STUDIES OF THE NAD METABOLISM OF HAEMOPHILUS INFLUENZAE

by

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INTRODUCTION

The genus *Haemophilus* is comprised of a group of small, gram-negative, aerobic bacteria characterized by its cocco-bacilliary (pleomorphic) shape and requirement for one or both of two growth factors, *viz*. X-factor (hemin) and V-factor (nicotinamide adenine dinucleotide (NAD)). All *Haemophilus* organisms are non-motile, non-sporeforming and are capable of forming capsules. Table I lists several species within this genus and their respective X- and V-factor requirements. Several of these organisms are pathogenic to man and other animals. *Haemophilus aegyptius* (Koch-Week's bacillus) infection causes contagious conjunctivitis, or pink eye. *Haemophilus ducreyi* is the primary cause of the venereal disease, soft chancre. *Haemophilus suis* and *Haemophilus gallinarium* are responsible for acute respiratory infections in pigs and chickens, respectively. *Haemophilus parahaemolyticus* (currently in the literature as *Haemophilus pleuropneumoniae*) causes a highly contagious, and usually fatal, respiratory infection in swine. This infection has become a major problem in North American swine production. The most extensively studied *Haemophilus* species, which serves as the prototype for the genus, is the human pathogen, *Haemophilus influenzae* .

TABLE I

X- and V-factor Requirements of
Species of Haemophilus

Species	V-factor Requirement	X-factor Requirement
H. influenzae	+	+
H. aegyptius	+	+
H. haemolyticus	+	+
H. ducreyi	-	+
H. aphrophilus	-	+
H. parainfluenzae	+	-
H. parahaemolyticus	+	-
H. paraphrophilus	+	-
H. suis	+	+
H. gallanarium	+	+

Haemophilus influenzae was initially isolated by Pfeiffer in 1892 (1) from the upper respiratory tracts of patients who had died during a massive flu epidemic. Pfeiffer incorrectly postulated that this organism was the primary cause of influenza. The organism was given the name, "influenza bacillus", by which it would appear in the literature for several decades. In the 1930s, a virus was identified as the primary cause of influenza (2), while Margaret Pittman correctly identified Haemophilus influenzae as the major cause of bacterial meningitis (3).

Haemophilus influenzae can exist in both encapsulated and noncapsulated forms. The noncapsulated forms are present as part of the normal nasopharyngeal bacterial flora in approximately 50-75% of all adults (4). These noncapsulated forms are frequently implicated as secondary pathogens, following respiratory disorders such as pneumonia or bronchitis. The encapsulated forms are primary pathogens and are implicated in several invasive diseases. They are subclassified into types a-f based on the nature of the carbohydrate moieties of their capsular polysaccharides. Haemophilus influenzae type b, which possesses a polyribophosphate (PRP) structure similar to the immunogenic capsule of Pneumococcus , is responsible for 10,000 to 20,000 cases of bacterial meningitis annually in the United

States alone (5). The disease is most frequently seen in children ages 1-3, a time when passively-acquired immunity from the child's mother is no longer operative and the child's own immune system is not fully developed. Haemophilus influenzae type b is also implicated as the second most frequent cause of otitis media (middle ear inflammation), affecting 95% of all children at least once by age five (6).

Therapeutic treatment of Haemophilus -induced meningitis has relied upon the use of antibiotics, particularly ampicillin and chloramphenicol. These antibiotics have proven very successful in limiting the morbidity of Haemophilus infections. However, recent developments have occurred which promote Haemophilus influenzae , once again, to the position of being a serious health hazard. In 1974, initial reports of ampicillin-resistant Haemophilus influenzae infections appeared in the literature (7). This problem has now increased to the point where an estimated 16% of all Haemophilus influenzae infections can no longer be treated with ampicillin (8). All of the resistant organisms investigated possessed beta-lactamase, an enzyme which catalyzes the hydrolysis of the beta-lactam rings of ampicillin, penicillin and other cephalosporin antibiotics. Focus then turned to the use of chloramphenicol, an

antibiotic whose use is usually avoided due to the potentially serious side effects of the drug. While chloramphenicol was initially effective in the treatment of ampicillin-resistant infections, reports of plasmid-mediated resistance to the antibiotic appeared in the literature (9).

Additional problems have contributed to the need for rapidly developing an effective method of treatment of Haemophilus influenzae . In the past, reports of the transfer of virulent Haemophilus influenzae between individuals were rare. However, numerous reports of the spreading of Haemophilus infections within the home, day-care centers and hospitals have led to the classification of Haemophilus influenzae type b infections as contagious (10). Increased incidents of Haemophilus influenzae meningitis in adults have recently been reported. One report attributed 20% of the meningitis cases in adults to Haemophilus influenzae type b as compared to earlier estimates of between 1-3% (11).

All of these developments have prompted the continuing efforts to develop alternative methods of treatment of Haemophilus infections. Most recently, work has focused on the development of vaccines (12) and the use of other antibiotics, the most promising of which has been moxalactam, a semi-synthetic oxa-beta-lactam which is

resistant to beta-lactamase catalyzed hydrolysis (13).

Biochemical analyses of Haemophilus influenzae metabolism have generally fallen into a narrow group of categories. The discovery of restriction endonucleases, which has provided a revolution in the field of molecular biology, was initially made using Haemophilus influenzae (14). Since the initial discovery in 1970, over 80 different restriction endonucleases have been purified from various microorganisms, with members of the genus Haemophilus producing 22 of these (15).

Haemophilus influenzae has also been observed to readily take up and incorporate foreign DNA (16). Extensive time and effort have been devoted to achieving an understanding of this phenomenon known as competence, or genetic transformation. This pursuit has led to extensive investigations into the nature of the Haemophilus influenzae inner and outer membranes (17). These studies have also necessitated the development of a defined media that could be used to determine what nutrients are needed specifically for the development of competence (18). As a result of these pursuits, amino acid, nucleoside, vitamin and mineral growth factors, in addition to the unusual X- and V-factors requirements, have been determined.

