STUDIES ON PYRIDINE NUCLEOTIDE-DEPENDENT PROCESSES IN HAEMOPHILUS INFLUENZAE

by

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(ABSTRACT)

Haemophilus influenzae and related species have a unique requirement for externally-provided NAD; therefore, several pyridine nucleotide-requiring enzymes become important for the survival of these pathogens.

Haemophilus influenzae ATP:NMN adenylyltransferase was partially purified 15-fold with a 27% yield using dye affinity chromatography. Affinity chromatography was also used to purify NAD kinase from *Haemophilus influenzae*, 18-fold with a 32% yield. Substrate specificity studies of these enzymes demonstrated the enzymes to function with 3-acetylpyridine analogs of their respective substrates.

A membrane-bound NMN glycohydrolase was demonstrated in *Haemophilus influenzae*. The enzyme functions with 3-acetylpyridine mononucleotide as a substrate, and is inhibited effectively by 3-aminopyridine mononucleotide. The possible involvement of this enzyme in the transport of NMN into the cytoplasm is discussed.

Growth inhibition studies demonstrated that 3-aminopyridine mononucleotide is a potent inhibitor of growth of the organism and could inhibit growth by inhibiting the transport of NMN. The previously reported inhibition of growth by the 3-aminopyridine adenine dinucleotide was attributed to the formation of the mononucleotide through the reaction catalyzed by the *Haemophilus influenzae* periplasmic nucleotide pyrophosphatase.

A cytosolic lactate dehydrogenase, specific for D(-)-lactate was purified to electrophoretic homogeneity 2100-fold with a 14% yield. The purified enzyme was demonstrated to be a tetramer of M_r = 135,000. It catalyzes essentially the reduction of pyruvate with very low activity observed for the oxidation of D(-)-lactate. An optimum pH of 7.2 was determined for the reduction of pyruvate with NADH as the coenzyme. Several NADH analogs, altered either in the pyridine or purine moiety, functioned as coenzymes. Coenzyme-competitive inhibition by adenosine derivatives demonstrated important interactions of the pyrophosphate region of the coenzyme in binding with the enzyme. Several structural analogs of NADH and pyruvate were evaluated as selective inhibitors of the enzyme. Chemical modification of the purified D-lactate dehydrogenase was effectively achieved by micromolar concentrations of several N-alkylmaleimides. Positive chainlength effects in the inactivation by maleimides indicated the presence of a hydrophobic region close to the sulfhydryl groups being modified. The product of the reaction catalyzed by D-lactate dehydrogenase, D(-)-lactate, provides the substrate for a membrane-bound D-lactate oxidase. The D-lactate oxidase converts D(-)-lactate back to pyruvate and transfers electrons to the respiratory chain. No cytosolic L(+)-lactate dehydrogenase was found in *Haemophilus influenzae*; however, the organism possesses a L-lactate oxidase associated with the cell membrane. The L-lactate oxidase is also part of the respiratory chain, and utilizes exogenous L(+)-lactate to give pyruvate for the organism to use as a carbon source.

To

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Table of Contents

List of Illustrations

List of Tables

List of Abbreviations

AAD 3-Aminopyridine adenine dinucleotide

ADP-ribose Adenosine diphosphoribose

AMN 3-Aminopyridine mononucleotide

AMP, ADP, and ATP Adenosine 5′-mono-, di-, and triphosphates

APAD(H) 3-Acetylpyridine adenine dinucleotide (reduced form)

APMN 3-Acetylpyridine mononucleotide

BHI Brain Heart Infusion media

Bicine N,N-Bis(2-hydroxyethyl)glycine

BSA Bovine serum albumin

CFE Cell-free extracts

DEPC Diethylpyrocarbonate

DHAP Dihydroxyacetone phosphate

D-LDH D(-)-Lactate dehydrogenase

DNA Deoxyribonucleic acid

DTT Dithiothretitol

EDTA Ethylenediaminetetraacetic acid, disodium salt

FAD Flavin adenine dinucleotide

FDA U.S. Food and Drug Administration

FMN Flavin mononucleotide

GTP Guanosine triphosphate

Hepes N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HLADH Horse liver alcohol dehydrogenase

HPLC High performance liquid chromatography

LDHs Lactate dehydrogenases

L-LDH L(+)-Lactate dehydrogenase

MES 2-[N-Morpholino]ethane sulfonic acid

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NA Nicotinic acid

NAD and NADH Nicotinamide adenine dinucleotide and its reduced form

deNAD Deamido nicotinamide adenine dinucleotide

NaMN Nicotinate mononucleotide

Nam Nicotinamide

NADP Nicotinamide adenine dinucletide phosphate

NADase NAD glycohydrolase

NBT Nitro blue tetrazolium

NEM N-Ethylmaleimide

NMN Nicotinamide mononucleotide

NMNase NMN glycohydrolase

NR Nicotinamide riboside

P Particulate fraction from high-speed centrifugation

PMS Phenazine methosulfate

PRP Polyribophosphate vaccine

PRP-D Polyribophosphate-diphteria toxin vaccine

QA Quinolinic acid

S Soluble fraction from high-speed centrifugation

SDS Sodium dodecyl sulfate

TLC Thin-layer chromatography

Tris Tris(hydroxymethyl)aminoethane

UV Ultraviolet

YADH Yeast alcohol dehydrogenase

CHAPTER I

INTRODUCTION

The genus *Haemophilus* includes a group of gram-negative facultative aerobic coccobacilli characterized by their unique requirement for one or both of two growth factors: X-factor (hemin) and V-factor, intact nicotinamide adenine dinucleotide (NAD) (1, 2). Table I lists several species within this genus and their respective X- and V-factor requirements. Several of these organisms are important pathogens not only of man but also of various economically important animals. *Haemophilus aegyptius* causes acute conjuctivitis in humans and *H. ducreyi* is the causative agent of the venereal disease soft chancre. *Haemophilus gallinarum* is responsible for acute respiratory infections in chickens. *Haemophilus suis* and *H. pleuropneumoniae* are important respiratory tract pathogens of swine causing Glässer's disease and pleuropneumonia, respectively, which are highly contagious and usually fatal infections.

Among the *Haemophilus* species, *H. influenzae* is clearly the most important in human medicine. Although not responsible for epidemic influenza, as the name suggests, it is involved in a variety of severe infections in man. These infections include meningitis in children, of which *H. influenzae* type b is the leading cause, as well as other septicemic conditions with local implications such as epiglottitis, pneumonia, urinary

Table 1. X- and V- factor Requirements of *Haemophilus* Species

Species	V-factor Requirement (NAD)	X-factor Requirement (hemin)
H. influenzae	+	+
H. aegyptius	+	+
H. haemolyticus	+	+
H. ducreyi	–	+
H. aphrophilus	–	+
H. parainfluenzae	+	–
H. pleuropneumoniae	+	–
H. paraphrophilus	+	–
H. suis	+	+
H. gallinarum	+	+

infections, and cellulitis. *Haemophilus influenzae* is the most extensively studied *Haemophilus* organism, and serves as the prototype for the genus. The encapsulated forms of this organism are primary pathogens and are subclassified into types a to f based on the nature of the carbohydrate moieties of their capsular polysaccharides. The capsules of all six capsular types consist of polysaccharides containing two different sugars, but the capsular material of type b, polyribophosphate (PRP), is unique among them in that both of its sugars are pentoses. *Haemophilus influenzae* type b is the primary cause of bacterial meningitis in humans, responsible for 8000 cases annually in the United States (3). The disease most frequently affects children under three years of age. Meningitis is not easily diagnosed in infancy, as the symptoms, fretfulness, fever and vomiting, are so common in infants that the early stages of the infection can easily be missed.

Treatment of *H. influenzae* infections has relied predominantly on the use of ampicillin and chloramphenicol; however, significant increase in strains resistant to these antibiotics has been reported (4 - 7). In addition to these antibiotic susceptibilities, there has been an alarming increase in adult meningitis cases attributed to *H. influenzae.* About 20% of the adult meningitis cases are now attributed to *H. influenzae*, compared to 1 - 3% estimated earlier (8, 9). Another aspect of the clinical importance of *Haemophilus* meningitis that has been recently explored is the contagious character of the disease. It has become clear that when a child has *Haemophilus* meningitis, this is of importance not only to him but potentially to his household or other close contacts, particularly young children (10). Secondary spread of *Haemophilus* infections in day-care centers is well-documented (11). All of these findings clearly show the need for rapid development of alternative and effective methods of treatment of *H. influenzae* infections.

Recently, work has focused on searching for new effective antibiotics against *Haemophilus* meningitis. The new cephalosporins, cefuroxime, moxalactam, cefotaxime, ceftriaxone, and ceftazidime, are being evaluated as first-line drugs for therapy for β-lactamase-positive strains (12). Considerable effort has been made to develop an effective vaccine against *Haemophilus* meningitis. In recent months much controversy has focused on interpretations of new data concerning the safety and efficacy of *H. influenzae* type b vaccines. The "plain" capsular polysaccharide vaccine (PRP) was assessed to be sufficiently effective in children older than 18 months, although only 25% of *H. influenzae* type b meningitis occurs in children of that age. The vaccine is recommended for a child older than 18 months if he(she) is at increased disease risk. The new diphtheria toxoid conjugate vaccine (PRP-D) has been approved by the U.S. Food and Drug Administration (FDA), but no efficacy study has been performed with PRP-D in children 18 months of age or older. The decision to license PRP-D was based on the enhanced immunogenicity of this vaccine in comparison to the response elicited by the unconjugated PRP (13). Evidently, more work has to be done to develop an effective vaccine against *Haemophilus* meningitis, since the vaccines available now are ineffective on infants younger than 18 months, the age at which 75% of all *H. influenzae* meningitis occurs (13, 14).

The clinical and pathological findings associated with *Haemophilus* infections are well-documented (15, 16); however, little is known about the biochemistry and physiology of the causative organism. *Haemophilus influenzae* is well-known for its restriction endonucleases. The discovery of restriction endonucleases, which has provided a revolution in the field of molecular biology, was initially made using *H. influenzae* (17). From all the restriction enzymes purified at this time from different microorganisms, 20% were derived from *Haemophili* species (18). *Haemophilus influenzae* has also been observed to incorporate foreign deoxyribonucleic acid (DNA), a phenomenon known

as competence or genetic transformation. Investigation of this mechanism led to extensive studies into the nature of *H. influenzae* inner and outer membranes (19, 20).

Haemophilli are nutritionally fastidious and require a source of pyridine nucleotides (V-factor) and/or hemin (X-factor) for growth. The X-factor requirement is caused by the loss of enzymatic capacity to synthesize protoporphyrin IX from δ-amino levulinic acid (21). The V-factor requirement is due to the absence of the nicotinamide phosphoribosyltransferase which is required for the formation of the nicotinamide-ribose bond. To provide for this requirement, media should be supplemented with NAD. However, a limited number of other pyridine compounds, including nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide mononucleotide (NMN), nicotinamide riboside (NR), and reduced nicotinamide adenine dinucleotide (NADH), can substitute for NAD and support growth of *H. influenzae*. Nicotinic acid, nicotinamide, quinolinic acid, and a mixture of ribose, adenosine 5′-monophosphate (AMP), and nicotinamide have been found to be ineffective as supporters of growth of the organism (22 - 25). Albritton (26) has confirmed that *H. influenzae* does not possess a functional *de novo* pathway, or a Preiss-Handler pathway for the biosynthesis of NAD.

Due to the unique requirement of *H. influenzae* and related species for externally-provided intact NAD, several pyridine nucleotide-requiring enzymes become important for the survival of these organisms. In this respect, little is known about the NAD metabolism in *H. influenzae*, and even less is known about various metabolic pathways in this organism. Selective inhibition or inactivation of any of the enzymes involved in the NAD metabolism could offer a good alternative for controlling growth of the organism.

Recently, a nucleotide (NAD) pyrophosphatase activity was found in *H. influenzae* periplasm (25). The enzyme catalyzes the hydrolysis of external NAD to AMP and NMN. The transport of NMN into the cell has not been investigated. The

present studies demonstrate the presence of a membrane-bound NMN glycohydrolase (NMNase) that cleaves NMN to give nicotinamide and ribose 5-phosphate. Substrate specificity, inhibition, and other properties of this enzyme, as well as its possible involvement in the transport of NMN will be discussed.

The intracellular NMN is utilized to resynthesize NAD inside the cell. The ATP:NMN adenylyltransferase (NMN adenylyltransferase), responsible for the synthesis of NAD from NMN and adenosine triphosphate (ATP), was found in *H. influenzae* sonicates. The NAD kinase that catalyzes the phosphorylation of NAD to produce NADP was also observed in *H. influenzae* sonicates. These two enzyme activities determine the NAD/NADP ratio and are crucial for the survival of the organism. The present study includes the partial purification of these important enzymes and studies of some of their properties. Of special interest are the studies of substrate specificities and inhibition since they relate directly to questions concerning the maintenance of coenzyme levels with respect to meeting the V-factor requirement of the organism.

Two NMN analogs, altered in the pyridinium moiety were prepared, *i.e.* 3-acetylpyridine mononucleotide, which bears an electron-withdrawing group in the 3-position, and 3-aminopyridine mononucleotide, with an electron donor group at the same position. The ability of these mononucleotides to support or inhibit growth of *H. influenzae* was investigated and correlated with their functioning as substrates or inhibitors of NMN adenylyltransferase and NMNase. Numerous studies of various compounds that either act as V-factor or as competitive inhibitors of V-factor, are presented in this work.

Early studies of the 3-aminopyridine adenine dinucleotide inhibition of growth of *H. influenzae* prompted the investigation into dehydrogenases that could be the target of this inhibition. A lactate dehydrogenase specific for D(-)-lactate was found in *H. influenzae* sonicates and the enzyme was used to further investigate the mechanism of

growth inhibition by the 3-aminopyridine analog of NAD. The present study describes the purification and characterization of *H. influenzae* D-lactate dehydrogenase with respect to substrate specificity, inhibition, chemical and kinetic properties. The reaction catalyzed by this dehydrogenase is essentially unidirectional, converting pyruvate to D(-)-lactate specifically. Therefore the enzyme seems to function as a pyruvate reductase rather than a lactate oxidase. It was of interest to investigate other mechanisms of lactate oxidation in *H. influenzae* and correlate them with the D-lactate dehydrogenase (NAD-dependent) activity. The present study demonstrates the presence of two lactate oxidase activities (NAD-independent) in *H. influenzae*, one specific for D(-)-lactate and the other specific for the L(+)-isomer. Several properties of these lactate oxidases as well as their involvement in the respiratory chain of aerobically grown *H. influenzae* will be discussed.

CHAPTER II

INTERCONVERSION OF PYRIDINE NUCLEOTIDES IN HAEMOPHILUS INFLUENZAE

INTRODUCTION

Nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate are compounds of great importance in cellular metabolism. They function in numerous anabolic and catabolic processes. The structures of these compounds are presented in Figure 1. Their participation in oxidation-reduction processes has been recognized as essential in over 300 enzymatically-catalyzed redox reactions (27).

The discovery that NAD serves as an energy source for deoxyribonucleic acid ligase in procaryotes demonstrated that in addition to its classical role as an oxidation-reduction cofactor, NAD can also serve as a substrate for an enzyme with a clearly-defined metabolic function. In contrast to oxidation-reduction reactions in which there is no net consumption of NAD, DNA ligase cleaves NAD at the pyrophosphate bond, giving AMP and NMN. The enzyme plays an important role in DNA synthesis, repair and recombination (28). NAD also serves as a substrate in reactions that produce adenosine diphosphoribose (ADP-ribose) (29, 30). These reactions are catalyzed by

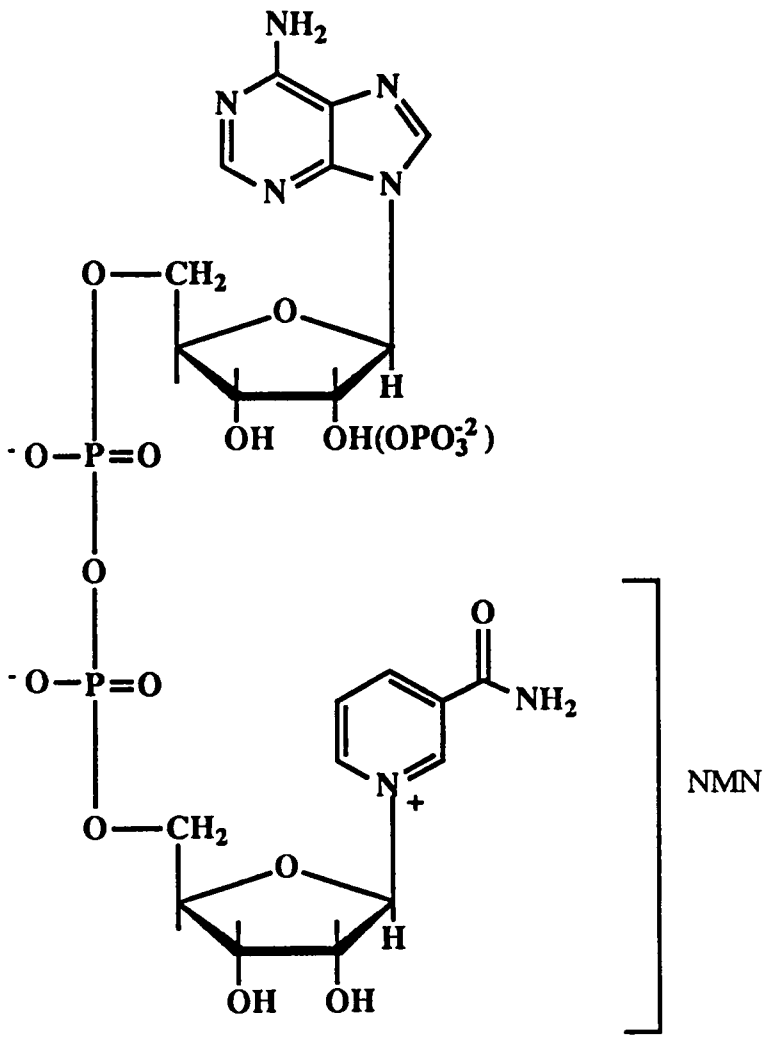

Figure 1. Chemical Structures of Nicotinamide Adenine Dinucleotide (NAD) and Nicotinamide Adenine Dinucleotide Phosphate (NADP): Parentheses indicate the location of the third phosphate group in NADP. The structure of nicotinamide mononucleotide (NMN) is indicated.

NAD glycohydrolases, giving nicotinamide and ADP-ribose. Certain enzymes which possess glycohydrolase activity have also been shown to transfer the ADP-ribose to various acceptors. This ADP-ribosylation can occur in eucaryotic as well as in procaryotic cells. A third type of NAD reaction, poly-ADP-ribosylation of nuclear proteins, plays a role in DNA repair, chromatin condensation and regulation of growth through modulation of the cell cycle (31).

Reduced pyridine nucleotide coenzymes also play an important role in the regulation of amphibolic pathways such as the tricarboxylic acid cycle and the pentose pathway (32). Therefore, research into the synthesis, recycling, and regulation of pyridine nucleotides is of importance in understanding the biochemistry of all cells. Extensive research on the biosynthesis of NAD has been undertaken in mammalian and lower eucaryotic systems, but to a lesser extent in procaryotes. A review of the literature reveals two main *de novo* biosynthetic pathways to NAD (Figure 2). One pathway involves the aerobic degradation of tryptophan by mammalian cells and some lower eucaryotes. A second pathway used by most procaryotes, is anaerobic and synthesizes the pyridinium ring of NAD from low molecular weight precursors (aspartate and dihydroxyacetone phosphate or formate). Both pathways lead to the formation of quinolinic acid. Quinolinic acid phosphoribosyltransferase uses phosphoribosylpyrophosphate to produce nicotinate mononucleotide. All organisms capable of performing *de novo* NAD biosynthesis use the same steps to convert quinolinic acid to NAD (33). These steps include the formation of nicotinic acid adenine dinucleotide from the mononucleotide, and subsequent amidation of the dinucleotide to give NAD. Several organisms prefer to use nicotinic acid as a substrate for NAD biosynthesis. The three-step pathway from nicotinic acid to NAD, elucidated by Preiss and Handler in 1958 (34), is identical to the final steps of the *de novo* pathway, but using nicotinic acid rather than quinolinic acid to form nicotinic acid mononucleotide.

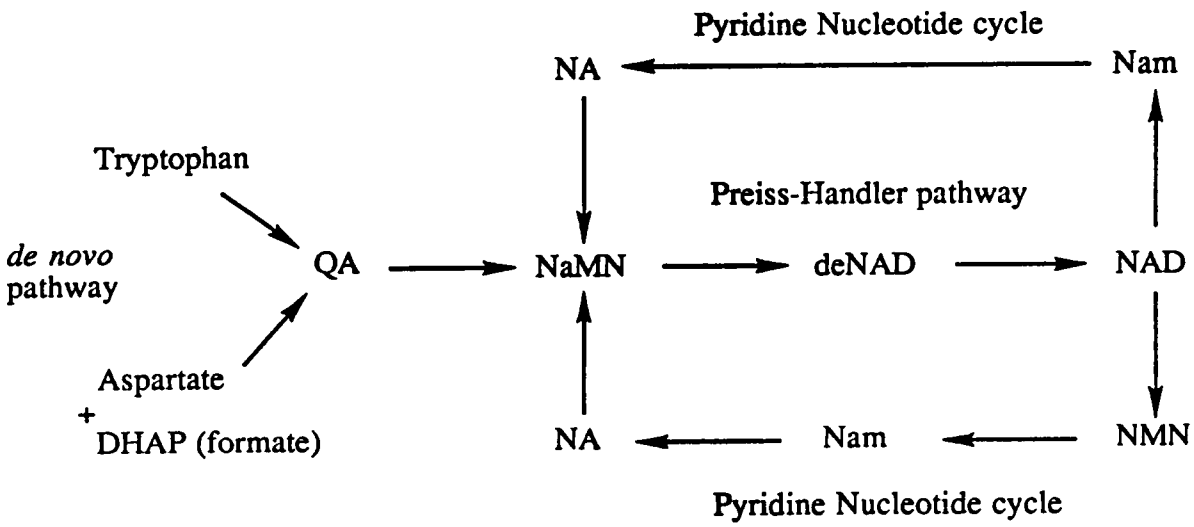

Figure 2. The Three Known Pathways of NAD Biosynthesis: a) *de novo* biosynthesis (from low molecular weight precursors), b) the Preiss-Handler pathway (from nicotinic acid), and c) the pyridine nucleotide cycles. Abbreviations: NaMN, nicotinate mononucleotide; Nam, nicotinamide; NA, nicotinic acid; DHAP, dihydroxyacetone phosphate; QA, quinolate; deNAD, deamido nicotinamide adenine dinucleotide.

An alternative to the *de novo* biosynthesis and the Preiss-Handler pathway is the use of pyridine nucleotide cycles or salvage pathways. These cycles, shown in Figure 2, allow some cells to utilize preformed pyridine bases that are obtained from intracellular breakdown or from exogenous sources. Most procaryotes and eucaryotes are capable of synthesizing NAD *de novo* or produce NAD from nicotinic acid by the Preiss-Handler pathway. Certain *Haemophilus* species are of special interest since they lack these pathways, therefore requiring exogenous NAD for growth. This NAD requirement was discovered many years ago by Davis (35). In 1920, it was confirmed by Thjotta *et al.* (36) that *Haemophilus influenzae* required two growth factors and they were named V- and X-factor. Lwoff and Lwoff (37) determined the nature of the V-factor to be NAD and proved that NADP could also support growth of the organism. The reduced forms of these pyridine nucleotides (NADH and NADPH) were also shown to be acceptable replacements for the V-factor. In 1944, Gingrich and Schlenck (22) provided evidence that nicotinamide riboside could also serve as V-factor while nicotinamide, ribose and AMP could not. Only a limited number of pyridine compounds were known to support growth of *Haemophili, i.e.* NAD, NMN, NR, NADP, and the hypoxanthine analog of NAD, whereas nicotinic acid adenine dinucleotide, quinolinic acid, nicotinic acid, nicotinate mononucleotide, nicotinic acid riboside, and a mixture of nicotinamide, D-ribose, and AMP were ineffective.

The lack of the *de novo* and Preiss-Handler pathways for the synthesis of NAD in *Haemophilus* species was confirmed by O'Reilly and Niven (38) in *H. parasuis* and *pleuropneumoniae*; Cynamon *et al.* (39) in *H. parainfluenzae*; Kasarov and Moat (40) in *H. haemoglobinophilus*; and Albritton (26) in *H. influenzae*.

Recent studies of the utilization of externally-added intact NAD for growth of *H. influenzae* demonstrated the presence of a periplasmic nucleotide pyrophosphatase (NAD pyrophosphatase, EC 3.6.1.9). This enzyme was purified and characterized (25)

and demonstrated to catalyze the hydrolysis of external NAD to AMP and NMN, the latter being utilized for the intracellular resynthesis of NAD. In this study, the ATP:NMN adenylyltransferase (NMN adenylyltransferase, EC 2.7.7.1) activity required for intracellular resynthesis of NAD was observed in cytoplasmic fractions of *H. influenzae*. In the same manner, ATP:NAD 2′-phosphotransferase (NAD kinase, EC 2.7.1.23) activity, responsible for the synthesis of NADP from NAD, was also found in *H. influenzae* sonicates. The coupled activities of NAD pyrophosphatase, NMN adenylyltransferase, and NAD kinase are crucial to the survival of the organism. Therefore, characterization of these enzymes would provide important information concerning the maintenance or inhibition of growth of *H. influenzae*.

In studies of the periplasmic NAD pyrophosphatase (25), NAD analogs such as 3-acetylpyridine adenine dinucleotide and thionicotinamide adenine dinucleotide served as substrates for the nucleotide pyrophosphatase as well as supporting growth of the organism in the absence of NAD. These results would indicate that the corresponding acetylpyridine and thionicotinamide mononucleotides could be available inside the cell. The organism could satisfy its requirement for NAD by intracellularly synthesizing the corresponding dinucleotides that could be used by dehydrogenases. Although the thionicotinamide analog of NAD could undergo a slow chemical conversion to NAD, the chemical stability of the 3-acetylpyridine analog suggests the possibility that cytosolic dehydrogenases utilize this unnatural coenzyme in support of important oxidation-reduction reactions. Studies of the substrate specificities of NMN adenylyltransferase and NAD kinase will demonstrate the possibility for intracellular synthesis of these NAD(P) analogs.

In studies of *H. influenzae* growth inhibition with NAD as a V-factor (25), 3-aminopyridine adenine dinucleotide was found to be a potent inhibitor. Significant inhibition of growth was observed at 0.2 μM concentration of the dinucleotide. This

NAD analog was also found to be a fairly good substrate for the NAD pyrophosphatase, which suggests the availability of the 3-aminopyridine mononucleotide inside the cell. This mononucleotide might interact with cytosolic enzymes causing inhibition of growth of the organism. In addition, 3-aminopyridine adenine dinucleotide(phosphate) could be resynthesized by NMN adenylyltransferase and NAD kinase and the interaction of these dinucleotides with dehydrogenases could be the point of growth inhibition observed by 3-aminopyridine adenine dinucleotide. Again, the characterization of *H. influenzae* NMN adenylyltransferase and NAD kinase would help to elucidate the mechanism of growth inhibition by 3-aminopyridine adenine dinucleotide.

The present study includes the partial purification of *H. influenzae* NMN adenylyltransferase and NAD kinase, and the investigation of their properties. Of special interest are the studies of substrate specificity and inhibition in an effort to answer the questions addressed above.

It has been shown repeatedly that *Haemophili* can not grow on nicotinamide as the V-factor (22, 23, 38 - 41). However, O'Reilly and Niven (38, 41) as well as Cynamon (39) observed that extrinsic NAD or NMN were catabolized by *Haemophilus* species to yield extracellular nicotinamide. No ADP-ribosylation was observed in *H. parasuis* and *H. pleuropneumoniae* when arginine was used as the ADP-ribose acceptor (42). Although the participation of other acceptors can not be ruled out to count for the formation of nicotinamide, the presence of NAD glycohydrolase (NADase, EC 3.2.2.5) or NMN glycohydrolase (NMNase, EC 3.2.2.14) activities should also be considered. Even though it seems a waste of energy for an organism that requires externally-provided NAD for growth to have an NADase, this enzyme activity was investigated in *H. influenzae* sonicates. No NADase was found, but NMNase activity was demonstrated.

The membrane localization, substrate specificity, inhibition, and other characteristics of *H. influenzae* NMNase are described in this study.

EXPERIMENTAL PROCEDURES

MATERIALS

Haemophilus influenzae strain Rd was obtained from Dr. William L. Albritton of the University of Saskatchewan, Saskatoon. Brain Heart Infusion (BHI) and triethanolamine were obtained from Fisher Scientific. Protamine sulfate, streptomycin sulfate, hemin, and histidine were obtained from Sigma. Phenazine methosulfate (PMS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-methyl resorcinol monohydrate (orcinol), tris-(hydroxymethyl)aminomethane (Tris), bovine serum albumin (BSA), and dithiothreitol (DTT) were purchased from Sigma. Matrex gel Green A was obtained from Amicon, and Dowex AG 1X-8 was purchased from Bio-Rad. 3-Acetylpyridine adenine dinucleotide (APAD) was purchased from Sigma, and 3-aminopyridine adenine dinucleotide (AAD) was prepared by a published procedure (43). 3-Aminopyridine (AMN) and 3-acetylpyridine (APMN) mononucleotides were prepared as described in the following Methods section. Nicotinamide mononucleotide, ATP, ADP, guanosine triphosphate (GTP), 5-phosphoribosyl pyrophosphate, ribose, ribose 5-phosphate, D-glucose 6-phosphate, yeast alcohol dehydrogenase (YADH), horse liver alcohol dehydrogenase, yeast glucose 6-phosphate dehydrogenase, and nucleotide pyrophosphatase from *Crotalus adamanteus* venom were purchased from Sigma. Precoated Merck silica gel 60 aluminum-backed plates for thin-layer chromatography (TLC) were from Bodman Chemicals. Cellulose TLC sheets without fluorescent indicator were purchased from Eastman Kodak, and DEAE-cellulose TLC sheets were from J.T. Baker Chemical Co.. Magnesium chloride ($MgCl_2$) and ehylenediaminotetraacetic acid, disodium salt (EDTA) were purchased from J.T. Baker

Chemical Co. Propylene glycol was obtained from Aldrich Chemical Co., Inc.. 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 27.8 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 μg/ml) and 7.5 ml of a hemin-histidine solution were then added aseptically 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 (0.6 mg/ml) was added directly to the cooled autoclaved media. Growth was initiated by inoculation with a 3-ml 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 rapidly in an isopropanol-dry ice bath and stored at -70°C until time of use. Cells were grown in a New Brunswick G-25R incubator with shaking at 210 cycles/min for 10 hours. The cells (22 - 28 g wet weight/6 Fernbachs) were harvested by centrifugation at 18,000 x g for 12 min, washed in 50 mM potassium phosphate buffer, pH 7.0, resuspended with a minimal amount of the same buffer, divided, and stored at -70°C until used.

Growth studies - The ability of a compound to serve as a V-factor was investigated using 250-ml nepheloculture flasks containing 50 ml of BHI media supplemented with hemin and the indicated amount of the compound to be tested as V-factor. Growth was initiated by addition of 0.5 ml of freshly grown cells in late log phase at 37°C and 200 cycles/min. Growth was followed turbidimetrically by measuring absorbance at 660

nm over a 10 to 12 hour-period (late log phase). The rate of growth was expressed as doubling time values, determined as the time needed for the absorbance at 660 nm to rise from a value of 0.2 to 0.4.

The ability of a compound to inhibit growth of the organism was investigated, in a similar fashion, using 250-ml nepheloculture flasks containing 50 ml of BHI media supplemented with hemin, 0.1 μg/ml NAD (or NMN), and the indicated amount of the inhibitor to be tested. Growth was initiated by addition of 0.5 ml of freshly grown cells in late log phase at 37°C and 200 cycles/min. Growth was followed turbidimetrically at 660 nm and the rate of growth was expressed as doubling time values (defined above).

Enzyme assays - To measure NMN adenylyltransferase activity, a coupled YADH assay was developed to measure activity, even in crude preparations where other interfering enzyme activities were present. The adenylyltransferase was assayed at room temperature in 1-ml reaction mixtures containing 75 mM Tris-HCl, pH 8.0, 0.5% ethanol, 15 mM $MgCl_2$, 2.5 mM ATP, 1.3 mM NMN, 3 units of YADH and the protein aliquot to be assayed. The reaction was started by the addition of NMN and the absorbance increase at 340 nm was monitored. One unit is the amount of enzyme that produces one μmol of NAD per min. A quick spot test assay, developed to monitor activity during chromatographic enzyme purification, was a modification of the NADH assay method reported by Abdallah and Biellmann (44). The reaction mixture (1 ml) contained 50 mM Tris-HCl, pH 8.5, 0.5% ethanol, 15 mM $MgCl_2$, 2.5 mM ATP, 1.3 mM NMN, 20 μg YADH , 0.5 mM MTT, 0.5 mM PMS, and the appropriate amount of protein to be assayed. If the enzyme activity was present, MTT was reduced as indicated by the development of a purple solution in the spot test plate. A negative control, where NMN was omitted, was run in parallel.

The NAD kinase activity was measured in a 1-ml reaction mixture containing 50 mM Tris-HCl, pH 8.0, 0.4 mM $MgCl_2$, 0.2 mM ATP, 0.4 mM NAD, 5 mM glucose

6-phosphate, 0.4 unit glucose 6-phosphate dehydrogenase, and the protein aliquot to be assayed. The reaction was followed by the increase in absorbance at 340 nm. A spot test to monitor activity of the NAD kinase was also developed, similar to the one described for NMN adenylyltransferase. NAD pyrophosphatase was assayed using the YADH assay as decribed by Kahn and Anderson (25).

NMNase (NADase) activity was determined spectrophotometrically by following the rate of NMN (NAD) hydrolysis, using a modification of the cyanide-addition method described by Kaplan *et al.* (45). The assay mixture (2.6 ml) for NMNase activity contained 50 mM Tris-HCl, pH 8.0, 1.25 mM NMN, and the protein to be assayed, and was incubated at 37 °C. At timed intervals, a 0.2-ml aliquot was withdrawn and mixed with 1.8 ml 1 M potassium cyanide (KCN). The absorbance at 327 nm was measured for each sample, plotted, and the amount of NMN hydrolyzed over the incubation time course was calculated using 6.2×10^3 as the molar extinction coefficient of the cyanide adduct.

Nicotinamide phosphoribosyltransferase (EC 2.4.2.12) activity was assayed by measuring the formation of NMN by the cyanide-addition method. The reaction mixture (3 ml) contained 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM nicotinamide, 20 mM 5-phosphoribosyl pyrophosphate, 10 mM ATP, and 0.2 ml of the supernate fraction (100,000 x g, 1 hr, 4 °C) that had very little NMNase activity. Aliquots (0.2 ml) were withdrawn with time and mixed with 1 M KCN (1.8 ml). The absorbance at 327 nm was measured for each timed sample, plotted and the amount of NMN synthesized was calculated.

Thin-layer chromatography (TLC) - Ascending one-dimensional chromatography was performed at room temperature using the standard technique worked out by Stahl (46). Solutions of the compounds to be separated were introduced with a Hamilton syringe about 1 cm from the lower edge of the plate. After the solvent

had evaporated, the plates were placed in a chromatography tank filled to a depth of about 0.5 cm with a suitable developing solvent, and previously saturated with the solvent vapor. The plates were removed from the tank after the solvent front had advanced about 10 cm from the starting line. The plates were dried and the separated compounds were detected by fluorescence, UV absorption, or by spraying developing reagents. The rate of advance of each compound in a given solvent is expressed as the R_f value, that is, the ratio of the distance advanced by the compound, to the distance advanced by the solvent front. Compounds were identified by comparing their R_f values to those of known standards run at identical conditions.

NMNase product analysis - The products of the NMNase reaction were investigated by TLC using silica gel 60 aluminum-backed sheets with 95% ethanol:1 M ammonium acetate, pH 7.4 (5:2) as the developing solvent. The reaction mixture (1 ml) contained 50 mM Tris-HCl, pH 8.0, 0.9 mM NMN, and 0.1 ml of 20% *H. influenzae* sonicate. Aliquots (10 μl) of the reaction mixture were removed with time and spotted on silica gel plates. Ultraviolet-absorbing materials were located with a Mineralight UVS-12, and the pentose phosphates were detected by spraying the plates with the orcinol/sulfuric acid reagent (0.2% orcinol in 2 M H_2SO_4), brief heating in 110°C oven, and detection of sugar spots.

Protein determination - Protein concentrations were determined by the microprotein assay of the Coomassie blue method (47) using BSA as a standard.

Ultrafiltration - Concentration of protein fractions by ultrafiltration was carried out using Amicon PM-10 membranes at 4°C.

Spectral measurements - Ultraviolet and visible absorbance measurements were performed on Beckman Acta MVI and Shimadzu UV 2100 recording spectrophotometers.

pH Measurements - All pH measurements were made at room temperature on a Radiometer digital PHM 52 pH meter equipped with a GK-2321C combination electrode.

High performance liquid chromatography (HPLC) - HPLC was performed on a Spectra-Physics SP8000 chromatograph instrument equipped with a Spectra-Physics model 770 variable wavelength detector. An ion-exchange HPLC column was used (4.6 x 250 mm) packed with Alltech RSIL-AN resin, five micron particle size and equilibrated in 100 mM potassium phosphate buffer, pH 3.8. The column was maintained at 45 °C and the flow rate was 1.5 ml/min. Absorbance of the column eluate was monitored at 260 nm. Compounds were identified by comparing their retention times to those of standards run under identical conditions.

Preparation and purification of nicotinamide mononucleotide analogs - Nicotinamide mononucleotide analogs were prepared by the method described by Christ and Cooper (48) with some modifications. A 10-ml reaction mixture containing 50 mM Tris-HCl, pH 8.5, 100 mg NAD analog, 30 mg $MgCl_2$, and 5 units nucleotide pyrophosphatase from *Crotalus adamanteus*, was incubated at 37 °C with stirring. The pH was adjusted to 7.4 by addition of NaOH and kept at that pH until the reaction was completed. Aliquots were withdrawn with time to monitor the disappearance of the NAD analog. 3-Acetylpyridine adenine dinucleotide disappearance was assayed using horse liver alcohol dehydrogenase by following the increase in absorbance at 365 nm. 3-Aminopyridine adenine dinucleotide (AAD) disappearance was monitored concomitantly with the appearance of 3-aminopyridine mononucleotide (AMN) by TLC on cellulose plates with a solvent system ethanol:0.1 M acetic acid (1:1). After incubation (9 and 12 hours for 3-acetylpyridine and 3-aminopyridine mononucleotide, respectively), the crude reaction mixtures were applied, at room temperature, to a Dowex AG 1X-8, formate anion exchange column and the column was washed with distilled

water. Elution of the mononucleotides was performed with a linear gradient of 0 to 0.1 M ammonium formate. Fractions containing mononucleotide were pooled, and lyophilized.

RESULTS

Partial purification of *H. influenzae* NMN adenylyltransferase

Frozen cells (20 g) were dispersed with 100 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM DTT at 4°C using a glass homogenizer. The homogenate was sonicated in an ice-salt bath using the microprobe tip of an Ultrasonics Sonifier Cell Disruptor at 70 W for five min. Cell debris was removed by centrifugation for 20 min at 17,000 x g.

Protamine sulfate was added to the 20% sonicate supernate at 4 °C to give a 0.2% final concentration of protamine sulfate. This mixture was stirred for 30 min in an ice-salt bath, and centrifuged at 39,000 x g for 10 min. The pellet was discarded and the supernate was dialyzed against 3 liters of 20 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM DTT. The dialyzed protamine sulfate fraction was applied to a Matrex gel Green A column (1 x 10 cm) previously equilibrated at 4°C in 50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM DTT. After washing the column with the equilibration buffer, a linear salt gradient, 0.05 to 0.8 M KCl in the same buffer, was applied. The NMN adenylyltransferase was eluted later by using 1 M KCl in the same buffer. Fractions (2.5 ml) were checked for the presence of NMN adenylyltransferase using the spot test described under Methods, and those fractions containing the enzyme were assayed for NMN adenylyltransferase activity using the enzyme activity assay described in Methods. Protein concentration was monitored by 280 nm absorbance. The elution profile is shown in Figure 3. The results of the purification procedure are summarized in Table II. The enzyme was purified 15-fold with a 27% yield and a final specific activity of 0.02 units per mg protein.

