH (102).

Since NAD is a limiting factor in the growth of *H. influenzae*, selective inhibition of the utilization of NAD by cytosolic dehydrogenases in conjunction with inhibition of the periplasmic nucleotide pyrophosphatase, could amplify the effect of such compounds in controlling growth of the organism. As effective inhibitors of *H. influenzae* MDH, 3-pyridylcarbinol adenine dinucleotide,  $\alpha$ -ketoglutarate, and Procion Blue HB were demonstrated to inhibit growth of the organism at concentrations observed to affect the catalytic activity of the purified MDH (Table XIII). As a substrate for the periplasmic nucleotide pyrophosphatase, the 3-pyridylcarbinol adenine dinucleotide would, upon hydrolysis, yield the 3-pyridylcarbinol mononucleotide, and this

mononucleotide may serve as a substrate for the intracellular NMN:ATP adenylyltransferase. At present, the substrate specificity of the adenylyltransferase has not been determined. Growth inhibition by Procion Blue HB, a large polar molecule incapable of diffusing into the cells, could be attributed to inhibition of the nucleotide pyrophosphatase located in the periplasmic space. This is supported by the fact that affinity chromatography on Matrex gel Blue A was a very effective step in the purification of the pyrophosphatase (13).

Several compounds exhibiting coenzyme-competitive inhibition of 6-PGDH were also analyzed for their ability to inhibit the growth of *H. influenzae* organism. 3-Aminopyridine adenine dinucleotide phosphate (AADP) at a concentration of 1  $\mu\text{g/ml}$  completely inhibits the growth of the organism. The inhibition of growth by 2',5'-ADP and 2'P-ADP-ribose occurred in a concentration range similar to that previously observed with AMP which inhibited the periplasmic nucleotide pyrophosphatase (13).

From previous experiments (13) on NAD metabolism of *H. influenzae*, the 3-acetylpyridine and thionicotinamide analogs of NAD supported growth of the organism, indicating that cytosolic dehydrogenases should be able to function with these analogs for cellular oxidation-reduction processes. The present studies confirmed that these analogs of NAD and NADP functioned as coenzymes for the two metabolically important dehydrogenases investigated.

The growth inhibition by AAD and AADP occurred at concentrations much lower than that required to inhibit the MDH and 6-PGDH. The effectiveness of these analogs as growth inhibitors can not be attributed to interactions with the two dehydrogenases investigated in this study. Other dehydrogenases as well as the NMN:ATP adenylyltransferase and NAD kinase could be considered prime targets for inhibition by the 3-aminopyridine analogs. Since AAD served as substrate for the nucleotide pyrophosphatase, the inhibition of growth by AAD(P) might be related to the

3-aminopyridine mononucleotide formed. 3-Aminopyridine mononucleotide, a product of the nucleotide pyrophosphatase reaction, might interact directly with other cytosolic enzymes or 3-aminopyridine adenine dinucleotide might be resynthesized by the adenylyltransferase and subsequently converted to 3-aminopyridine adenine dinucleotide phosphate by NAD kinase. Future experiments on substrate specificities and inhibitor profiles of the purified adenylyltransferase and NAD kinase should help distinguish between these different possibilities.

## CHAPTER VI

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