In-depth analysis of the X-factor (hemin) requirement of

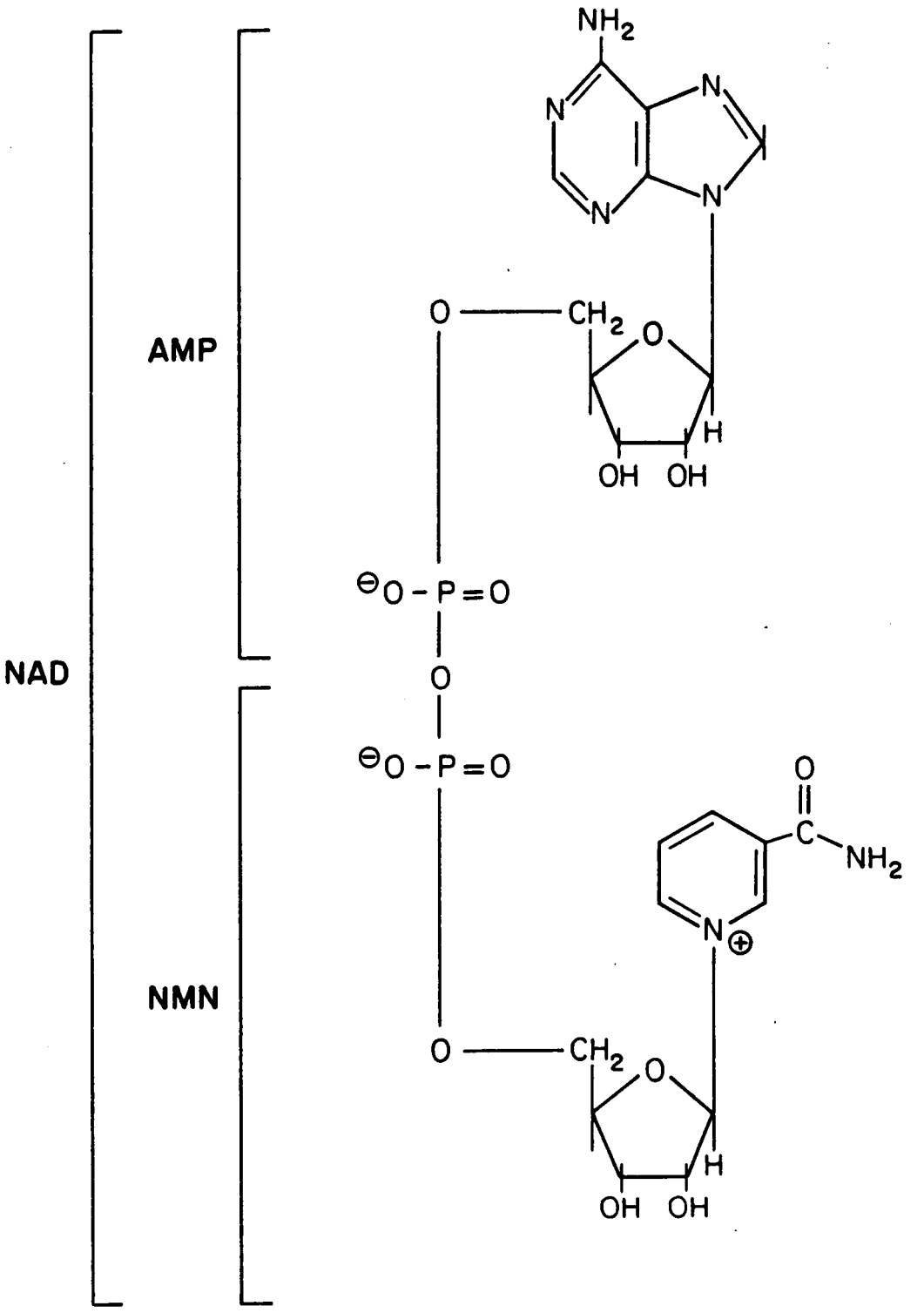
Haemophilus influenzae produced a substantial portion of our understanding of the structure and function of the respiratory chain (19). In comparison, very little has been done to explore the nature of the V-factor requirement. It has therefore been the overall goal of this project to acquire a more thorough understanding of the V-factor requirement, a characteristic unique to the genus Haemophilus . Assuming that an enzymic process is involved in the internalization of the V-factor, NAD, it is proposed to isolate and purify the enzyme of importance, to characterize the enzyme to permit manipulation of its catalytic activity and to correlate the kinetic properties of the enzyme with the manipulation of growth of the organism. The work that has been done in investigation of the V-factor requirement, as well as other pertinent literature, will now be reviewed.

LITERATURE REVIEW

The pyridine nucleotides are essential for both anabolic and catabolic pathways. In 1935, Warburg et al. (20) discovered that the pyridine nucleotides are essential cofactors in several biological oxidation reactions, undergoing reversible oxidation and reduction while in association with several enzymes. Today, the coenzymes are recognized as essential in the function of over 300 enzymatically-catalyzed redox reactions (21).

Recent work has focused on the role pyridine nucleotides play in other reactions not involving oxidation-reduction. It has recently been discovered that NAD is an essential substrate for deoxyribonucleic acid (DNA) ligase in prokaryotes. Cleavage of NAD (Figure 1) at the pyrophosphate bond is involved in the action of this enzyme, which plays an important role in DNA synthesis, repair and recombination (22). Reactions involving the hydrolysis of the nicotinamide-ribose bond have also been the subject of numerous investigations (23). This reaction catalyzed by NAD glycohydrolases produces nicotinamide and adenosine diphosphoribose (ADPR). Certain enzymes which possess glycohydrolytic activity have also been shown to catalyze the transfer of ADPR to various acceptors.

Figure 1. The structure of beta-nicotinamide adenine dinucleotide (NAD). Cleavage of the pyrophosphate bond of this compound produces nicotinamide mononucleotide (NMN) and adenosine 5'-monophosphate (AMP).


NAD
AMP
NMN
NH2
N
N
N
N
O
CH2
O
H
OH
OH
⊖O-P=O
O
⊖O-P=O
O
C
NH2
N⊕
O
CH2
O
H
OH
OH

Mono-ADP-ribosylation reactions, in which a single ADPR is transferred to an acceptor protein, have been observed to be catalyzed by the toxins of Cornybacterium diphtheriae (24), Vibrio cholerae (25) and Bordetella pertussis (26). In eukaryotes, it has been observed that several proteins of the mammalian liver are ADP-ribosylated in vivo (27) and recently, Moss et al. have described the purification and characterization of two eukaryotic enzymes that catalyze the mono-ADP-ribosylation of various acceptors (28).

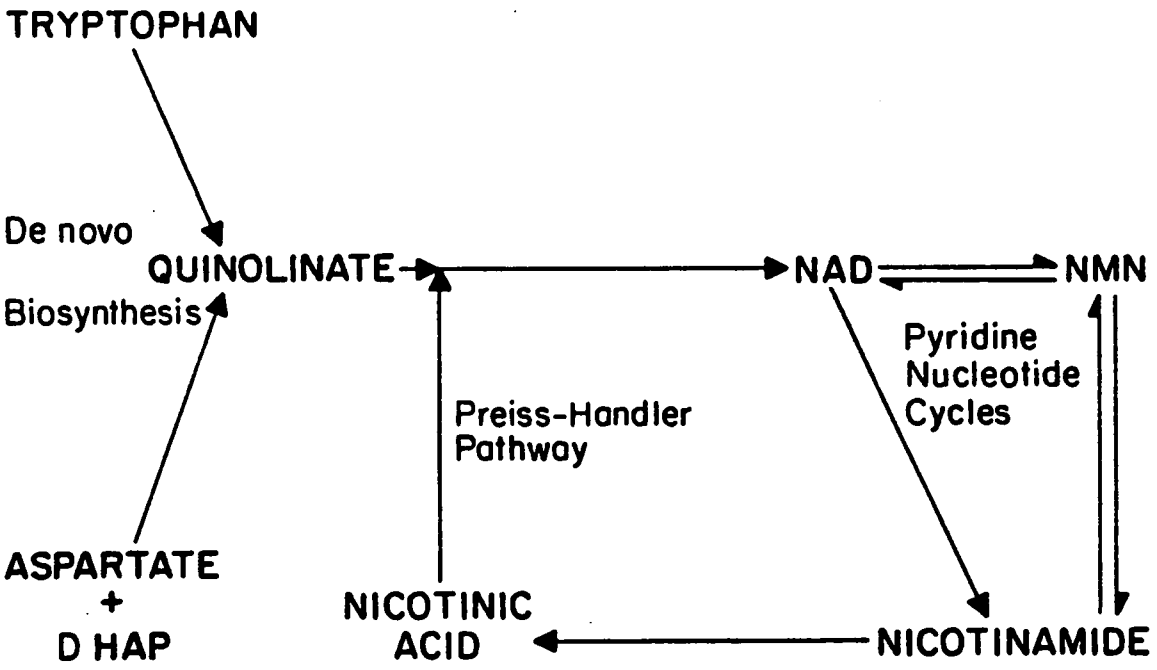
The eukaryotic enzyme, poly-ADPR synthetase, catalyzes the synthesis of long polymers of ADPR which are typically transferred to various nuclear protein acceptors, particularly histones. The exact role of poly-ADPR is still unknown, but evidence is accumulating that the poly-ADP-ribosylation of nuclear protein is involved in DNA repair, chromatin condensation and regulation of growth through modulation of the cell cycle (29). In addition to these reactions involving pyridine nucleotides, the reduced pyridine nucleotides are known to serve as allosteric effectors in various pathways, such as the citric acid cycle (30). The synthesis, recycling and regulation of pyridine nucleotides therefore represents a major concern for all cells.