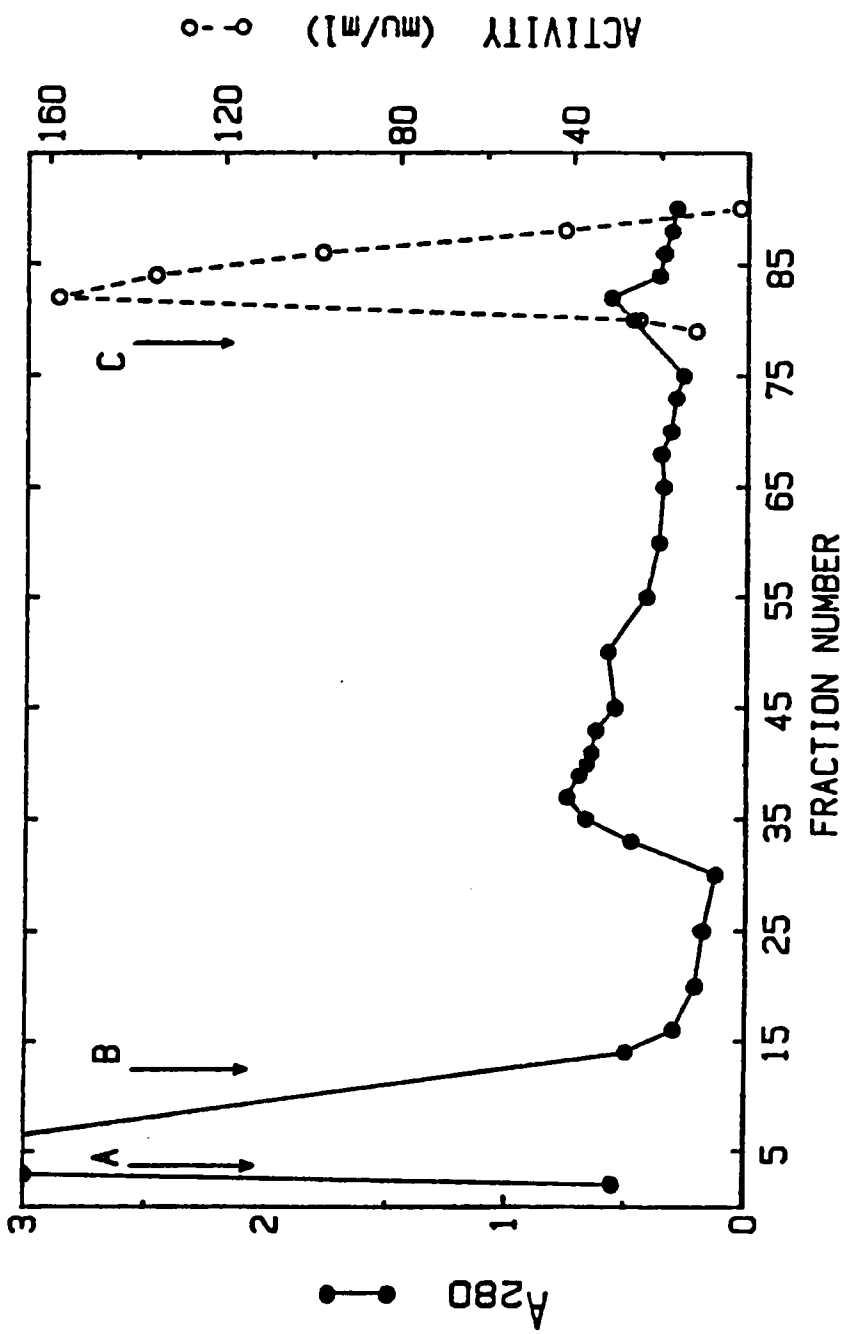

Figure 3. Affinity Chromatography on Matrex Gel Green A for the Purification of NMN Adenylyltransferase: The dialyzed protamine sulfate fraction was applied to a Matrex gel Green A (1 x 10 cm) column previously equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM DTT. Arrow A indicates the point 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 same buffer. Arrow C indicates the point were 1 M KCl in the same buffer was applied. Protein concentration is measured as absorbance at 280 nm. The NMN adenylyltransferase activity is reported as milliunits per ml.

Table II. Partial Purification of NMN Adenylyltransferase

FRACTION	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (units)	SPECIFIC ACTIVITY (units/mg)	YIELD %	FOLD PURIF.
20% Sonicate supernate	2400	3.0	0.001	100	1.0
0.2% Protamine sulfate supernate	1320	2.5	0.002	83	1.6
Matrex gel Green A	45	0.8	0.018	27	14.8

Properties of *H. influenzae* NMN adenylyltransferase

The partially purified enzyme in 50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM DTT lost 30% of its activity after 4 days at -15°C. Attempts to stabilize the enzyme using 2 mM EDTA, 10% propylene glycol, 0.1% bovine serum albumin, 5 mM $MgCl_2$, or 0.1 M KCl, failed. However, the fraction from the Green A column in 50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM DTT, and 1 M KCl was observed to maintain the enzyme activity for one week at -15°C. With this enzyme, the NAD synthesis from NMN was absolutely dependent upon the presence of magnesium ions, with a 30 mM optimum concentration.

Enzyme kinetics - Using the YADH-coupled assay to measure NMN adenylyltransferase activity, initial velocities were measured in reaction mixtures containing varying amounts of NMN and ATP. When the concentration of NMN was varied at fixed concentrations of ATP, the double reciprocal plots for initial velocities converged on the X-axis and replots of slopes and intercepts were linear (Figure 4). The initial velocity patterns were consistent with a sequential mechanism for the reaction catalyzed by NMN adenylyltransferase. Kinetic parameters determined graphically were: K_m for NMN, 60 μM; K_m for ATP, 0.5 mM; and V_{max} , 9 μmol/min/mg protein.

NMN analogs as substrates for NMN adenylyltransferase - The partially purified NMN adenylyltransferase was used for substrate specificity studies. The reaction mixtures (1 ml) contained 75 mM Tris-HCl, pH 8.0, 0.5% ethanol, 2.5 mM ATP, 15 mM $MgCl_2$, 3 units YADH, 0.3 ml enzyme aliquot (0.2 mg protein/ml), and the indicated amount of the mononucleotide to be studied. When 3-acetylpyridine mononucleotide was investigated as a substrate for the enzyme at a concentration of 4.3 mM, a slight increase in absorbance at 365 nm was observed after serveral minutes of reaction. The

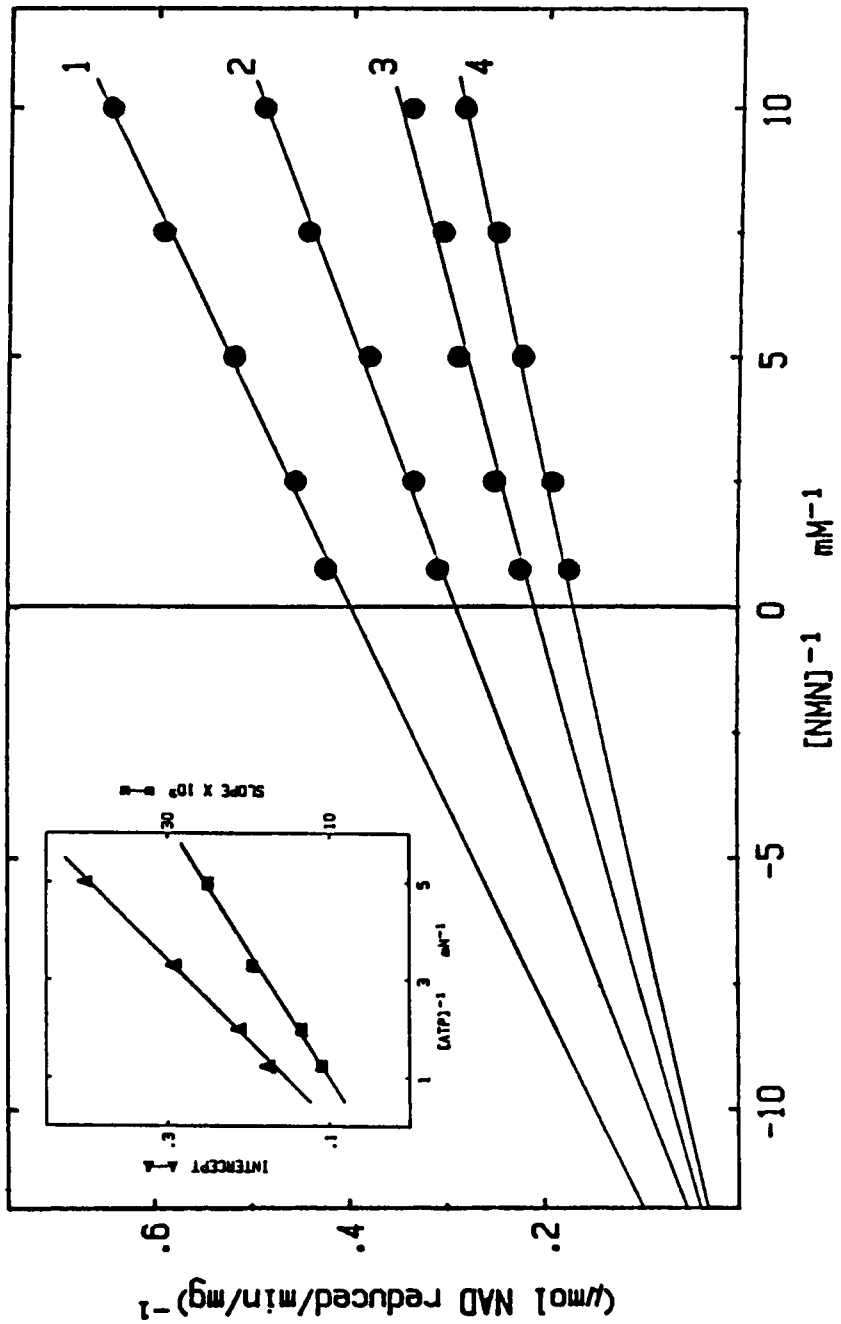

Figure 4. **Effect of NMN and ATP Concentrations on NMN Adenylyltransferase Initial Velocities:** Assay mixtures contained concentrations of NMN varying from 0.1 to 1.3 mM at four fixed concentrations of ATP in 50 mM Tris-HCl buffer, pH 8.0. The ATP concentrations used were: line 1, 0.2 mM; line 2, 0.3 mM; line 3, 0.5 mM, and line 4, 0.8 mM. Inset is a replot of slopes (■) and intercepts (▲).

recorded spectra of the reaction mixture from 300 to 420 nm showed the formation of a peak at 360 nm with time. To confirm that the 3-acetylpyridine adenine dinucleotide (APAD) was being formed, an aliquot of the reaction mixture was applied to an ion-exchange HPLC column (RSIL-AN) as described under Methods. The presence of 3-acetylpyridine adenine dinucleotide in the reaction mixture was demonstrated by comparing the retention times of the reaction mixture peaks with a standard mixture sample containing ATP, 3-acetylpyridine adenine dinucleotide, and 3-acetylpyridine mononucleotide. When 3-aminopyridine mononucleotide (AMN) was investigated as a substrate for the enzyme at a concentration of 1.7 mM, the progress of the reaction was followed by TLC. Thin-layer chromatography was performed on cellulose plates with a solvent system of 95% ethanol:0.1 M acetic acid (1:1), and the compounds were located by UV absorbance and fluorescence using known AMN and AAD standards. The disappearance of a spot at R_f = 0.70 corresponding to the 3-aminopyridine mononucleotide, and the appearance of a fluorescent spot at R_f = 0.48 corresponding to the dinucleotide analog was observed. An aliquot of the reaction mixture was also applied to an RSIL-AN ion-exchange HPLC column to confirm the presence of 3-aminopyridine adenine dinucleotide as a product of the reaction. Guanosine triphosphate (GTP) was demonstrated not to substitute for ATP in the NMN adenylyltransferase-catalyzed reaction.

Inhibition of NMN adenylyltransferase by 3-aminopyridine mono and dinucleotide - The 3-aminopyridine mononucleotide was shown to be a substrate for the enzyme. It was also investigated at higher concentrations as a possible inhibitor of the NAD synthesis catalyzed by the NMN adenylyltransferase. No inhibition of the enzyme activity was observed at concentrations as high as 0.25 mM. The 3-aminopyridine adenine dinucleotide was investigated as an inhibitor of the NMN adenylyltransferase and was found not to inhibit the enzyme at concentrations as high as 0.7 mM.

Growth studies

3-Acetylpyridine mononucleotide was investigated as a V-factor, and growth studies were performed as described in Methods using various concentrations of the 3-acetylpyridine mononucleotide (0.05 - 3.0 μg /ml). The growth observed at different concentrations of the 3-acetylpyridine mononucleotide is shown in Figure 5.

The ability of the 3-aminopyridine mononucleotide to inhibit growth of the organism was investigated. Growth studies were performed as described in Methods with NAD (0.1 μg/ml) as the V-factor and various concentrations of the 3-aminopyridine mononucleotide (0 - 3 μg/ml). The same experiment was performed using NMN as the V-factor at a concentration of 0.1 μg/ml. The inhibition observed at different concentrations of the 3-aminopyridine mononucleotide when NAD was used as a V-factor is shown in Figure 6. The same inhibition pattern was observed when cells were grown with NMN as the V-factor (Figure 7). 3-Aminopyridine adenine dinucleotide (AAD) proved to be a potent inhibitor of growth when *H. influenzae* cells were grown on either NAD or NMN. Growth studies were conducted using NMN as V-factor (0.1 μg/ml), AAD as the inhibitor (1 μg/ml), and in the presence of 10 μM ADP in order to inhibit the NAD pyrophosphatase. As shown in Figure 8, no inhibition of growth by AAD was observed when conversion of this dinucleotide to the mononucleotide was blocked. The results of growth studies and inhibition of growth are summarized in Tables III and IV.

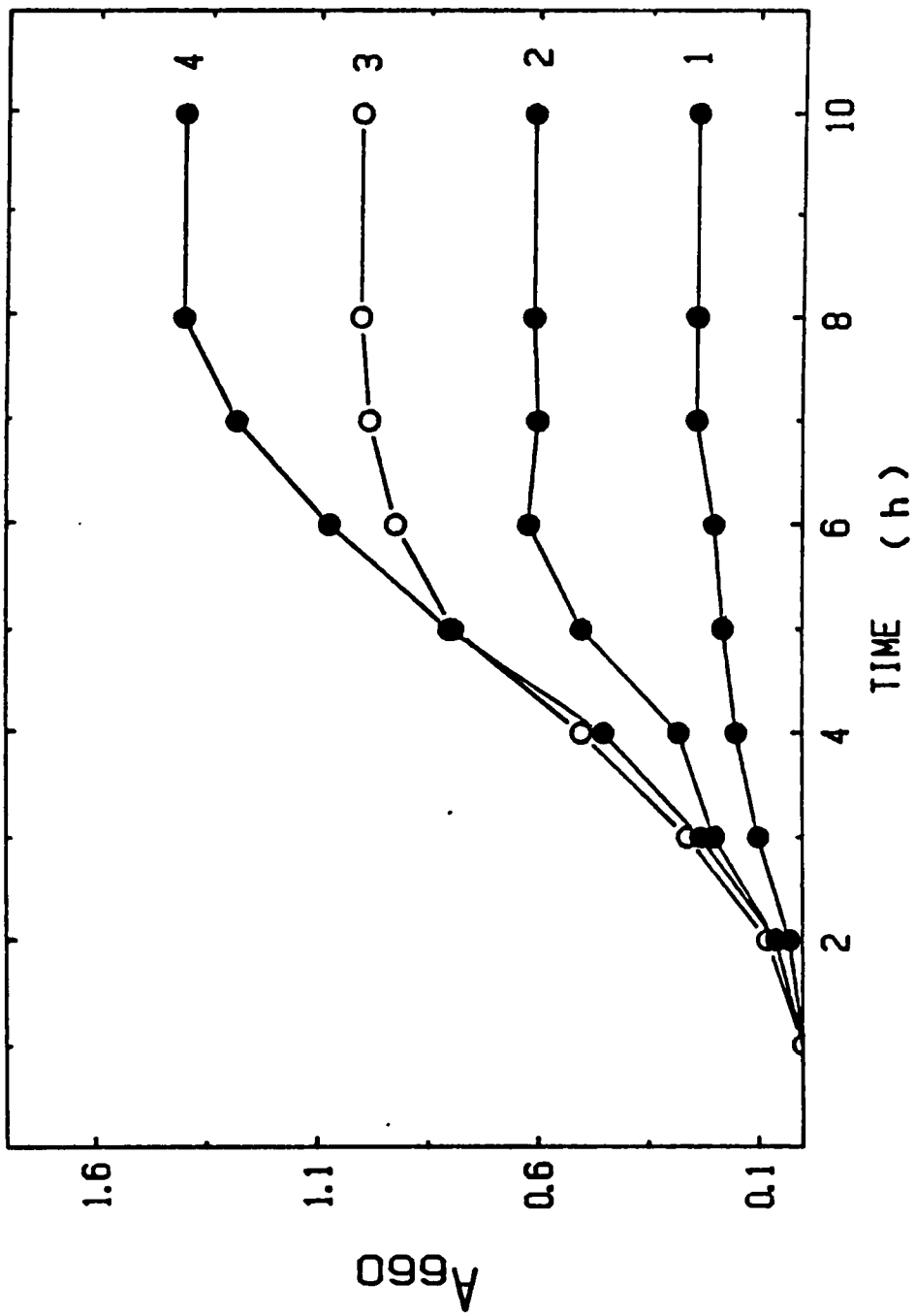

Figure 5. **Growth of *H. influenzae* with 3-Acetylpyridine Mononucleotide (APMN) as V-factor:** The growth of the organism was conducted as described under Methods. The concentrations of APMN used were: line 1, 0.05 μg/ml; line 2, 1.0 μg/ml; and line 4, 3.0 μg/ml. Line 3 represents growth with NMN as V-factor at a concentration of 1.0 μg/ml.

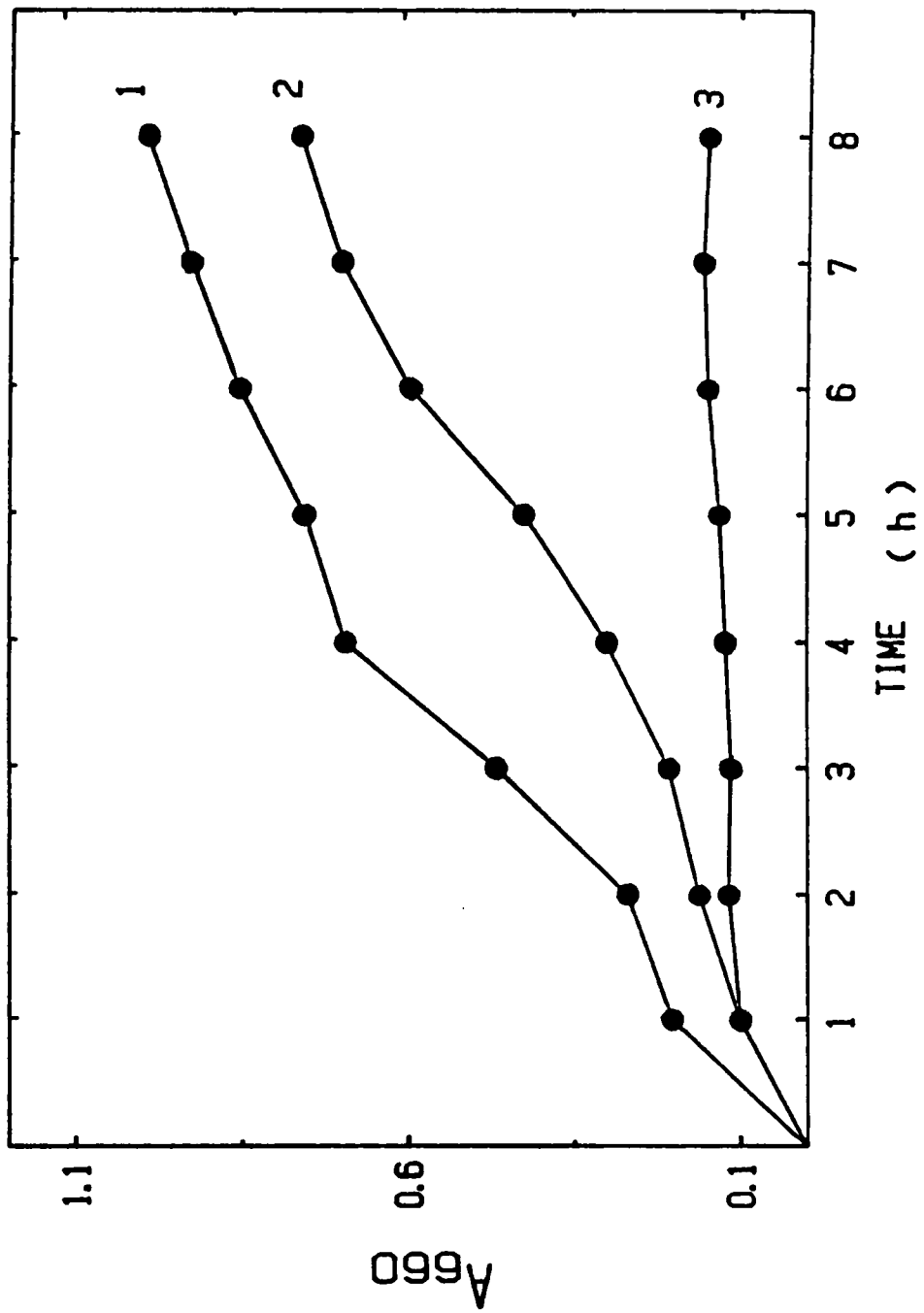

Figure 6. Inhibition of Growth of *H. influenzae* with NAD as V-factor by 3-Aminopyridine Mononucleotide (AMN): The growth inhibition experimental procedure is described under Methods. The concentrations of AMN used were: line 1, none; line 2, 0.1 μg/ml; and line 3, 1 μg/ml.

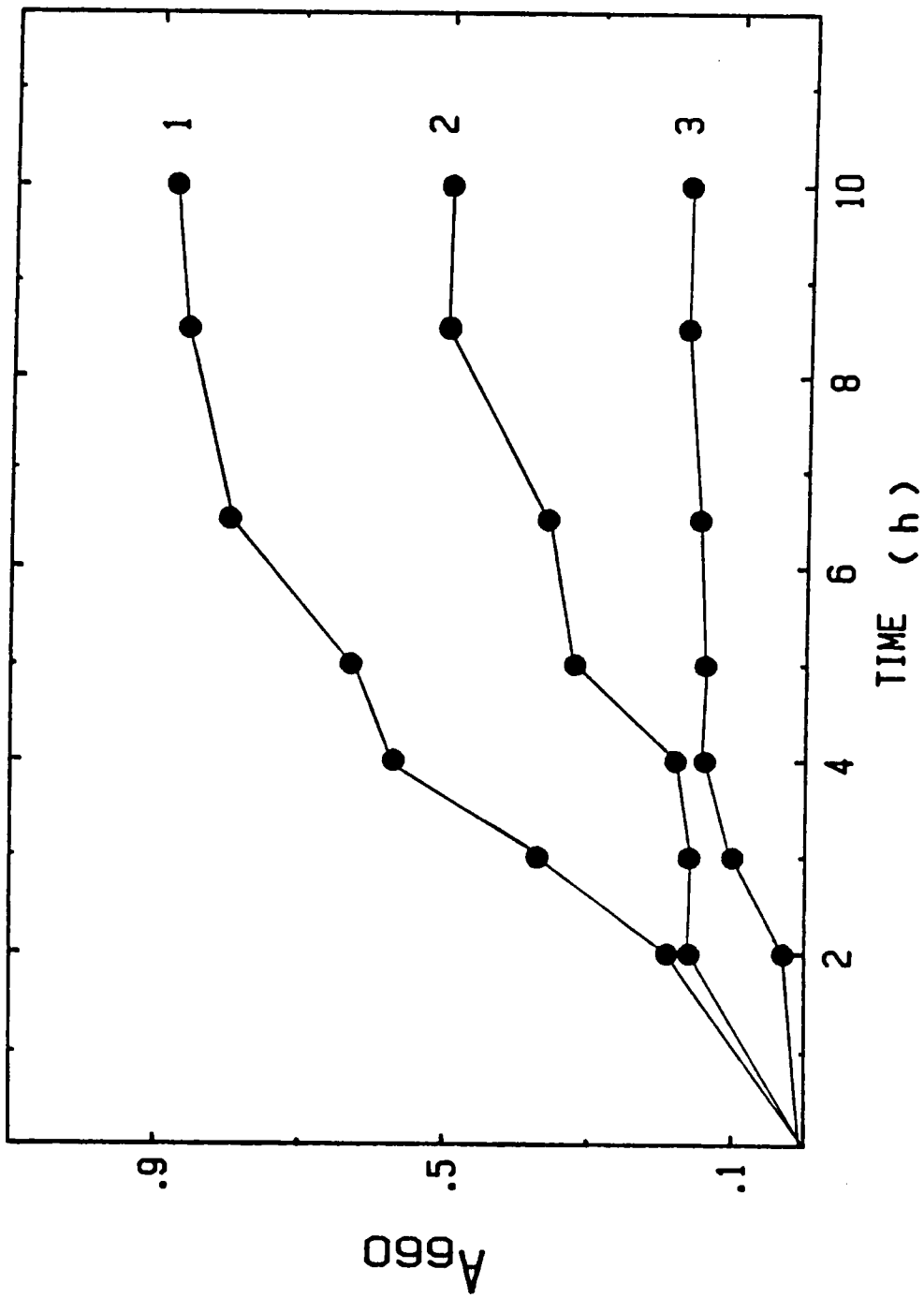

Figure 7. Inhibition of Growth of *H. influenzae* with NMN as V-factor by 3-Aminopyridine Mononucleotide (AMN): The growth inhibition experimental procedure is described under Methods. The concentrations of AMN used were: line 1, none; line 2, 0.1 μg/ml; and line 3, 1 μg/ml.

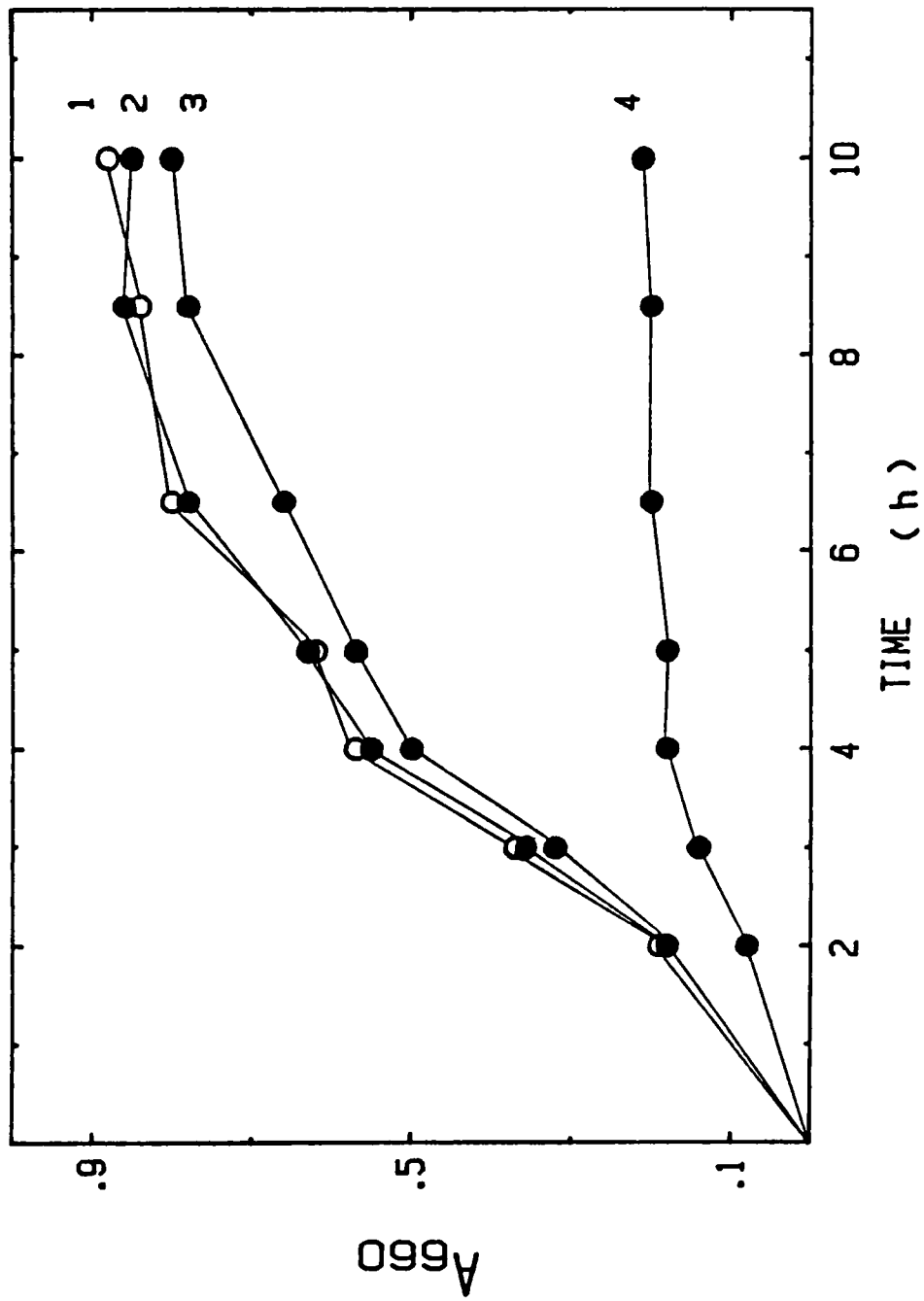

Figure 8. Growth of *H. influenzae* in the Presence of ADP and 3-Aminopyridine Adenine Dinucleotide (AAD) with NMN as V-factor: The growth of the organism was conducted as described under Methods. Line 2, NMN (0.1 μg/ml) and ADP (10 μM); line 3, NMN (0.1 μg/ml), ADP (10 μM), and AAD (1 μg/ml); and line 4, NMN (0.1 μg/ml) and AAD (1 μg/ml). Line 1 represents growth with NMN as V-factor at a concentration of 0.1 μg/ml.

Table III. The Ability of Various Compounds to Serve as V-factor

COMPOUND	CONCENTRATION (μg/ml)	DOUBLING TIME (min)
NAD	1.0	39
3-Acetylpyridine adenine dinucleotide	1.0	250
NMN	1.0	54
3-Acetylpyridine mononucleotide	1.0	93
3-Acetylpyridine mononucleotide	3.0	60

Table IV. Inhibition of Growth with NAD or NMN as V-factor (0.1 μg/ml)

INHIBITOR	CONCENTRATION (µg/ml)	V-FACTOR	DOUBLING TIME (min)
---	---	NAD	65
AAD	0.1	NAD	85
AAD	1.0	NAD	NG*
AMN	0.1	NAD	198
AMN	1.0	NAD	NG*
---	---	NMN	73
AAD	0.1	NMN	135
AAD	1.0	NMN	NG*
AMN	0.1	NMN	270
AMN	1.0	NMN	NG*
ADP	4.5	NMN	74
ADP / AAD	4.5/1.0	NMN	80

* NG = No growth observed.

Partial purification of *H. influenzae* NAD kinase

Frozen cells (20 g) were dispersed with 100 ml of 50 mM potassium phosphate buffer, pH 7.0 at 4°C using a glass homogenizer. The homogenate was sonicated in an ice-salt bath using the microprobe tip of an Ultrasonics Sonifier Cell Disruptor at 70 W for five min. Cell debris was removed by centrifugation for 20 min at 17,000 x g. Approximately 80% of the supernate was carefully removed and centrifuged at 100,000 x g for 60 min at 4 °C.

The high-speed centrifugation supernate was applied to a Matrex gel Green A column (1 x 11 cm) previously equilibrated at 4°C in 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT. After washing the column with the equilibration buffer, the enzyme was eluted by applying a linear salt gradient, 0 to 0.5 M KCl. Fractions (2.5 ml) were assayed for NAD kinase activity using the enzyme assay described under Methods, and the protein concentration was monitored by 280 nm absorbance. The elution profile is shown in Figure 9.

The tubes containing NAD kinase activity were pooled and dialyzed against 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT. The dialyzed enzyme fraction was applied to a Matrex gel Blue A column (1 x 10 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT. The NAD kinase activity was eluted by washing the column with the equilibration buffer. The elution profile is shown in Figure 10. The results of the purification procedure are summarized in Table V. The enzyme was purified 18-fold with a 32% yield and a final specific activity of 0.05 units per mg of protein.

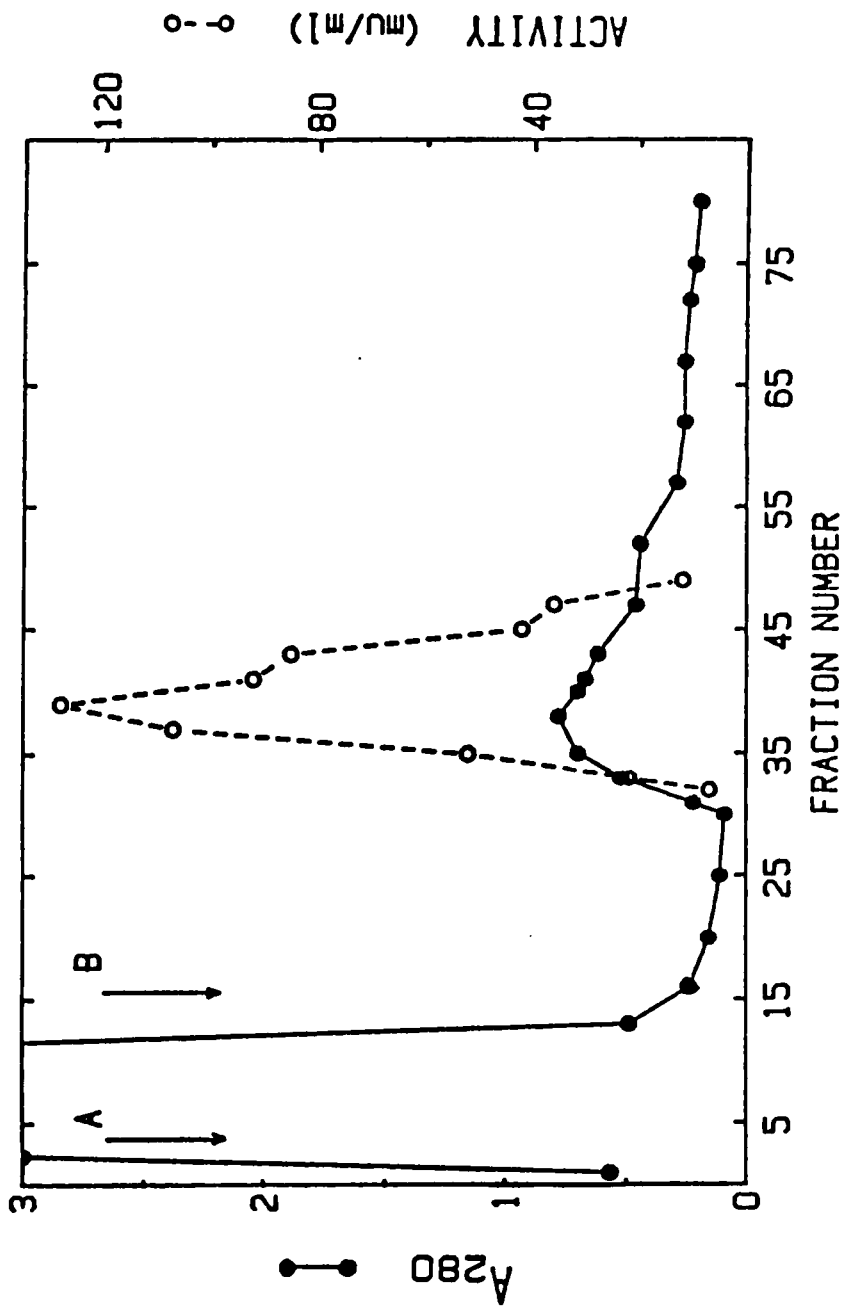

Figure 9. Affinity Chromatography on Matrex Gel Green A for the Purification of NAD Kinase: The high-speed centrifugation supernate was applied to a Matrex gel Green A column (1 x 10 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT. Arrow A indicates the point of initiation of washing with the equilibration buffer. Arrow B indicates the initiation of a salt gradient of 0 to 0.5 M KCl in the same buffer. Protein concentration is measured as absorbance at 280 nm and NAD kinase activity is reported as milliunits per ml.

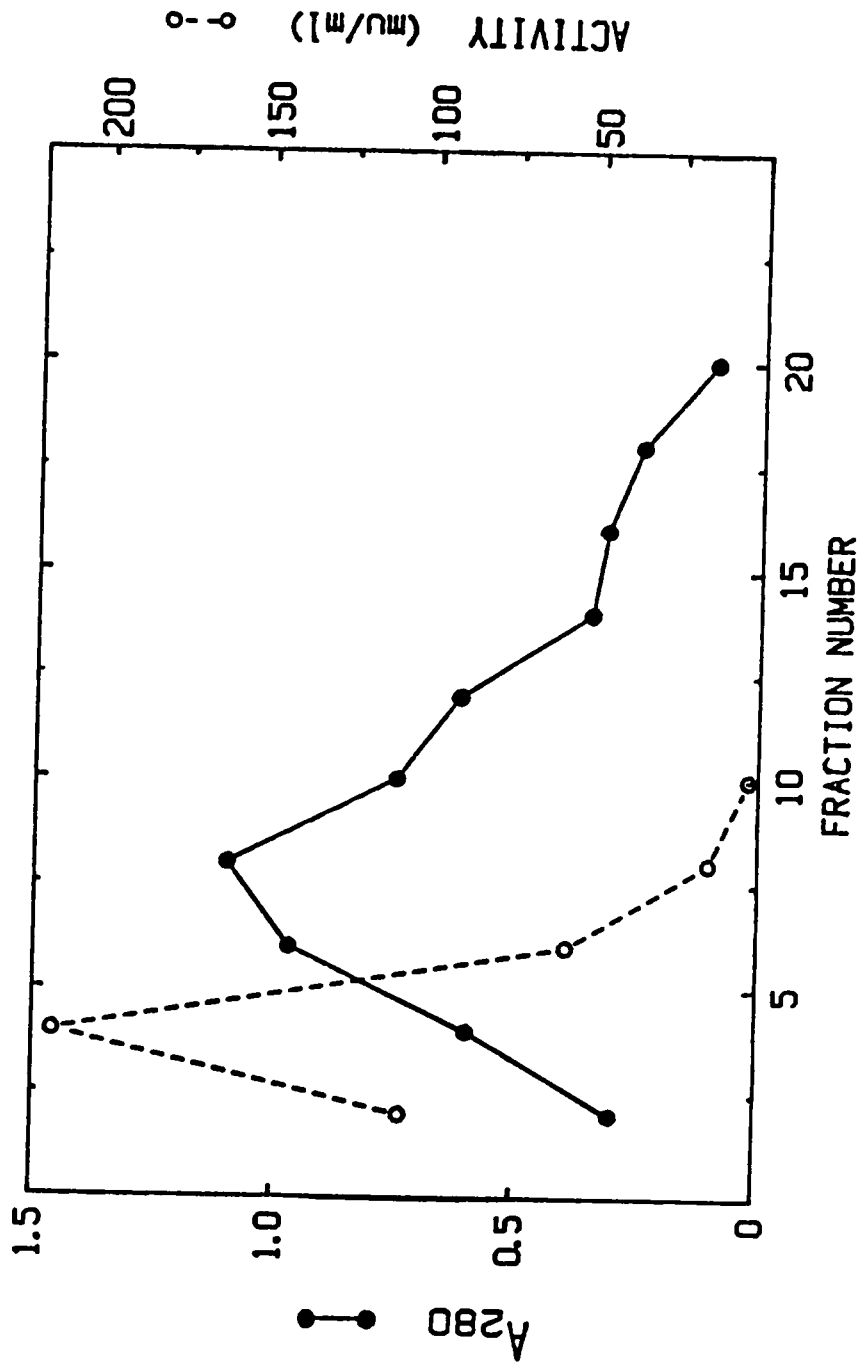

Figure 10. Affinity Chromatography on Matrex Gel Blue A for the Purification of NAD Kinase: The pooled fraction of NAD kinase activity from the Green A column was dialyzed and applied to a Matrex gel Blue A column (1 x 10 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT. The NAD kinase activity was eluted by washing the column with the equilibration buffer.

Table V. Partial Purification of NAD Kinase

FRACTION	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (units)	SPECIFIC ACTIVITY (units/mg)	YIELD %	FOLD PURIF.
20% Sonicate supernate	858	2.1	0.002	100	1.0
Ultracentrifugation	465	3.4	0.007	157	2.8
Matrex gel Green A	38	0.9	0.023	43	9.2
Matrex gel Blue A	15	0.7	0.046	32	18.4

Properties of *H. influenzae* NAD kinase

The enzyme was shown to be cytosolic. More than 100% of the activity was recovered in the supernate after high-speed centrifugation (100,000 x g, 60 min, 4°C). It appears that an inhibitor or a competing enzyme activity was removed in this procedure since a higher NAD kinase activity was found (157% yield). The enzyme exhibited an absolute dependence on magnesium ions for activity. Maximum activity was achieved when the ratio Mg:ATP was 1:2.

Enzyme kinetics - The effect of various concentrations of ATP (25 - 500μM) on NAD kinase activity at a fixed saturating concentration of NAD (0.4 mM) is shown in Figure 11. Substrate inhibition was observed at ATP concentrations higher than 0.2 mM. An apparent K_m for ATP was calculated from the linear portion of the curve at the higher ATP concentrations. The effect of various NAD concentrations (40 - 125 μM) on NAD kinase initial velocities at 0.2 mM ATP concentration is shown in Figure 12. The kinetic parameters determined graphically were: K_m for ATP, 30 μM; K_m for NAD, 55 μM; and V_{max} , 8 μmol NADP formed per min per mg of protein.

Substrate specificity of NAD kinase - 3-Acetylpyridine adenine dinucleotide was investigated as a substrate for the enzyme. The reaction mixtures (1 ml) contained 50 mM Tris-HCl, pH 8.0, 7.5 mM $MgCl_2$, 0.2 mM ATP, 5 mM glucose 6-phosphate, 0.4 unit of glucose 6-phosphate dehydrogenase, 0.1 ml of the partially purified enzyme (0.1 mg protein/ml), and various concentrations of the NAD analog (0.12 - 2 mM). The reaction was followed by measuring the increase in absorbance at 365 nm. Reciprocal plots of initial velocities versus NAD analog concentrations are presented in Figure 13. An apparent K_m for 3-acetylpyridine adenine dinucleotide of 0.5 mM and a V_{max} of 16 μmol of 3-acetylpyridine adenine dinucleotide phosphate (APAD) formed per min per mg protein were determined.

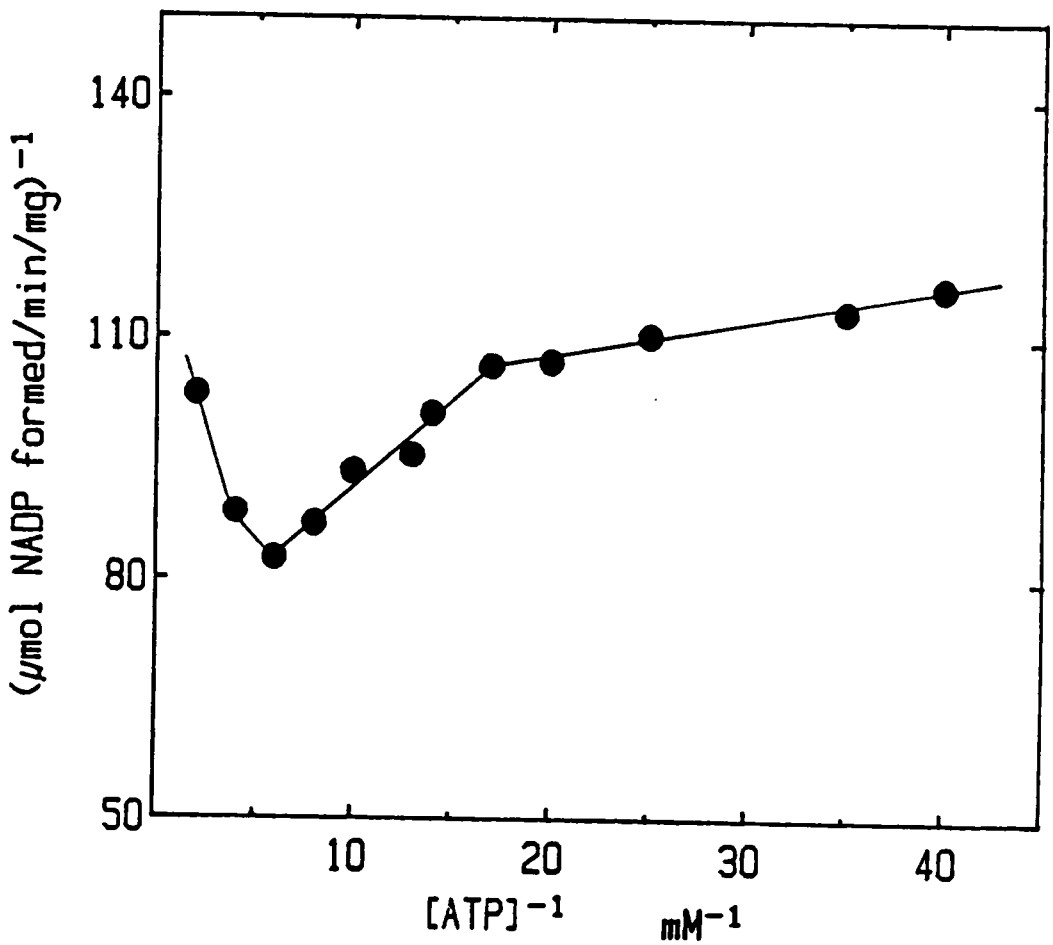

Figure 11. Effect of ATP Concentrations on NAD Kinase Initial Velocities: The assay mixtures contained concentrations of ATP varying from 25 to 500 μM at a saturating concentration of NAD (0.4 mM) in 50 mM Tris-HCl buffer, pH 8.0.

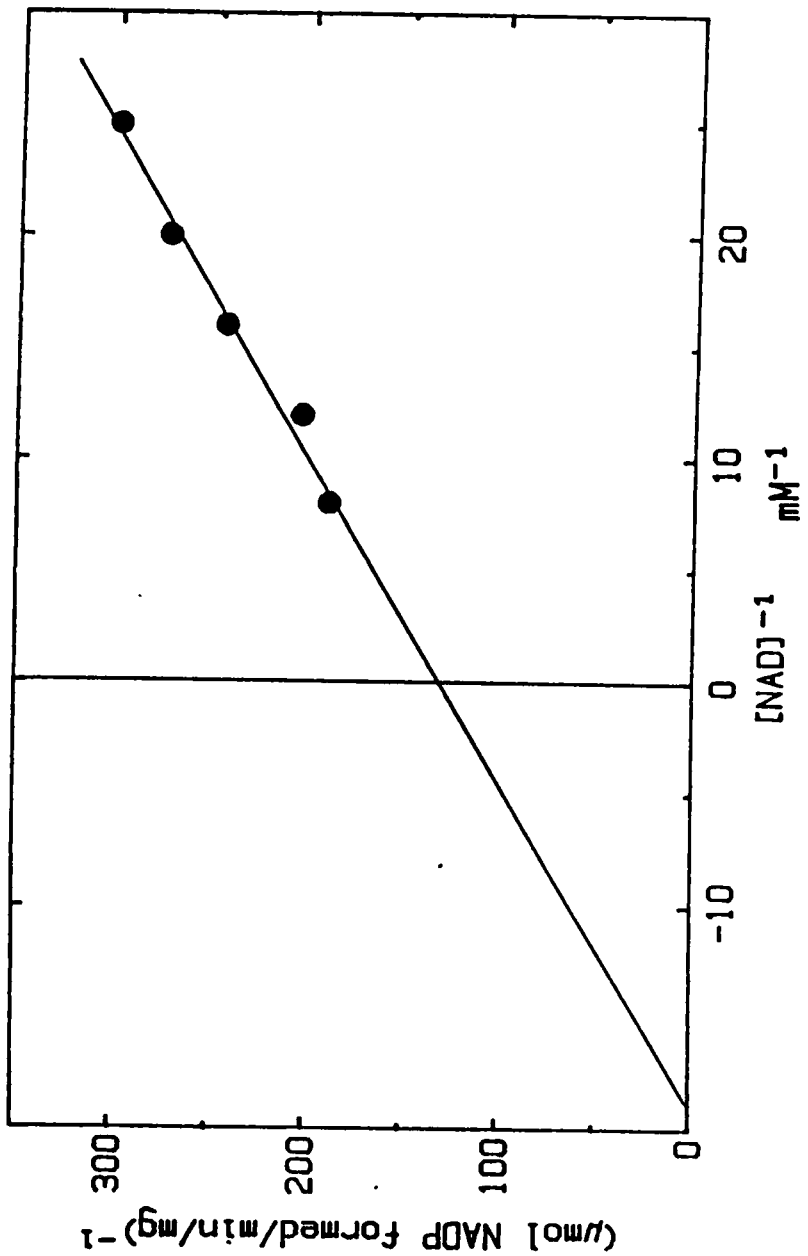

Figure 12. Effect of NAD Concentrations on NAD Kinase Initial Velocities: The assay mixtures contained concentrations of NAD varying from 40 to 125 μM at saturating concentrations of ATP (0.2 mM) in 50 mM Tris-HCl buffer, pH 8.0.

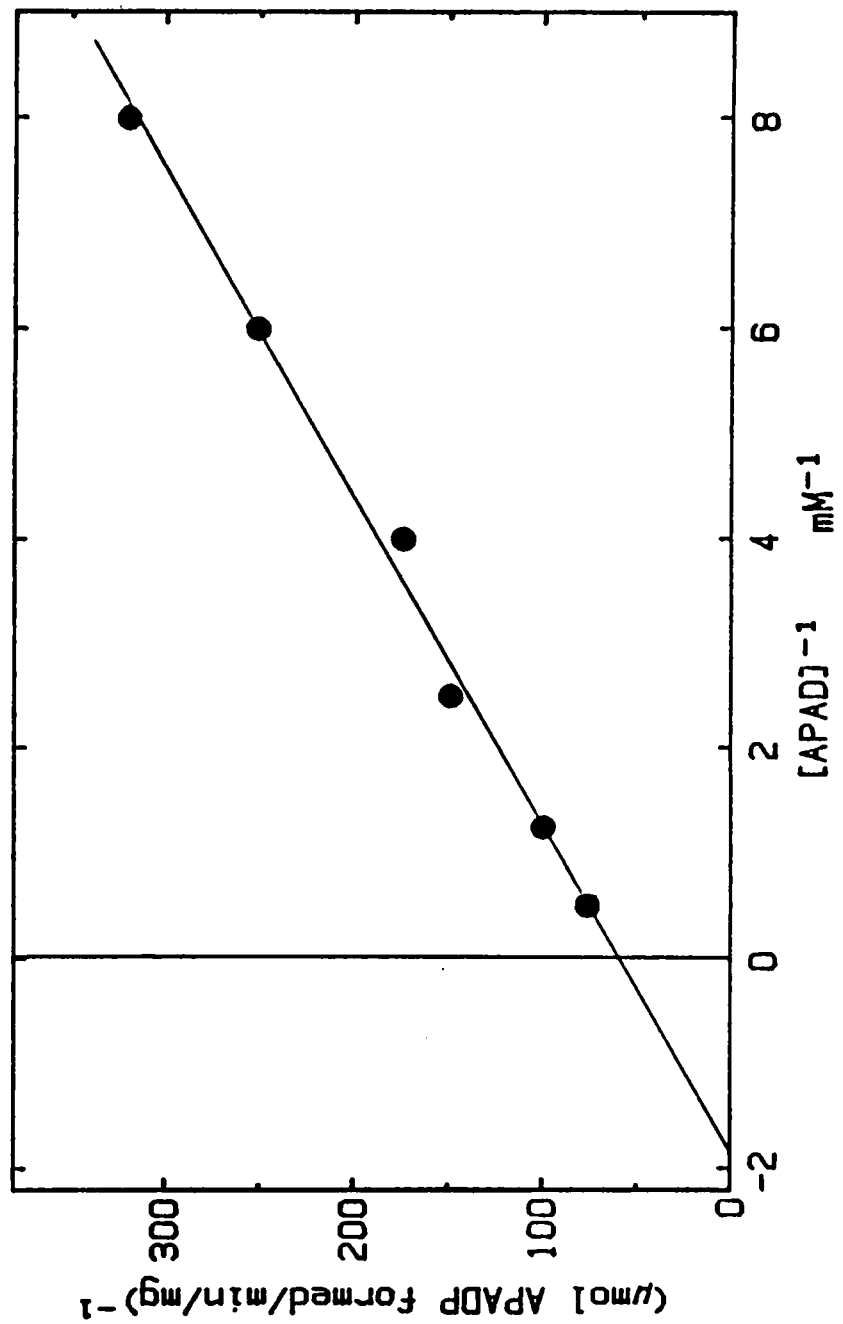

Figure 13. Effect of 3-Acetylpyridine Adenine Dinucleotide (APAD) Concentrations on NAD Kinase Initial Velocities: The assay mixtures contained concentrations of APAD varying from 0.12 to 2 mM at saturating concentrations of ATP (0.2 mM) in 50 mM Tris-HCl buffer, pH 8.0.

The 3-aminopyridine analog of NAD was also tested as a possible substrate for the NAD kinase. The reaction mixture (1 ml) contained 50 mM Tris-HCl, pH 8.0, 7.5 mM $MgCl_2$, 0.2 mM ATP, 0.1 ml of the partially purified enzyme (0.1 mg protein/ml), and 2 mM 3-aminopyridine adenine dinucleotide. The reaction was monitored by TLC, using DEAE-cellulose plates and a developing solvent of 95% ethanol:1 M ammonium acetate, pH 7.4, (1:1). The compounds were detected by UV absorbance and fluorescence. No disappearance of 3-aminopyridine adenine dinucleotide, nor formation of 3-aminopyridine adenine dinucleotide phosphate, was observed compared with standards run under the same conditions, indicating that 3-aminopyridine adenine dinucleotide is not an effective substrate for the NAD kinase. This result was confirmed by HPLC. An aliquot of the reaction mixture was applied to an ion-exchange column (RSIL-AN) and only the peaks of 3-aminopyridine analog of NAD and ATP were detected.

Since NAD kinase activity, specific for NADH has been found in some organisms (49), NADH was investigated as a substrate for the *H. influenzae* NAD kinase. No activity was found when NADH was used as the substrate at concentrations as high as 0.1 mM.

Inhibition of NAD kinase - Inhibition of NAD kinase by 3-aminopyridine adenine dinucleotide was investigated. The reaction mixtures (1 ml) contained 50 mM Tris-HCl, pH 8.0, 7.5 mM $MgCl_2$, 0.2 mM ATP, 50 μM NAD, 0.1 ml of the partially purified enzyme (0.1 mg protein/ml), and 3-aminopyridine adenine dinucleotide at concentrations as high as 1 mM. No inhibition was observed under these conditions.

NMNase activity in *H. influenzae*

Haemophilus influenzae sonicates were first tested for the presence of NADase activity using the cyanide-addition assay described under Methods. Hydrolysis of NAD was observed when this activity was assayed using the cyanide-addition method, and a lag phase was observed before any breakdown of NAD was determined. When the disappearance of NAD in the reaction mixture was followed simultaneously by both the cyanide-addition and the YADH methods (described under Methods), different results were obtained. Immediate disappearance of NAD was observed when assayed by the YADH method, but a lag phase was again observed when assayed by the cyanide-addition method. These results suggested that NAD was initially hydrolyzed by NAD pyrophosphatase, yielding NMN which was further hydrolyzed by an NMNase activity. When NMN was used as the substrate, hydrolysis of the nicotinamide-ribose bond occurred without a lag phase. When NAD was used as the substrate, and 10 μM ADP was added to the reaction mixture in order to inhibit any NAD pyrophosphatase activity, no hydrolysis of the nicotinamide-ribose bond of NAD was observed. When NMN was used as the substrate only 12% inhibition was observed with 10 μM ADP. Collectively, these results were consistent with the presence of an NMNase activity in *H. influenzae* sonicates. The sonicate was also investigated for the presence of a heat-activated NADase. *Haemophilus influenzae* sonicate was heated at 100 °C for one min, centrifuged, and assayed for NADase activity. This procedure had been used before to reveal the presence of heat-activated NAD-hydrolyzing enzymes in other bacterial systems (49, 50). No NAD hydrolysis was observed in *H. influenzae* even after heat activation.

Properties of *H. influenzae* NMNase

Haemophilus influenzae NMNase appears to be associated with the cell membrane since 78% of the activity is recovered in the particulate fraction after high-speed centrifugation (100,000 x g, 60 min, 4 °C). The products of the NMNase reaction were identified by TLC on silica gel plates. The incubation mixture (1 ml) contained 50 mM Tris-HCl, pH 8.0, 0.9 mM NMN, and 0.1 ml of *H. influenzae* sonicate. Aliquots (10 μl) of the reaction mixture were removed with time and spotted on silica gel aluminum-backed plates. The developing solvent used was 95% ethanol:1 M ammonium acetate, pH 7.4 (5:2). Only one UV-absorbing band was detected as a reaction product with a R_f = 0.63, identical to the one observed for the nicotinamide control sample. When the same TLC plate was stained with the orcinol/sulfuric acid reagent, two bands became visible as reaction products. The R_f for these two bands were 0.20 and 0.52, corresponding with those of standards of ribose and ribose 5-phosphate, respectively. An observed hydrolysis of p-nitrophenylphosphate confirmed the presence of phosphatase activity in the sonicate. Thus, the results indicated that the enzyme hydrolyzed NMN to give nicotinamide and ribose 5-phosphate, the latter being further hydrolyzed to ribose by phosphatase activity present in the sonicate.

Enzyme kinetics - The kinetic parameters for the NMNase were determined from the double reciprocal plots of initial velocities versus NMN concentrations (0.3 - 1.25 mM) (Figure 14). The K_m for NMN was calculated to be 0.5 mM and the V_{max}, 1 nmol NMN hydrolyzed per min per mg protein.

Substrate specificity of NMNase - 3-Acetylpyridine mononucleotide was investigated as a substrate for the NMNase. Lineweaver-Burk plots for this substrate (0.4 - 3.6 mM) are shown in Figure 15. The K_m for 3-acetylpyridine mononucleotide was cal-

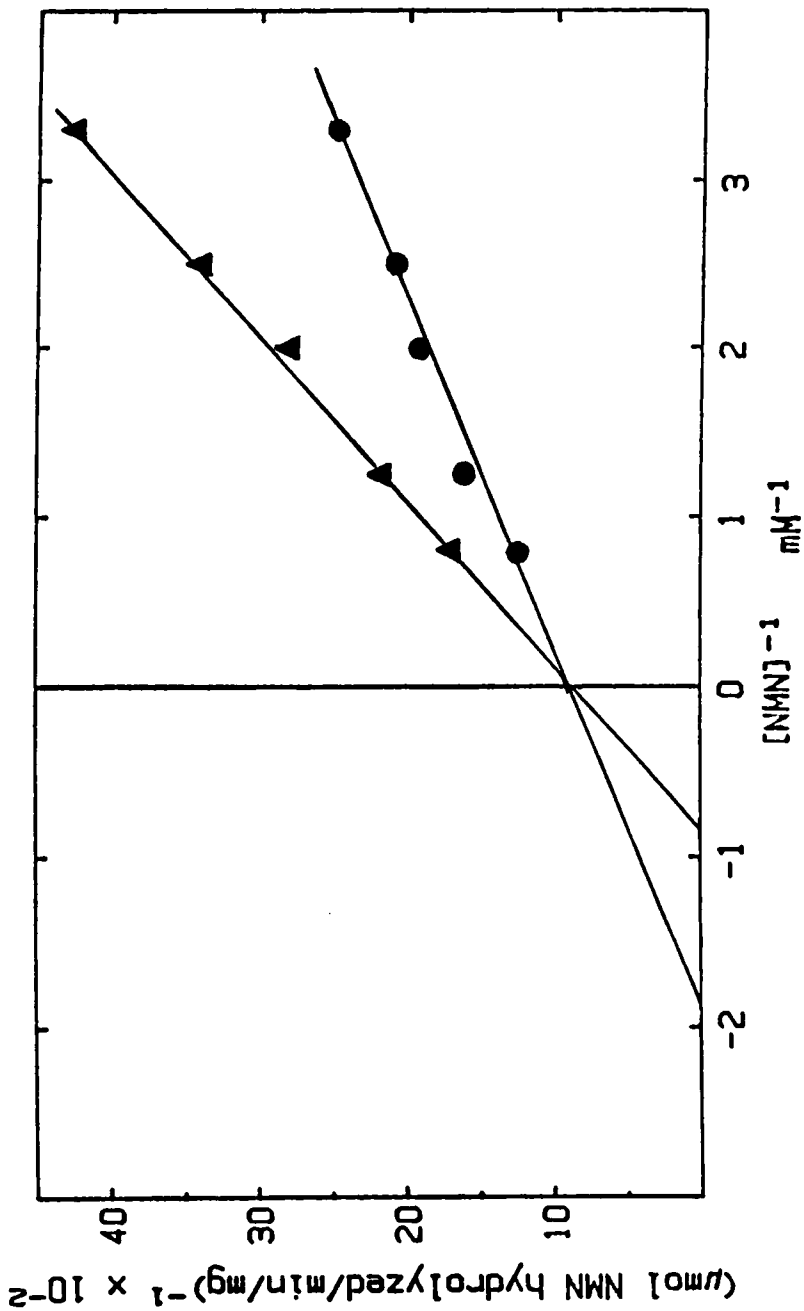

Figure 14. Effect of NMN Concentrations on NMNase Initial Velocities: The assay mixtures contained concentrations of NMN varying from 0.3 to 1.25 mM in 50 mM Tris-HCl buffer, pH 8.0, in the absence (●) and in the presence (▲) of 1 mM 3-aminopyridine mononucleotide.

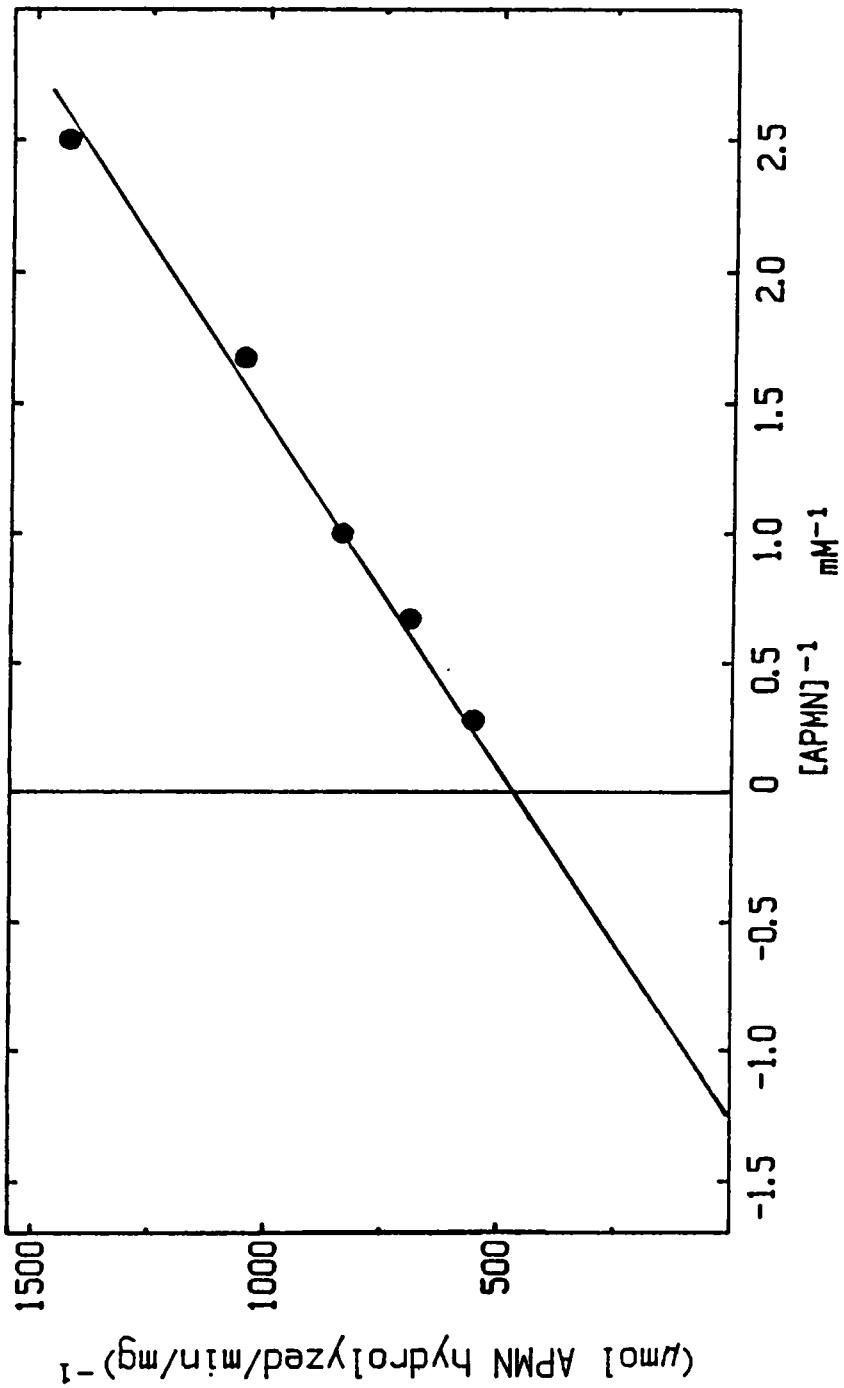

Figure 15. Effect of 3-Acetylpyridine Mononucleotide (APMN) on NMNase Initial Velocities: The assay mixtures contained concentrations of APMN varying from 0.4 to 3.6 mM in 50 mM Tris-HCl buffer, pH 8.0.

culated to be 0.8 mM and the V_{max}, 2 nmol 3-acetylpyridine mononucleotide hydrolyzed per min per mg protein.

Inhibition of NMNase - 3-Aminopyridine mononucleotide did not serve as a substrate for the NMNase. This analog (1 mM) was investigated as a possible inhibitor of the NMNase activity, and it was shown to be a relatively good competitive inhibitor of the enzyme, with a K_i of 1.2 mM (Figure 14).

NMNase was shown to be completely inhibited by ATP at 1 mM concentrations. Since the NMNase preparation contained other interfering enzyme activities, the observed inhibition by ATP was not clear. No inhibition of NMNase was observed at 0.1 M nicotinamide, a product of the reaction.

The possibility of a base-exchange reaction (transglycosidation) catalyzed by the NMNase was investigated at 37°C in 2.6-ml incubation mixtures containing 50 mM Tris-HCl, pH 8.0, 0.96 mM NMN, 50 mM 3-acetylpyridine (free base), and 0.4 ml of 20% *H. influenzae* sonicate. Aliquots (0.2 ml) were withdrawn with time and mixed with 1 M KCN (1.8 ml). Absorbance at 327 nm (λ max of the NMN-cyanide adduct), and 343 nm (λ max of the 3-acetylpyridine mononucleotide-cyanide adduct) were measured and the absorbance ratio 343/327 calculated. The ratio remained constant with time, indicating that no base-exchange reaction took place. In addition, inhibition of NMNase by 3-acetylpyridine (free base) was investigated, and no inhibition was observed at 0.1 M concentration.

The presence of an NMNase in *H. influenzae* which produces free nicotinamide, raises the question of whether or not this organism is capable of synthesizing NMN from nicotinamide in the cytoplasm. The enzyme responsible for this synthesis would be nicotinamide phosphoribosyltransferase, which is present in all members of the genus *Haemophilus* that do not require a V-factor for growth (40). Although *H. influenzae* is a V-factor organism, it was still important to investigate the possible presence of a

nicotinamide phosphoribosyltransferase activity. No such activity was found when 10 mM nicotinamide and 20 mM 5-phosphoribosylpyrophosphate were used as substrates in the presence of 10 mM ATP, and assayed as described under Methods.

DISCUSSION

The NMN adenylyltransferase, also called NAD pyrophosphorylase, was first described by Kornberg (52) and catalyzes the synthesis of NAD from NMN with concomitant formation of pyrophosphate. The enzyme has been extensively studied in eucaryotic systems (53 - 57), and it was found to be located in the cell nucleus associated with the chromatin (58, 59). Since this discovery, the NAD turnover at the nuclear level has been suggested to be of biological significance, with NAD being involved in the regulation of cell proliferation (60, 61). In procaryotes, the enzyme has not been studied as extensively. NMN adenylyltransferase activity has been found in *Lactobacillus fructosus* (62), *E. coli* (63), and *H. haemoglobinophilus* (40). No studies on the homogeneous enzyme from bacteria have been reported, due principally to difficulties in the purification procedure and the stability of the enzyme activity. The NMN adenylyltransferase from *H. influenzae* was partially purified by protamine sulfate fractionation and dye affinity chromatography. The results of the purification procedure are summarized in Table II. The enzyme was purified 15-fold with a 27% yield and a final specific activity of 0.02 units/mg protein. No further purification of the enzyme was pursued due to the instability of the enzyme activity and the low amount of enzyme present in the sonicates at the start of the purification. The partially purified NMN adenylyltransferase from *H. influenzae* was observed to have a much higher affinity for NMN than ATP (K_m for NMN, 60 μM, and K_m for ATP, 0.5 mM). This is also the case for the NMN adenylyltransferase from *Lactobacillus fructosus* with K_m values of 0.67 mM and 2.7 mM reported for NMN and ATP, respectively (62). The K_m values reported for most eucaryotic NMN adenylyltransferases were in the micromolar region (100 - 500 μM) and were similar for both ATP and NMN.

The presence of NAD kinase is well-established in mammalian systems (64 - 70), as well as in plants (71 - 73), yeast (49, 74), and bacteria (64, 75 - 78). A consensus is evident in the literature that NAD kinase catalyzes the only known biochemical reaction available for the synthesis of NADP. The NAD kinase activity, specific for NAD is essentially a cytosolic enzyme, but it was also found in the heavy mitochondrial fractions of some eucaryotic systems (49, 79, 80). Studies with *Saccharomyces cerevisiae* suggested the presence of another NAD kinase specie that is specific for NADH and is also mitochondrial (49). NAD kinase activity, specific for NAD was found in *H. influenzae* sonicates. The enzyme was observed to be cytosolic and was partially purified using dye affinity chromatography. The results of the purification procedure are summarized in Table V. The enzyme was purified 18-fold with a 32% yield and a final specific activity of 0.05 units/mg protein. No further purification of the enzyme was pursued due to the instability of the enzyme activity and the low amounts of enzyme present in the original sonicate. K_m values of 55 μM and 30 μM were found for NAD and ATP, respectively. At higher concentrations of ATP (> 0.2 mM), the partially purified enzyme was found to be inhibited by this substrate, see Figure 11. In addition, the enzyme displayed negative cooperativity at low concentrations of ATP (< 0.06 mM), indicating that the enzyme is tightly regulated by ATP levels in the cell (Figure 11). Lundquist and Olivera also found that NAD kinase from *E. coli* was inhibited by high ATP levels (78). These authors have presented data which suggest that the interconversion of NAD and NADP is important in the regulation of pyridine nucleotide cycles in this organism.

The ability of various compounds to serve as V-factor or inhibitors of growth of *H. influenzae* was studied by Kahn and Anderson (25). A good correlation has been found between substrates for the NAD pyrophosphatase and supporters of growth of the organism. NAD analogs such as 3-acetylpyridine and thionicotinamide adenine dinucleotide have been found to substitute for NAD as V-factor. They are also suitable

substrates for the NAD pyrophosphatase, which would indicate that the corresponding mononucleotides could be available inside the cell. The substrate specificities of the NMN adenylyltransferase and NAD kinase were of special interest to investigate the intracellular formation of these NAD analogs. The present work demonstrates that 3-acetylpyridine mononucleotide can be used by the *H. influenzae* NMN adenylyltransferase to yield the corresponding dinucleotide. The 3-acetylpyridine adenine dinucleotide can also be used to synthesize the corresponding dinucleotide phosphate (3-acetylpyridine adenine dinucleotide phosphate) by NAD kinase (Figure 13). The 3-acetylpyridine analog of NAD and NADP could then be available in the cytoplasm and could be used by NAD- and NADP-dependent dehydrogenases. Malate and 6-phosphogluconate dehydrogenases in *H. influenzae* have been shown to function with the 3-acetylpyridine analog of NAD(P) (81, 82).

Another NAD analog, 3-aminopyridine adenine dinucleotide, was found to be a very potent inhibitor of growth of *H. influenzae* (25). This analog has an electron donor group at the 3-position of the pyridine ring and is not reduced in dehydrogenase reactions. Kahn and Anderson (25) found that 3-aminopyridine adenine dinucleotide not only inhibits growth of *H. influenzae* at submicromolar concentrations, but also is a fairly good substrate for the periplasmic NAD pyrophosphatase. Therefore, the corresponding mononucleotide could be intracellularly available. The substrate specificity study of the NMN adenylyltransferase demonstrated that the 3-aminopyridine mononucleotide served as a substrate for this enzyme, yielding the 3-aminopyridine adenine dinucleotide. No inhibition of NMN adenylyltransferase activity was observed by 3-aminopyridine mononucleotide, and 3-aminopyridine adenine dinucleotide, at concentrations as high as 0.3 and 0.7 mM, respectively. Therefore, the very effective inhibition of growth by 3-aminopyridine adenine dinucleotide can not be explained by interactions with the NMN adenylyltransferase, and, since the NAD analog is synthe-

sized by this enzyme, it could be available to interact with cytosolic NAD-dependent dehydrogenases and the NAD kinase. The substrate specificity of NAD kinase was studied, and 3-aminopyridine adenine dinucleotide was found not to be an effective substrate for the enzyme. This result suggests that 3-aminopyridine adenine dinucleotide phosphate is not formed in the cytoplasm. Therefore, the mechanism of inhibition of growth by 3-aminopyridine adenine dinucleotide is not likely due to any interaction with NADP-dependent dehydrogenases.

The ability of 3-acetylpyridine mononucleotide to serve as a V-factor was investigated since it was shown to be a substrate for the NMN adenylyltransferase. As expected, this mononucleotide supported growth of *H. influenzae* but less efficiently than NMN at the same concentration (Table III). These and earlier studies of 3-acetylpyridine adenine dinucleotide (25) indicate that intracellular 3-acetylpyridine adenine dinucleotide(phosphate) must serve as coenzyme for *H. influenzae* dehydrogenases, although only a sampling of dehydrogenases, *H. influenzae* malate dehydrogenase (81), 6-phosphogluconate dehydrogenase (82), and D-lactate dehydrogenase (Chapter III), have been shown to function with 3-acetylpyridine analogs as coenzymes.

Inhibition of growth of *H. influenzae* by ADP (10 μM) was observed when NAD was used as V-factor; however, the inhibitory effect of ADP disappeared when cells were grown on NMN as V-factor, see Figure 8. The concentration of ADP used in this experiment (10 μM) was sufficient to inhibit the periplasmic NAD pyrophosphatase (K_i = 1.6 μM); therefore, ADP was found to inhibit growth of the organism by inhibiting the first step in the internalization of NAD. When cells are grown on NMN, ADP is no longer inhibitory of growth, indicating an alternate mechanism of internalization of NMN that does not involve the participation of the NAD pyrophosphatase.

3-Aminopyridine adenine dinucleotide was observed to be a potent inhibitor of growth of *H. influenzae* when NAD or NMN were used as V-factors. This NAD analog at 1 μg/ml concentration was investigated as a growth inhibitor using NMN as V-factor (0.1 μg/ml) in the presence of ADP to inhibit the periplasmic NAD pyrophosphatase. In this case, 3-aminopyridine adenine dinucleotide was found not to be inhibitory of growth. Thus, it was concluded that the 3-aminopyridine analog of NAD did not inhibit growth when it could not be hydrolyzed to the 3-aminopyridine mononucleotide. 3-Aminopyridine mononucleotide was prepared as described in Methods and its ability to inhibit growth of *H. influenzae* was invesigated using NAD or NMN as V-factors (Figures 6 and 7). In either case this mononucleotide was shown to be a potent inhibitor of growth, being as effective as 3-aminopyridine adenine dinucleotide at the same molar concentrations (Table IV). The required growth inhibitor is then the 3-aminopyridine mononucleotide, or the corresponding dinucleotide that could be resynthesized intracellularly by NMN adenylyltransferase.

NMNase activity was found in *H. influenzae* sonicates. The enzyme catalyzed the breakdown of NMN to give nicotinamide and ribose 5-phosphate, and was found to be associated with the cytoplasmic membrane. Hydrolysis of NMN by NMNase activity has not been observed in animal tissues, but the presence of NMNase has been reported in bacterial systems (84 - 86). 3-Acetylpyridine mononucleotide, a supporter of growth of *H. influenzae*, was found to be an effective substrate for the *H. influenzae* NMNase, with a K_m of 0.8 mM and a V_{max} of 2 nmol 3-acetylpyridine mononucleotide hydrolyzed/min/mg protein (Figure 15). As expected from the known chemical stability of 3-aminopyridine mononucleotide, this derivative did not serve as a substrate for the enzyme. This NMN analog, instead, was found to be a good competitive inhibitor of the NMNase activity with a K_i of 1.2 mM (Figure 14). No inhibition of NMNase ac-

tivity was observed by nicotinamide at 0.1 M concentration. Only 12% inhibition was achieved by 10 μM ADP.

It is difficult to explain the presence of an NMNase activity in *H. influenzae*, an organism that requires a V-factor for growth and that was shown repeatedly incapable of growing on nicotinamide as the V-factor. The enzyme responsible for the synthesis of NMN from nicotinamide would be nicotinamide phosphoribosyltransferase, which is present in all members of the genus *Haemophilus* that do not depend on an external V-factor source for growth. As expected, such activity was not found in *H. influenzae* sonicates. In *Salmonella typhimurium* and *E. coli*, NMN was suggested to be transported by the membrane-associated NMNase, which, in the process of transport, converts NMN to nicotinamide (86). We can speculate that NMNase in *H. influenzae* is also a transporter of NMN. One could think that the NMNase hydrolyzes NMN in order to transport the mononucleotide across the membrane, and once facing the cytoplasm, it could resynthesize NMN using free nicotinamide through a transglycosidation reaction. Other pyridine nucleotide glycohydrolases, such as NADases, have been reported to catalyze a base exchange (or transglycosidation) reaction with a variety of pyridine bases (87 - 89), and more than 70 NAD analogs have been synthesized using this reaction (91). Investigation of a transglycosidation reaction catalyzed by *H. influenzae* NMNase was carried out as described in Methods. Such activity in *H. influenzae* NMNase would suggest a mechanism of transporting intact NMN across the membrane, and, in addition, would be a novel method for the synthesis of several NMN analogs. No transglycosidation activity was observed for *H. influenzae* NMNase when 3-acetylpyridine (free base) was used at 50 mM concentration. It is possible that this enzyme hydrolyzes NMN to nicotinamide only when it is detached from the membrane, and displays a different activity *in vivo*. If the NMNase is truly an NMN transporter, the inhibition observed by 3-aminopyridine mononucleotide becomes significant. Al-

though NMN supports growth of *H. influenzae* at micromolar concentrations, the K_m for NMN determined for the NMNase was 0.5 mM. On the other hand, 3-aminopyridine mononucleotide could inhibit growth of *H. influenzae* at micromolar concentrations, but exhibited a K_i of 1 mM with NMNase. Therefore, if NMNase is a transporter for NMN but the activity being measured is not the one displayed *in vivo*, 3-aminopyridine mononucleotide could inhibit growth of *H. influenzae* by inhibiting the transport of NMN into the cell. Further investigation is needed in order to understand the biological functioning of NMNase in *H. influenzae*.

CHAPTER III

HAEMOPHILUS INFLUENZAE D-LACTATE DEHYDROGENASE PURIFICATION AND CHARACTERIZATION

INTRODUCTION

Early studies on the 3-aminopyridine adenine dinucleotide inhibition of growth of *H. influenzae* (25) prompted the investigation of cytosolic dehydrogenases that could be the target of this inhibition. Selected enzyme activities were measured in 20% *H. influenzae* sonicates (Table VI), and malate and 6-phosphogluconate dehydrogenases were observed to be the major dehydrogenase activities present in the sonicates. These enzymes have recently been purified and characterized in our laboratory (81, 82, 90). It has been shown that these dehydrogenases are not inhibited by the 3-aminopyridine analog of NAD, and/or 3-aminopyridine adenine dinucleotide phosphate at concentrations sufficient to inhibit growth of the organism. Therefore, the effectiveness of 3-aminopyridine adenine dinucleotide as a growth inhibitor can not be attributed to interactions with these two dehydrogenases. A lactate dehydrogenase specific for D(-)-lactate (EC 1.1.1.28) was found in *H. influenzae* sonicates, and it was purified and characterized to investigate further the mechanism of inhibition of growth of the organism by 3-aminopyridine adenine dinucleotide.

Microorganisms normally possess a dehydrogenase specific for D-lactate, whereas most vertebrates have L-lactate-specific enzymes (91, 92). It is generally found that the mammalian L(+)- lactate dehydrogenases are tetrameric, with high molecular

Table VI. Selected Enzyme Activities in 20 % *H. influenzae* Sonicates

Enzyme	Activity (units/ml)
Malate dehydrogenase	9.5
L-lactate oxidase	7.8
6-Phosphogluconate dehydrogenase	2.4
D-lactate oxidase	1.5
D-lactate dehydrogenase	1.2
Succinate dehydrogenase	1.1
Glucose 6-phosphate dehydrogenase	0.66
Glutamate dehydrogenase	0.38
Glutathione reductase	0.35
Fumarase	0.32
Alcohol dehydrogenase	--*
L-α-Glycerophosphate dehydrogenase	--*
L-Lactate dehydrogenase	--*
3-Phosphoglycerate dehydrogenase	--*

* Not detected.

weights (140,000) and that pyruvate is involved in the regulation of their activity (91,93). It is also well-known that the reaction catalyzed by L-lactate dehydrogenase follows an ordered bi-bi sequential mechanism, with NADH as the leading substrate and NAD as the last product released (94, 95). Considerably less information is available concerning D-lactate-specific dehydrogenases from microorganisms. The lack of information is in part due to the fact that most of these enzymes are very unstable when purified. D-Lactate dehydrogenases (D-LDHs) have been partially purified from several species of the genus *Lactobacillus* (96, 97); however, the predominant lactate dehydrogenase (LDH) found in *Lactobacillus* is specific for the L(+)-lactate. All *Leuconostoc* species form D(-)-lactate from glucose (98), and have been found to contain D-LDH but no L-lactate dehydrogenase (L-LDH) (99). *Pediococcus cerevisiae* forms D(-)-lactate only late in the growth cycle, when the pH lowers (100). A D-LDH from *E. coli* was purified and characterized by Tarmy and Kaplan in 1968 (101, 102).

The present work describes the purification and characterization of D(-)-lactate dehydrogenase from *H. influenzae*. The enzyme differs markedly from the animal lactate dehydrogenases in many of its chemical and catalytic properties, but shows great similarities with the D-LDH from *E. coli*. Many interesting properties of the enzyme, such as irreversibility of the reaction catalyzed, sensitivity to autoxidation, and rapid inactivation by maleimides were observed and will be discussed.