Three mechanisms are known which organisms use to

fulfill their requirement for NAD. The three mechanisms, as shown in Figure 2, include: 1) de novo biosynthesis, 2) conversion of nicotinic acid (niacin) to NAD (the Preiss-Handler pathway) and 3) the use of pyridine nucleotide cycles.

Many organisms are able to synthesize NAD de novo from various precursors. The prokaryotic and eukaryotic pathways are clearly delineated by the nature of the precursor(s) used. The recognition of tryptophan as a precursor to NAD was first suggested as a result of nutritional studies which showed that humans suffering from pellagra (niacin deficiency) could be effectively treated by dietary supplementation with either niacin or tryptophan (31). Less than one decade after this observation, the majority of the steps involved in the conversion of tryptophan to NAD were described, primarily by Yanofsky et al. from their work with Neurospora (32). This anabolic pathway, found in most eukaryotes, resembles very closely the catabolic pathway used by many procaryotes to obtain energy from the oxidation of tryptophan. The first two steps of both pathways are, in fact, identical. The great similarity between the two pathways has led Gaertner and Shetty (33), among others, to propose that the anabolic pathway represents a divergent evolutionary step from the catabolic pathway.

Figure 2. The three known mechanisms of acquiring pyridine nucleotides: 1) de novo biosynthesis, 2) the Preiss-Handler pathway and 3) the pyridine nucleotide cycles.


TRYPTOPHAN
De novo
QUINOLINATE
Biosynthesis
NAD
NMN
Pyridine
Nucleotide
Cycles
Preiss-Handler
Pathway
ASPARTATE
+
D HAP
NICOTINIC
ACID
NICOTINAMIDE

An alternative method to de novo biosynthesis of NAD from tryptophan has been observed in many prokaryotes and in some plants (34,35). These systems involve the synthesis of the pyridine ring from aspartate and other compounds. The most widely observed pathway involves the condensation of aspartate with dihydroxyacetone phosphate (DHAP) leading to the formation of quinolinic acid (36). A second pathway involving aspartate has been observed in Clostridium butylicum . In this organism aspartate reacts with formate and acetyl coenzyme A to form quinolinate (37). One unusual feature of de novo NAD biosynthesis is the fact that all known pathways lead to a common intermediate, quinolinic acid. In addition, the steps which are used in the conversion of quinolinate to NAD are also identical in all organisms capable of performing de novo NAD biosynthesis (38). These steps include the formation of nicotinic acid mononucleotide from quinolinate, conversion of the mononucleotide to nicotinic acid adenine dinucleotide and the amidation of this compound to produce NAD.

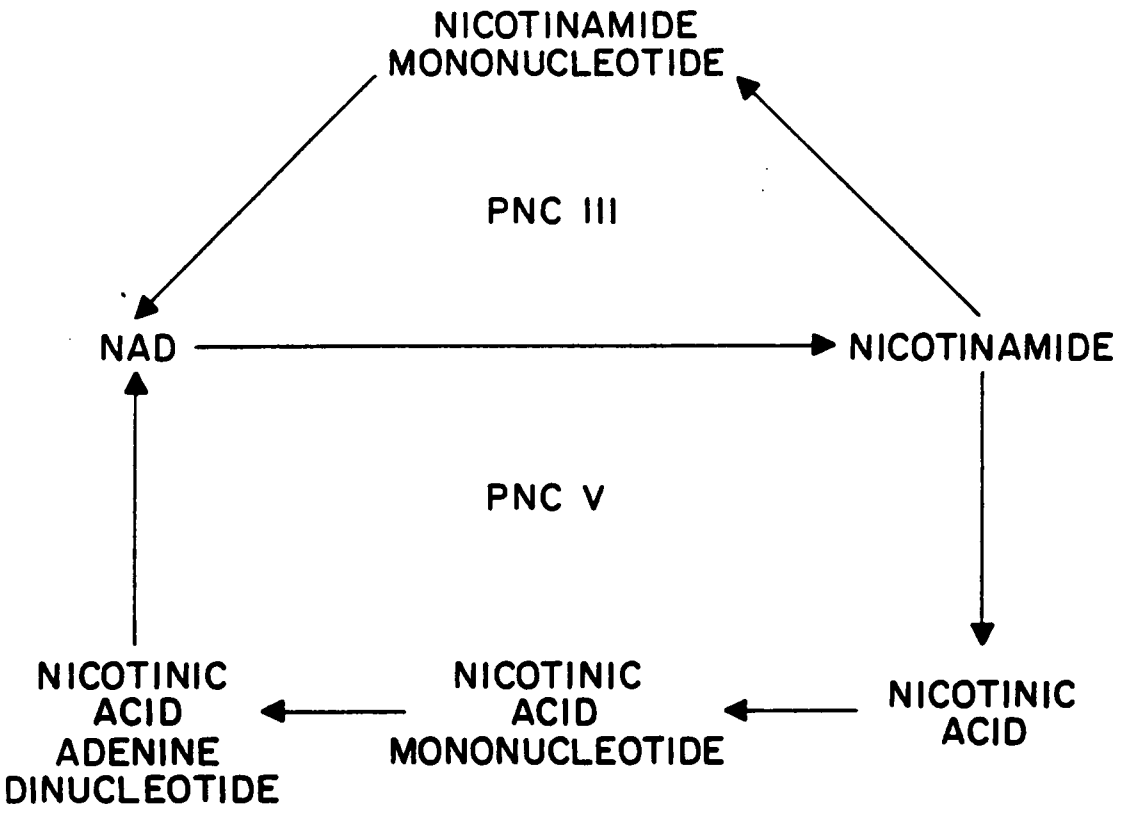
The preferred substrate for NAD biosynthesis in several organisms is nicotinic acid (39). The three-step pathway from nicotinic acid to NAD, elucidated by Preiss and Handler in 1958 (40), is identical to the final steps of de novo biosynthesis except that nicotinic acid, rather than

quinolinate, is used to form nicotinic acid mononucleotide. In eukaryotes, formation of nicotinic acid adenine dinucleotide occurs in the nucleus, while all other steps occur in the cytoplasm (41). Another difference between prokaryotes and eukaryotes is the source of nitrogen for the final amidation step. Prokaryotes use ammonium ion while eukaryotes use glutamine (42).