EXPERIMENTAL PROCEDURES

MATERIALS

Haemophilus influenzae strain Rd was obtained from Dr. William L. Albritton of the University of Saskatchewan, Saskatoon. Brain Heart Infusion (BHI) and triethanolamine were obtained from Fisher Scientific. Protamine sulfate, streptomycin sulfate, hemin, histidine, 2-[N-morpholino]-ethane sulfonic acid (MES), Bicine, and Hepes buffers were obtained from Sigma. Acrylamide, N,N'-methylenebisacrylamide, and Coomassie Brilliant Blue G-250 were purchased from Eastman Kodak. Coomassie Brilliant Blue R-250 was purchased from Bio-Rad. Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), protein molecular weight standards, dithiothreitol (DTT), Sephacryl-200, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), pyruvate, oxalacetate, lithium D(-)- and L(+)-lactate, adenosine, ATP, ADP, AMP, ADP-ribose, glyoxylate, benzoylformate, α-ketoisovalerate, α-ketoglutarate, α-ketobutyrate, fructose 1,6-diphosphate, malonate, malate, 6-phosphogluconate, D-glucose 6-phosphate, 3-phosphoglycerate, L-α-glycerophosphate, glutathione (oxidized), iodoacetic acid, iodoacetamide, and diethylpyrocarbonate (DEPC) were obtained from Sigma. Procion Blue HB (Cibacron blue F3GA), 2,4-pentanedione, and Norit A pellets were purchased from Aldrich Chemical Co.. Matrex gel Green A and Blue A were obtained from Amicon. Phenyl-Sepharose CL-4B and blue dextran 2000 were purchased from Pharmacia. All dinucleotides were purchased from Sigma except 3-aminopyridine adenine dinucleotide, thionicotinamide adenine dinucleotide, and nicotinamide $1,N^6$-ethenoadenine dinucleotide which were prepared by published procedures (43, 103, 104).

Ethylene glycol was obtained from Fisher Scientific. Succinate was purchased from J. T. Baker Chemical Co.. D(-)-Lactate dehydrogenase (EC 1.1.1.28) from *Leuconostoc mesenteroides*, l(+)-Lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle, and horse liver alcohol dehydrogenase (HLADH) were purchased from Sigma. A partially purified D-lactate oxidase from *H. influenzae* was obtained as described below (Chapter IV). N-Ethylmaleimide (NEM) was purchased from Sigma. N-Propylmaleimide was obtained from Lancaster Synthesis Ltd., and N-butylmaleimide from Nutritional Biochemicals Inc.. All other N-alkylmaleimides (pentyl - heptyl, inclusive) were prepared by a published procedure (105). All other chemicals were of reagent grade.

METHODS

Growth of the organism - *Haemophilus influenzae* was grown as previously described under Methods in Chapter II.

Enzyme activity assay - D-Lactate dehydrogenase (D-LDH) was routinely assayed at room temperature in the direction of pyruvate reduction. The standard assay mixture (1 ml) contained 50 mM Hepes buffer, pH 7.0, 0.1 mM NADH, and 4 mM pyruvate. The reaction was initiated by addition of the enzyme and initial velocities of pyruvate reduction were determined by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH. A unit of enzyme activity is defined as the oxidation of one μmol of NADH per min. D-Lactate dehydrogenase activity in the forward direction (D-lactate oxidation) was investigated at room temperature in a 1-ml cuvette containing 45 mM glycine-NaOH buffer, pH 9.0, 20 mM lithium D(-)-lactate, and 0.54 mM NAD. The reaction was initiated by the addition of enzyme and the increase in absorbance at 340 nm was monitored.

Protein determination - Protein concentrations were determined by the microprotein assay of the Coomassie blue method (47) using BSA as a standard.

Ultrafiltration - Concentration of protein fractions by ultrafiltration was carried out at 4°C using Amicon PM-10 membranes.

Polyacrylamide Gel Electrophoresis - Polyacrylamide disc gel electrophoresis was performed by the methods of Davis (106), and Weber and Osborn (107). The concentration of acrylamide was 7.5% in the separating gel and 2.5% in the stacking gel. Electrophoresis was carried out at 2.5 mA per gel at 4°C. Gels were stained overnight in 0.25% Coomassie Brilliant Blue G-250, 40% ethanol, and 15% acetic acid. Destaining was carried out with a solution containing 40% ethanol and 15% acetic acid for 4

hours. Stained gels were stored in 10% acetic acid. Activity staining for D-LDH was performed by a method analogous to the negative stain technique described by Kaplan (101). Gels were incubated in 50 mM potassium phosphate buffer, pH 7.0, 20 mM pyruvate, and 0.5 mM NADH for 30 min at room temperature. This solution was removed from the gel by aspiration and the staining solution was added. The staining solution contained 50 mM Tris-HCl, pH 8.6, 0.025 mM PMS, and 0.7 mM NBT. Gels were incubated in this solution for 30 min in the dark and then the reaction was stopped by addition of 5% acetic acid. Gels were rinsed and stored in aqueous solution. Wherever NADH was present, a purple-blue color developed, thus a clear band indicated that NADH had been oxidized and the D-LDH was localized at that band.

SDS-Polyacrylamide Gel Electrophoresis - The subunit molecular weight of the enzyme was determined using 12% SDS slab gels according to the method of Laemmli (108) but using DTT instead of β-mercaptoethanol in the sample buffer. Slab gel electrophoresis was carried out using a BRL vertical gel electrophoresis system. Gels were fixed in 50% methanol for 30 min and then stained in the staining solution for 30 min with constant gentle agitation (40-60 rpm). The staining solution contained 0.6 g Coomassie Blue R-250 in a mixture of 150 ml methanol, 30 ml acetic acid and 120 ml distilled water. The gels were washed with agitation in distilled water that was changed 2-3 times as it turned blue. Gels were placed in a destaining solution until the band(s) appeared. The destaining solution was 20% in methanol and 10% in acetic acid. Once the band(s) became visible, the gels were placed in 10% acetic acid. Molecular weight of the protein was determined relative to the molecular weight standards of phosphorylase b, 97,400; bovine serum albumin, 66,000; ovalbumin, 45,000; glyceraldehyde 3-phosphate dehydrogenase, 36,000; and carbonic anhydrase, 29,000.

Chemical modification of D-LDH - The D-LDH from *H. influenzae* was observed to be significantly inactivated by micromolar concentrations of maleimides. The purified

enzyme was stored in 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT. In maleimide inactivation studies, the DTT present to stabilize the enzyme on storage could readily consume the low concentration of maleimide before the enzyme was inactivat ed. Therefore, the enzyme was incubated with Norit A pellets to remove most of the DTT before the sufhydryl reagent was added (109). The time-dependent inactivation of D-LDH by N-alkylmaleimides was studied at 25°C in incubation mixtures (0.4 ml) containing 50 mM Hepes buffer, pH 7.0, 2.5 % ethanol (to solubilize the maleimide), 0.7 μg enzyme (in 10 mM potassium phosphate buffer pH 7.0, containing 1 mM DTT), and Norit A pellets in a 1:1 ratio (w/w, DTT/Norit). After 6 min of incubation at 25°C, required for the Norit to remove the DTT present, the maleimide was added and this was considered time zero. At timed intervals, aliquots (20-50 μl) were withdrawn from the incubation mixture and added to the standard assay mixture to measure D-LDH activity. The logarithm of the enzyme activity remaining at any given time was plotted against time of incubation, and the pseudo first-order rate constant was calculated from the equation $k_1 = 0.693 / t_{\frac{1}{2}}$. A control was run with an incubation mixture containing 50 mM Hepes buffer, pH 7.0, 2.5% ethanol, 0.7 μg enzyme (in 10 mM potassium buffer, pH 7.0, containing 1 mM DTT), and Norit A pellets in the ratio indicated above, in the absence of N-alkylmaleimide. A slight loss in D-LDH activity was observed for the control incubation mixture over a 15 min period. The k_1 value obtained for this control incubation mixture was subtracted from all the k_1 values determined for each series of N-alkylmaleimide concentrations before plotting these constants against maleimide concentration. Apparent second-order rate constants (k_2) of enzyme inactivation were obtained from the slopes of the linear portion of these plots for the six N-alkylmaleimides studied (ethyl - heptyl, inclusive).

Inactivation of D-LDH by iodoacetic acid (0.3 - 1.8 mM) and iodoacetamide (10 - 60 mM) was carried out at 25°C in 0.2-ml reaction mixtures containing 50 mM Hepes

buffer, pH 7.0, 0.7 μg enzyme and the indicated amount of sulfhydryl reagent. No Norit A pellets were added since the concentrations used of these sulfhydryl reagents were well in excess with respect to the DTT present in the enzyme aliquot. The time of addition of the enzyme to the incubation mixture was considered time zero, and at various times thereafter, 20 μl aliquots were transferred to a standard assay mixture to measure D-LDH activity. The rate of inactivation of D-LDH by iodoacetic acid and iodoacetamide followed pseudo first-order kinetics. Second-order rate constants of inactivation were determined as explained above for N-alkylmaleimide inactivation.

Modification of lysine groups of D-LDH by 2,4-pentanedione was investigated at 25°C in 0.2-ml incubation mixtures containing 50 mM Hepes buffer, pH 7.0, 0.7 μg enzyme, and up to 100 mM of the reagent. Aliquots (20 μl) were removed with time and assayed for D-LDH activity.

Diethylpyrocarbonate (DEPC) was used to investigate the presence of essential histidine groups in the enzyme. Modification of D-LDH by DEPC was carried out at 15°C in 0.2-ml incubation mixtures containing 50 mM Hepes buffer, pH 7.0, 0.7 μg enzyme, and up to 20 mM DEPC. The DEPC reagent was kept at 4°C prior to use. Aliquots (20 μl) were removed from the incubation mixtures at timed intervals and D-LDH activity assayed.

Spectral measurements - UV and visible absorbance measurements were performed on a Beckman Acta MVI recording spectrophotometer. The zero suppress mode was used for measuring D-LDH-catalyzed reduction of pyruvate. Fluorescence measurements were made on a Perkin-Elmer 650-40 spectrofluorometer.

pH Measurements - All pH measurements were made at room temperature on a Radiometer digital PHM 52 pH meter equipped with a GK-2321C combination electrode.

Preparation of reduced NAD analogs - The reduction of NAD analogs structurally altered in the pyridine or purine moieties was performed as follows: 3-ml reaction mixtures were stirred at 25°C containing 37 mM Glycine-NaOH buffer, pH 9.3, 1 mg HLADH, 1 % ethanol, and 10 mg of the appropriate dinucleotide. The reduction of the dinucleotide was considered complete when no further increase in absorbance was observed at the appropriate wavelengths of the reduced dinucleotide. At this point the reaction mixture was ultrafiltered through an Amicon PM-10 membrane to remove the protein and UV spectra were taken in order to determine purity and concentration of the reduced NAD analog solution. The reduced NAD analog obtained was used immediately.

Determination of stereospecificity - The stereospecificity of the D-LDH reaction was determined using a homogeneous preparation of the enzyme. A reaction mixture containing limiting amounts of pyruvate (1 mM) and excess NADH (0.1 mM) was incubated at room temperature and NADH oxidation was followed spectrophotometrically until the absorbance at 340 nm became constant (the pyruvate was completely reduced). The reaction mixture was then boiled for 3 min, cooled, and centrifuged in a clinical centrifuge for 10 min. Aliquots (0.2 ml) were assayed for D- and L- lactate using the method described by Dennis (110). The assay mixture used to investigate for D(-)-lactate contained 50 mM Tris-HCl, pH 8.0, 0.54 mM 3-acetylpyridine adenine dinucleotide, 9 units of *Leuconostoc mesenteroides* D(-)-lactate dehydrogenase, and the 0.2-ml aliquot of the reaction mixture to be assayed. The reaction was followed by measuring the increase in absorbance at 365 nm. D(-)-Lactate was also assayed in a reaction mixture containing 50 mM Tris-HCl, pH 8.0, 0.5 mM MTT, 0.4 mM PMS, *H. influenzae* D-lactate oxidase (partially purified) and the 0.2-ml aliquot to be assayed. The reaction was followed by the increase in absorbance at 578 nm.

The assay mixture used to investigate for the L(+)-isomer contained 50 mM glycine-NaOH buffer, pH 8.5, 0.5 mM 3-acetylpyridine adenine dinucleotide, 18 units of rabbit muscle L(+)-lactate dehydrogenase and the 0.2-ml aliquot of the reaction mixture to be assayed. The reaction was followed by measuring the increase in absorbance at 365 nm.

RESULTS

Purification of *H. influenzae* D-LDH

Frozen cells (20 g) were thawed and dispersed with 100 ml of 50 mM potassium potassium phosphate buffer, pH 7.0 containing 1 mM DTT at 4°C using a glass homogenizer. The homogenate was sonicated in an ice-salt bath using the microprobe tip of an Ultrasonics Sonifier Cell Disruptor at 70 W for five min. Cell debris was removed by centrifugation for 20 min at 17,000 x g.

Protamine sulfate was added to the 20% sonicate supernate at 4°C to give a 0.2% final concentration of protamine sulfate. This mixture was stirred for 30 min in an ice-salt bath, and centrifuged at 39,000 x g for 10 min. The pellet was discarded and the supernate was adjusted to 60% ammonium sulfate saturation with continuous stirring at 4°C for 2 hours. The suspension was then centrifuged at 38,000 x g for 15 min. The pellet obtained was resuspended in 20 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT.

The ammonium sulfate fraction was applied to a Matrex gel Green A column (1.0 x 10 cm) previously equilibrated in 10 mM potassium potassium phosphate buffer, pH 7.0, containing 1 mM DTT. After washing the column with the equilibration buffer, the D-LDH was eluted using a linear salt gradient, 0.05-0.8 M KCl in the same buffer. Fractions (2.5 ml) were assayed for D-LDH activity, and the protein concentration was monitored by 280 nm absorbance. The elution profile is shown in Figure 16. Fractions containing D-LDH activity were pooled and used in the next purification step.

The pooled enzyme sample from the Green A column was applied to a phenyl-Sepharose hydrophobic column (1.5 x 12 cm) previously equilibrated with 10 mM

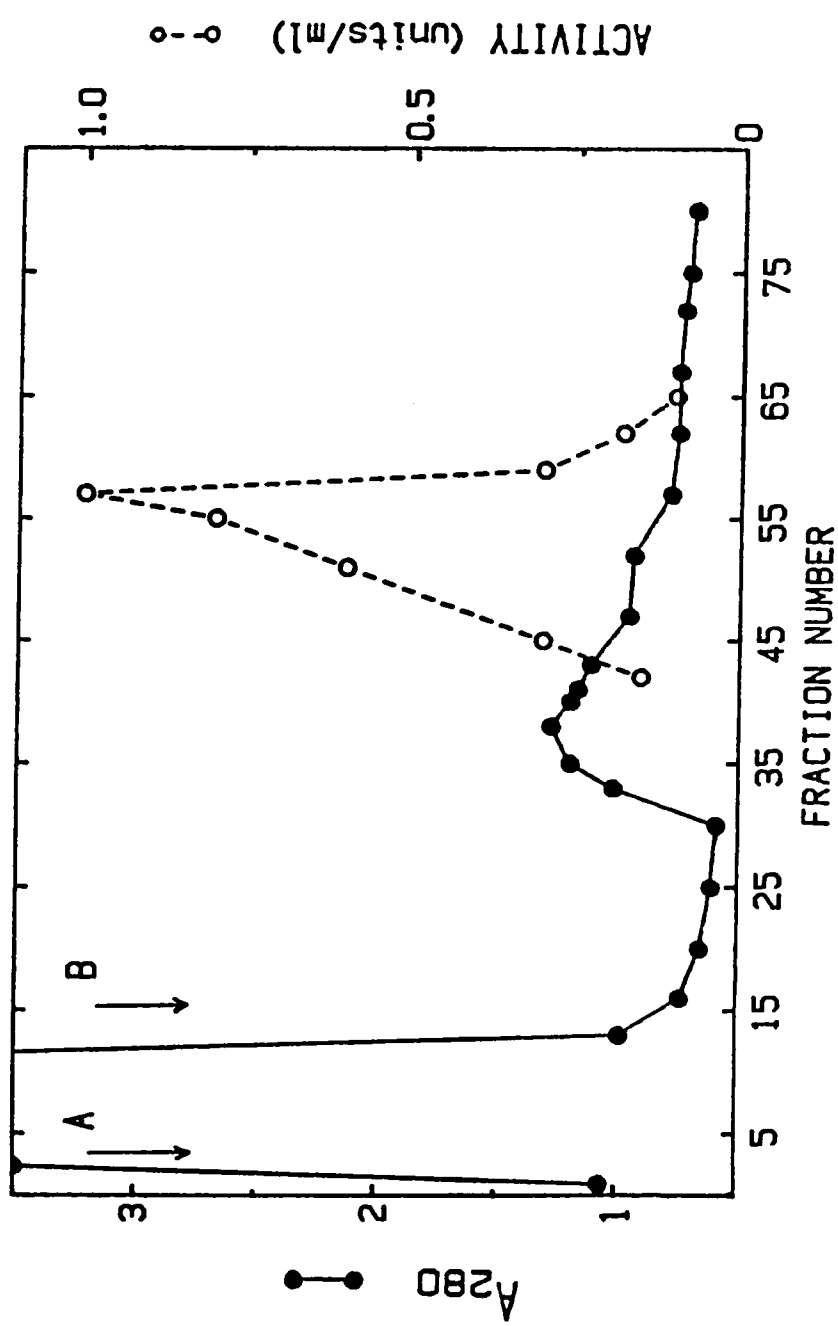

Figure 16. Affinity Chromatography on Matrex Gel Green A: The 60% ammonium sulfate pellet, resuspended in 20 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT was applied to a Matrex gel Green A (1 x 10 cm) previously equilibrated in the same buffer. Arrow A indicates the point of washing 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. Protein concentration is measured as absorbance at 280 nm. The D-LDH activity is reported as units per ml.

potassium phosphate buffer, pH 7.0, containing 1 mM DTT and 0.6 M KCl. Unbound proteins were washed from the column with equilibration buffer. Moderately hydrophobic proteins were eluted by the lower ionic strength 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT. The D-LDH was then eluted with the lower ionic strength buffer in the presence of 20% ethylene glycol. The elution profile is shown in Figure 17. Fractions (2.5 ml) containing D-LDH activity were pooled for further fractionation.

The sample from the phenyl-Sepharose column was applied to a second Matrex gel Green A column (1.0 x 10 cm) equilibrated with 10 mM potassium potassium phosphate buffer, pH 7.0, containing 1 mM DTT. After washing the column with equilibration buffer, the D-LDH was eluted by a linear salt gradient, 0-1 M KCl in the same buffer. The elution profile is shown in Figure 18. Fractions (2.5 ml) containing enzyme activity were pooled and the ionic strength reduced to 0.05 M KCl using ultrafiltration.

The desalted and concentrated sample from the second Green A column was applied to a Matrex gel Blue A column (1.0 x 6.0 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0 containing 1 mM DTT. After washing with equilibration buffer, the D-LDH was eluted by a linear salt gradient, 0-0.5 M KCl in the same buffer. The elution profile is shown in Figure 19.

The results of the purification procedure are shown in Table VII. The enzyme was purified 2100-fold with a 14 % yield and a final specific activity of 300 μmol per min per mg protein.

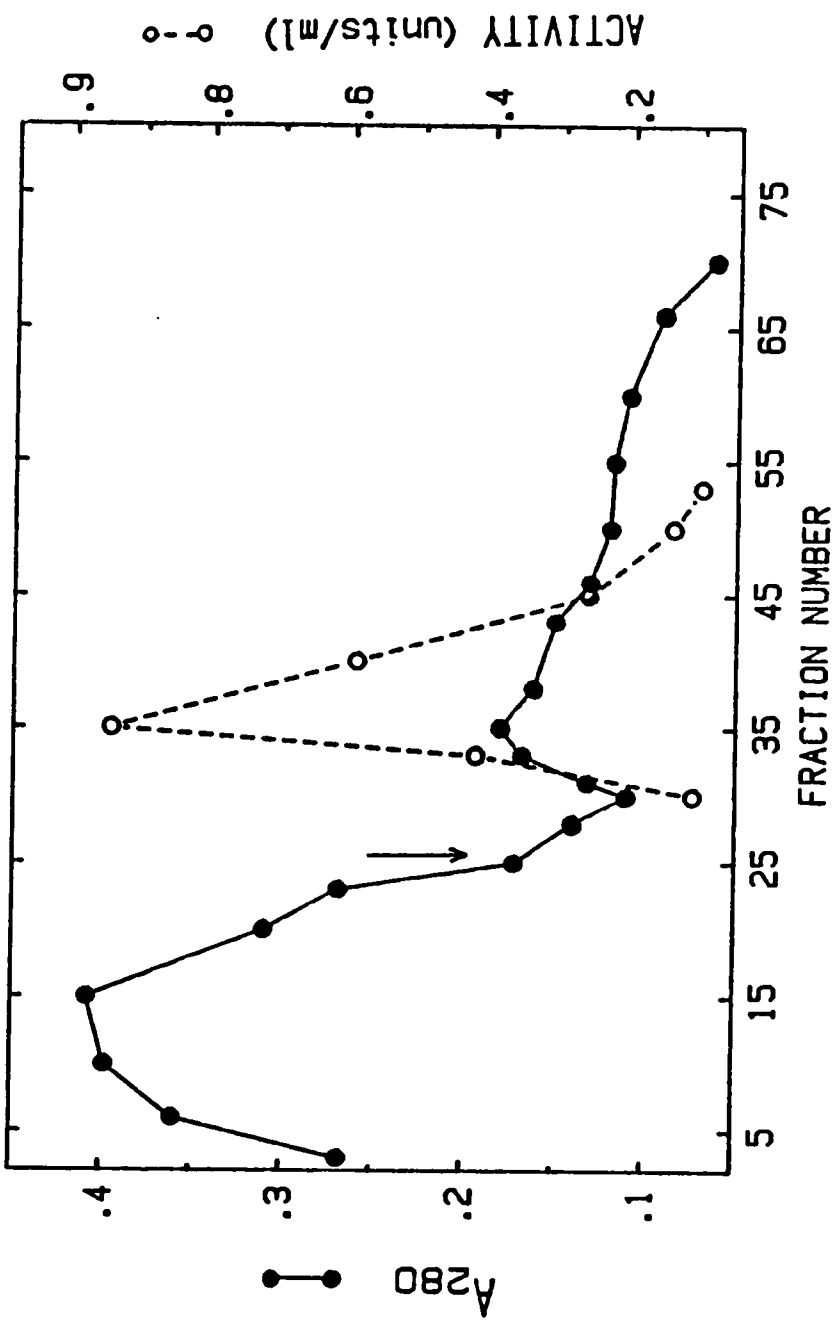

Figure 17. Phenyl-Sepharose CL-4B Chromatography: The pooled enzyme sample from the Green A column was applied to a phenyl-Sepharose hydrophobic column (1.5 x 12 cm) previously equlilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT and 0.6 M KCl. The arrow indicates the starting point of elution with 20% ethylene glycol containing buffer as stated in the text. Protein concentration is indicated as absorbance at 280 nm, and the D-LDH activity is reported as units per ml.

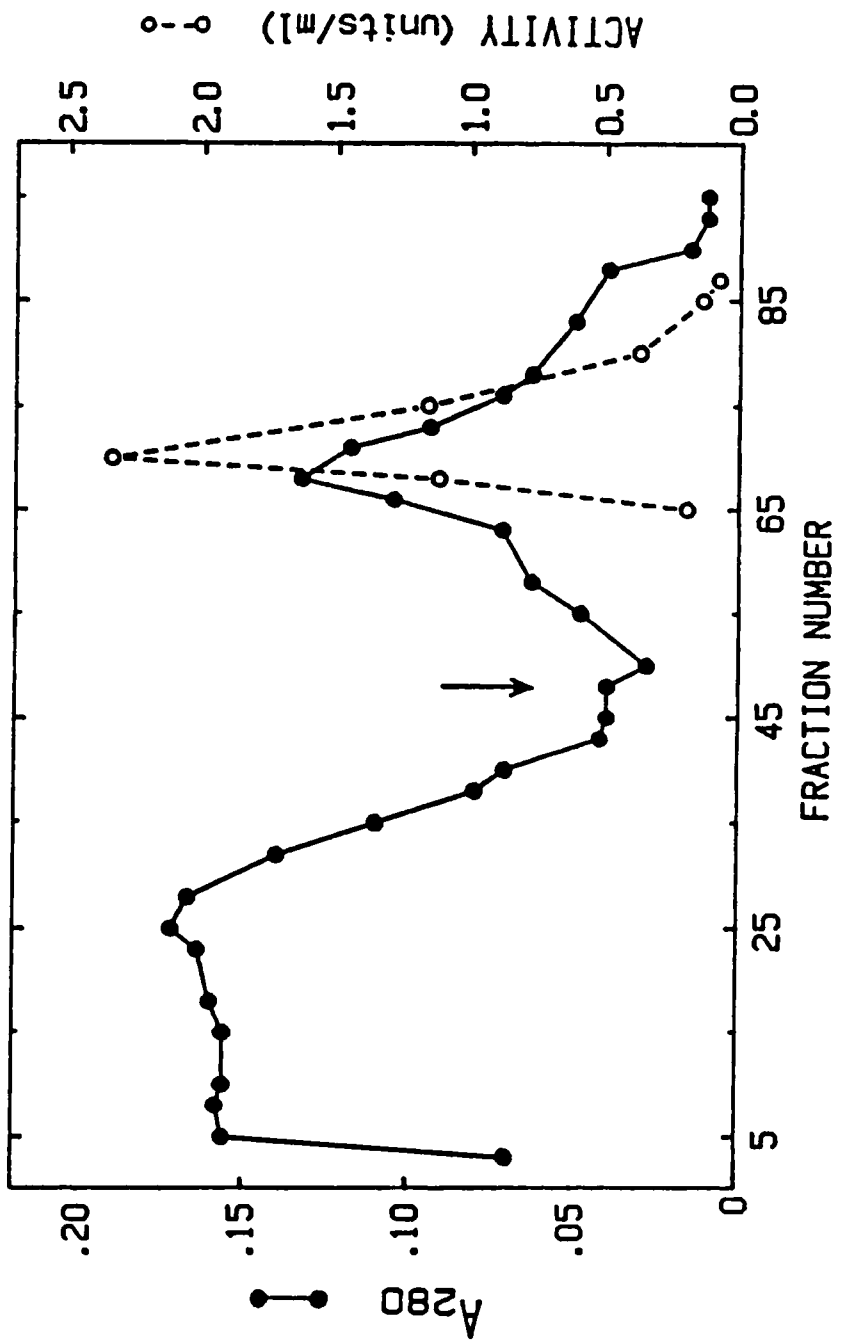

Figure 18. Affinity Chromatography on Matrex Gel Green A: The pooled enzyme sample from the phenyl-Sepharose column was applied to a Matrex gel Green A column (1 x 10 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT. The arrow indicates the point of initiation of a linear salt gradient of 0 to 1 M KCl in the equilibration buffer. Protein concentration is indicated as absorbance at 280 nm, and D-LDH activity is reported as units per ml.

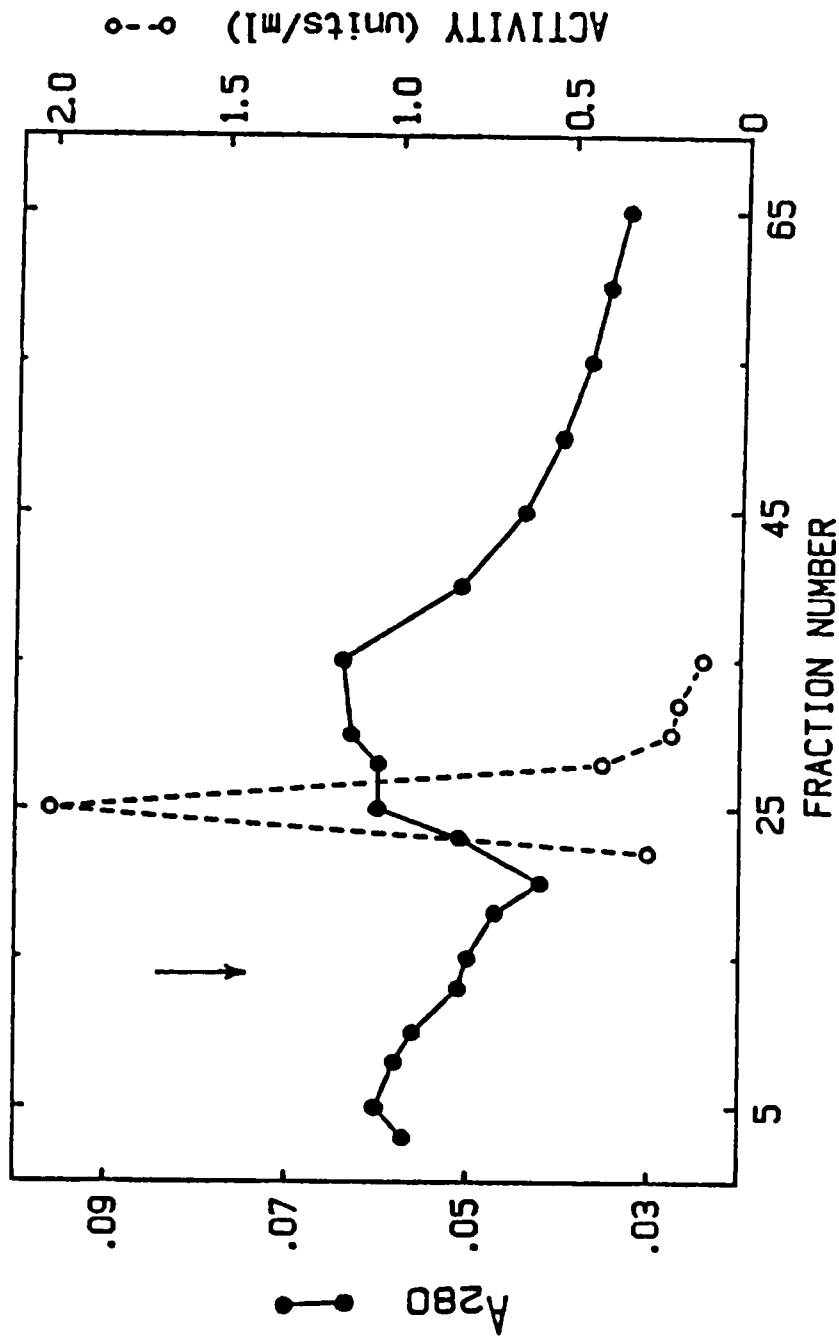

Figure 19. Affinity Chromatography on Matrex Gel Blue A: The pooled second Green A column enzyme sample, after desalting and concentration on Amicon PM-10 membrane, was applied to a Matrex gel Blue A column (1 x 6 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT. A linear gradient of 0 to 0.5 M KCl in the equilibrating buffer was applied as indicated by the arrow. Protein concentration is indicated as absorbance at 280 nm, and D-LDH activity is reported as units per ml.

Table VII. Purification of D-Lactate Dehydrogenase

FRACTION	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (units)	SPECIFIC ACTIVITY (units/mg)	YIELD %	FOLD PURIF.
Sonicate	903	125	0.14	100	1.0
Protamine Sulfate	533	81	0.15	65	1.0
Ammonium Sulfate Pellet	232	94	0.41	75	3
Matrex gel Green A	19.4	81	4.15	64	30
Phenyl-Sepharose	2.1	53	25.0	42	180
Matrex gel Green A	0.8	32	38.0	26	272
Matrex gel Blue A	0.06	18	300.0	14	2100

Properties of the purified D-LDH

The enzyme was purified to electrophoretic homogeneity. When analyzed by polyacrylamide gel electrophoresis at pH 8.3, the purified enzyme migrated as a single protein band which corresponded to D-LDH as determined by the activity staining technique.

The native molecular weight of the enzyme was determined under nondenaturating conditions of gel filtration on Sephacryl-200 at 4°C. The column (1.5 x 80 cm) was equilibrated with 100 mM potassium phosphate buffer, pH 7.5, containing 1 mM DTT. Elution volumes (V_e) of standard proteins and D-LDH were determined by measuring absorbance at 280 nm and enzyme activity, respectively. The void volume (V_o) of the column was determined using blue dextran. The calculated V_e/V_o value for the purified enzyme corresponded to an apparent M_r of 135,000 as shown in Figure 20 (line A). SDS-Polyacrylamide gel electrophoresis was used to determine the number and molecular weight of subunits. Electrophoretic conditions were essentially those reported by Davis, Weber and Osborn (106, 107), except that DTT was used instead of β-mercaptoethanol at the same concentration in the sample buffer. The apparent molecular weight obtained for the subunits of the purified enzyme under these denaturating conditions was 35,000 as shown in Figure 20 (line B).

The effect of pH on enzyme activity was investigated using three zwitterionic buffers having overlapping pH ranges: 50 mM MES-NaOH, 50 mM Bicine-NaOH, and 50 mM Hepes-NaOH. Initial velocities of NADH oxidation were measured as a function of pH between pH 6.2 and 8.5. V_{max}/K_m values were calculated for each pH when NADH concentrations were varied at saturation levels of pyruvate. The V_{max}/K_m values exhibited an optimum pH of 7.2 as shown in Figure 21.

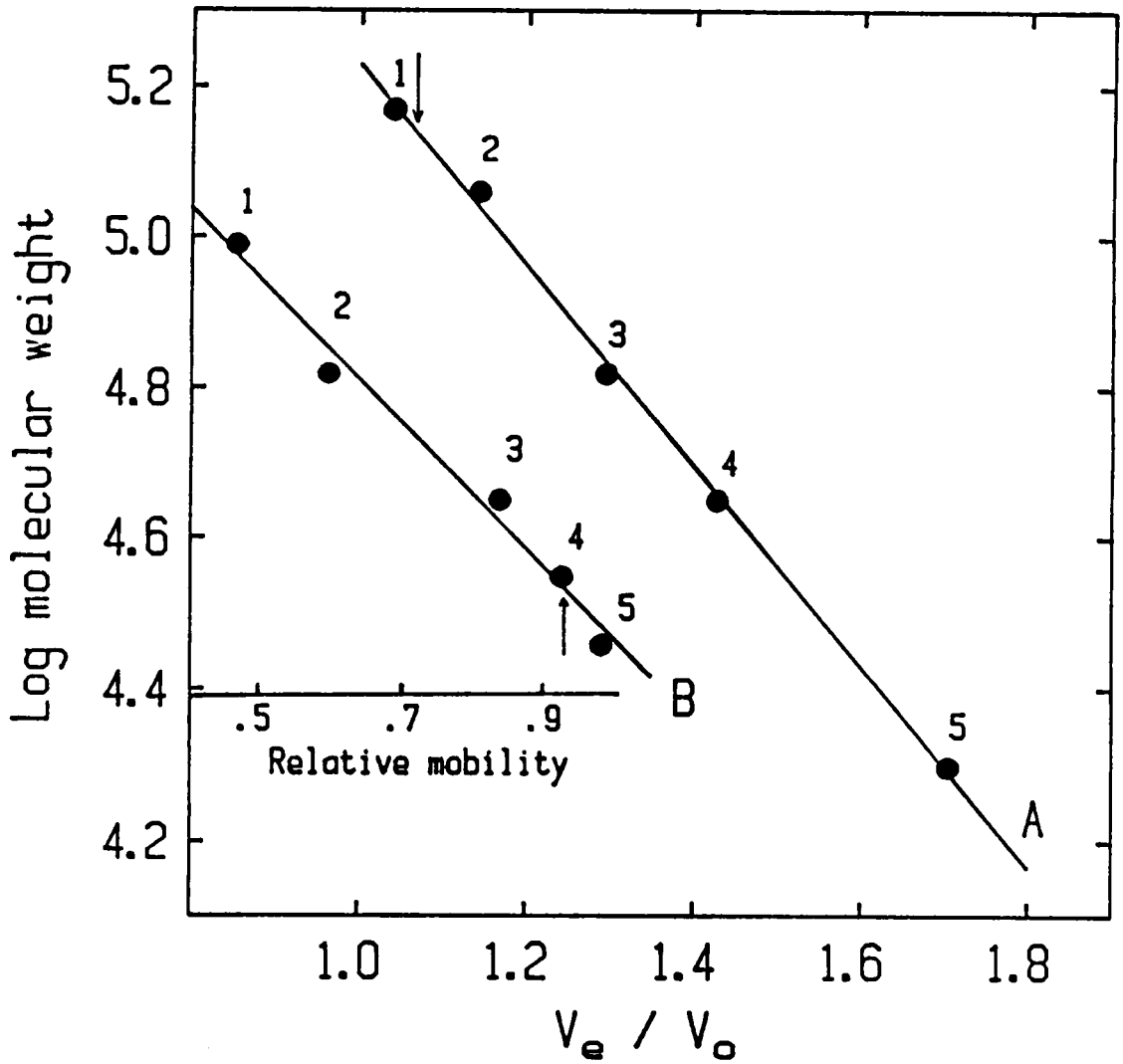

Figure 20. Molecular Weight Determination of D-lactate Dehydrogenase: A. Gel filtration on Sephacryl-200. Molecular weight standards were 1) yeast alcohol dehydrogenase, 2) β-galactosidase, 3) bovine serum albumin, 4) ovalbumin, and 5) soy bean trypsin inhibitor. B. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Molecular weight standards were 1) phosphorylase b, 2) bovine serum albumin, 3) ovalbumin, 4) glyceraldehyde-3-phosphate dehydrogenase, and 5) carbonic anhydrase. The arrows indicate the values obtained for D-lactate dehydrogenase.

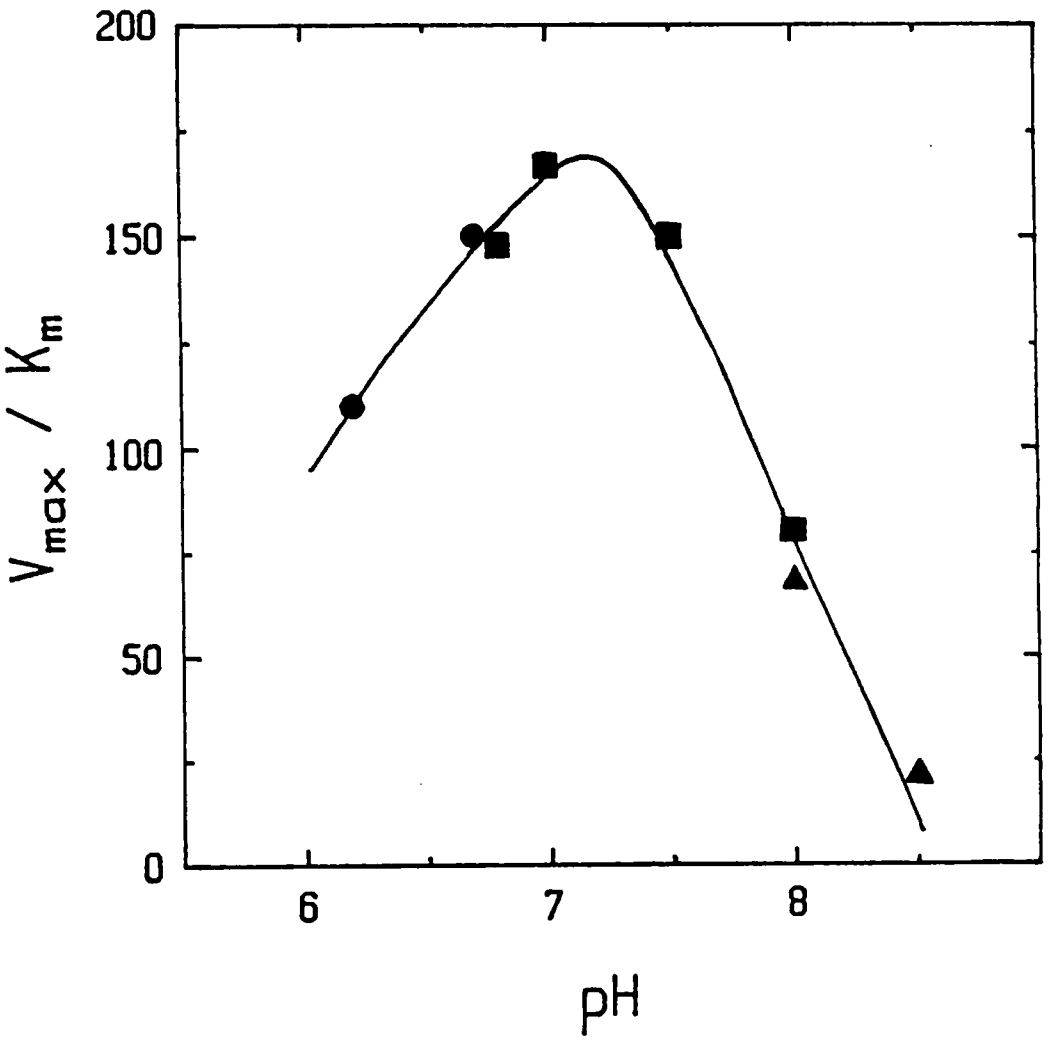

Figure 21. Effect of pH on Catalytic Activity of D-Lactate Dehydrogenase: The assay mixtures contained 4 mM pyruvate, and NADH concentrations varied from 0.1 to 0.017 mM at different pHs. Reactions were initiated by addition of the purified enzyme. Buffers used were 50 mM Bicine (▲), 50 mM Hepes (●), and 50 mM MES (■).

The effect of ionic strength on enzyme activity was investigated using KCl. Initial velocities were measured in 50 mM Hepes buffer, pH 7.0 and the ionic strength was increased by addition of KCl from 0 to 100 mM. As shown in Figure 22, KCl inhibits the reaction rate at unsaturating levels of pyruvate (0.2 - 3 mM). The addition of KCl to the standard NADH-pyruvate assay system resulted in a transposition of the curve of the initial velocity versus substrate concentration from hyperbolic to sigmoidal. The Hill plots for pyruvate show that KCl increases the slope of log v/V_{max} - v versus log [pyruvate] from 1.4 to 2.4 (Figure 23). The effect of several salts, other than KCl, on the enzyme activity was investigated. Initial velocities were measured in 50 mM Hepes buffer, pH 7.0, at saturating concentrations of the substrate and the coenzyme, and at various concentrations of the salt to be studied. The results obtained are shown in Figure 24.

The purified enzyme was routinely stored at 4°C at a protein concentration of 10 - 30 μg/ml in 10 mM phosphate buffer, pH 7.0 containing 1 mM DTT. Under these conditions, the enzyme was stable for at least three weeks without apparent loss of activity. However, when the enzyme solution was stored at -15°C overnight it lost more than 85% of its activity. The purified enzyme was denatured at moderate temperatures in 10 mM phosphate, pH 7.0 containing 1 mM DTT. The rate of thermal denaturation followed first-order kinetics as shown in Figure 25. The enzyme was incubated at different temperatures (44 - 58°C) and the D-LDH activity remaining in the incubation mixture was assayed at timed intervals. A half-life of 10.7 min was observed at 54°C. When NADH was included in the incubation mixture at a concentration of 50 μM, the half-life at this temperature was increased to 16.2 min. The presence of 50 μM NADH decreased the rate constant by 34%, indicating a 34% protection against thermal denaturation at 54°C. In contrast, the substrate pyruvate, at concentrations as high as 12 mM did not show any protection against thermal denaturation. The oxidized form

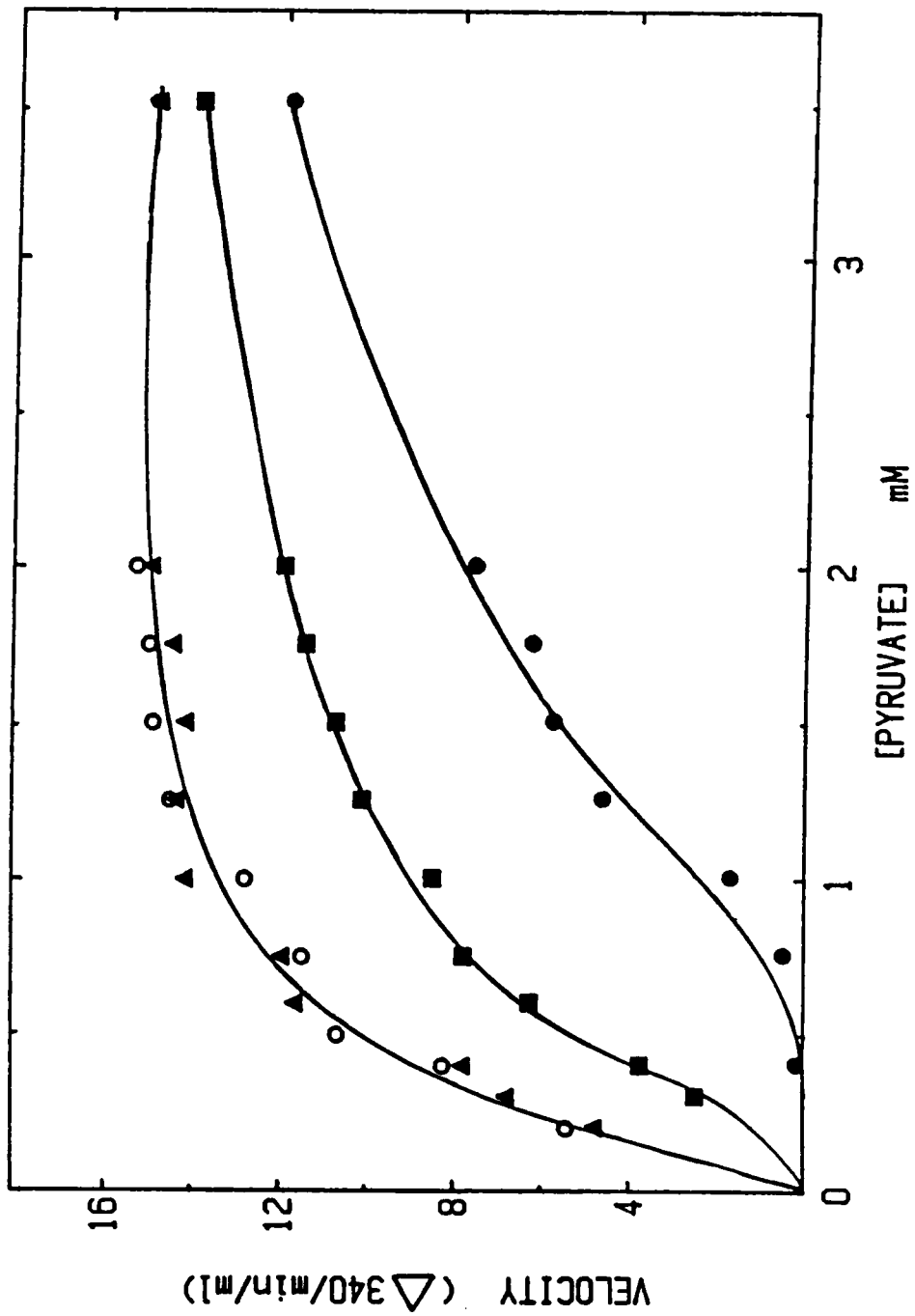

Figure 22. Effect of Salt Concentration on D-Lactate Dehydrogenase Activity: Initial velocities were measured in 50 mM Hepes buffer, pH 7.0 and increasing concentrations of KCl: (○), none; (▲), 30 mM KCl; (■), 60 mM KCl; (●), 100 mM KCl. The concentration of pyruvate in the assay mixture was varied from 0.2 to 3 mM and NADH was kept constant at 0.1 mM.

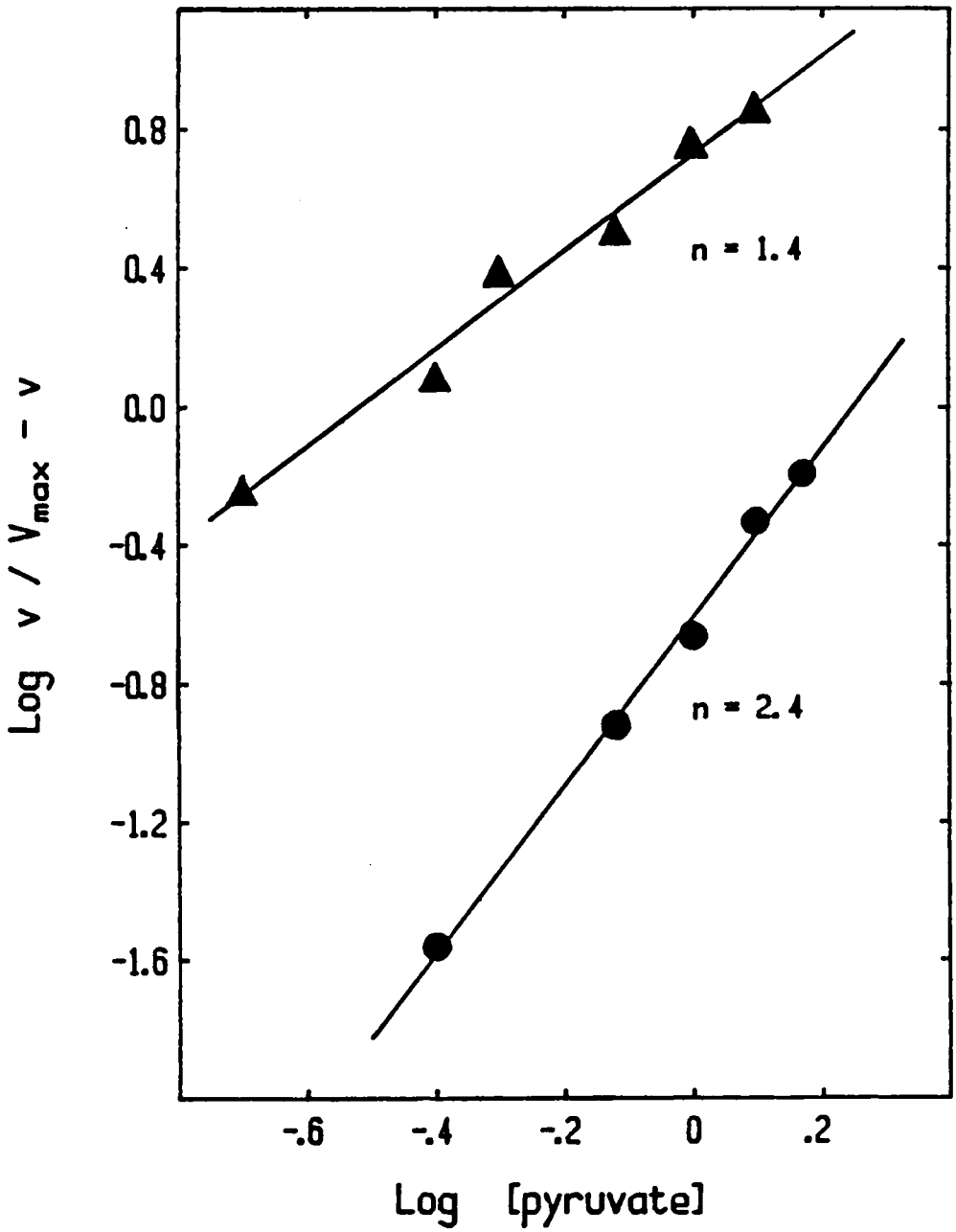

Figure 23. Hill Plots of the Effect of KCl on D-LDH Initial Velocities: Hill plots for pyruvate in the absence (▲) and in the presence (●) of 100 mM KCl. The slope (n) was increased from 1.4 to 2.4 by KCl.

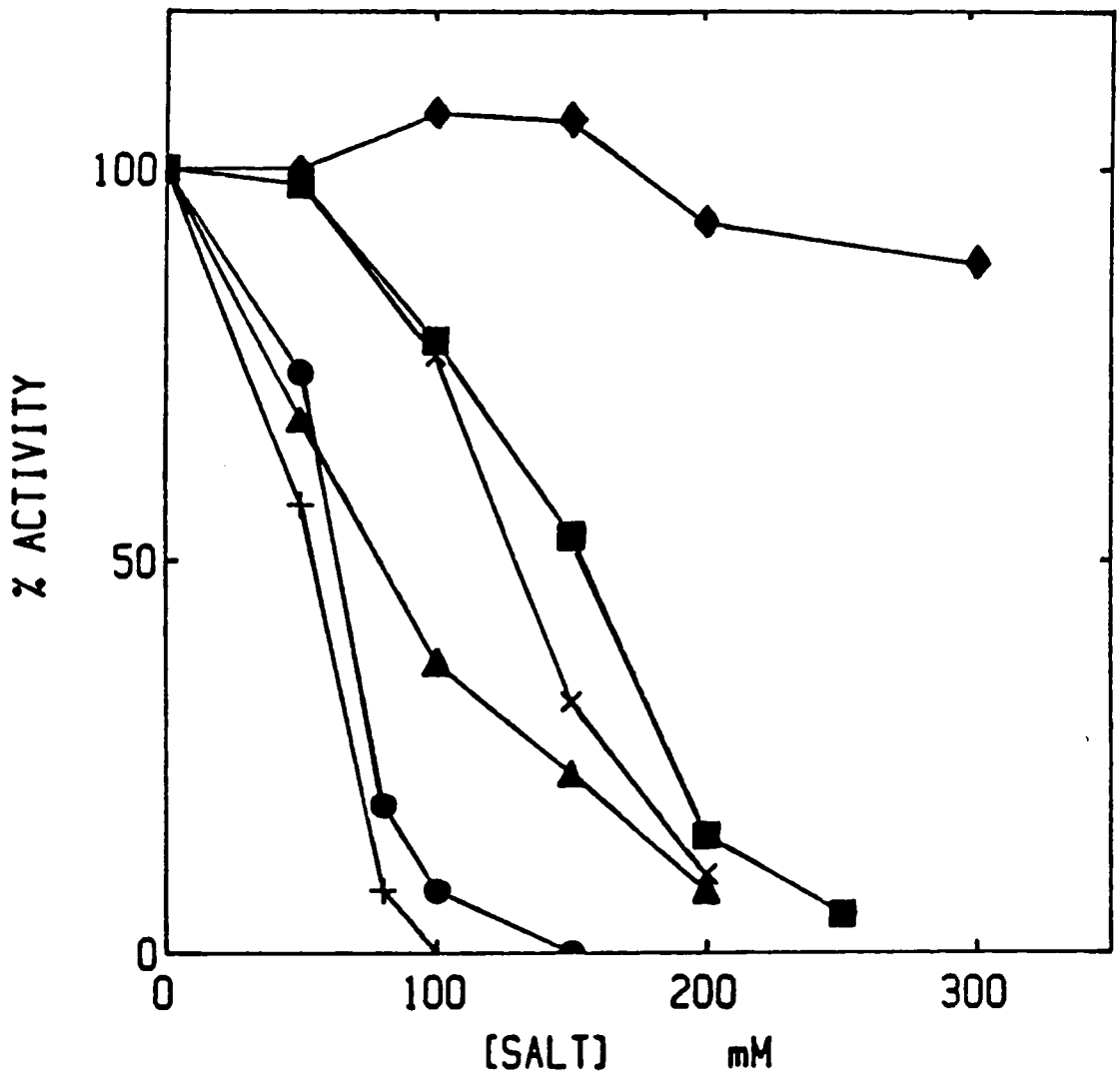

Figure 24. Effect of Different Salts on D-LDH Activity: Initial velocities were measured in 50 mM Hepes buffer, pH 7.0, at saturating concentrations of the substrates, and at various concentrations of the salt. The salts used were: (◆), sodium acetate; (×), NaCl; (■), KCl; (▲), Mg_2SO_4; (●), NaBr; and (+), NaI.

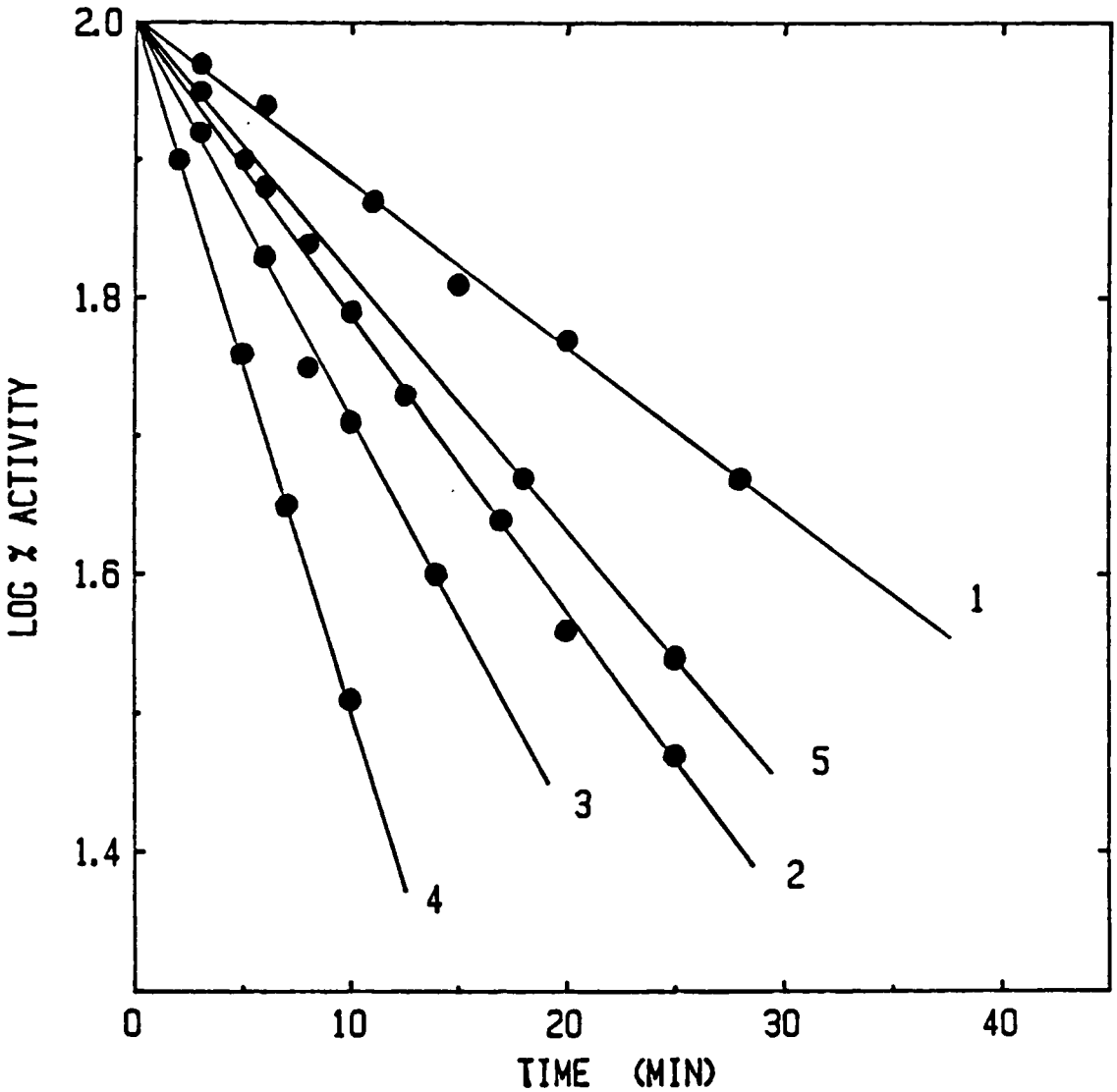

Figure 25. Thermal Denaturation of D-Lactate Dehydrogenase: The incubation mixtures contained 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT, and 1 μg of purified enzyme. Samples were withdrawn at timed intervals for activity assay. Line 1, 44°C, line 2, 49°C, line 3, 54°C, and line 4, 58°C. Line 5 indicates protection of D-LDH against denaturation at pH 7.0 and 54°C, by 50 μM NADH.

of the coenzyme, NAD, likewise did not protect the enzyme from denaturation when studied at a concentration of 3 mM.

The purified enzyme produced a typical UV-visible absorbance spectrum with a single absorbance maximum at 275 nm. The fluorescence spectrum of the purified enzyme (Figure 26) showed an excitation maximum at 285 nm and an emission maximum at 320 nm. This fluorescence profile is characteristic of the presence of tryptophan residues in the protein.

The enzyme catalyzes the reduction of pyruvate to give exclusively D(-)-lactate as shown by the experiments described under Methods. A reaction mixture containing limiting amounts of pyruvate and excess NADH was incubated at room temperature until the pyruvate was completely reduced. The reaction mixture was then boiled for 3 min to denature protein, cooled and centrifuged. The presence of D(-)-lactate in the supernatant fraction was confirmed using the enzymatic assay described by Dennis (110). No L(+)-lactate formation was observed. The reaction catalyzed is essentially unidirectional. The oxidation of D(-)-lactate with NAD as the coenzyme proceeds at approximately 0.15% of the rate of pyruvate reduction. When the 3-acetylpyridine analog of NAD was used as the coenzyme, a 3-fold increase in the rate of lactate oxidation was observed. No lactate oxidation was observed when the L(+)-isomer of lactate was used. The rate of NADH oxidation was determined to be directly proportional to the amount of enzyme present as shown in Figure 27.

The K_m and V_{max} values for both substrates for the pyruvate reduction reaction were determined under optimal assay conditions. The kinetic constants were calculated through initial velocity studies varying one substrate at several fixed concentrations of the second one (111). The initial velocities obtained by varying NADH at four fixed concentrations of pyruvate are shown in Figure 28.

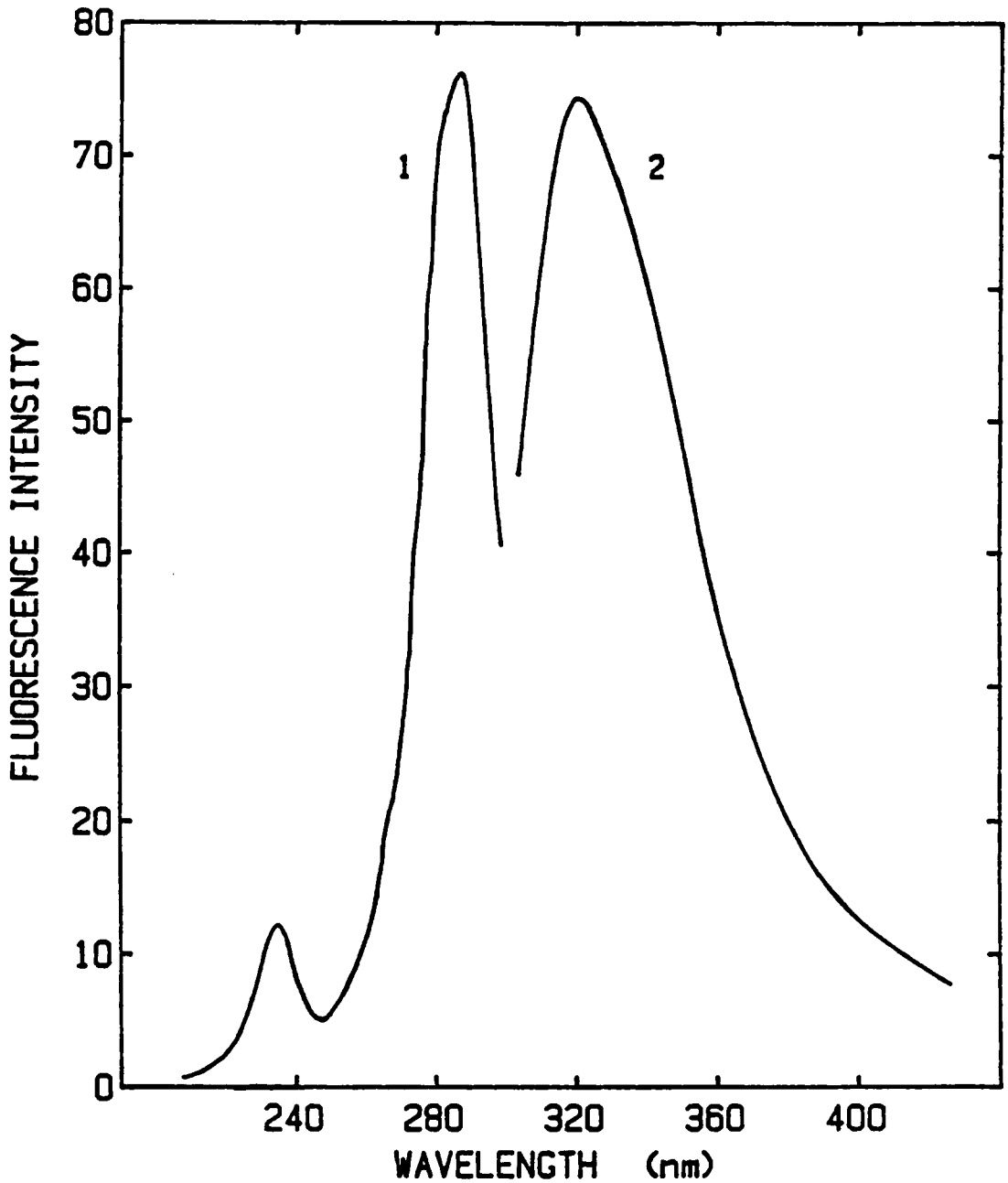

Figure 26. The Fluorescence Spectrum of D-lactate Dehydrogenase: The excitation spectrum (line 1) was determined with an emission wavelength at 320 nm and the emission spectrum (line 2) was determined with an excitation wavelength at 285 nm.

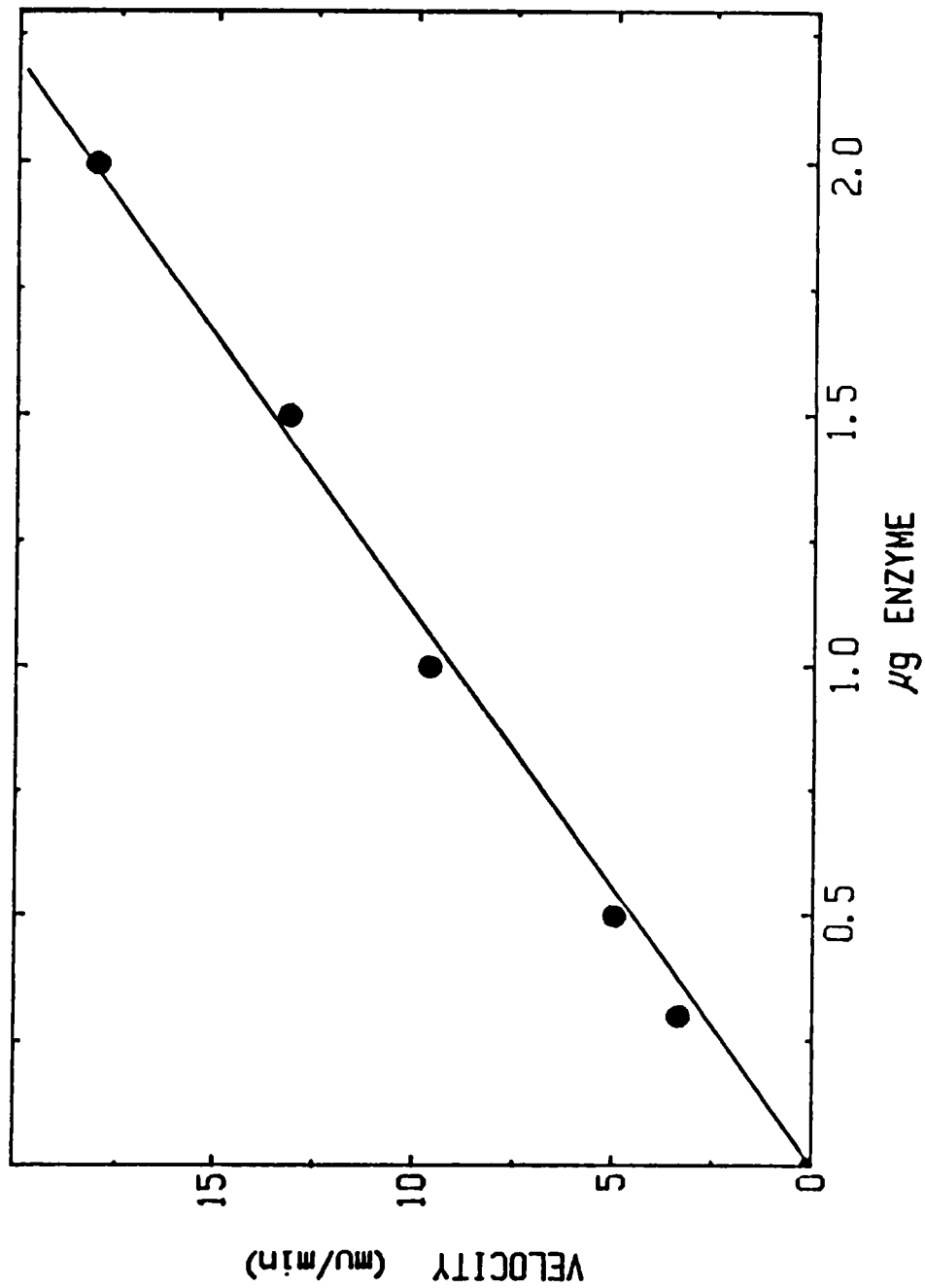

Figure 27. The Proportionality of the Reduction of Pyruvate to the Amount of D-Lactate Dehydrogenase Present: Reaction mixtures contained 50 mM Hepes buffer, pH 7.0, 0.1 mM NADH, 4 mM pyruvate, and the indicated amount of enzyme.

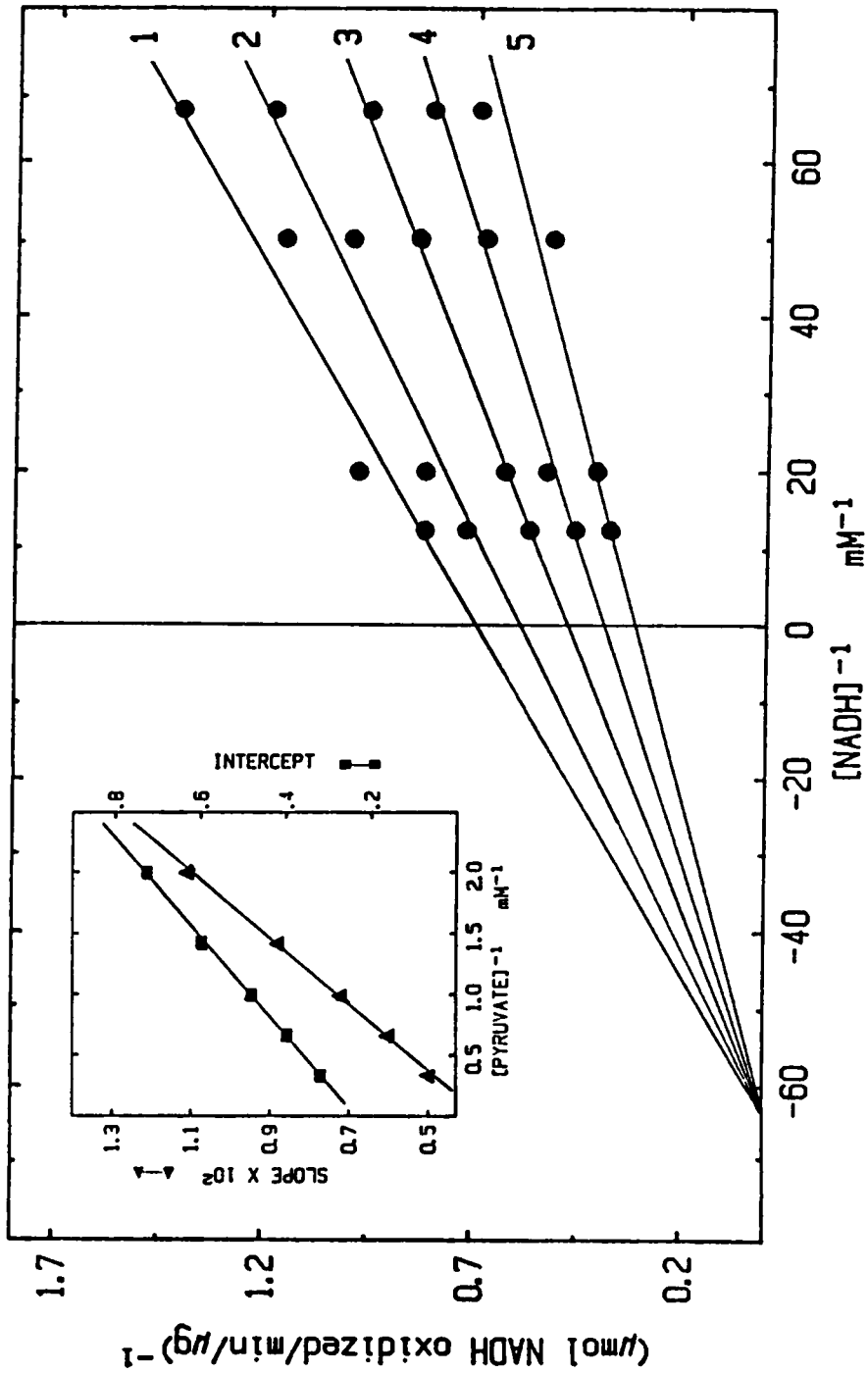

Figure 28. Effect of NADH and Pyruvate Concentration on D-Lactate Dehydrogenase Initial Velocities: Assay mixtures contained concentrations of NADH varying from 15 to 80 μM at five different concentrations of pyruvate in 50 mM Hepes buffer, pH 7.0. The pyruvate concentrations used were: line 1, 0.5 mM; line 2, 0.7 mM; line 3, 1.0 mM; line 4, 1.5 mM; and line 5, 3.0 mM. Inset is a replot of slope (▲) and intercepts (■).

Slope and intercept replots (Figure 28, inset) were used to determine the values of K_m and V_{max} (25). The results obtained by varying each substrate at different fixed concentrations of the appropriate second substrate gave converging line relationships (Figure 29) indicating a sequential reaction mechanism.

Substrate specificity of *H. influenzae* D-LDH

Several α-ketoacids were investigated as possible substrates for the enzyme. The rate of oxidation of NADH was measured at 340 nm under optimal assay conditions using 4 to 30 mM concentrations of the substrate studied and 20 to 100 ng of the purified enzyme. Table VIII shows the K_m and V_{max} values and the ratio V_{max}/K_m for those compounds observed to function as substrates.

Coenzyme specificity of *H. influenzae* D-LDH

Several NAD analogs were enzymatically reduced as described under Methods, and the reduced dinucleotides obtained were investigated as coenzymes for the D-LDH. The rates of oxidation of the NADH analogs were determined spectrophotometrically under optimal assay conditions containing 0.1 mM reduced dinucleotide and a saturating level of pyruvate. Those analogs observed to function as coenzymes were further studied at four concentrations between 0.02 and 0.25 mM, and the K_m and V_{max} values were determined as previously described. As an example, Figure 30 shows the effect of reduced 3-acetylpyridine adenine dinucleotide (APADH) concentrations on D-LDH activity.

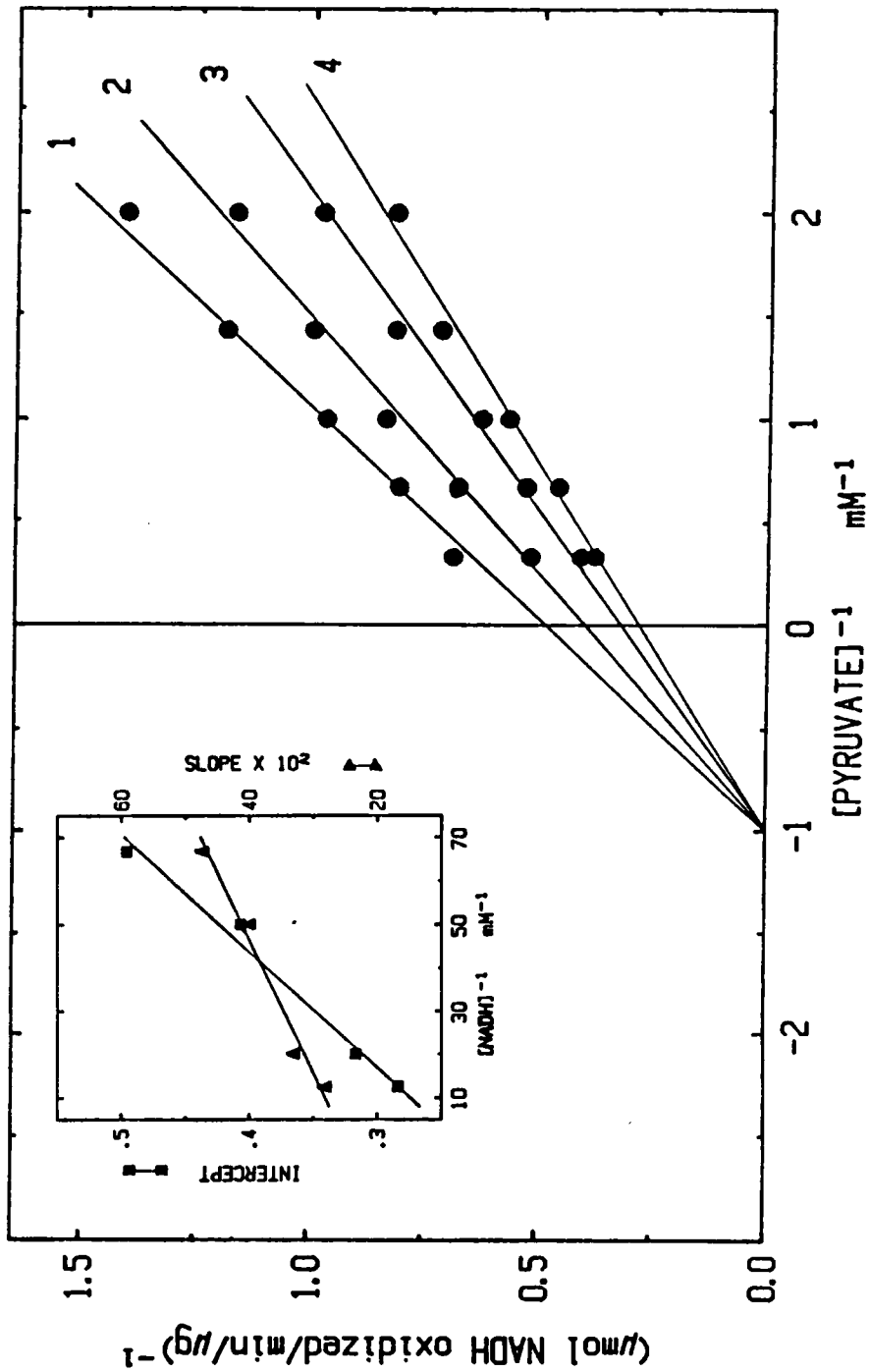

Figure 29. Effect of NADH and Pyruvate Concentrations on D-Lactate Dehydrogenase Initial Velocities: Assay mixtures contained concentrations of pyruvate varying from 0.5 to 3 mM at four fixed concentrations of NAD in 50 mM Hepes buffer, pH 7.0. The NADH concentrations used were: line1, 0.015 mM; line 2, 0.02 mM; line 3, 0.05 mM; and line 4, 0.08 mM. Inset is a replot of slopes (▲) and intercepts (■).

Table VIII. Substrate Specificities of *H. influenzae* D-Lactate Dehydrogenase

SUBSTRATE	K_m (mM)	V_{max} (units/ml)	V_{max}/K_m
Pyruvate	1.0	0.48	0.480
Glyoxylate	41.0	0.32	0.008
Benzoylformate	---*		
Oxalacetate	---*		
α-Ketoglutarate	---*		
α-Ketoisovalerate	---*		
α-Ketobutyrate	---*		

* Did not serve as substrate at concentrations as high as 30 mM.

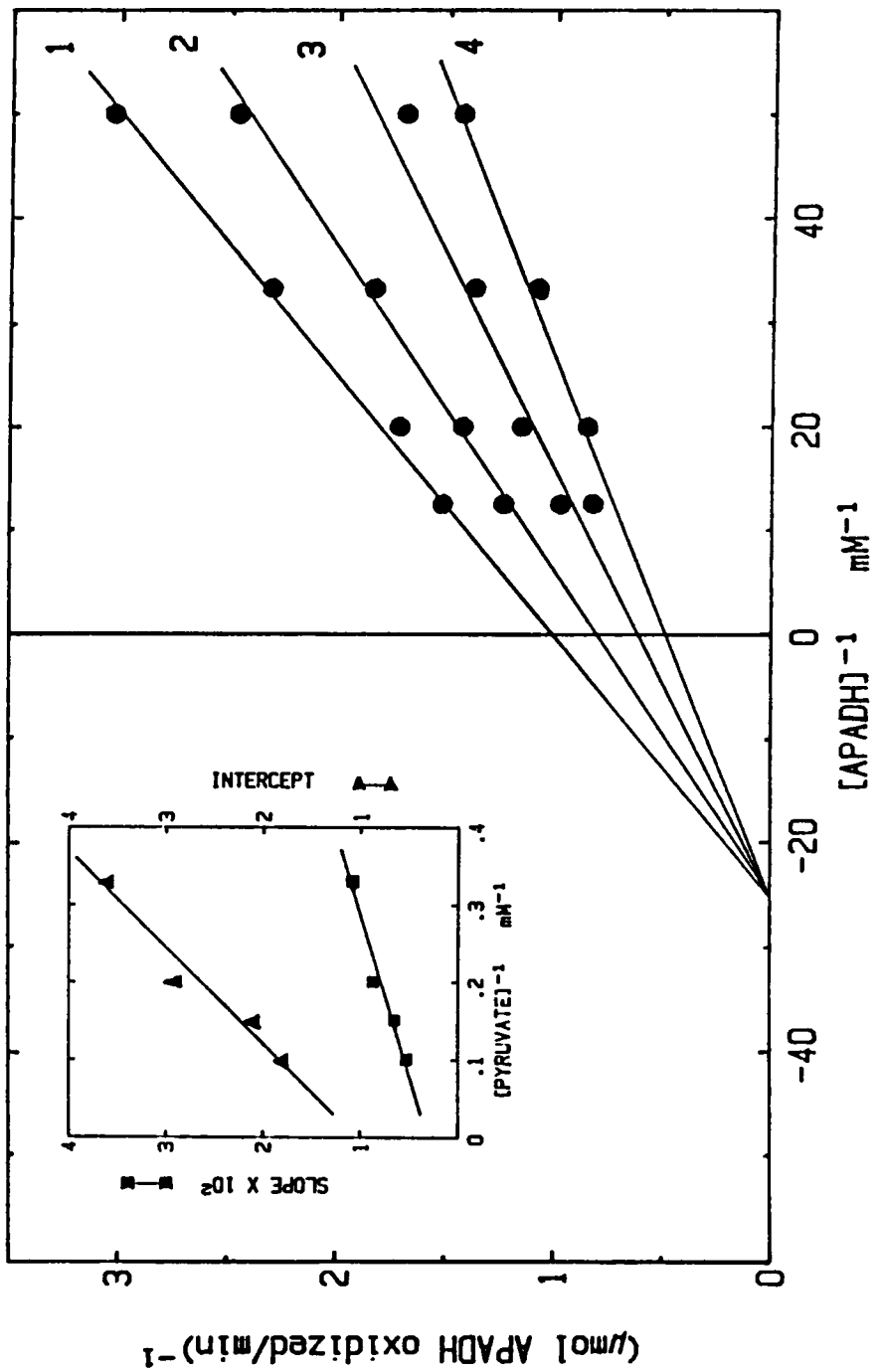

Figure 30. Effect of Reduced 3-Acetylpyridine Adenine Dinucleotide (APADH) Concentrations on D-LDH Initial Velocities: Assay mixtures contained concentrations of APADH varying from 0.02 to 0.08 mM at four fixed concentrations of pyruvate in 50 mM Hepes buffer, pH 7.0. The pyruvate concentrations used were: line 1, 3 mM; line 2, 5 mM; line 3, 6.7 mM; and line 4, 10 mM. Inset is a replot of slopes (■) and intercepts (▲).