An alternative to de novo biosynthesis and the Preiss-Handler pathway is the use of pyridine nucleotide cycles (PNCs) or salvage pathways. Pyridine nucleotide cycles are sequences of enzyme-catalyzed reactions resulting in the resynthesis of NAD from a compound produced by hydrolytic degradation of NAD. The Preiss-Handler pathway, although a component of several pyridine nucleotide cycles, is not considered to be a pyridine nucleotide cycle because nicotinic acid can not be produced directly from NAD.

Most eukaryotes possess either a five-membered pyridine nucleotide cycle (a PNC V), or a three-membered cycle (a PNC III) (Figure 3). The initial step in either cycle is cleavage of the nicotinamide-ribose bond of NAD, which is typically catalyzed by a NAD glycohydrolase or by enzymes which catalyze ADP-ribosylation (e.g. poly-ADPR synthetase). These cycles may internalize NAD in organisms which possess externally directed glycohydrolase activity,

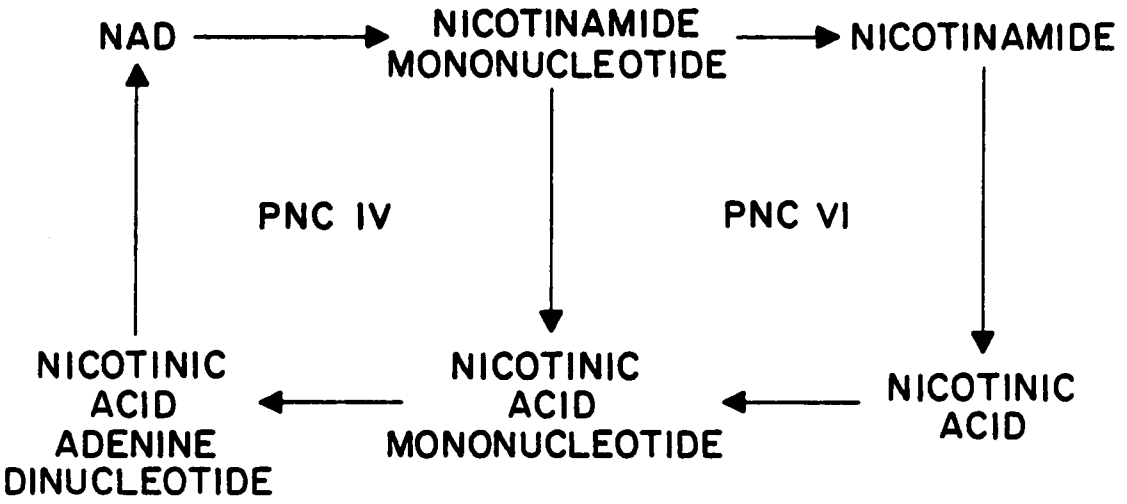
Figure 3. The three-membered and five-membered pyridine nucleotide cycles, PNC III and PNC V.


NICOTINAMIDE
MONONUCLEOTIDE
PNC III
NAD
NICOTINAMIDE
PNC V
NICOTINIC
ACID
ADENINE
DINUCLEOTIDE
NICOTINIC
ACID
MONONUCLEOTIDE
NICOTINIC
ACID

or they may function as a salvage pathway for recycling nicotinamide resulting from poly-ADPR synthetase activity. Recycling of nicotinamide to NAD is initiated by either a nicotinamide deamidase (for PNC V) or a nicotinamide phosphoribosyltransferase (PNC III). In the PNC V, the nicotinic acid that is produced now enters the Preiss-Handler pathway and is recycled to NAD. In the PNC III, the cycle is completed by reaction of the NMN with ATP as catalyzed by a NMN adenyl transferase to produce NAD.

The predominant pyridine nucleotide cycles in prokaryotes are the PNC IV and PNC VI. These pathways are both shown in Figure 4. Both cycles are initiated by hydrolysis of the pyrophosphate bond, producing NMN and AMP. Two enzymes that catalyze cleavage of the pyrophosphate bond appear to be primarily responsible for initiation of these cycles. In Escherichia coli , the enzyme DNA ligase has several roles including repair of damaged DNA, linkage of DNA during recombination, and the joining together of Okazaki fragments that are produced from discontinuous DNA replication (43). The mechanism of this enzyme is believed to involve the hydrolysis of the pyrophosphate bond of NAD with the subsequent formation of NMN and an adenylated intermediate of the DNA involved in the DNA-DNA linkage (44). The second enzyme known to contribute to the

Figure 4. The four-membered and six-membered pyridine nucleotide cycles, PNC IV and PNC VI.


NAD
NICOTINAMIDE MONONUCLEOTIDE
NICOTINAMIDE
PNC IV
PNC VI
NICOTINIC ACID ADENINE DINUCLEOTIDE
NICOTINIC ACID MONONUCLEOTIDE
NICOTINIC ACID

initiation of the PNC IV and PNC VI is NAD pyrophosphatase. This enzyme is responsible for the extracellular production of NMN in E. coli (66) and Salmonella typhimurium (45).

The recycling of NMN back to NAD in the PNC VI proceeds by hydrolysis of the nicotinamide-ribose bond to produce free nicotinamide, a step which is catalyzed by NMN glycohydrolase. This enzyme has been observed to be of both cytoplasmic (46) and membrane-bound (47) location. The membrane-bound NMN glycohydrolases appear to be involved in the transport of nicotinamide into the cell where the remaining enzymes of PNC VI participate in the resynthesis of NAD. These are the same enzyme activities which recycle nicotinamide back to NAD in the eukaryotic PNC V. The combined actions of NAD pyrophosphatase (or DNA ligase) and NMN glycohydrolase in the PNC VI therefore replace the action of NAD glycohydrolase in eukaryotes.

An alternative method for recycling NMN into NAD (i.e. the PNC IV) has now been established in five microorganisms. Clostridium sticklandii (48), Azotobacter vinelandii (49), Propionibacterium shermanii (50), S. typhimurium (51) and E. coli (52) all possess the enzyme NMN deamidase, which catalyzes the amidohydrolysis of NMN, producing nicotinic acid mononucleotide. The nicotinic acid mononucleotide is then converted to NAD via the same steps used in the

Preiss-Handler pathway and de novo biosynthesis. This pyridine nucleotide cycle is therefore unique in that the cycle does not involve free nicotinamide or nicotinic acid. The majority of microorganisms analyzed possess both the PNC IV and the PNC VI, and one, A. vinelandii , appears to possess PNC IV, PNC V, and PNC VI (46). Investigations with E. coli have led to an estimate that of the NAD turnover in this organism, 72% is recycled through the PNC IV and 28% is through PNC VI (52). Similar studies with S. typhimurium produced estimates of between 60-69% of NAD turnover via PNC IV and 31-40% by PNC VI (53).

Investigations into the NAD metabolism of Haemophilus influenzae originated with the recognition by Davis in 1917 that two growth factors were required by "influenza bacilli" (54). In 1920, this observation was confirmed by Thjotta et al., and the two growth factors were named X- and V-factor (55). In 1937, the nature of V-factor was correctly described by Andre' and Marguerite Lwoff (56). In analyzing the properties of V-factor which had been extracted from yeast, a profile developed which was noticed to be very similar to that of "cozymase" (NAD). The Lwoffs then determined that the V-factor requirement could be met by using Warburg's coenzyme (NADP). In addition, a preparation of yeast cozymase was also able to function as V-factor.

These pyridine nucleotides were then chemically reduced and growth studies proved that NADPH and NADH were also acceptable replacements for V-factor. Further investigations showed that adenylic acid (AMP), nicotinic acid and nicotinamide were unable to function as V-factor. The Lwoffs correctly hypothesized that the V-factor-requiring Haemophilus organisms are unable to synthesize functional pyridine nucleotide coenzymes. They also postulated that this decrease in the ability to synthesize coenzymes represents a "physiological evolution," an idea that would be reiterated 40 years later by Gaertner and Shetty (33).

In the 1940's, Gingrich and Schlenck provided evidence that in addition to NAD and NADH serving as V-factor, nicotinamide riboside could serve as V-factor while nicotinamide, ribose and AMP did not (57,58). They suggested that the key biosynthetic step which Haemophilus influenzae cannot perform is the linkage of nicotinamide to ribose. This work also described, for the first time, the successful use of a NAD analog, nicotinamide hypoxanthine dinucleotide, as V-factor.

While investigating the synthesis of pyridine nucleotides by human erythrocytes from nicotinic acid, Leder and Handler found that NMN, in the presence of "limiting quantities" of NAD, served as V-factor for Haemophilus

parainfluenzae (59).

In an investigation into the phenomenon of satellitism, a compound excreted by a pseudomonad was seen to function as V-factor in several species of Haemophilus (60). In an effort to identify this compound, it was reconfirmed that NMN is a suitable replacement for V-factor. Characterization of the secreted growth factor showed that a carbohydrate, possibly ribose, was released on acid hydrolysis. The growth factor was also seen to be free of phosphate, and both before and after acid hydrolysis migrated differently from nicotinic acid, nicotinamide, nicotinamide riboside, NMN and NAD on paper chromatographic analysis. The authors concluded that the substance secreted by the pseudomonad which serves as V-factor is "a nicotinamide riboside with unknown substitutions."

Recent work by Albritton (122) has confirmed previous observations that Haemophilus influenzae does not possess functional de novo or Preiss-Handler pathways. Haemophilus influenzae , as well as other V-factor-requiring Haemophilus species were found to be incapable of growing with either quinolinic acid or nicotinic acid as V-factor.

In summary, in the absence of functional de novo or Preiss-Handler pathways, it is probable that the V-factor-requiring Haemophilus organisms obtain pyridine

nucleotides by either direct uptake of the dinucleotide or by hydrolysis of NAD to a transportable fragment, followed by internal resynthesis of the compound (i.e. a PNC). Evidence favors the latter of the two possibilities. First, the plasma membrane in gram-negative bacteria is considered to be impermeable to dinucleotides (61). It has been shown specifically in E. coli (62) and S. typhimurium (45) (both gram-negative organisms) that when these organisms possess mutations that disallow the use of both pyridine nucleotide cycles and de novo biosynthesis of NAD, they are unable to grow on intact NAD, so that diffusion of intact NAD into these cells is minimal. The well-documented ability of Haemophilus influenzae to substitute nicotinamide riboside or NMN as V-factor indicates that the organism must possess the biosynthetic pathway(s) required to produce NAD from these compounds.

In the majority of microorganisms investigated, initiation of pyridine nucleotide cycles occurs by cleavage of the pyrophosphate bond of NAD. The predominant enzyme catalyzing extracellular hydrolysis of NAD is a nucleotide (NAD) pyrophosphatase. This activity has been observed in several microorganisms (63-66). These enzymes generally have broad substrate specificities, pH optima of between pH 7-9, require divalent cations and are inhibited by EDTA and

5'-nucleotides.

The specific aim of the work that is presented was to acquire a more thorough understanding of the NAD metabolism of Haemophilus influenzae . A nucleotide pyrophosphatase, which we believe to be involved in the initial step(s) of NAD internalization, was purified and its properties investigated. At the cellular level, numerous studies of the growth of Haemophilus influenzae in the presence of various compounds that either act as V-factor, or as competitive inhibitors of V-factor, are presented. The growth of the organism was manipulated by the use of specific substrates and inhibitors of the nucleotide pyrophosphatase.

EXPERIMENTAL PROCEDURES

Materials

Haemophilus influenzae strain Rd was obtained from Dr. William L. Albritton of the University of Saskatchewan in Saskatoon. Brain Heart Infusion was obtained from Fisher Scientific. Reagent grade Tris(hydroxymethyl)aminomethane, hemin, histidine, streptomycin sulfate and lysozyme were purchased from the Sigma Chemical Company. Ethylenediaminetetracetic acid (EDTA), obtained as the tetrasodium salt, and alkyl glucosides were from Calbiochem. All nucleosides, mono- and dinucleotides were purchased from Sigma except 3-aminopyridine adenine dinucleotide (AAD), nicotinamide $1,N^6$-ethenoadenine dinucleotide, 3-aminopyridine $1,N^6$-ethenoadenine dinucleotide, pyridine adenine dinucleotide, 3-methylpyridine adenine dinucleotide, thionicotinamide adenine dinucleotide, 3-pyridylcarbinol adenine dinucleotide, 3-pyridylacetonitrile adenine dinucleotide and 4-aminopyridine adenine dinucleotide, which were prepared by published procedures (67-70). Nicotinamide and nicotinic acid were from Eastman Organic Chemicals. P11 cellulose phosphate was obtained from Whatman, Sephacryl S-200 from Sigma, and Matrex Green gel A and Matrex Blue gel A from Amicon. Dowex AG 1X-8 was purchased from Bio-Rad.

A from Amicon. Dowex AG 1X-8 was purchased from Bio-Rad. Acrylamide was also obtained from Bio-Rad. N,N'-Methylenebisacrylamide and Coomassie Brilliant Blue G-250 were purchased from Eastman Organic Chemicals. Coomassie Brilliant Blue R and N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Sigma while the ammonium persulfate was from Mallinckrodt. Sodium dodecyl sulfate (SDS) was from K and K Laboratories. All protein standards used were obtained from Sigma. 2,3-Butanedione and 2,4-pentanedione were purchased from the Aldrich Chemical Company. Woodward's Reagent K, iodoacetamide, N-ethylmaleimide and bis-(paranitrophenyl) phosphate (bis-PNPP) were obtained from Sigma.