Table IX shows the K_m and V_{max} values for those analogs observed to function as coenzymes relative to the values obtained for NADH. No reaction was observed when NADPH was investigated as the coenzyme at concentrations as high as 0.25 mM.

Studies of D-LDH inhibition.

Several compounds, structurally analogous to either the substrate or the coenzyme, were investigated for their ability to inhibit the enzyme. Inhibition patterns were determined from double reciprocal plots using two fixed concentrations of the inhibitor and varying one substrate at saturating concentrations of the second substrate.

In studies of coenzyme-competitive inhibition of D-LDH, initial velocities were determined by varying the concentration of NADH (15 - 80 μM) at several fixed concentrations of various inhibitors and at 4 mM of pyruvate. The importance of the pyrophosphate region in binding of the coenzyme to the enzyme was demonstrated using several adenosine derivatives as inhibitors. Adenosine, AMP, ADP, ATP, and ADP-ribose proved to be effective coenzyme-competitive inhibitors. An example of the inhibition observed with all these adenosine compounds is shown in Figure 31 for ADP-ribose. The K_i values obtained (listed in Table X) decrease in the order of adenosine > AMP > ADP > ATP > ADP-ribose.

The *H. influenzae* D-LDH was shown to bind effectively to the blue affinity column Matrex gel Blue A. Therefore, the free ligand, Procion Blue HB was investigated as a coenzyme-competitive inhibitor. As anticipated, the dye proved to be a competitive inhibitor with a K_i value of 27 nM, which was by far the most effective inhibitor of the enzyme observed. 3-Aminopyridine adenine dinucleotide, a potent inhibitor of growth of *H. influenzae*, did not show any inhibition of D-LDH at a concentration of 0.76 mM, that is 50 times higher than the K_m for NADH.

Table IX. Coenzyme Specificities of *H. influenzae* D-Lactate Dehydrogenase

REDUCED DINUCLEOTIDE	K_m (μM)	RELATIVE MAXIMUM VELOCITIES (%)
Nicotinamide adenine dinucleotide	15	100
3-Acetylpyridine adenine dinucleotide	38	128
Thionicotinamide adenine dinucleotide	400	65
Nicotinamide hypoxanthine adenine dinucleotide	36	400
Nicotinamide 1,N^6-ethenoadenine dinucleotide	20	115
Nicotinamide adenine dinucleotide phosphate	---*	---*

* No reaction observed.

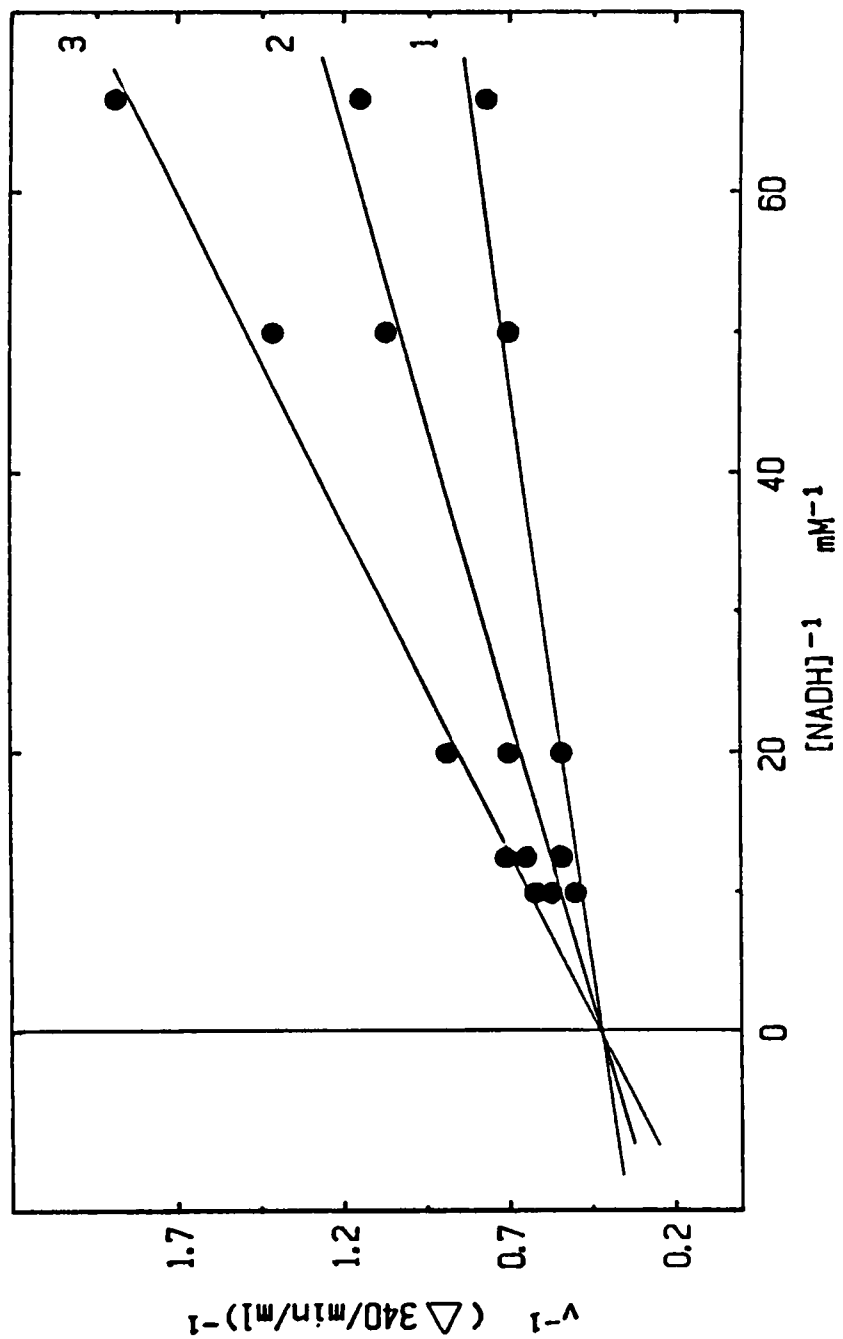

Figure 31. Coenzyme-competitive Inhibition of D-LDH by ADP-ribose: Reaction mixtures contained 50 mM Hepes buffer, pH 7.0, 4 mM pyruvate, 0.18 μg of enzyme, and 15 to 80 μM NADH at three different concentrations of ADP-ribose. The concentrations of ADP-ribose used were: line 1, none; line 2, 0.1 mM; and line 3, 0.2 mM.

Table X. Inhibition of D-Lactate Dehydrogenase by Adenosine Derivatives

INHIBITOR	K_i (mM)
Adenosine	13.3
AMP	1.6
ADP	0.3
ATP	0.2
ADP-ribose	0.062

In studies of substrate-competitive inhibition of D-LDH, initial velocities were determined varying the concentration of pyruvate (0.5 to 4 mM) at several fixed concentrations of various inhibitors and at 0.1 mM NADH. Table XI lists all of the compounds tested as inhibitors as well as the K_i values and type of inhibition obtained. The inhibition by oxamate, a known inhibitor of all mammalian D-LDHs, was shown to be noncompetitive against pyruvate (Figure 32).

Product inhibition was investigated by double reciprocal plots using different fixed concentrations of one product, and varying one substrate at unsaturating concentrations of the second substrate. Product inhibition by NAD against NADH was shown to be competitive with a K_i value of 1.8 mM. NAD inhibition against pyruvate, and D-lactate inhibition against NADH and pyruvate, displayed noncompetitive inhibition patterns. The K_i values obtained for D-lactate were unusually high, which is in accordance with the one-way character of the enzyme.

Chemical modification of the D-LDH

The chemical modification of *H. influenzae* D-LDH was investigated to identify possible amino acid residues implicated in the catalytic process. The need of the thiol reagent DTT in the enzyme preparations in order to maintain the enzymatic activity, suggested the importance of sulfhydryl groups in the catalytic process. Several reagents relatively selective for the sulfhydryl group of cysteine residues, such as iodoacetamide, iodoacetic acid, and N-alkylmaleimides, were used to investigate the role of sulfhydryl groups in the catalytic process. In order to measure the rate of inactivation of the enzyme by N-alkylmaleimides, the purified enzyme in 1 mM DTT was treated with Norit A pellets to remove DTT (109) prior to the addition of micromolar concentrations of the N-alkylmaleimide. The concentration of maleimide used varied with different maleimide

Table XI. Inhibition of *H. influenzae* D-Lactate Dehydrogenase

INHIBITOR	VARIABLE SUBSTRATE	TYPE OF INHIBITION	K_{is} (mM)	K_{ii} (mM)
D-Lactate	Pyruvate	NC	27.0	64.0
NAD	Pyruvate	NC	0.9	1.1
ADP-ribose	Pyruvate	NC	1.3	0.3
Oxamate	Pyruvate	NC	18.4	13.1
L-Lactate	Pyruvate	Competitive	130.0	--
Succinate	Pyruvate	Competitive	23.0	--
Malate	Pyruvate	--	--*	--
Malonate	Pyruvate	Competitive	14.0	--
Oxalacetate	Pyruvate	Competitive	8.0	--
α-Ketoglutarate	Pyruvate	Competitive	9.0	--
D-Lactate	NADH	NC	60.0	514.0
NAD	NADH	Competitive	1.8	--
Procion blue	NADH	Competitive	27×10^{-6}	--
AAD	NADH	--	--**	--

* No inhibition at concentrations as high as 25 mM.

** No inhibition at 0.76 mM 3-aminopyrydine adenine dinucleotide

NC= Noncompetitive Inhibition

AAD= 3-Aminopyridine adenine dinucleotide

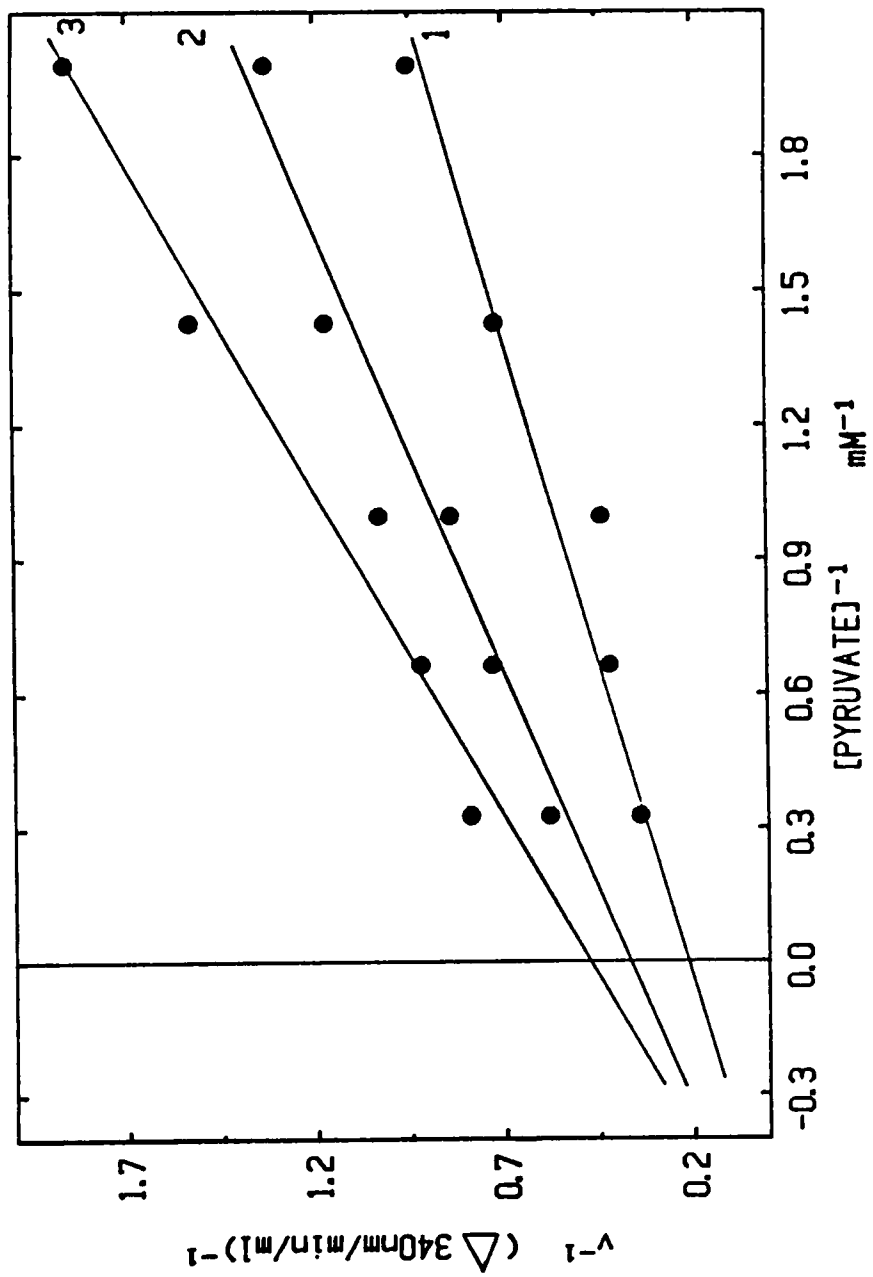

Figure 32. Inhibition of D-Lactate Dehydrogenase by Oxamate: Reaction mixtures contained 50 mM Hepes buffer, pH 7.0, 0.18 μg enzyme, 0.1 mM NADH, 0.5 to 3 mM pyruvate, and three different concentrations of oxamate. The concentrations of oxamate used were: line 1, none; line 2, 10 mM; and line 3, 20 mM.

derivatives (see Table XII), but was always maintained in excess, relative to the enzyme concentration. The time-dependent loss of D-LDH activity at 25°C and pH 7.0, by N-alkylmaleimides followed pseudo first-order kinetics and the pseudo first-order rate constant (k_1) for each of the maleimides studied (ethyl - heptyl, inclusive) was calculated as described in Methods. Inactivation of D-LDH by six concentrations of NEM is shown in Figure 33. The same pattern was observed for all the N-alkylmaleimides studied. Inactivation of the enzyme by concentrations of N-heptylmaleimide ranging from 15 to 80 μM is shown in Figure 34.

In the inactivation of D-LDH by N-alkylmaleimides, there was an instantaneous loss of enzyme activity followed by a pseudo first-order kinetics of inactivation. This early, rapid loss of enzyme activity was demonstrated to be reversed by dilution. For example, an incubation mixture containing 0.7 μg of the purified enzyme and 300 μM NEM, that lowers activity to 28% of the enzyme instantaneously, was reversed back to 64% active enzyme after dilution (1:1 in 50 mM Hepes buffer, pH 7.0).

The effect of NEM concentration on the pseudo first-order rate constant of enzyme inactivation is shown in Figure 35. A linear relationship was observed at a given concentration range (0 - 800 μM); however, at higher NEM concentrations a deviation from linearity appeared, indicating saturation kinetics. The slopes of the linear portions of these curves were considered as apparent second-order rate constants (k_2). Such constants obtained for all the alkylmaleimides studied are listed in Table XII. The logarithms of the apparent second-order rate constants of inactivation (k_2) plotted against the number of carbons in the respective alkyl chains gave a linear relationship (Figure 36).

The lack of linearity in second-order rate plots observed at the higher concentrations of maleimides suggested the involvement of saturation kinetics. The fact that

Table XII. Apparent Second-order Rate Constants (k_2) of Inactivation of D-LDH by N-Alkylmaleimides

N-ALKYLMALEIMIDE	CONCENTRATION RANGE (μM)	k_2 (M^{-1} min^{-1})
Ethyl	50 - 400	414
Propyl	40 - 300	726
Butyl	30 - 200	844
Pentyl	30 - 160	1100
Hexyl	15 - 80	1400
Heptyl	15 - 80	2181

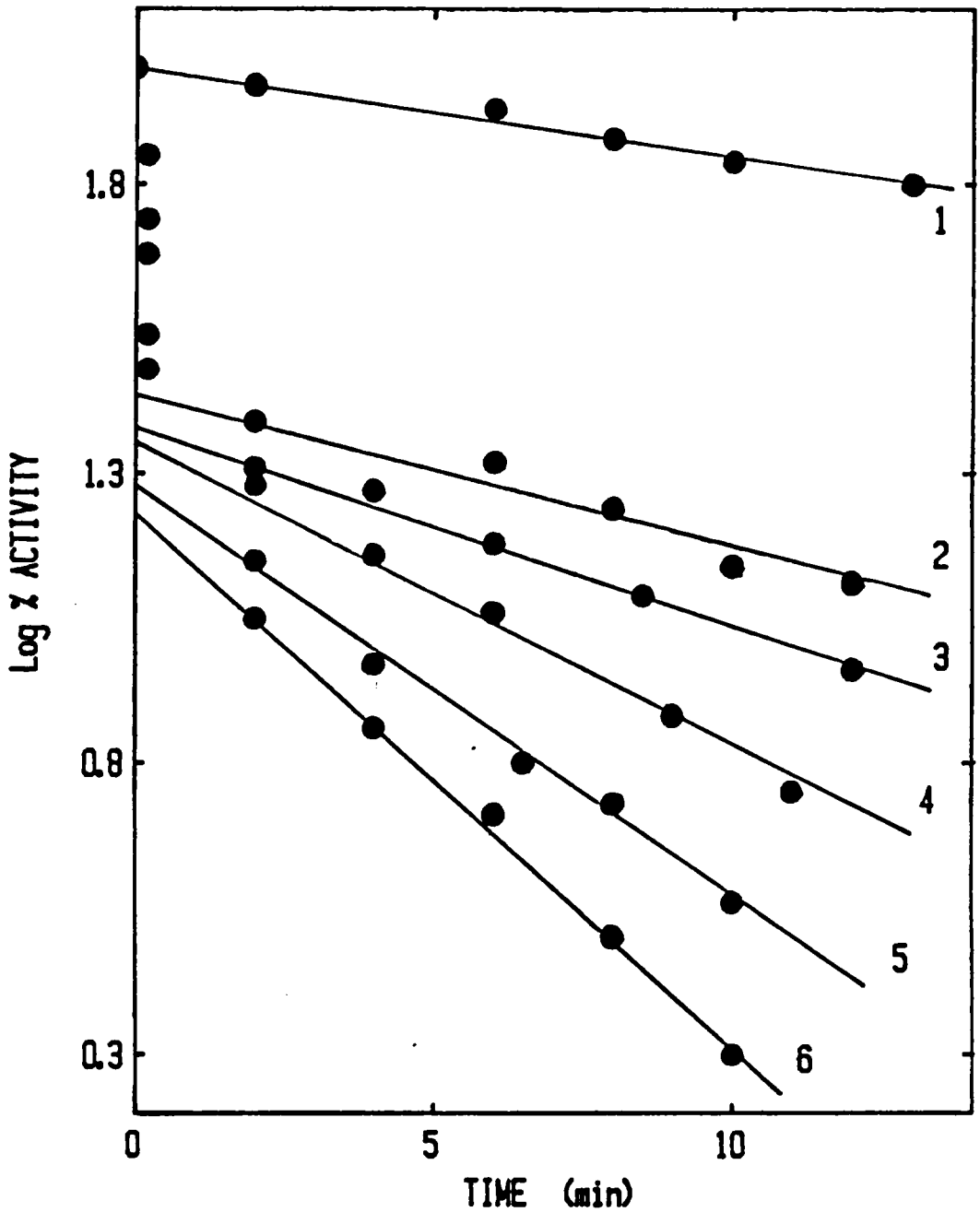

Figure 33. Time-dependent Inactivation of D-LDH by N-Ethylmaleimide (NEM): Enzyme inactivation and assay mixtures were described under Methods. The NEM concentrations used were: line 1 , none; line 2, 50 μM; line 3, 100 μM; line 4, 200 μM; line 5, 300 μM; and line 6, 400 μM.

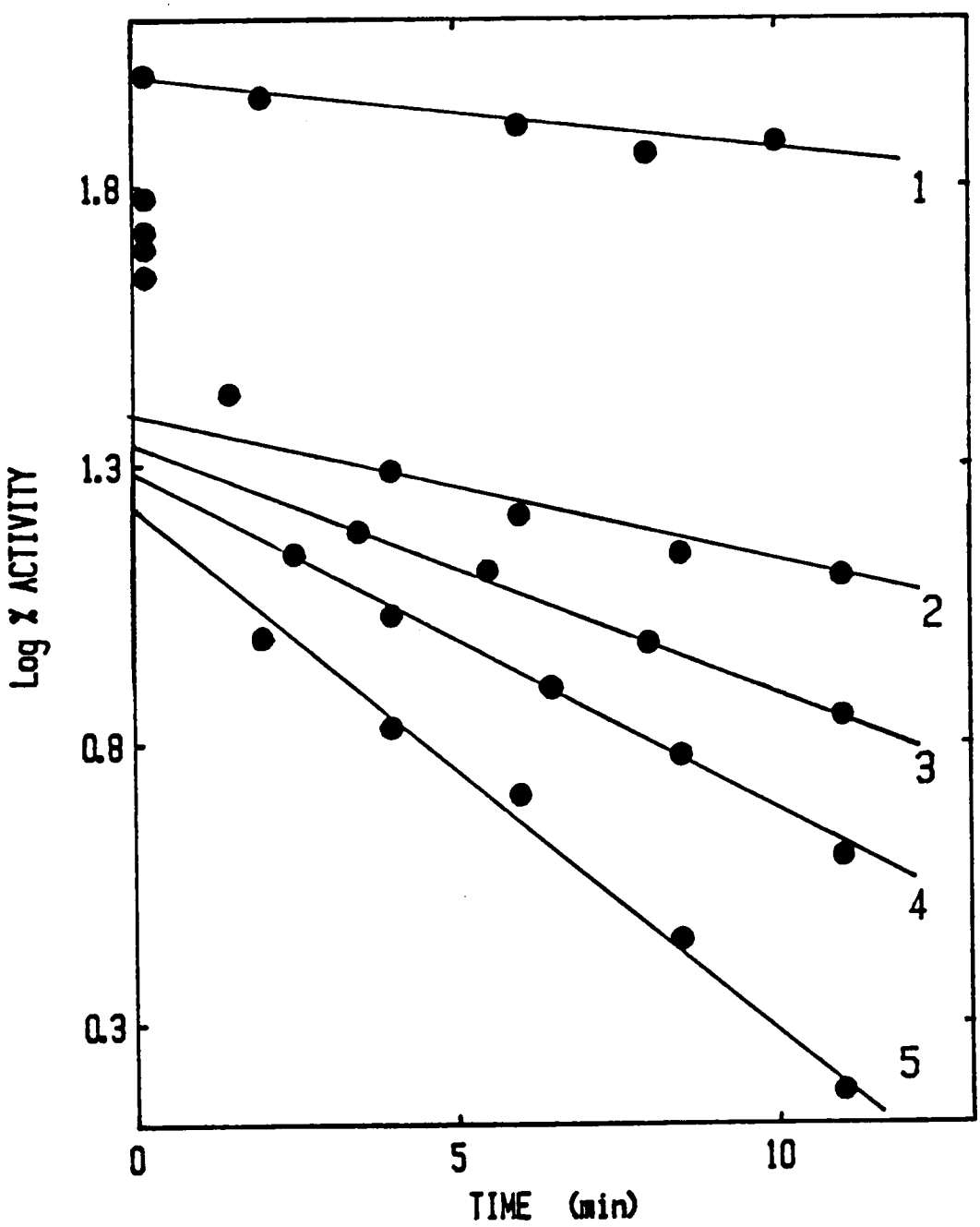

Figure 34. Time-dependent Inactivation of D-LDH by N-Heptylmaleimide: Enzyme inactivation and assay mixtures were described under Methods. The concentrations of N-heptylmaleimide used were: line 1, none; line 2, 15 μM; line 3, 30 μM; line 4, 50 μM; and line 5, 80 μM.

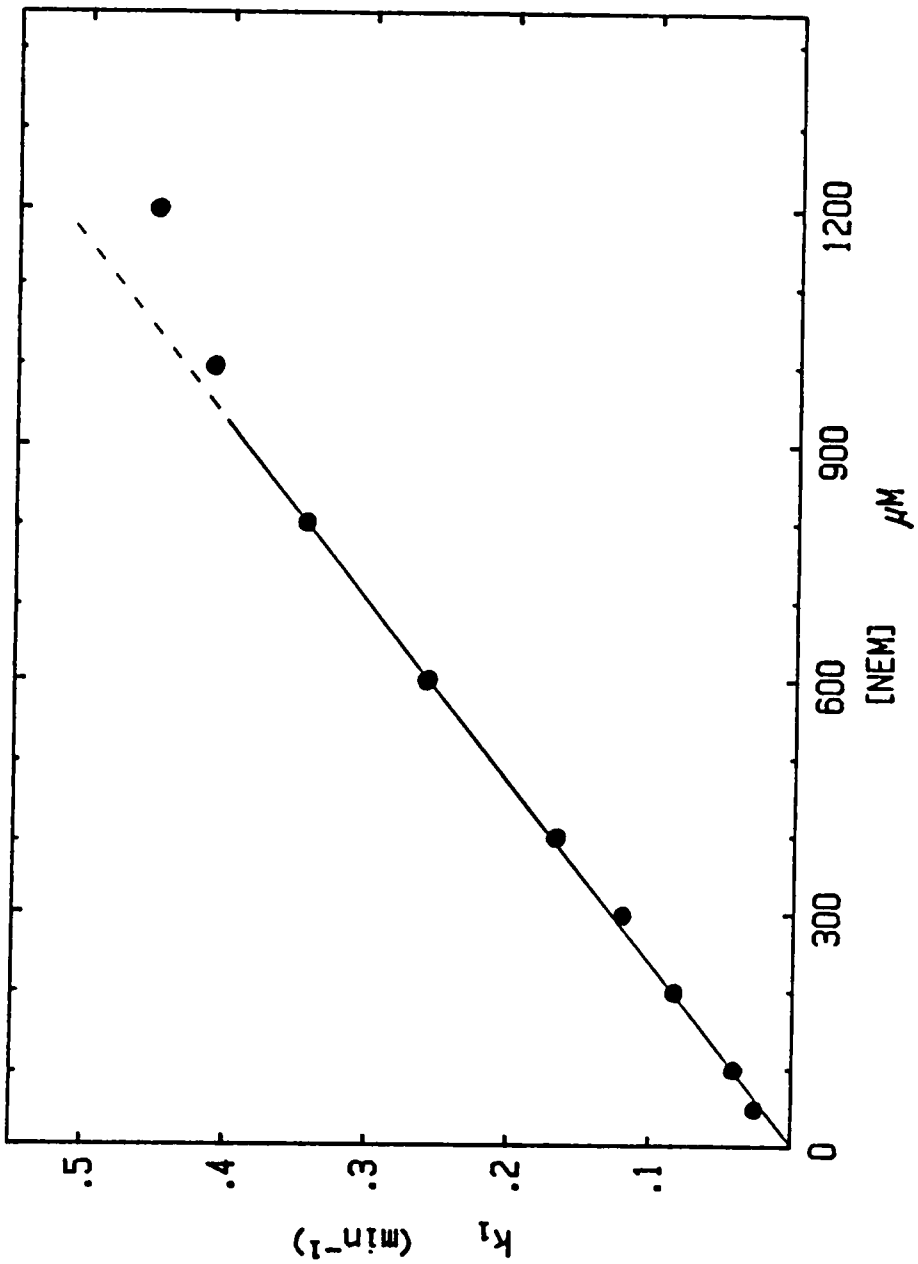

Figure 35. The Effect of N-Ethylmaleimide (NEM) Concentration on the Pseudo First-order Rate Constants of Inactivation of D-LDH

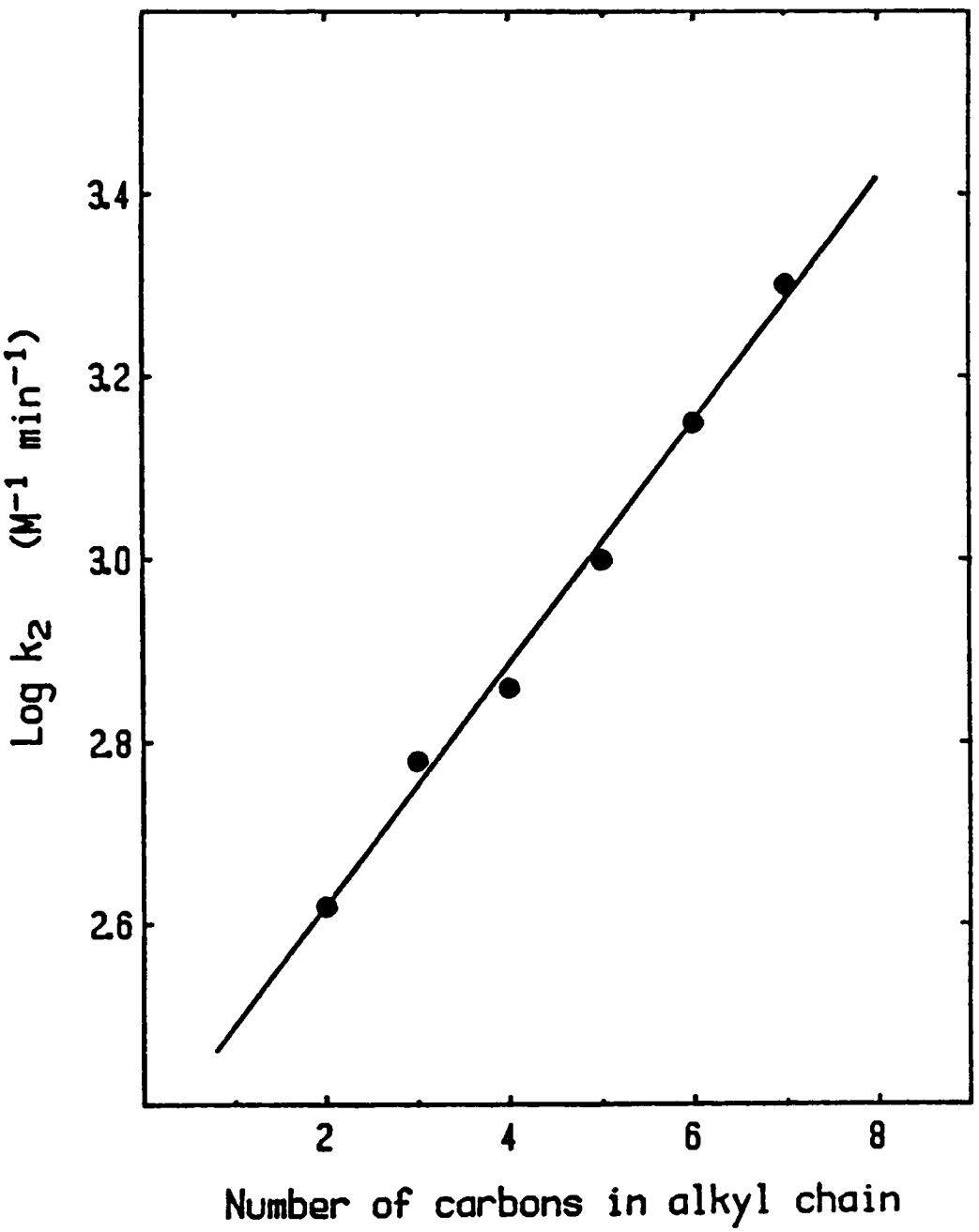

Figure 36. Positive Chainlength Effect of Inactivation of D-LDH by N-Alkylmaleimides: The relationship of the logarithm of the apparent second-order rate constants of inactivation (k_2) to the chainlength of the alkyl substituents of the maleimides.

saturation kinetics resulted from the formation of an enzyme-maleimide complex was demonstrated by plotting the data according to equation 1:

$$\frac{1}{k_1} = \frac{K_d}{k'} \cdot \frac{1}{[Mal]} + \frac{1}{k'}$$

where K_d is the dissociation constant for the enzyme-maleimide complex, k', the first-order rate constant at maleimide saturation, and k_1, the pseudo first-order rate constant observed. Plots of the reciprocal of the pseudo first-order rate constants ($1/k_1$) versus the reciprocal of the maleimide concentrations are shown in Figure 37, for N-ethyl-, N-pentyl-, and N-heptylmaleimides. The first-order rate constant at saturation (k') was obtained from the intercept of the double reciprocal plots. The same value of 0.6 min^{-1} was obtained for all three N-alkylmaleimides. The dissociation constants (K_d) for the N-alkylmaleimides were determined from the slope of the linear plots, and are listed in Table XIII.

The purified D-LDH was also inactivated at 25°C and pH 7.0 by iodoacetic acid and iodoacetamide following pseudo first-order kinetics. Apparent second-order rate constants (k_2) of 1.3 and 20 M^{-1} min^{-1} were found for iodoacetamide and iodoacetic acid inactivation, respectively. In the inactivation of D-LDH by iodoacetamide, an instantaneous loss of enzyme activity, followed by a pseudo first-order kinetics of inactivation was observed, in the same manner that occurred with N-alkylmaleimide inactivation (Figure 38). No such early, rapid loss of enzyme activity was observed when the D-LDH was inactivated by iodoacetic acid (Figure 39). No modification of D-LDH was observed by incubation with 2,4-pentanedione or diethylpyrocarbonate under the conditions described under Methods.

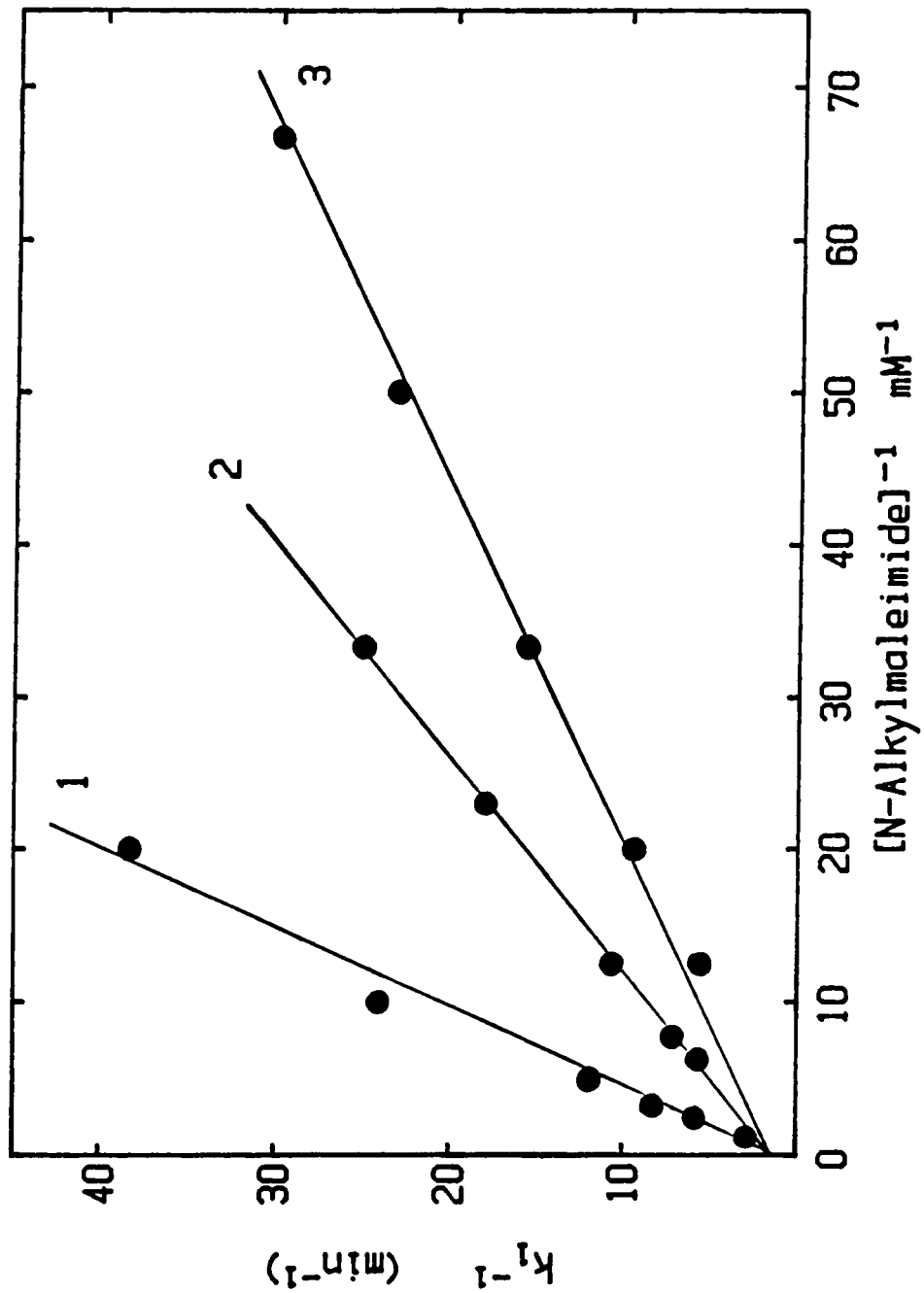

Figure 37. The Relationship of the Reciprocal of the Pseudo First-order Rate Constants to the Reciprocal of the N-Alkylmaleimide Concentrations: Line 1, N-ethylmaleimide, line 2, N-pentylmaleimide, and line 3, N-heptylmaleimide.

Table XIII. Dissociation Constants for N-Alkylmaleimides at pH 7.0 and 25°C

N-ALKYLMALEIMIDE	K_d* (mM)
Ethyl	1.12
Propyl	0.60
Butyl	0.51
Pentyl	0.44
Hexyl	0.38
Heptyl	0.25

* Dissociation constants were determined from plots as shown in Figure 37 .

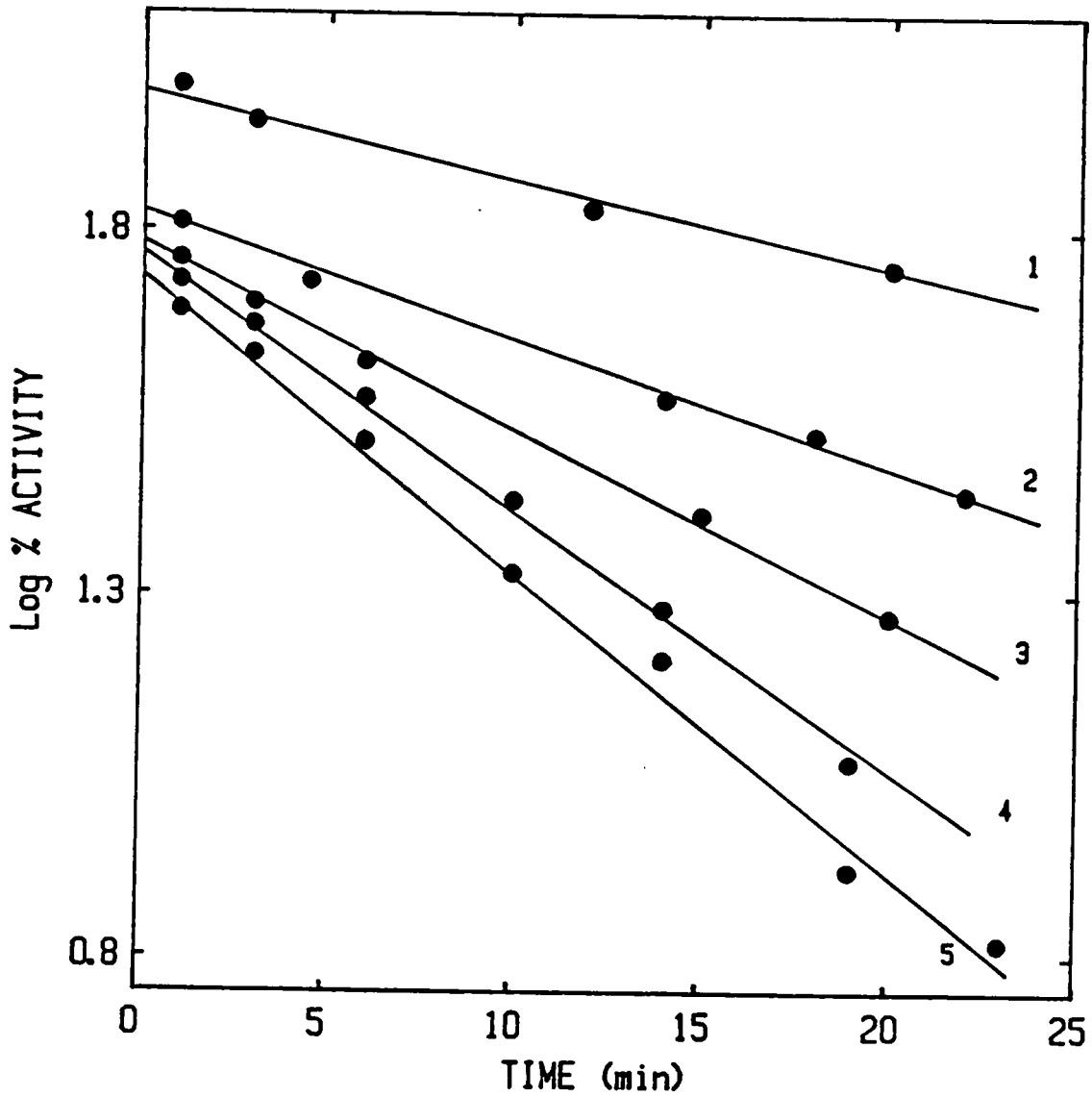

Figure 38. Time-dependent Inactivation of D-LDH by Iodoacetamide: Enzyme inactivation and assay mixtures were described in Methods. The iodoacetamide concentrations used were: line 1, none; line 2, 9.8 mM; line 3, 29.8 mM; line 4, 44.8 mM; and line 5, 59.8 mM.

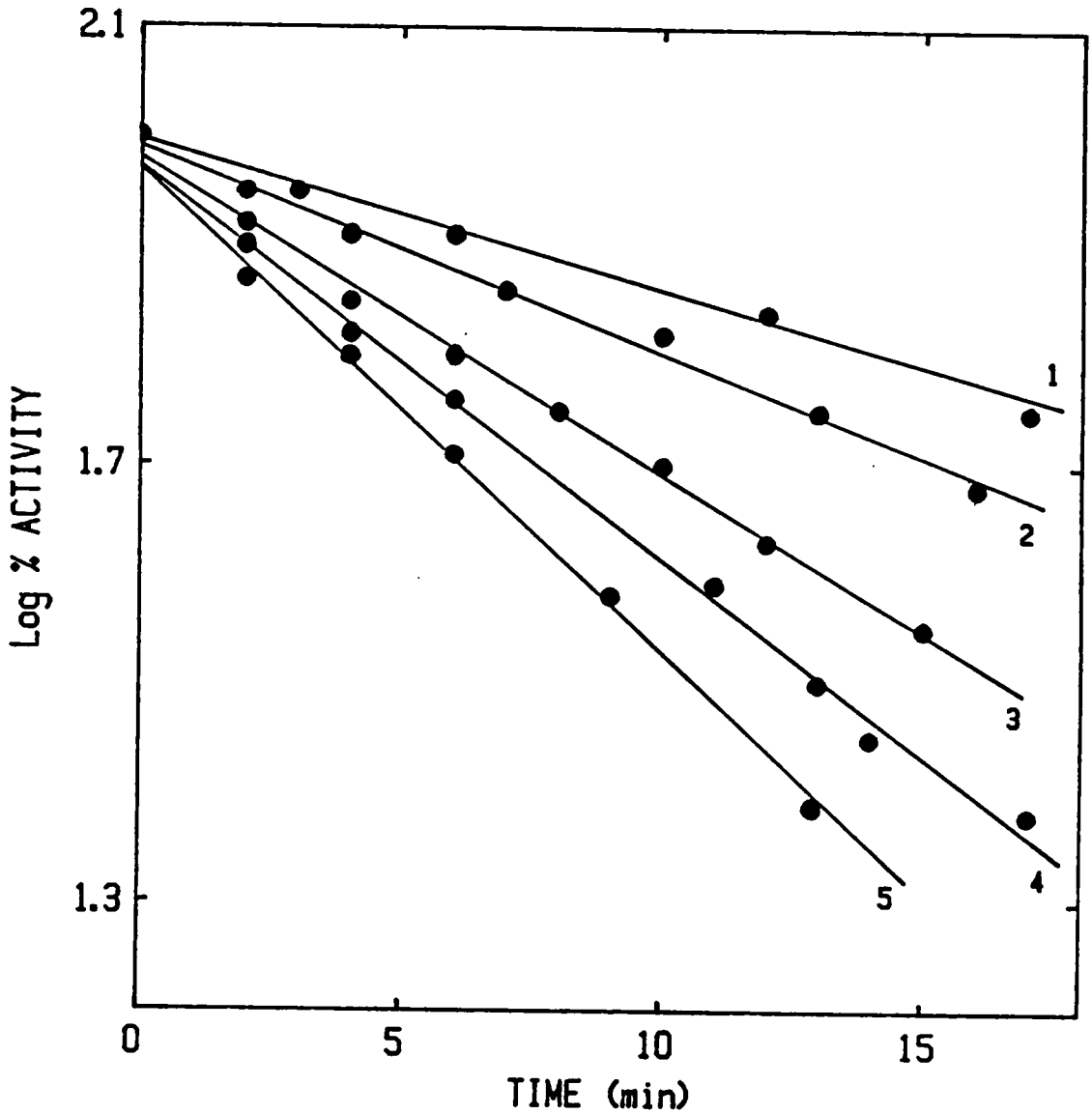

Figure 39. Time-dependent Inactivation of D-LDH by Iodoacetic Acid: Enzyme inactivation and assay mixtures were described in Msethods. The iodoacetic acid concentrations used were: line 1, none; line 2, 0.3 mM; line 3, 0.8 mM; line 4, 1.3 mM; and line 5, 1.8 mM.

DISCUSSION

A wide variety of different lactate dehydrogenases (LDHs) has been found among bacteria, in contrast to the similarity observed among the mammalian LDHs. Bacterial LDHs differ in such properties as stereospecificity, cofactor and activator requirements, reversibility, sensitivity to inhibitors, stability and molecular weight. Bacterial LDHs are of two types, one NAD-linked, the other flavin-linked. Each of these two types exists in a stereospecific D- and L- form. The NAD-dependent LDH activity found in *H. influenzae*, was specific for D-lactate. No LDH activity specific for the L-isomer was observed. However, activities for NAD-independent D- or L- LDH were found in this organism, and some characteristics of these enzymes will be discussed in Chapter IV.

The D-LDH from *H. influenzae* was purified to electrophoretic homogeneity through a 5-step procedure involving salt fractionation, hydrophobic and dye affinity chromatography. Several D-LDHs from lactic acid bacteria have been purified using affinity chromatography as a final step in the purification (oxamate-Sepharose 4B or adenosine-5′-monophosphate-Sepharose 4B affinity resins). Triazinyl dye affinity chromatography has not been used with these enzymes before but proved to be effective in the purification of D-LDH from *H. influenzae*. The enzyme could be eluted selectively by a mixture of NADH and pyruvate; however, KCl was used as the eluent to avoid interference by substrates in subsequent enzyme characterization. The results of the purification procedure are shown in Table VII. The enzyme was purified 2100-fold with a 14% yield and a final specific activity of 300 μmol NADH oxidized per min per mg protein. The specific activities of purified and partially purified preparations of bacterial D-LDHs show a wide variation in values from a low specific activity of 1.6 for

Pediococcus cerevisiae (100) to a value of 1,510 for *Leuconostoc mesenteroides* (112). The specific activity of the purified enzyme from *E. coli* (101) and *Lactobacillus leichmannii* (96) were reported to be 250 and 260, respectively, similar to the values obtained for *H. influenzae* D-LDH.

The native molecular weight of the purified D-LDHs was determined by gel-filtration chromatography using Sephacryl-200 (Figure 20). The enzyme eluted from this column with an apparent molecular weight of 135,000. Under denaturating conditions of SDS-polyacrylamide gel electrophoresis, a subunit molecular weight of 35,000 was observed, indicating a tetrameric structure in the native enzyme (Figure 20). Bacterial L-LDHs have been demonstrated to be tetrameric with molecular weights around 140,000 (100, 113 - 116), whereas D-LDHs from most invertebrates and microorganisms were found to be dimeric, with molecular weights approximately 70,000 (100, 117 - 119). Therefore, the D-LDH from *H. influenzae* resembles the bacterial L-lactate-specific dehydrogenases in molecular weight and quaternary structure. D-Lactate dehydrogenases from *Aerobacter aerogenes* (120) and *E. coli* (101) were also found to be larger than most bacterial D-LDHs with molecular weights of 112,000 and 115,000 to 140,000, respectively.

The optimum pH for the reduction of pyruvate by D-LDHs has been often reported to be below 7.0, mainly because the enzymes studied are from lactic acid bacteria and it is reasonable to expect these enzymes to be active at acidic pH values. An optimum pH of 7.2 was found for *H. influenzae* D-LDH (Figure 21). The concentration of the buffers used for the pH study were sufficiently low to give no ionic strength effect on enzyme activity.

The effect of ionic strength on enzyme activity was investigated using KCl. Initial velocities were measured in 50 mM Hepes buffer, pH 7.0 at saturating levels of NADH and various concentrations of pyruvate, and the ionic strength was increased by

addition of KCl from 0 to 100 mM. Concentrations of KCl higher than 50 mM showed an inhibitory effect on D-LDH activity at non-saturating levels of pyruvate. The enzyme responded to increasing concentrations of KCl by deviating from simple Michaelis-Menten kinetics to a sigmoidal behavior, indicating positive cooperativity (Figure 22). Hills plots (121) of these data showed that KCl changes the apparent kinetic order of the reaction with respect to pyruvate from first-order to second-order (Figure 23). These results suggested that the tetrameric D-LDH from *H. influenzae* was an allosteric protein which was potentially capable of being regulated by one or more physiological effector ligands. It has been shown that fructose 1,6-bisphosphate is a specific activator of LDHs from most streptococcal species (122, 123) and a few *lactobacilli*, and α-ketoglutarate, as well as other tricarboxylic cycle intermediates, have been reported to stimulate rabbit muscle LDH activity (124). Neither of these metabolites, however, had any effect on the activity of D-LDH from *H. influenzae.* Even though ATP was found to be a competitive inhibitor of the enzyme with respect to NADH, it did not mimic the effect of KCl on the enzyme activity.

The effect of several salts other than KCl on the enzyme activity was investigated (Figure 24). At saturating concentrations of the substrate and the coenzyme, 50% inhibition of D-LDH was obtained with salt concentrations ranging from 50 to 160 mM. Apparently, the effect of the cation is less important since NaCl and KCl inhibited the enzyme to the same extent. The data indicate that the inhibition is related to the nature of the anion. The fact that Mg_2SO_4 did not inhibit the enzyme to a greater extent than NaI or NaBr, and the absence of effects by sodium acetate, demonstrated that a general ionic strength effect is not involved.

The D-LDH from *H. influenzae* was unstable in the absence of a protective thiol reagent. Dithiothreitol (DTT) at 1 mM concentration was found to be effective in stabilizing the enzyme and it was used through all of the purification and experimental

procedures. The D-LDH from *E. coli* was also found to require 0.1 M mercaptoethanol or 0.01 M DTT in order to maintain enzymatic activity (101). In contrast to animal LDHs that are quite stable in buffers containing no reducing agent, the LDH from *H. influenzae* requires DTT in the buffer to mantain the enzyme in a fully active form. The purified D-LDH was observed to be stable when stored at 4°C in 10 mM phosphate buffer, pH 7.0, containing 1 mM DTT; however, the enzyme could not withstand freezing, like most other LDHs. The rate of thermal denaturation of the enzyme at moderate temperatures (44 to 58°C) followed first-order kinetics (Figure 25). Slight protection against denaturation was observed with 50 μM NADH; however, no protection was observed with pyruvate or NAD. Although bacterial LDHs vary greatly in their sensitivities to heat, *H. influenzae* D-LDH showed a higher sensitivity to moderate temperatures than most of the bacterial D-LDHs (96, 97, 100, 112, 117, 118).

The reaction catalyzed by the D-LDH is the reduction of pyruvate in the presence of NADH to give exclusively D-lactate. The reaction catalyzed is essentially irreversible since oxidation of D-lactate with NAD as the coenzyme proceeded at approximately 0.15% of the rate of pyruvate reduction. It seems that the enzyme is designed to reduce pyruvate rather than oxidize lactate. Most D-LDHs are kinetically reversible (96 - 98, 100) but a few have been reported to be unidirectional using only pyruvate as the substrate (101, 125, 126). An explanation for the apparent kinetic irreversibility of the reaction could be that the enzyme exhibits poor affinity for D-lactate and/or the coenzyme NAD. The high K_i values obtained for D-lactate and NAD in product inhibition studies were consistent with this hypothesis.

The kinetic mechanisms involved in mammalian LDH reactions have been studied in detail (92). All mammalian LDH reactions followed an ordered sequential mechanism with NADH as the leading substrate and NAD as the last product released. With the *H. influenzae* enzyme, initial velocities of pyruvate reduction carried out by

varying one substrate at several fixed concentrations of the second substrate led to converging lines in double reciprocal plots, indicating a sequential reaction mechanism (Figure 28 and 29). The K_m values determined for pyruvate and NADH were 1 mM and 15 μM, respectively. Product and dead-end inhibition were investigated and the results are summarized in Table XI. The results obtained were not conclusive enough to indicate whether the sequential mechanism of the reaction was of an ordered or random type; however, the protection against thermal denaturation by NADH and not by pyruvate would be consistent with an ordered mechanism. Substrate inhibition by pyruvate was observed at concentrations higher than 5 mM. Inhibition by pyruvate is seldom reported, but the LDH of *S. aureus* was also found to be inhibited by high concentrations of pyruvate (127).

Several reduced pyridine dinucleotides, altered in either the pyridine or purine moiety, were studied as coenzymes for *H. influenzae* D-LDH. Kinetic parameters for five NADH analogs exhibiting coenzyme activity are listed in Table IX. The purified enzyme was observed to function with several NADH analogs altered in either the purine or pyridine portion of the dinucleotide. Nicotinamide 1,N^6-ethenoadenine dinucleotide in the reduced form serves as a coenzyme for the D-LDH reaction almost as effectively as NADH. Therefore, an alteration in the purine portion of the dinucleotide did not affect the effectiveness of the coenzyme. Of special interest was the functioning of the 3-acetylpyridine, hypoxanthine and thionicotinamide analogs, which were observed previously to support growth of *H. influenzae* in the absence of NAD (25). The substitution of groups at the 3-position of the pyridine moiety of NADH resulted in higher K_m and V_{max} values compared to those obtained for NADH. The exception was the reduced thionicotinamide analog that exhibited a much higher K_m and a somewhat lower V_{max} , giving a V_{max} /K_m ratio 30 times lower than the one obtained for NADH. The presence of a sulfur atom at the 3-position definitely altered the binding

of the coenzyme to the enzyme. The purified D-LDH could be classified strictly as an NAD-dependent dehydrogenase since no activity was observed with NADPH as the coenzyme.

Substrate specificity of the enzyme was studied using several α-ketoacids. Table VIII shows the kinetic parameters obtained for those compounds observed to function as substrates. *Haemophilus influenzae* LDH is highly specific for pyruvate as are most D-LDHs. Glyoxylate was found to serve as a substrate but with much lower affinity; the V_{max}/K_m ratio was 125-times lower than the one obtained for pyruvate.

Selective inhibition of enzymes involved in NAD metabolism in *H. influenzae* could have a pronounced effect on growth of the organism. Recent inhibition studies of *H. influenzae* NAD pyrophosphatase (25) led to the recognition of several effective inhibitors of the enzyme that were also effective inhibitors of growth of the organism in the same concentration range. In the present study, selective inhibition of D-LDH was investigated in an effort to determine important interactions in the binding of ligands at the coenzyme and substrate sites of the enzyme. Several adenosine derivatives 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 plots using several fixed concentrations of the inhibitor. The results demonstrated that all the adenosine derivatives studied were coenzyme-competitive inhibitors of the enzyme. The K_i values obtained decreased in the order: adenosine > AMP > ADP > ATP > ADP-ribose (Table X). The significant increase in binding of ADP and ATP relative to adenosine indicated the importance of ionic interactions at the pyrophosphate region of the NADH binding site. The enhanced binding of ADP-ribose compared with that of ADP could reflect the importance of a region on the enzyme which interacts with the additional ribose group on the ADP-ribose molecule. The binding of ADP-ribose is very effective as indicated by the low K_i observed; however, the

K_i obtained for NAD was exceptionally high. These values would indicate an appreciable unfavorable effect by the positive charge on the pyridinium moiety of NAD on binding of the coenzyme. Competitive inhibition with respect to NADH by ATP and ADP was also observed in other bacterial D-LDHs (100, 102, 117, 126, 128), but the K_i values observed for the *H. influenzae* enzyme were much lower. It is worth mentioning that ATP which is often a good inhibitor of bacterial LDHs does not have the same effect on mammalian LDHs.

In early studies it was observed that NAD analogs that do not possess a sufficient electron withdrawing group at the 3-position of the pyridine ring are not reduced enzymatically (129). 3-Aminopyridine adenine dinucleotide which bears an electron donor group at the 3-position of the pyridine ring, does not serve as a coenzyme for the D-LDH, but it was investigated as a possible competitive inhibitor with respect to NADH. The 3-aminopyridine analog of NAD was observed not to inhibit the enzyme at concentrations as high as 0.76 mM. The ineffectiveness of this dinucleotide as an inhibitor of *H. influenzae* LDH was of special interest since inhibition of growth by this dinucleotide occurred at submicromolar concentrations (25). The mechanism of inhibition of growth by this NAD analog could not be explained by interactions of the analog with the D-LDH. In general, this analog of NAD has been demonstrated consistently to be a rather poor coenzyme-competitive inhibitor of dehydrogenases, functioning in the millimolar range (43, 81). A very effective coenzyme-competitive inhibitor of the enzyme (K_i = 27 nM) was found to be Procion Blue HB (Cibacron blue F3GA), a ligand that was successfully used for affinity chromatography in the purification of D-LDH. Procion Blue HB is a water-soluble triazine dye that binds to the nucleotide-binding folds of many enzymes (130). It has been used in crystallographic studies to identify the coenzyme-binding region of dehydrogenases (131, 132). Strong binding of Procion Blue HB to NAD(P)-dependent dehydrogenases has been frequently observed

(133), but many other enzymes also interact with this dye, such as NAD glycohydrolase (134, 135), NAD kinase (136), adenylate cyclase (137), and phosphorylase a (138) to mention some. *Haemophilus influenzae* malate dehydrogenase has been observed to be efficiently inhibited by this dye with a K_i of 0.2 μM (81).

Structural analogs of pyruvate were tested as competitive inhibitors of D-LDH, and the K_i values obtained are listed in Table XI. Oxamate is a well-known inhibitor of mammalian and some bacterial LDHs. The effect of oxamate is relevant to the use of affinity chromatography in the purification of these enzymes. *Haemophilus influenzae* D-LDH was only slightly inhibited by oxamate in a noncompetitive manner, with K_i values of 18 and 13 mM for the slope and intercept effects, respectively (Figure 32). The D-LDH of *Lactobacillus plantarum* was found to be unaffected by oxamate (97).

The participation of different amino acid residues in the catalytic activity of *H. influenzae* D-LDH was investigated. The need for DTT in enzyme preparations to maintain the enzyme active, suggested the importance of sulfhydryl groups in the overall catalytic process. The enzyme was chemically modified by reagents relatively selective for sulfhydryl groups such as iodoacetic acid, iodoacetamide, and N-alkylmaleimides. The inactivation of *H. influenzae* D-LDH with N-alkylmaleimides followed pseudo first-order kinetics (Figures 33 and 34). The rates of inactivation were shown to increase with increasing chainlength of the maleimide derivative (Figure 36). The apparent second-order rate constant for enzyme inactivation by N-heptylmaleimide was approximately 5 times higher than that obtained with N-ethylmaleimide. A chainlength effect of this magnitude is not expected in simple addition reactions to the double bond of the maleimide ring. For example, no rate differences were observed in the reactions of N-heptylmaleimide and N-ethylmaleimide with cysteine (105). Therefore, the positive chainlength effects observed in enzyme inactivation have been interpreted to indicate that the N-alkylmaleimides interact with a nonpolar region of the enzyme which is lo-

cated near the essential sulfhydryl groups. The degree to which nonpolar interactions play a role in the inactivation of different enzymes was evaluated through the comparison of ratios of second-order rate constants for the N-heptyl and N-ethylmaleimides, k_2 (heptyl)/k_2 (ethyl). Table XIV shows the value of this ratio calculated for several enzymes. A ratio of 1.0 such as that observed for yeast glucose 6-phosphate dehydrogenase indicated no chainlength effect in the inactivation process, whereas a ratio higher than 4.8 was observed for many enzymes exhibiting positive chainlength effects. The k_2 (heptyl)/k_2 (ethyl) ratio for *H. influenzae* D-LDH was calculated to be 5.3, comparable with values of 4.8 for beef heart LDH (139) and 6.6 for yeast glutathione reductase (140), but not as marked an effect as the one found in beef muscle LDH (139) or papain (141) with ratios of 11.0 and 18.4, respectively.

The same pattern of inactivation of *H. influenzae* D-LDH was observed for six different N-alkylmaleimides. The data obtained are most consistent with a two-step inactivation process. The first step would be a rapid noncovalent binding of the maleimide in the vicinity of the sulfhydryl groups followed by an irreversible alkylation of the sulfhydryl groups which results in the total inactivation of the enzyme. Saturation kinetics was observed at higher concentrations of N-ethylmaleimide (Figure 35), suggesting that a maximum rate of inactivation of the enzyme could be approached by increasing the concentration of the maleimide. Extrapolation of reciprocal plots of pseudo first-order rate constants versus maleimide concentrations (Figure 37), yielded the same maximum rate of inactivation for each maleimide, *i.e.* 0.6 min^{-1}. Therefore, the inactivation process can not be described in terms of simple second-order kinetics, but rather should take into account an enzyme saturation effect observed by the prior binding of maleimide derivatives. The process could be described as follows:

Table XIV. Second-order Rate Constants (k_2) for Maleimide Reactions

Sulfhydryl compound or enzyme	N-Ethylmaleimide (k_2) (M^{-1} min^{-1})	N-Heptylmaleimide (k_2) (M^{-1} min^{-1})	$\frac{k_2 \text{ (heptyl)}}{k_2 \text{ (ethyl)}}$
Cysteine, pH 5.0 (105)*	1210.0	1210.0	1.0
Glutathione, pH 5.0 (105)	1545.0	1545.0	1.0
Hog Kidney D-Amino Acid Oxidase (142)	27.0	282.0	10.4
Papain (141)	166.0	3050.0	18.4
Yeast Alcohol Dehydrogenase (105)	13.0	112.0	8.6
Rat Ovarian 20α-Hydroxysteroid Dehydrogenase (143)	3.4	38.0	11.2
Beef Heart Lactate Dehydrogenase (H_4) (139)	9.9	48.0	4.8
Beef Muscle Lactate Dehydrogenase (M_4) (139)	7.7	85.0	11.0
Yeast Glutathione Reductase (140)	2340.0	15500.0	6.6
Sheep Liver Sorbitol Dehydrogenase (146)	26.1	235.0	9.0
H. influenzae D-Lactate Dehydrogenase	414.0	2181.0	5.3
Yeast Glucose 6-phosphate Dehydrogenase (105)	2.4	3.2	1.3
Yeast 6-Phosphogluconate Dehydrogenase (144)	18.5	10.2	0.6
Chicken Liver Phosphoglycerate Dehydrogenase (145)	570.0	600.0	1.1

* Numbers in parentheses are References.

$$E + Mal \rightleftharpoons E \cdot Mal \xrightarrow{k'} E - Mal$$

$$K_d = \frac{[E]\ [Mal]}{[E \cdot Mal]}$$

where E.Mal is the reversible complex, E--Mal, the inactive enzyme, K_d, the dissociation constant for the enzyme-maleimide complex, and k', the first-order rate constant obtained at saturation. The first step, or binding process, is suggested to involve the interaction of the maleimide with a nonpolar region of the enzyme. Thus, the enzyme-maleimide complex would be stabilized by hydrophobic interactions, and a higher concentration of the complex would be formed with the longer chain derivatives. The dissociation constant (K_d) for the E.Mal complex was determined for all of the N-alkylmaleimides studied. The values obtained decreased with increasing length of the alkyl chain. The second step, the irreversible inactivation of the enzyme, is rate-limiting and is not influenced by the length of the alkyl chain. This is to be expected since there is no chainlength effect on the rate of reaction of maleimides with sulfhydryl compounds, such as cysteine and glutathione (105). In the first step of the inactivation process, a rapid loss of activity was observed. Some enzyme activity lost in this first step was recovered in an active form after dilution, indicating a reversible process. For example, in an incubation mixture containing 0.7 μg of the purified enzyme, the addition of 300 μM N-ethylmaleimide, lowers the enzyme activity to 28% instantaneously; this was reversed back to 64% active enzyme after 1:1 dilution. This is consistent with a reversible binding process prior to the chemical modification of the enzyme.

Iodoacetamide also modified the *H. influenzae* D-LDH, but less effectively than N-alkylmaleimides. An early, rapid loss of enzyme activity was observed followed by pseudo first-order kinetics of inactivation. The second-order rate constant (k_2) for

iodoacetamide inactivation was determined to be 1.3 M^{-1} min^{-1}, much lower than the one determined for N-ethylmaleimide inactivation, 414 $M^{-1}min^{-1}$. Inactivation of D-LDH by iodoacetic acid also followed pseudo first-order kinetics, but no instantaneous drop in enzyme activity was observed. A k_2 of 20 M^{-1} min^{-1} was determined for iodoacetic acid inactivation. The fact that iodoacetic acid did not inactivate the enzyme in a biphasic manner as was observed for maleimides and iodoacetamide, indicates that the first step in the inactivation of D-LDH by the latter sulfhydryl reagents is a binding process rather than the modification of a very reactive sulfhydryl group. The highly polar molecule of iodoacetic acid is not likely to bind effectively to the hydrophobic region of the enzyme close to the essential sulfhydryl group(s).

It has been shown previously that maleimides can react with functional groups of proteins other than sulfhydryl groups (144 - 149) , and, for this reason, the inactivation of D-LDH described earlier was studied under conditions where reactions with other functional groups were minimized. No inactivation of the enzyme was observed by diethylpyrocarbonate, a reagent that modifies essential histidine residues of proteins. In addition, no chemical modification of the D-LDH was observed by incubation with 100 mM 2,4-pentanedione, a reagent that modifies lysine residues.

In vertebrates, the pyruvate/lactate ratio is regulated by the five LDH isozymes composed of different subunits. The predominant form in muscle, LDH (M_4), is active under physiological conditions when the concentration of pyruvate is high, thus permitting the oxidation of NADH under the anaerobiosis developed during muscle contraction. On the other hand, in highly aerobic tissues such as the heart, the LDH (H_4) isozyme predominates. This H_4 isozyme is inhibited by relatively high concentrations of pyruvate and its main enzyme function is to convert lactate to pyruvate. In *H. influenzae*, the failure of the D-LDH to catalyze a significant rate of NAD reduction with lactate seems to indicate that its physiological role is not the degradation of lactate but

rather the formation of D-lactate from pyruvate and NADH. Indeed, it is shown later in this work (Chapter IV) that the organism degrades D-lactate using an NAD-independent LDH system (D-lactate oxidase) which is part of the respiratory chain. The *H. influenzae* D-LDH may serve to provide reducing equivalents (D-lactate) for the respiratory chain as well as serving to reoxidize NADH for further metabolic needs. In the absence of a complete tricarboxylic acid cycle (150), an alternative means of using pyruvate for energy production would be advantageous to this organism.

CHAPTER IV

STUDIES ON LACTATE OXIDASES IN HAEMOPHILUS INFLUENZAE

INTRODUCTION

The presence of an NAD-dependent D-lactate dehydrogenase was demonstrated in *H. influenzae* (Chapter III). The reaction catalyzed by this enzyme is essentially unidirectional, converting pyruvate to D(-)-lactate exclusively, in the presence of NADH. Because the enzyme appears to function as a pyruvate reductase rather than a lactate oxidase, it was of interest to investigate other mechanisms of lactate oxidation in *H. influenzae.*

The oxidation of lactate has long been observed in both mammalian and microbial systems. In mammalian tissues, and in some microbial systems, lactate oxidation is primarily NAD-dependent (91) and is specific for the L(+)-stereoisomer (151). In addition, a flavoprotein-dependent type of lactate oxidase has been observed almost exclusively in microorganisms (152 -156). The flavoprotein-catalyzed reaction can be specific for either the L- or the D- isomer of lactate and is usually associated with

the cytoplasmic membranes. These reactions can be linked through cytochromes for the transfer of electrons to oxygen (152).

A review of the literature on lactate oxidation reveals considerable confusion in the nomenclature of the enzymes involved. Two types of LDHs have been found in bacteria, one dependent on NAD, and the other independent of the coenzyme. The NAD-linked LDHs are cytoplasmic and use NADH to convert pyruvate to L- or D-lactate (EC 1.1.1.27 and EC 1.1.1.28, respectively). These enzymes have been studied mainly in lactic acid bacteria where they play an important role as these organisms are restricted to the use of carbohydrates for growth and energy. Another type of LDH, which does not use NAD/NADH as coenzyme, is found in a variety of bacteria. Such enzymes are known as NAD-independent LDHs (EC 1.1.1.99) or lactate oxidases. These enzymes are all flavin-containing proteins associated with the cell membrane and linked with electron transport. In general, they are found in bacteria which grow well aerobically, such as *Enterobacteria* and *Staphilococci*; however, they have been found also in anaerobes such as *Propionibacterium pentosacerum* (157). The membrane-bound, NAD-independent, LDH from *E.coli* has been purified and extensively studied as shown in the reviews by Ho (158) and Ingledew (159). Lactate oxidases catalyze the oxidation of lactate to pyruvate and transfer the reducing equivalents to the electron transport system. When the enzyme is bound to the membrane, interacting with cytochromes, the activity displayed is generally called lactate oxidase activity. As soon as the enzyme is detached from the membrane, and the activity measured with an artificial electron acceptor, it is frequently called a lactate dehydrogenase (NAD-independent). In the following study, the name lactate oxidase will be used to refer to the NAD-independent LDHs and differentiate them from the NAD-dependent LDH (D-LDH) discussed in Chapter III.

The present studies demonstrate the presence of two lactate oxidase activities in *H. influenzae*, one specific for D(-)-lactate and the other specific for the L(+)-isomer. The membrane localization, kinetic properties, respiratory activities, and other characteristics of these lactate oxidases are described in this study.

EXPERIMENTAL PROCEDURES

MATERIALS

Haemophilus influenzae strain Rd was obtained from Dr. William L. Albritton of the University of Saskatchewan, Saskatoon. Brain Heart Infusion (BHI), treethanolamine, potassium cyanide (KCN), and sodium azide (NaN_3) were obtained from Fisher Scientific. Streptomycin sulfate, hemin, and histidine were obtained from Sigma. Phenazine methosulfate (PMS), sodium dithionite, pyruvate, oxamate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nicotinamide mononucleotide (NMN), NAD, NADH, D(-)- and L(+)-lactate (lithium salts), L(+)-lactate dehydrogenase from rabbit muscle, bovine serum albumin (BSA), and Tris and Hepes buffers were purchased from Sigma. All other chemicals were of reagent grade.

METHODS

Preparation of bacterial extracts - *H. influenzae* cells were grown as described previously (Methods, Chapter II). Cells at early stationary phase were harvested by centrifugation (18,000 x g, 10 min, 4 °C), and washed with 50 mM potassium phosphate buffer, pH 7.0. The cells were resuspended in the same buffer to make a 20% (w/v) homogenate, using a glass homogenizer. The homogenate was sonicated in an ice-salt bath using the microprobe of an Ultrasonics Sonifier Cell Disruptor at 70 W for five min. Unbroken cells and large debris were sedimented by centrifugation (12,000 x g, 10 min, 4 °C), and the top 90% of the supernatant fraction was carefully removed; this

fraction is referred to as cell-free extract (CFE). The cell-free extract was either stored on ice or subjected to further fractionation by high-speed centrifugation (100,000 x g, 60 min, 4 °C). The supernatant fraction was carefully removed and stored on ice. This fraction is referred to as the soluble fraction (S). The pellet of high-speed centrifugation was resuspended using a glass homogenizer with a volume of phosphate buffer equal to half the volume of the original cell-free extract , and is referred to as the particulate fraction (P).

Enzyme assays - D(-)-Lactate oxidase (NAD-independent D-lactate dehydrogenase) activity was measured at room temperature in a 1-ml cuvette containing 50 mM Tris-HCl, pH 8.0, 2.5 mM D(-)-lactate, and 0.5 mM MTT. The reaction was started by addition of the enzyme aliquot and increase in absorbance at 578 nm was followed. The L(+)-lactate oxidase activity was measured in the same manner, using 0.75 mM L(+)-lactate as the substrate. Lactate oxidase activities were also measured by following the oxygen uptake polarographically at 30 °C. A Clark electrode (Yellow Springs Instrument Co.) was used, coupled to a chart recorder (Fisher Scientific Recordall Series). The electrode was calibrated using buffer saturated with air (240 μM in oxygen) and sodium dithionite (zero in oxygen). The reaction mixtures (2 ml) contained 50 mM potassium phosphate buffer, pH 7.0, 5 mM D(-)-lactate or 0.5 mM L(+)-lactate, and the enzyme aliquot to be assayed was added last to initiate the reaction. Rates of oxygen uptake were reported as nmol oxygen consumed per min per mg protein. D(-)-Lactate racemase activity was measured as described by Dennis (160).

Protein determination - Protein concentrations were determined by the micro-protein assay of the Coomassie blue method (47) using BSA as a standard.

pH Measurements - All pH measurements were made at room temperature on a Radiometer digital PHM 52 pH meter equipped with a GK-2321C combination electrode.

Spectral measurements - The UV-visible absorbance measurements were performed on Beckman Acta MVI and Shimadzu UV2100 recording spectrophotometers.

Determination of the products of the lactate oxidase reactions - The product of the D(-)- as well as the L(+)- lactate oxidase reactions were investigated using *H. influenzae* cell-free extracts as the source of the enzymes. Reaction mixtures (2 ml) containing 50 mM Tris-HCl, pH 8.0, 5 or 10 μmol D(-)-lactate or L(+)-lactate, and 1 ml CFE (11 mg protein/ml) were stirred at room temperature with vigorous agitation. After one hour of reaction, the crude mixtures were boiled for 3 min, cooled, and centrifuged in a clinical centrifuge. Aliquots (10 μl) of the supernate were used to enzymatically assay for pyruvate. A control was run in parallel that contained no D(-)- or L(+)-lactate. A mixture containing 15 μmol of pyruvate in 50 mM Tris-HCl, pH 8.0 was treated in the same way as the reaction mixtures in order to verify that pyruvate was not destroyed in the procedure. The assay mixture used to determine the amount of pyruvate formed consisted of 50 mM Hepes buffer, pH 7.0, 0.1 mM NADH, 9 units of rabbit muscle L(+)-lactate dehydrogenase and a 10-μl aliquot of the reaction product sample in a total volume of 1 ml. The decrease in absorbance at 340 nm was monitored. The amount of pyruvate formed for each reaction mixture was calculated from a standard curve run under the same conditions and with various concentrations of pyruvate.

Determination of respiratory rates - Respiration was measured at 30 °C with a Clark oxygen electrode, calibrated using buffer saturated with air (240 μM in oxygen) and the addition of excess of sodium dithionite for zero oxygen. The incubation mixtures contained cell-free extract, soluble or particulate fraction in 50 mM potassium phosphate buffer, pH 7.0 in a total volume of 2.0 ml. The reaction was initiated by addition of 20 μl of the substrate to give final concentrations of 5 mM D(-)-lactate, 0.5 mM L(+)-lactate, 5 mM succinate, 5 mM formate, or 0.5 mM NADH. The results were corrected for autooxidation and endogenous respiration activity obtained under identical

conditions of assay. The values presented are the average of four separate preparations. Inhibitors were added when oxygen tension was greater than 100 μM. The final concentrations of inhibitors used were: 37 mM sodium azide, 5 mM potassium cyanide, and 20 mM sodium oxamate. Percentage of inhibition was calculated from the initial rate of oxygen consumption with substrate alone, and the slowest rate achieved within 1 min after addition of the inhibitor.

Cytochrome spectra - Cytochrome spectra were measured at room temperature in a Perkin-Elmer Model 557, dual-wavelength, double-beam spectrophotometer. Samples were diluted as necessary with 50 mM potassium phosphate buffer, pH 7.0. Reduced minus oxidized difference spectra were obtained by reducing the content of one cuvette with a few grains of sodium dithionite, either 0.5 μM or 7.5 mM L(+)-lactate, or either 0.5 mM or 5 mM D(-)-lactate, and oxidizing the contents of the other cuvette by exposure to air. KCN was added to both cuvettes to a final concentration of 10 mM to inhibit the terminal oxidase. Spectra were recorded before (for base line calibration) and after the addition of the substrate to the second cuvette, until the absorbance of the peaks attained a maximum.

RESULTS

Lactate oxidase activities in *Haemophilus influenzae*

Lactate oxidase activities specific for either D- or L-lactate were found in *H. influenzae* cell-free extracts. The oxidation of lactate was not dependent on addition of NAD, flavin adenine dinucleotide (FAD), or flavin mononucleotide (FMN). Either MTT or oxygen could serve as terminal electron acceptors for these enzymes. Both enzymes were membrane-associated since 70 to 76% of the activities were recovered in the particulate fraction after high-speed centrifugation (100,000 x g, 60 min, 4°C), see Table XV. The product of both lactate oxidase reactions was demonstrated to be pyruvate. For these studies, an excess of *H. influenzae* cell-free extract was added to the reaction mixture to insure complete and rapid oxidation of different concentrations of D(-)- or L(+)-lactate. When the substrate was completely oxidized, samples were assayed enzymatically with L(+)- lactate dehydrogenase from rabbit muscle for the formation of pyruvate. Results of a typical experiment are given in Table XVI.

In order to determine the presence of two different enzymes, one specific for D(-)-lactate and the other specific for the L(+)- isomer, rather than one enzyme capable of accepting as a substrate either lactate isomer, thermal denaturation studies were conducted. *Haemophilus influenzae* cell-free extracts were incubated at 34 and 39 °C, and both, L- and D-lactate oxidase activities were measured with time. The incubation mixtures (1 ml) contained 0.2 ml of cell-free extract (9 mg protein/ml) in 50 mM Tris-HCl, pH 8.0. Aliquots (10 - 20 μl) were withdrawn with time and assayed for both enzyme activities. The rate of thermal denaturation was different for each enzyme, but, in both

Table XV. Lactate Oxidase Activities in Subcellular Fractions from *H. influenzae*

CFE = cell-free extracts, P = particulate fraction, S = soluble fraction.

Fraction	Vol. (ml)	Protein (mg/ml)	D-Lactate oxidase (units)*	Specific activity (units/mg)	L-Lactate oxidase (units)*	Specific activity (units/mg)
CFE	31	23	46.5	0.06	248	0.35
P	15	15	32.5	0.14	189	0.84
S	30	11	6	0.02	21	0.06

* A unit of enzyme activity is defined as the oxidation of one μmol of lactate per minute. Lactate oxidase activities were measured using MTT as the electron acceptor as described under Methods, Chapter IV.

Table XVI. **Reaction Stoichiometry for the Oxidation of D(-)- and L(+)-Lactate by *H. influenzae* Cell-free Extracts**

SUBSTRATE	CONCENTRATION (μmol/2 ml)	PYRUVATE FORMED * (μmol/2 ml)
D-Lactate	0	0
	5	3.9
	10	9.2
L-Lactate	0	0
	5	4.9
	10	9.5

* Assayed using rabbit muscle L-LDH as explained under Methods, Chapter IV.

cases, followed first-order kinetics as shown on Figure 40. A half-life of 110 min was observed for L(+)-lactate oxidase at 34 and 39°C. However, the D(-)-lactate oxidase was more sensitive to moderately high temperatures, exhibiting a half-life of 42 min at 34°C that decreased to 15 min when incub 39°C.

Respiratory activities in *H. influenzae*

Cell-free extracts derived from *H. influenzae* oxidized NADH, succinate, formate, and D- and L-lactate (Table XVII). L(+)-Lactate was shown to be the substrate most rapidly oxidized by *H. influenzae* cell-free extracts. The rate of D(-)-lactate oxidation was shown to be similar to that of NADH. When cell-free extracts were fractionated by high-speed centrifugation, particulate fractions oxidized all of the above substrates, while soluble fractions just slowly oxidized all of the substrates except succinate (Table XVII). These data confirmed the membrane-bound character of the oxidases. The effect of known respiratory chain inhibitors such as KCN and sodium azide is shown in Table XVIII.

In most cases, the inhibition by cyanide and azide was not instantaneous but developed slowly. Maximal inhibition was observed within 1 min after the addition of the inhibitor to the cell extracts and never exceeded 90%, except when formate was used as the reductant. Oxamate, a known inhibitor of mammalian lactate oxidases (161, 162) had no effect on lactate-stimulated respiration in *H. influenzae* cell-free extracts. Potassium cyanide was tested for the ability to inhibit the D- or L-lactate oxidase activities (activity measured as MTT reductase). The concentration of inhibitor used was 2 mM which inhibited oxygen uptake by 50%. Only 15% inhibition of MTT reductase activity was achieved when D(-)-lactate was the substrate, and no inhibition at all was observed when L(+)-lactate was used as the substrate. These results suggest that KCN inhibits the

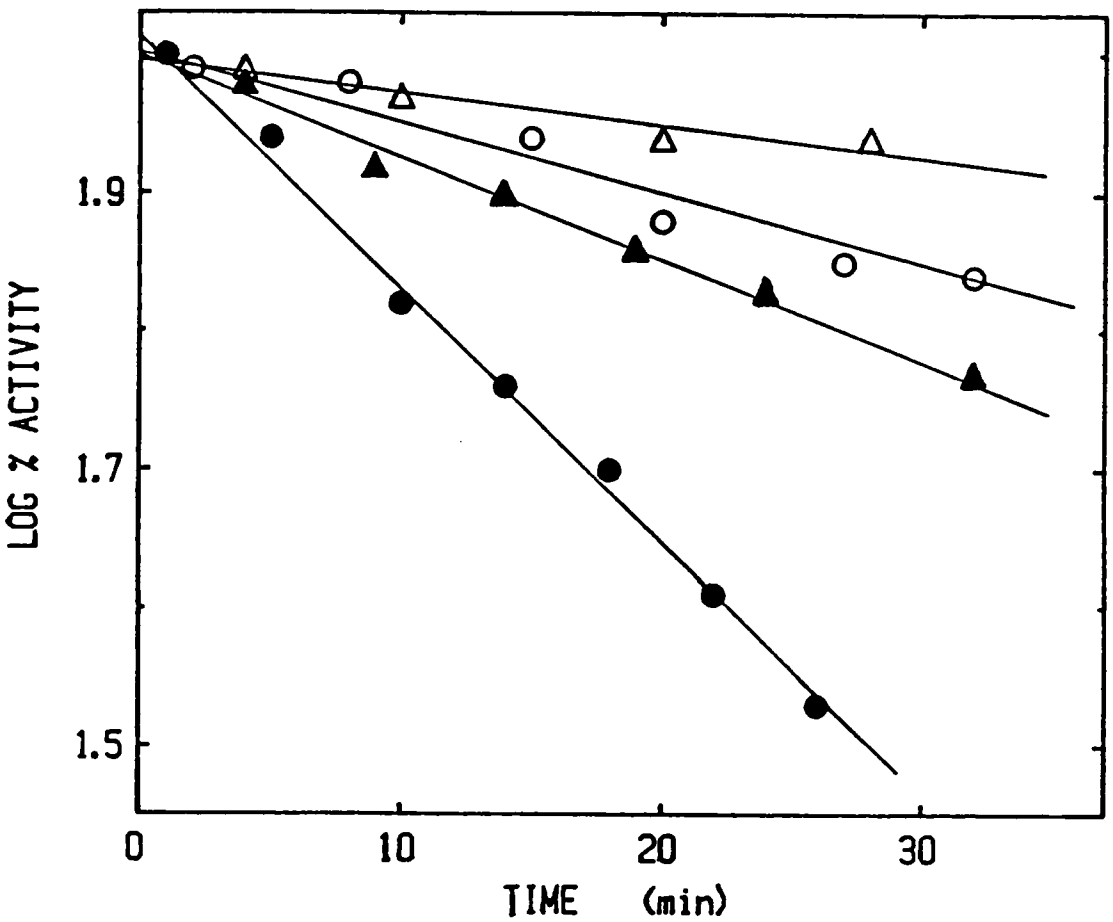

Figure 40. Thermal Denaturation of D- and L-lacate Oxidases: The enzymes were incubated in 50 mM Tris-HCl buffer, pH 8.0 at two different temperatures: (▲), 34°C, and (●), 39°C. Open symbols represent L-lactate oxidase activity. Solid symbols represent D-lactate oxidase activity.