Methods

Growth of the Organism on Liquid Media

Cells were grown in 750 ml of medium in 2.8 l Fernbach flasks at 37°C in a New Brunswick G-25R incubator with shaking at 120 cycles/min. The media were prepared by dissolving 28 g of solid Brain Heart Infusion in 750 ml of distilled water. This mixture was stirred with heating until all the solid material was dissolved. This solution was then autoclaved for 15 min at 120°C under 15 pounds per square inch of pressure. After autoclaving, the solution was cooled to room temperature and 7.5 ml of a NAD solution (300 ug/ml)

were added. A hemin-histidine solution (7.5 ml) was then added to the broth. The hemin-histidine solution was prepared by dissolving 10 mg each of hemin and histidine in 4.6 ml of distilled water. To this solution, 0.4 ml of triethanolamine was added and the mixture was placed in a water bath at 55°C for 10 min. After this time, the mixture was removed from the water bath and 5.0 ml of distilled water were added. Both the NAD and hemin-histidine solutions were added to the broth after filter sterilization by passing the solutions through a 0.2 micron Gelman Acrodisc disposable filter assembly. Streptomycin sulfate (450 mg) was added directly to the prepared medium. Growth was initiated by addition of a previously prepared innoculum. Innocula were prepared by taking 2.5-ml aliquots of the medium containing the bacteria grown to late log phase and adding them to 0.5 ml of glycerol. These fractions were frozen rapidly in a dry ice-ethanol bath and stored at -70°C until used to innoculate sterile media. For studies of the nucleotide pyrophosphatase, the cells were harvested by centrifugation at 18,000 x g for 10 min, washed twice in 50 mM Tris-HCl, pH 8.5, resuspended in a minimal amount of this buffer and stored at -15°C until used. Cells for the initial studies of the fate of extracellular NAD were prepared as follows. Freshly grown cells in 500 ml of brain heart

infusion broth were harvested in late linear phase by centrifugation at 18,000 x g for 10 min and washed twice by suspension in approximately 80 ml of 50 mM Tris-HCl, pH 9.0, followed by centrifugation at 18,000 x g for 10 min. The final pellet was resuspended in 5.0 ml of te buffer and the entire cell suspension was added to 1.0 ml of 5 mM magnesium chloride and enough solid NAD for a final NAD concentration of 2 mM. This mixture was incubated at 37°C and at timed intervals, 1.5 ml aliquots were removed and placed in a clinical centrifuge for several minutes at the highest speed setting. The supernatant solution was then filtered using a 0.2 micron Gelman Acrodisc disposable filter assembly. Fifty microliters of the filtrate were diluted with 200 µl of 100 mM potassium phosphate, pH 4.0, and analyzed by ion-exchange HPLC as described in Experimental Procedures. For studies of the NAD pyrophosphorylase, cells from 1.5 l of broth were harvested and washed as described above and were then disrupted by sonication using four 30-sec pulses. A soluble fraction was obtained by centrifugation at 100,000 x g for one hour at 4°C. In growth studies in which the ability of a compound to serve as V-factor was analyzed, 48.5 ml of sterile broth were placed in a 250 ml Erlenmeyer flask. To this solution the appropriate amount of nucleotide was added in a volume of one ml and growth was initiated by addition

of 0.5 ml of freshly grown cells in late log phase. The innocula in all of these experiments, except those where NMN was used as V-factor, contained 1 μg/ml NAD. In experiments with NMN as V-factor the innocula were grown in the presence of 1 μg/ml of NMN. In growth inhibition experiments, the flasks were prepared with 0.1 μg/ml NAD, the indicated amount of inhibitor and broth in a total volume of 50 ml. In all of the experiments, the organisms were grown at 37°C and shaken at 120 cycles/min.

Solubilization of the Nucleotide Pyrophosphatase with Detergents

Cells (1.5 g, wet weight) from 750 ml of liquid broth were harvested and washed twice in 50 mM Tris-HCl, pH 8.5. The cells were then resuspended in 5.0 ml of the same buffer and sonicated at 4°C using four 30-sec pulses. This material was then centrifuged for 10 min at 18,000 x g. The pellet was then resuspended in 5.0 ml of the buffer and one ml of this preparation was added to 0.11 ml of detergent and incubated for one hour at 4°C. The incubation mixture was then centrifuged for one hour at 100,000 x g. The supernatant was then assayed for activity using the yeast-alcohol dehydrogenase assay.

Lysozyme Digestion

Release of the nucleotide pyrophosphatase for

purification was accomplished by using a slight modification of the procedure of Malamy and Horecker (71). Cells (24 g, wet weight) from 6 l of broth that had been prepared and frozen as described in Experimental Procedures were thawed and suspended in one liter of 33 mM Tris-HCl, pH 8.0, 20% sucrose. Eighty-four ml of 100 mM EDTA, pH 8.0, was added followed by addition of 3.6 mg of lysozyme (48,000 U/mg). This mixture was then placed in a water bath at 37 C for one hour. After the incubation, the solution was centrifuged at 19,000 x g for 15 min. The supernatants were pooled and used for purification of the nucleotide pyrophosphatase.

The Yeast Alcohol Dehydrogenase Assay

This assay was used in early investigations concerning the location and properties of the enzyme and was typically used with crude and partially-purified preparations from Haemophilus influenzae . The assay used was based on the procedure of Kornberg (72) and can be used to determine the presence of several different hydrolytic activities towards NAD. The assay is based on the fact that NADH possesses an absorption maximum at 340 nm. By monitoring the absorbance at this wavelength of a sample in the presence of excess ethanol, both before and after the addition of the yeast alcohol dehydrogenase (ADH), the difference in absorbance can be related to the amount of NAD present in the sample.

Typically an incubation mixture containing 2.5 mM Tris-HCl, pH 8.5, 2 mM NAD, 1 mM magnesium chloride, and the sample to be assayed in a total volume of 1.0 ml was prepared and incubated at 37°C. At timed intervals, an aliquot was removed from the mixture and the reaction was stopped by adding it to trichloroacetic acid (TCA) to give a final concentration of 6.7% TCA (w/v). The precipitated protein was then removed by centrifugation in a clinical centrifuge set at the highest speed setting for two minutes. A 0.1-ml sample of the supernatant was then added to a cuvette containing 0.88 ml of a reagent which contained 90 mM unbuffered Tris and 2.85% ethanol (v/v). The absorbance at 340 nm was determined and then 20 µl of yeast-ADH (10 mg/ml, 365 U/mg in 60 mM potassium phosphate, pH 7.5) were added and the absorbance was redetermined. The difference in the two readings can be equated to the amount of NAD present using the extinction coefficient for NADH, 6.25 O.D./mM (73). In this manner, the time-dependent loss of NAD in the presence of the nucleotide pyrophosphatase was observed. All spectrophotometric measurements were made using a Beckman Acta MVI spectrophotometer.

The Fluorimetric Assay

A fluorimetric assay was developed to be used as a rapid, sensitive technique for the assay of column fractions

during the purification of the nucleotide pyrophosphatase. The assay was based on the observation that on total hydrolysis of flavin adenine dinucleotide (FAD), a seven-fold increase in fluorescence intensity is produced when monitored at an excitation wavelength of 465 nm and an emission wavelength of 510 nm. All fluorescence measurements were made using a Perkin-Elmer 650-40 fluorescence spectrophotometer. The reaction mixtures contained 25 mM Tris-HCl, pH 8.5, 1 mM magnesium chloride, 300 nm FAD, and enzyme in a total volume of 3.0 ml. Reactions were conducted at room temperature.