Table XVII. Respiratory Activities of Subcellular Fractions from *H. influenzae*

CFE = cell-free extracts, P = particulate fractions, S = soluble fractions.

SUBSTRATE	CONC. (mM)	Respiratory activities *		
		CFE	P	S
L-Lactate	0.5	256	538	18
D-Lactate	5.0	54	112	5
NADH	0.5	62	135	7
Succinate	5.0	28	52	0
Formate	5.0	45	98	3

* Respiratory activities are expressed as nmol oxygen per minute per mg of protein.

Table XVIII. Inhibition of Respiration of Cell-free Extracts from *H. influenzae*

SUBSTRATE	CONC. (mM)	% inhibition KCN (5 mM)	NaN3 (37 mM)	Oxamate (20 mM)
L-Lactate	0.5	89	75	0
D-Lactate	5.0	80	86	0
NADH	0.5	78	80	0
Succinate	5.0	76	75	0
Formate	5.0	100	86	0

D- and L-lactate oxidation at a site beyond the flavoprotein region (presumably at the terminal oxidase site).

Kinetic parameters for D- and L-lactate oxidases in *H. influenzae*

The effect of various D(-)-lactate concentrations on D(-)-lactate oxidase activity using oxygen or MTT as the electron acceptors is shown in Figure 41. Lineweaver-Burk plots for the MTT or oxygen reductase activities at different concentrations of L(+)-lactate are shown in Figure 42. K_m and V_{max} values were determined from these double reciprocal plots and are listed in Table XIX.

Cytochrome reduction in *H. influenzae*

Difference spectra (reduced minus oxidized) of *H. influenzae* cell-free extracts were taken using different substrates. Dithionite-reduced minus air-oxidized spectra exhibited peaks at 554 nm and 523 nm corresponding to the reduction of a c-type cytochrome, and shoulders at approximately 558 and 528 nm which suggested the involvement of a b-type cytochrome (Figure 43). The Soret peak at 420 nm observed is due to the fused γ peaks of the b- and c- type cytochromes. When NADH was used as reductant, the difference spectrum recorded was similar to that obtained with dithionite. The presence of b- and c- type cytochromes was also demonstrated when L(+)-and D(-)-lactate were used as substrates. However, the cytochrome reduction observed due to D(-)-lactate was much lower than the reduction due to the L(+)-isomer.

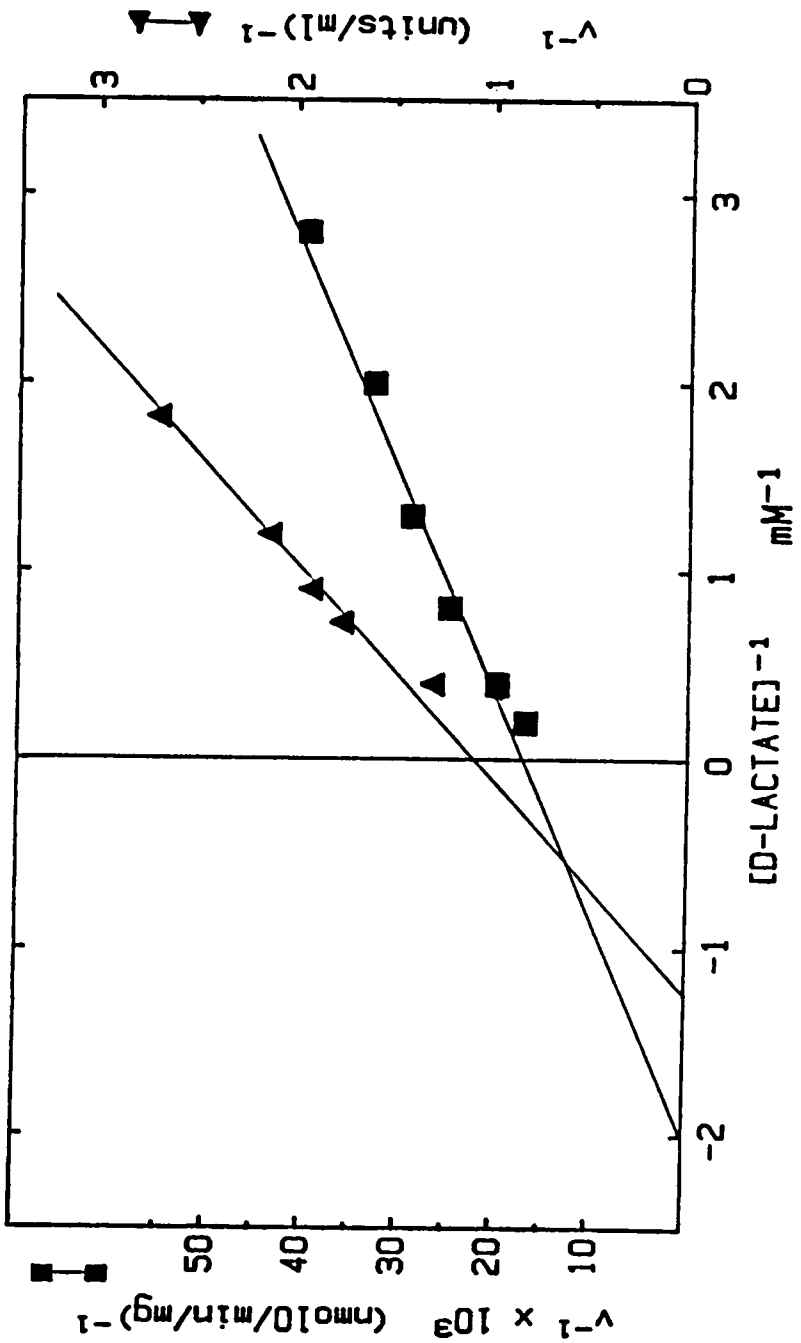

Figure 41. Effect of D(-)-Lactate Concentrations on the D-Lactate Oxidase Activity: D-Lactate oxidase activity was measured using oxygen (■) or MTT (▲) as electron acceptors.

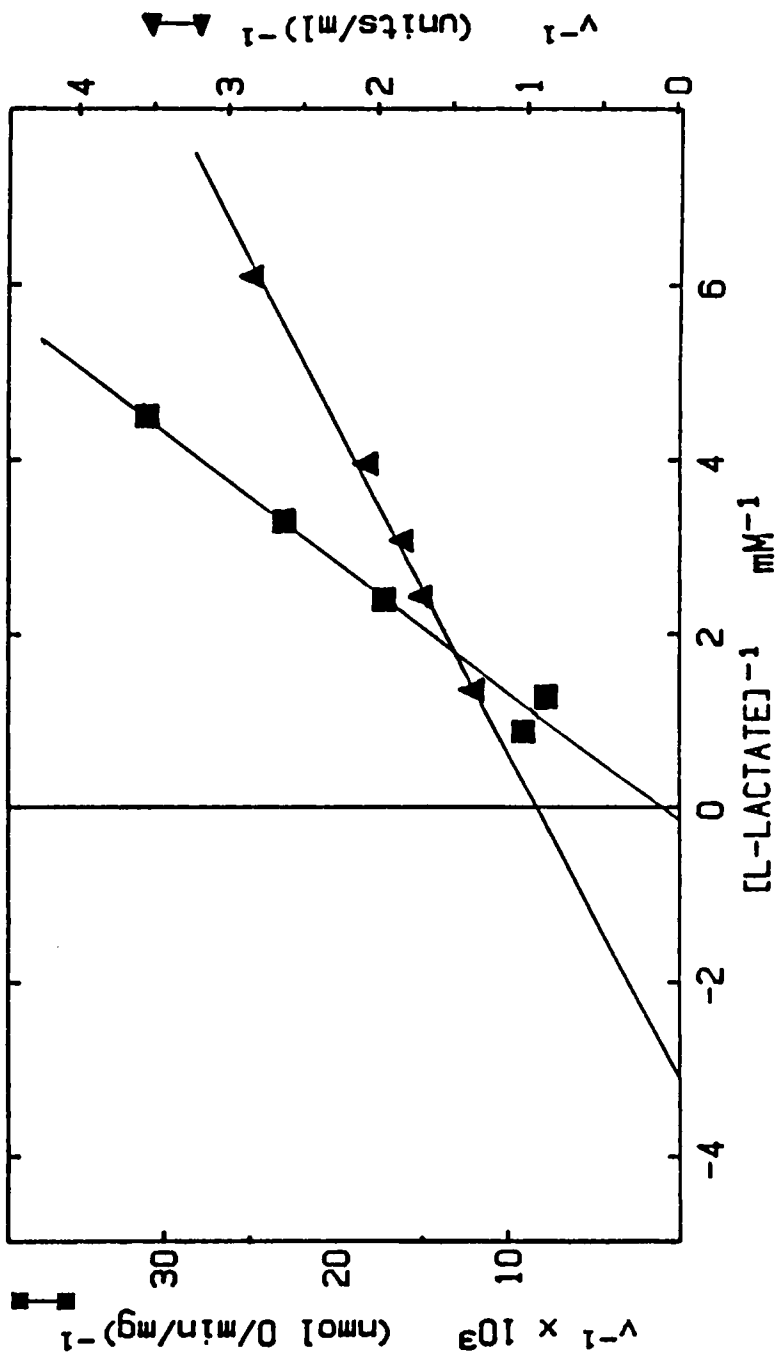

Figure 42. Effect of L(+)-Lactate Concentrations on the L-Lactate Oxidase Activity: L-Lactate oxidase activity was measured using oxygen (■) or MTT (▲) as electron acceptors.

Table XIX. Kinetic Parameters for D- and L-Lactate Oxidases

SUBSTRATE	MTT reductase activity		O_2 reductase activity	
	K_m (mM)	V_{max}*	K_m (mM)	V_{max}**
L(+)-Lactate	0.3	1	5.0	800
D(-)-Lactate	0.8	1	0.5	60

* Velocities determined as µmol MTT reduced per minute per mg of protein.

** Velocities determined as nmol O_2 consumed per minute per mg of protein.

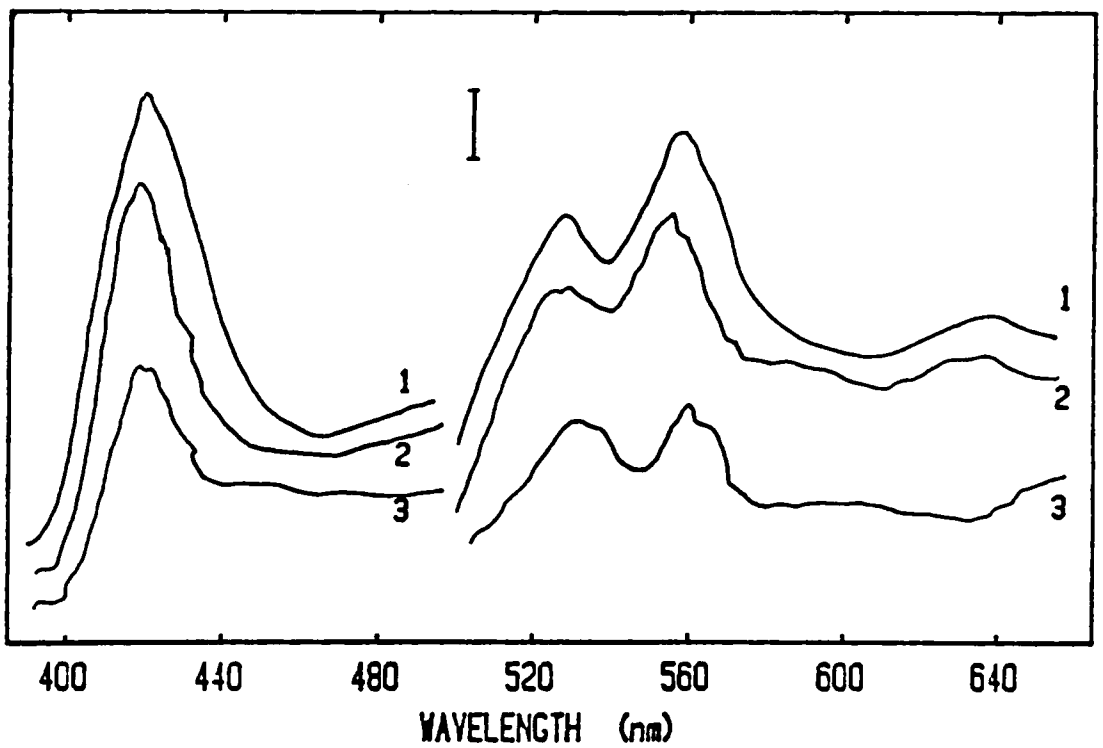

Figure 43. Reduced Minus Air-oxidized Difference Spectra of Cell-free Extracts of *H. influenzae*: For traces 1 to 3 the reductants were $Na_2S_2O_4$, 7.5 mM L(+)-lactate, and 0.5 mM D(-)-lactate, respectively. The bar represents 0.01 absorbance units between 660 and 500 nm, and 0.05 absorbance units between 500 and 400 nm.

DISCUSSION

Enzymes responsible for the oxidation of lactic acid appear to be ubiquitous in living organisms. The classical NAD-dependent, soluble L-LDHs of animal tissues have been studied extensively and information regarding the mechanism and properties of these enzymes have been accumulated. Two types of LDHs were found in bacteria, one that is dependent on NAD (NAD-linked LDHs), and the other type, which does not require NAD/NADH as coenzyme, will be called lactate oxidase rather than LDH. Both types of LDHs in bacteria can be specific for either D(-)- or L(+)- lactate. Some organisms have been found to contain both specificities; for example, *Lactobacillus arabinosus* and *Lactobacillus fructosus* were found to possess four different LDHs; two lactate oxidases and two NAD-dependent LDHs, and each one specific for D- or L-lactate (97, 156, 163).

A LDH specific for D-lactate was found in *H. influenzae* cytoplasm (Chapter III). The reaction catalyzed by this enzyme was essentially irreversible, reducing pyruvate to D-lactate in the presence of NADH. No LDH specific for L(+)-lactate was found in *H. influenzae* sonicates. However, the *H. influenzae* cells were shown to possess both D- and L-lactate oxidase activities associated with the cell membrane (Table XV). The lactate oxidase activities were measured either using oxygen, the natural electron acceptor, or MTT, an artificial electron acceptor, to monitor the reaction.

The product of the lactate oxidase reactions was investigated. Limiting amounts of D- or L-lactate were oxidized by *H. influenzae* cell-free extracts in the presence of an excess of oxygen. The product formed was determined enzymatically using rabbit muscle L-LDH and NADH, and was demonstrated to be pyruvate for both enzymatic reactions (Table XVI). This result raised the question of whether two different lactate

oxidases were present, or just one enzyme with the ability of handling as substrates, either L- or D-lactate. The differences observed in heat sensitivity for these enzymes were consistent with two separate lactate oxidases (Figure 40). *Haemophilus influenzae* D-lactate oxidase was found to be more sensitive to moderately high temperatures than the L-specific enzyme. The same result was observed for D- and L-lactate oxidases in *E. coli* (164).

Several investigators reported racemases frequently found in D,L-lactate forming bacteria. The racemases of *Lactobacillus sake* (165), *Leuconostoc curvatus* (166), *Clostridium butylicum* (110, 167), and *Staphilococcus aureus* (168), have been isolated. D-Lactate racemase activity was investigated in *H. influenzae* sonicates but no such activity was found.

Kinetic parameters for both lactate oxidases were determined using Lineweaver-Burk plots and the values obtained are listed in Table XIX. The V_{max}/K_m ratios were different, depending on the electron acceptor used in the activity assay. D- Lactate oxidase showed higher affinity for D(-)-lactate than the L-lactate oxidase for L(+)-lactate, when molecular oxygen was used as the electron acceptor; however, a much faster reaction rate was achieved by L-lactate oxidase. When D-lactate oxidase activity was assayed using MTT as the electron acceptor, double reciprocal plots (Figure 41) showed a K_m for D(-)-lactate of 0.8 mM, which is similar to the K_m reported for the purified D-lactate oxidase from *E. coli* (169). All the bacterial lactate oxidases (NAD-independent LDHs) have been observed to be membrane-bound, flavin-containing proteins (FAD or FMN), and catalyze the conversion of D- or L-lactate to pyruvate (170). In the present study, the D- and L-lactate oxidases from *H. influenzae* are shown to be membrane-bound proteins, and to convert lactate (D- or L-) to pyruvate, transferring electrons to the respiratory chain. Addition of FAD or FMN to *H. influenzae* sonicates did not result in a burst of D- or L-lactate oxidase activity. Al-

though spectral studies are needed on the purified enzymes in order to identify their prosthetic groups, these oxidases will be considered flavoproteins in the following discussion.

Respiratory studies with subcellular fractions derived form *H. influenzae* demonstrated that this organism could effectively oxidize NADH, formate, succinate, D- and L-lactate. The oxidation of these substrates was carried out more efficiently by the particulate fractions than the cell-free extracts, indicating the membrane-bound nature of the corresponding oxidases. The presence of a particulate formate dehydrogenase has also been found in *H. parainfluenzae* (167) but formate did not serve as an electron donor for *H. parasuis* cells (168). L(+)-Lactate was shown to be the substrate most effectively oxidized by *H. influenzae* while D(-)-lactate was oxidized at a rate similar to that of NADH.

Inhibition of respiratory activities by KCN (Table XVIII) and cytochrome reduction studies (Figure 43) were consistent with the oxidation of D- and L-lactate in *H. influenzae* coupled to an electron transport system. Potassium cyanide, a known inhibitor of the electron transport was shown to be inhibitory of D- and L-lactate oxidation. When these activities were measured as MTT reductases rather than oxygen reductases, no inhibition by the same concentration of KCN was observed for the L-LDH and only 15% inhibition for the D-LDH. These data suggest that KCN does not inhibit the LDHs themselves but rather inhibits the oxidation of lactate somewhere beyond the flavoprotein site. Oxamate, a known inhibitor of mammalian LDHs had no effect on lactate oxidation by cell-free extracts from *H. influenzae*. In addition, the cytoplasmic NAD-linked D-LDH was also observed to be relatively insensitive to oxamate, see Chapter III.

Reduced minus air-oxidized spectra of *H. influenzae* cell-free extracts suggested the presence of b- and c- type cytochromes. The reductants used were dithionite,

NADH, and D- and L-lactate. The same type of cytochromes have been found in other *Haemophilus* species (167, 168). The maximum cytochrome reduction achieved by D-lactate was observed to be lower than the one obtained with L-lactate as the reductant. This result would indicate that L-lactate is a better substrate for the respiratory chain, or that different cytochromes are being reduced by each isomer. Further studies of the cytochromes reduced by these substrates are needed to explain the difference.

Since no NAD-dependent LDH, specific for L-lactate, has been found in *H. influenzae* cytoplasm, the presence of an L-lactate oxidase associated with the membrane suggests that its function is the utilization of exogenous L-lactate as a carbon source. The membrane-bound L-lactate oxidase would convert extracellular L-lactate to pyruvate, transferring reducing equivalents to the respiratory chain. The net result would be the production of energy as ATP and the availability of pyruvate in the cytoplasm to be used as a precursor in the synthesis of other needed metabolites.

The D-lactate oxidase of the membrane provides an alternate means of using pyruvate for energy production since the NAD-dependent D-LDH (Chapter III) predominantly converts pyruvate to the required substrate, D-lactate.

CHAPTER V

CONCLUDING REMARKS

Haemophilus influenzae type b is the primary cause of bacterial meningitis in infants, responsible for more than 8,000 cases annually in the United States (3). In addition to childhood infections, an alarming increase of *H. influenzae* infections in adults has been reported in the literature (8, 9). Ampicillin and chloramphenicol were the antibiotics most commonly used for the treatment of *H. influenzae* infections. However, *Haemophilus* strains resistant to these antibiotics have been reported in the literature since 1974 (4 - 7), and this led to the search for new effective antibiotics and to the development of a vaccine to prevent these infections. The antibiotics of the cephalosporin type are being evaluated for the therapy of β-lactamase-positive strains (12). So far, no effective vaccine has been developed for children younger than 18 months old, the group of children most affected with *H. influenzae* meningitis (13, 14). Even though *Haemophilus* is a serious pathogen of humans and economically important animals, not much effort has been made to study the biochemistry of these organisms.

The unique requirement of *H. influenzae* for two growth factors has been known since 1920 (36). These unusual growth factors were named X- and V- factors and identified as hemin and NAD, respectively. Most microorganisms synthesize NAD *de novo*

from low molecular-weight precursors such as dihydroxyacetone phosphate, aspartate, and formate. Several organisms prefer to use nicotinic acid as a substrate for NAD biosynthesis, using the Preiss-Handler pathway (Figure 2). *Haemophilus influenzae* is of special interest since it lacks these pathways, therefore requiring exogenous NAD for growth. The inability to synthesize NAD from the usual precursors and the fact that intact NAD in the environment could be somewhat limited, suggest that the organism must have developed unique mechanisms to satisfy metabolic needs for NAD. Thus, the enzymes involved in the pyridine nucleotide metabolism of *Haemophilus* may also exhibit unique properties. These enzymes would include those for resynthesis of the coenzymes, dehydrogenases of catabolic and anabolic pathways, and oxidases responsible for the reoxidation of reduced coenzymes and energy generation.

Recent studies of the utilization of externally-added intact NAD for growth of *H. influenzae* demonstrated the presence of a periplasmic NAD pyrophosphatase. This enzyme was purified and characterized (25), and demonstrated to catalyze the hydrolysis of external NAD to AMP and NMN. The NMN produced in the pyrophosphatase reaction can be resynthesized to NAD intracellularly in a reaction with ATP, catalyzed by the NMN adenylyltransferase enzyme. Adenylyltransferase activity was demonstrated in cytoplasmic fractions of *H. influenzae*, as was the NAD kinase activity, responsible for the phosphorylation of NAD to give NADP.

In studies of the periplasmic NAD pyrophosphatase (25), NAD analogs such as 3-acetylpyridine adenine dinucleotide and thionicotinamide adenine dinucleotide, that functioned as substrates for the enzyme, also supported growth of the organism in the absence of NAD. In the present study, the functioning of 3-acetylpyridine mononucleotide as a substrate for the NMN adenylyltransferase, and the functioning of 3-acetylpyridine adenine dinucleotide as a substrate for the NAD kinase, was demonstrated. Therefore, the intracellular existence of both 3-acetylpyridine adenine

dinucleotides could occur through the catalytic activities of these two enzymes. Two cytosolic dehydrogenases in *H. influenzae*, the malate (NAD-dependent) and the 6-phosphogluconate (NADP-dependent) dehydrogenases were proven to function with the 3-acetylpyridine analog of NAD and NADP, respectively (81, 82).

In studies of *H. influenzae* growth inhibition with NAD or NMN as the V-factor, 3-aminopyridine adenine dinucleotide was found to be a potent inhibitor of growth. This NAD analog was also found to be a fairly good substrate for the periplasmic NAD pyrophosphatase (25), indicating the availability of the corresponding mononucleotide inside the cell. The effectiveness of 3-aminopyridine adenine dinucleotide as a growth inhibitor could be due to the inability of the organism to use the 3-aminopyridine mononucleotide produced and /or inhibitory effects of this mononucleotide on intracellular enzymes. The substrate specificities and inhibition studies of NMN adenylyltransferase and NAD kinase presented in this work are important to better understand this mechanism of inhibition by 3-aminopyridine adenine dinucleotide. No inhibition of the NMN adenylyltransferase was observed with 3-aminopyridine mononucleotide at 0.3 mM concentration; the NMN adenylyltransferase was also shown to use the 3-aminopyridine mononucleotide as a substrate, thus allowing for the resynthesis of the dinucleotide inside the cell. No inhibition of NMN adenylyltransferase was observed with 3-aminopyridine adenine dinucleotide at 0.7 mM concentration, and the 3-aminopyridine adenine dinucleotide formed from the corresponding mononucleotide by NMN adenylyltransferase, could function as a coenzyme-competitive inhibitor of NAD-dependent cytosolic dehydrogenases. The NAD-dependent malate dehydrogenase, the major dehydrogenase activity found in *H. influenzae* sonicates, was found to be poorly inhibited by 3-aminopyridine adenine dinucleotide, with a K_i of 2.5 mM (90). The characterization of *H. influenzae* D-LDH presented in this work demon-

strated that this NAD-dependent dehydrogenase is not inhibited by 3-aminopyridine adenine dinucleotide at concentrations as high as 0.76 mM.

The intracellular 3-aminopyridine adenine dinucleotide could also interact with NAD kinase, functioning either as a substrate, or as an inhibitor of this enzyme activity. The results obtained on the substrate specificity and inhibition of *H. influenzae* NAD kinase indicated that the 3-aminopyridine adenine dinucleotide does not function as a substrate for the enzyme, indicating that no 3-aminopyridine adenine dinucleotide phosphate should be formed in the cytoplasm. Therefore, the mechanism of inhibition of growth by 3-aminopyridine adenine dinucleotide can not be explained by interactions with NADP-dependent dehydrogenases. Yoon and Anderson (82) purified NADP-dependent 6-phosphogluconate dehydrogenase from *H. influenzae* and demonstrated the 3-aminopyridine adenine dinucleotide phosphate not to be a good coenzyme-competitive inhibitor of the enzyme. The K_i observed for this dinucleotide phosphate was 0.1 mM, whereas the obtained K_m for NADP was 16 μM.

In previous work (25) it was shown that AMP and ADP could inhibit growth of *H. influenzae* at micromolar concentrations. The concentrations of AMP and ADP that inhibit growth also effectively inhibit the NAD pyrophosphatase, indicating the importance of this enzyme for the processing of extracellular NAD in satisfying the growth requirement. Kahn and Anderson (25) made an interesting observation that AMP was no longer an inhibitor of growth of *H. influenzae* when NMN was used as V-factor. In the present study, ADP was also demonstrated to be ineffective as a growth inhibitor when the organism is grown on NMN (Figure 8). These results provided evidence of at least two different mechanisms for the organism to acquire pyridine nucleotides. The effectiveness of 3-aminopyridine adenine dinucleotide as a growth inhibitor of *H. influenzae* was investigated when cells were grown on NMN instead of NAD as the V-factor (Chapter II). This dinucleotide proved to be a potent inhibitor of growth either

with NAD or NMN as V-factors. When the effect of 3-aminopyridine adenine dinucleotide on growth was investigated with NMN as V-factor and in the presence of the NAD pyrophosphatase inhibitor, ADP (10 μM), no inhibition of growth was observed (Table IV). The ADP concentrations used were sufficient to inhibit the periplasmic NAD pyrophosphatase (K_i = 1.6 μM), indicating that 3-aminopyridine adenine dinucleotide could not be hydrolyzed to the corresponding mononucleotide. These results indicate that 3-aminopyridine adenine dinucleotide is not an inhibitor of growth of *H. influenzae* if it can not be hydrolyzed to the mononucleotide. Thus the actual growth inhibitor is 3-aminopyridine mononucleotide or the corresponding dinucleotide that could be synthesized intracellularly by NMN adenylyltransferase.

An NMNase activity associated with the cytoplasmic membrane was demonstrated in *H. influenzae* (Chapter II). The membrane-bound NMNase could be the site of inhibition of extracellular 3-aminopyridine mononucleotide. This enzyme may be involved in the transport of NMN to the cytoplasm as has been suggested for *Salmonella typhimurium* (169). It was shown repeatedly that most *Haemophili* can not grow on nicotinamide as the source of pyridine nucleotide (22, 23, 38 - 41). The enzyme responsible for the synthesis of NMN from nicotinamide, nicotinamide phosphoribosyltransferase, is present only in members of the genus *Haemophilus* that do not depend on an external V-factor for growth. As expected, such activity was not found in *H. influenzae* sonicates, therefore, it becomes difficult to explain the presence of an NMNase activity in *H. influenzae*, a V-factor organism. It is possible, however, that the physiological activity of the enzyme is different from the one measured. The enzyme could catalyze the hydrolysis of NMN when is detached from the membrane, but display another activity when it is part of the membrane and interacts with phospholipids and other proteins. Foster *et al.* (85) have presented data for *Salmonella typhimurium* that indicate NMN is transported across the cell membrane by a

membrane-bound NMNase. The presence of an NMNase as an NMN transporter, was also suggested for *E. coli* (84, 170). If the NMNase found in *H. influenzae* is truly an NMN transporter, the inhibition observed of NMNase activity by 3-aminopyridine mononucleotide becomes significant. Although 3-aminopyridine mononucleotide inhibits growth of *H. influenzae* at micromolar concentrations, the K_i determined for this mononucleotide for the NMNase was in the millimolar range (1.2 mM). The same relationship was observed for NMN as a supporter of growth of *H. influenzae* (micromolar concentrations) and as a substrate for the NMNase (K_m = 0.5 mM). Therefore, it is possible that extracellular 3-aminopyridine mononucleotide inhibits growth of *H. influenzae* by inhibiting the transporter activity of the membrane-bound NMNase.

Further studies are needed to investigate the biological functioning of NMNase in *H. influenzae* and its inhibition by 3-aminopyridine mononucleotide. These studies should be directed to the purification and characterization of *H. influenzae* NMNase, with special interest focused on its possible functioning as a transporter protein. In this respect, the development of *H. influenzae* mutants, defective in this enzyme, will help elucidate the mechanism of NMN transport in this organism.

Based on all the observations described above, a model for the utilization of exogenous NAD by *H. influenzae* is proposed and presented in Figure 44. When NAD is provided as the V-factor, the dinucleotide diffuses into the periplasm and is hydrolyzed to NMN and AMP by the NAD pyrophosphatase (25). The direct involvement of this enzyme in the internalization of NMN has not been ruled out. Inhibition of growth of *H. influenzae* is achieved by ADP by inhibiting the NAD pyrophosphatase. However, when NMN is used as the pyridine nucleotide source no inhibition by ADP is observed. Therefore, there is another possible mechanism by which NMN is internalized besides the NAD pyrophosphatase.

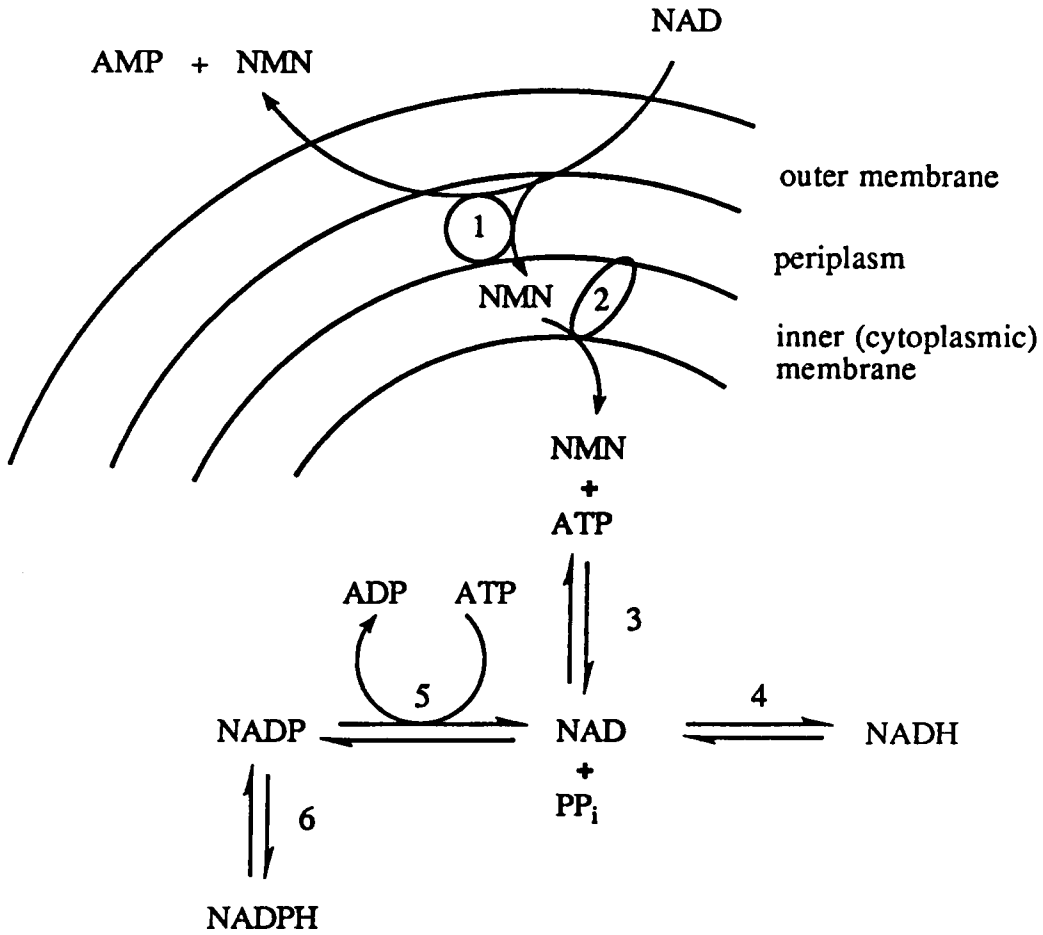

Figure 44. Model for the Utilization of Exogenous NAD by *H. influenzae*: 1 = NAD pyrophosphatase, 2 = NMNase (NMN transporter), 3 = NMN adenylyltransferase, 4 = NAD-dependent dehydrogenases, 5 = NAD kinase, and 6 = NADP-dependent dehydrogenases.

NMN could diffuse to the inner membrane and bind to the NMNase which facilitates the internalization of the mononucleotide, as has been observed in *Salmonella typhimurium* (169). This mechanism would be unaffected by ADP, thus explaining the ineffectiveness of ADP as an inhibitor of growth when cells are grown on NMN instead of NAD. This process would also be saturable and would therefore account for the saturation effect observed on the rates of growth with increasing concentrations of NMN, that does not occur with NAD as the V-factor (25).

An alternative model for the facilitated uptake of NMN obtained from NAD could involve a protein-protein interaction between the NAD pyrophosphatase and the NMN transporter (NMNase). The NAD pyrophosphatase would cleave NAD at the pyrophosphate bond and, while NMN is still bound to the enzyme, interact with the membrane-bound NMN transporter, transferring NMN to the cytoplasm. It may be that the NMN transporter, when it is not interacting with the pyrophosphatase or is not part of the membrane, binds NMN and catalyzes the hydrolysis to nicotinamide and ribose 5-phosphate. The hydrolysis of NMN was the NMNase activity observed in the present study.

Once NMN is in the cytoplasm, *H. influenzae* NMN adenylyltransferase utilizes it to resynthesize NAD intracellularly, and cytosolic NAD kinase phosphorylates NAD to give NADP. Internal NAD and NADP are used as coenzymes by the numerous cytosolic dehydrogenases.

A peculiar NAD-dependent D-LDH was found in *H. influenzae* that prompted the present study on lactate metabolism in *H. influenzae*. Chapter III describes the purification and characterization of this enzyme with respect to molecular weight, substrate and coenzyme specifity, pH profile, thermal stability, functional group involvement, and selective inhibition. The D-LDH in *H. influenzae* has been found to have notable similarity with the one found in *E. coli* (101, 102). *Haemophilus influenzae* D-LDH activity

provides the substrate for an NAD-independent D-LDH (D-lactate oxidase) that was found associated with the membrane. The D-lactate oxidase catalyzes the oxidation of D-lactate to pyruvate, and transfers the reducing equivalents to the electron transport system, with the final reduction of oxygen and concomitant formation of ATP. The electrochemical proton gradient generated across the membrane by electron transfer reactions in the respiratory chain could be used not only to drive ATP synthesis but also for solute uptake and other energy-requiring, membrane-associated processes. The D-lactate oxidase (NAD-independent D-LDH) in *E. coli* has been shown to catalyze the oxidation of D-lactate in electron transfer reactions, coupled to active transport of various amino acids and sugars (171). The D-lactate oxidase in *H. influenzae* could also be involved in active transport of amino acids and sugars.

The coupled activities of the D-LDH and D-lactate oxidase found in *H. influenzae* provide the organism with energy in the form of ATP and regeneration of NAD to be used by other dehydrogenases. In addition, these enzymes provide a way to utilize pyruvate derived from various carbohydrate sources to produce the energy-rich compound ATP. In a recent study (150) of glucose metabolism in *Haemophilus* species, a partial tricarboxylic acid cycle was indicated involving the carboxylation of phosphenolpyruvate to oxalacetate and, through malate and fumarate, to a major excretory product, succinate. Activities of the enzymes required for this pathway were demonstrated with malate dehydrogenase as the only NAD-dependent dehydrogenase in the sequence which catalyzes the reduction of oxalacetate to malate, with concomitant oxidation of NADH to NAD. In the same manner, the flavin-dependent succinate dehydrogenase reduces fumarate to succinate and reduced flavin adenine dinucleotide is oxidized. The presence in *H. influenzae* of a partial tricarboxylic acid cycle like the one described above, would limit the amount of reducing equivalents that could be transferred to the electron transport system. The coupled activities of the two D-lactate

enzymes described in this work offer a means of channeling the reducing equivalents of NADH to the respiratory chain for the production of energy.

No NAD-dependent LDH, specific for L(+)-lactate was found in the *H. influenzae* cytoplasm. However, an NAD-independent L-LDH (L-lactate oxidase) was found associated with the membrane. This enzyme catalyzes the oxidation of L(+)-lactate to give pyruvate, and transfers electrons to the respiratory chain. The net result would be the production of energy as ATP and the availability of pyruvate in the cytosol to be used as a precursor in the synthesis of other needed metabolites. The membrane-bound L- lactate oxidase could offer a means of utilization of exogenous L(+)-lactate as a carbon source for the organism.

A model for the utilization of lactate in *H. influenzae* is presented in Figure 45 based on the observations addressed above.

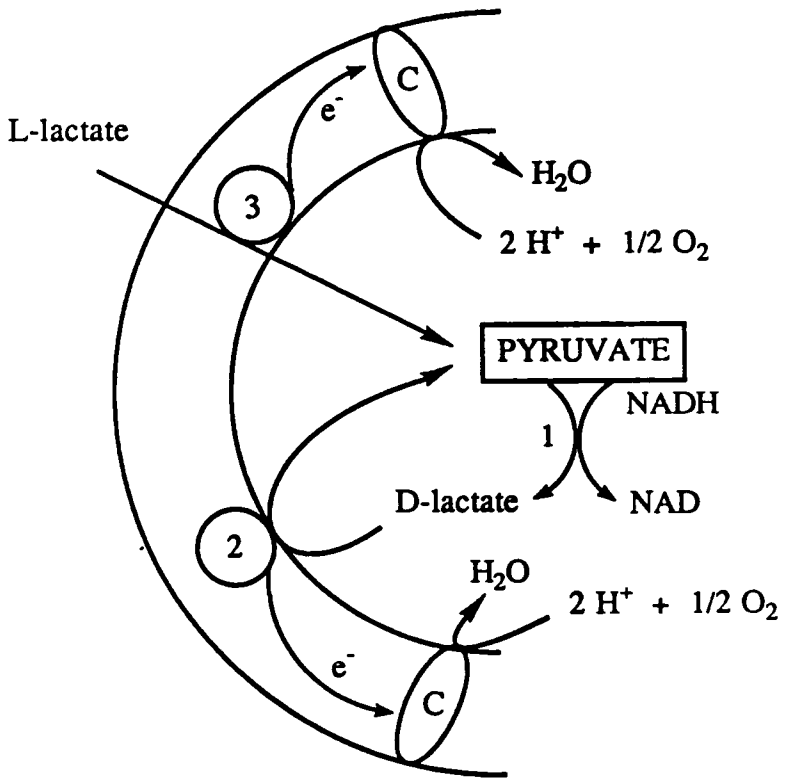

Figure 45. Model for the Utilization of Lactate in *H. influenzae*: 1 = NAD-dependent D-lactate dehydrogenase, 2 = D-lactate oxidase (NAD-independent D-LDH), 3 = L-lactate oxidase (NAD-independent L-LDH), and c = cytochromes.

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