The Titrimetric Assay

The titrimetric assay was used in all studies involved in the characterization of the purified enzyme. The assay is based on the fact that on cleavage of diesterified pyrophosphate moieties, two protons are released and two equivalents of base are therefore needed to maintain a constant pH. By monitoring the rate of base addition performed by the titrimeter, a direct assay of the initial velocity of the reaction is produced. All measurements of this kind were made using a Radiometer Copenhagen PHM82 pH meter equipped with a GK2320C combination electrode, TTT80 titrator, and ABU80 autoburette using a burette volume of 0.25 ml. Fresh titrant was prepared every day at a

concentration of 0.5 mM NaOH. After each day, the pH of the titrant was determined and used to calculate the exact concentration of base that had been used. The autoburette was set so that titrant would be delivered at maximum speed. The end point for all titrations, except those involved in the investigation of the effect of pH on activity, was 8.0. All reaction mixtures were placed in a vessel that allowed for continuous stirring over the course of the reaction with a magnetic stirrer. The vessel was placed in a water jacket connected to a Haake FE constant temperature circulator which maintained the temperature of the incubation mixture at 37°C. Reaction mixtures contained 50 mM potassium chloride, substrate and enzyme in a total volume of 3.0 ml. The pH of all solutions containing mono- and dinucleotides was adjusted to neutrality before using them. The starting pH of all reaction mixtures was adjusted to 8.0 prior to addition of the enzyme by manual addition of dilute sodium hydroxide. All reactions were initiated by the addition of enzyme to the reaction mixture. Under the conditions employed, the reaction rates were linear over the 2-3 minute period used to calculate the initial velocities.

The NAD Pyrophosphorylase Assay

The assay for NAD pyrophosphorylase was based on the yeast-ADH assay described earlier. Incubation mixtures

contained 100 mM Tris-HCl, pH 7.5, 15 mM magnesium chloride, 5 mM ATP, 2.5 mM NMN and enzyme in a total volume of 1.0 ml at 37°C. At timed intervals, aliquots were removed and the reaction was stopped with TCA as described earlier. The supernatant of this material was then analyzed for NAD by the yeast-ADH assay.

The Cyclic Phosphodiesterase Assay

Phosphodiesterase activity was monitored using a spectrophotometric assay which is based on the absorption maximum at 405 nm exhibited by the paranitrophenolate anion. Reaction mixtures contained 80 mM Tris-HCl, pH 8.0, 200 μM bis-PNPP and enzyme in a total volume of 1.0 ml at room temperature.

The Assay of Marker Enzymes

Enzymic markers for the cytoplasm (glutathione reductase), the inner membrane (succinate dehydrogenase) and periplasmic space (2',3'-cyclic phosphodiesterase) were assayed according to published procedures (74,75,137).

Protein Determination

The quantity of protein in various samples was determined by the method of Bradford (76) with crystalline bovine serum albumin (BSA) used as a standard.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed

according to Weber and Osborn (77). Samples were prepared for electrophoresis by adjusting them to 2% (v/v) SDS and 1.4 M beta-mercaptoethanol. These samples were then placed in a water bath at 100°C for 10 min. Electrophoresis was carried out at 8 mA/gel at 4°C for two hours. Proteins were visualized by placing the gels in a staining solution of 0.4% (w/v) Coomassie Brilliant Blue R in 50% (v/v) methanol, 9.2% acetic acid for two hours. The gels were destained by placing them in a bath of 50% methanol, 9.2% acetic acid for ten hours, then 5% methanol, 7.5% acetic acid for ten hours and finally in 7.5% acetic acid for ten hours.

Nicotinamide Riboside Synthesis

Nicotinamide riboside (NR) was prepared from NMN by incubating 10 mg of NMN with 20 mg of wheat germ acid phosphatase (0.36 U/mg) in 10 ml of 5 mM sodium acetate, pH 5.0 for 30 hours at room temperature with constant stirring. After this time, the incubation mixture was applied to a column (1.2 x 8.0 cm) of Dowex AG 1 X8 ion exchange resin and the NR was eluted from the column with distilled water. The compound which eluted from the column was identified as NR by thin-layer chromatography (78). Eastman 6064 cellulose chromatographic plates without fluorescence indicator were used and the solvent was one part of 10.7 g ammonium chloride, 0.69 g citric acid, 256 g sodium citrate in 100 ml

water to three parts 95% ethanol. The concentration of NR was determined by the cyanide addition assay (79).

High Performance Liquid Chromatography (HPLC)

Initial studies of the extracellular hydrolysis of NAD were conducted with analysis of the extracellular contents by ion-exchange HPLC. The column (4.6 x 250 mm) was packed with Alltech RSIL-AN resin, 5 micron particle size, and was equilibrated in 100 mM potassium phosphate, pH 4.0.

Product analysis was carried out by using reverse phase (ion-pair) chromatography. The column (4.6 x 250 mm) was packed with Alltech RSIL-C18-HL resin with a particle size of five microns. The column was equilibrated in buffer containing 35 mM potassium phosphate, 2.8 mM tetrapropylammonium hydroxide and 30% (v/v) methanol adjusted to a pH of 5.5. The column was maintained at 50°C and the flow rate was 0.75 ml/min. Both the ion-exchange and reverse phase chromatographic analyses were done using a Spectra-Physics SP8000 chromatograph equipped with a Spectra-Physics model 770 variable wavelength detector. Absorbance of the column eluant was monitored at 260 nm. Compounds were identified by comparing their retention times to those of standards run under identical conditions.

Gel filtration HPLC was done using a Bio-Rad TSK-250 column that was 7.5 x 300 mm. The column was used in a

Spectra-Physics SP3500B liquid chromatograph with a flow rate of 1.0 ml/min. The column was maintained at 33°C. The elution buffer was 0.1 M sodium sulfate. A Spectra-Physics model 770 detector was used to detect proteins eluting from the column. Absorbance of the eluant was monitored at 233 nm.

Amino Acid Analysis

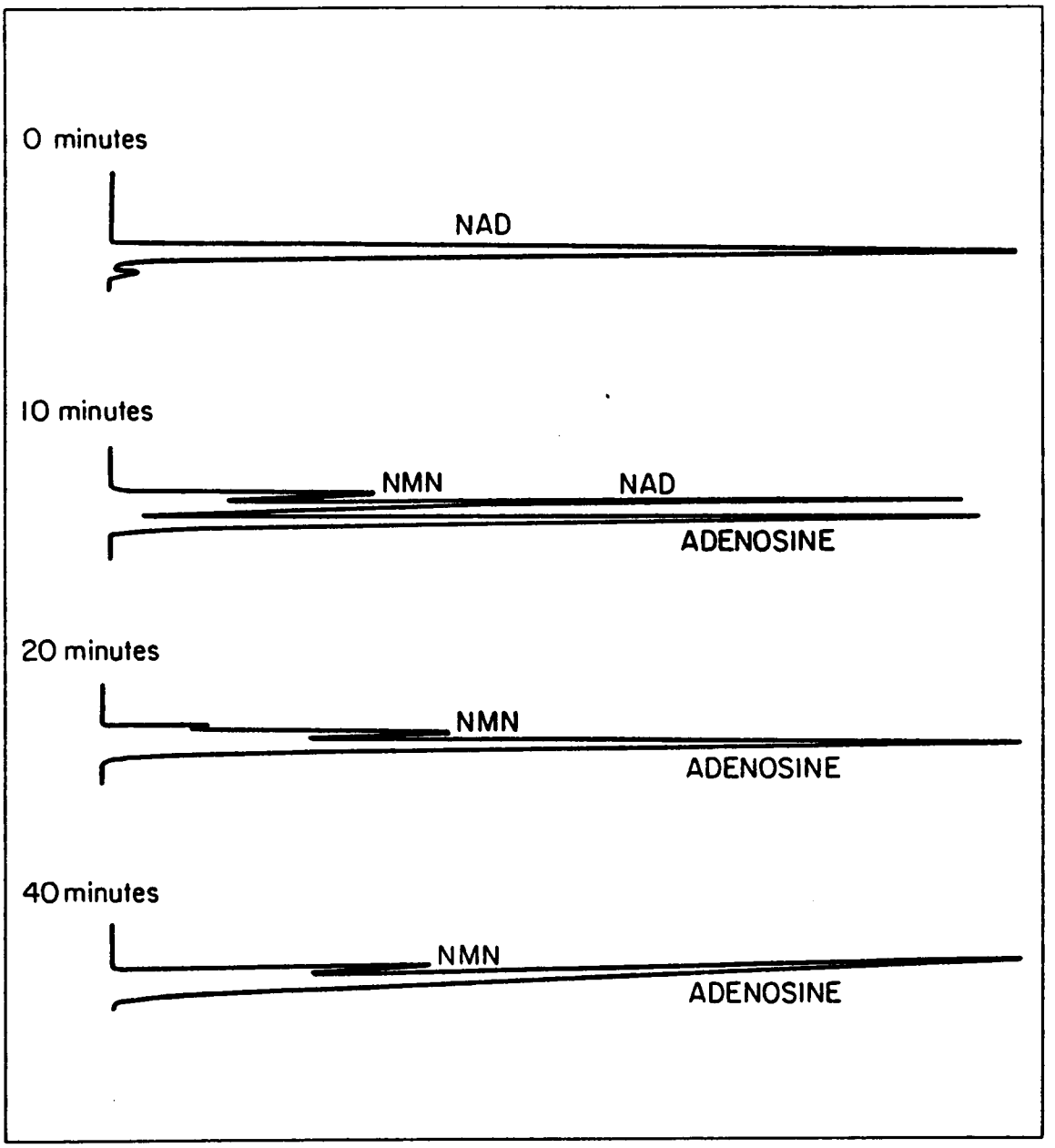
Samples were prepared for amino acid analysis by acid hydrolysis in 6 N HCl in sealed, evacuated tubes. Analyses were performed using a Beckman HPLC amino acid analyzer equipped with a sodium cation exchange column which was obtained from Pickering. The column was eluted first with 0.2 N sodium citrate buffer, pH 3.28 and then with sodium eluent from Pickering. Post-column detection of amino acids was done by reacting the eluent with ninhydrin, and the eluent was monitored at 440 and 570 nm. Amino acids were identified by comparing the retention times of peaks from the protein hydrolysate to those of amino acid standards.

RESULTS

Demonstration of an Externally-directed Nucleotide Pyrophosphatase Activity

The pyrophosphatase activity was initially demonstrated using incubations of intact cells of Haemophilus influenzae with NAD which were prepared as described in Experimental Procedures. As seen in Figure 5, the Haemophilus influenzae cells degraded the NAD with time. After 20 min, no NAD could be detected in the external medium. Two products appeared with time that had retention times identical to NMN and adenosine. The production of adenosine, rather than AMP, from cleavage of the pyrophosphate bond of NAD was shown to be the result of cleavage of the 5'-phosphate ester as catalyzed by either the Haemophilus influenzae alkaline phosphatase or 5'-nucleotidase. Since AMP and NAD comigrate in the HPLC procedure used, transient formation of AMP was not detected. However, similar studies conducted with ADPR demonstrated that after 10 min, 23% of the 260 nm-absorbing material resided in a peak corresponding to AMP. After 40 min, 80% of the 260 nm-absorbing material occurred in the peak corresponding to adenosine, while less than 2% was associated with AMP. Hydrolysis of NAD was confirmed by analysis of the filtrate using the yeast-ADH assay described

Figure 5. The hydrolysis of NAD by cells of Haemophilus influenzae. Incubation mixtures were constructed as described in Results.


0 minutes
NAD
10 minutes
NMN
NAD
ADENOSINE
20 minutes
NMN
ADENOSINE
40 minutes
NMN
ADENOSINE

earlier. Due to the apparent lack of nicotinamide and/or ADPR production, it was concluded that Haemophilus influenzae does not possess an externally directed glycohydrolase activity.

The predicted stoichiometry from cleavage of the pyrophosphate bond of NAD (one mole of NAD hydrolyzed producing one mole of NMN and adenosine) can be compared with the experimentally determined stoichiometry obtained by calculating the area under each of the peaks from the chromatogram. Due to the rapid hydrolysis of NAD by the cell suspension, a zero time sample was prepared by exactly the same procedure as was previously described with buffer, rather than cell suspension, added to the NAD. Using this procedure, it was determined that the area of the NAD peak at 0 time was 85 units. Using the extinction coefficients for NAD and adenosine (73), the predicted area of the adenosine peak on total hydrolysis of the NAD would be equal to the product of the ratio of extinction coefficients (Adenosine/NAD), multiplied by 85.0. This calculation, done for adenosine, gives a value of 72.7. As seen in Table II, this predicted value is in good agreement with the value observed at 20 min, when the NAD was apparently 100% hydrolyzed. A similar calculation can be made for the NMN. The predicted area of the NMN peak on total hydrolysis of

TABLE II

Hydrolysis of NAD by
Cells of Haemophilus influenzae

Time	Peak Area		
	NAD	NMN	Adenosine
min		units	
0	85.0	0.0	0.0
10	35.8	14.1	37.8
20	0.0	16.8	75.6
40	0.0	12.4	76.7

the NAD would be 26.0. At 20 min, the area of the NMN peak is 65% that of the predicted value and, unlike the area of the adenosine peak which remains relatively constant from 20-40 min, the area of the NMN peak decreases to 48% of the predicted value. These data suggest that NAD external to the cell is entirely degraded by an externally-directed enzyme as shown by the time dependent elimination of NAD and the recovery of the predicted amount of adenosine. Similar studies conducted with ADPR demonstrate the transient production of AMP followed by hydrolysis to adenosine. It therefore appears that Haemophilus influenzae possesses an active 5'-nucleotidase or alkaline phosphatase which converts the AMP produced from hydrolysis of the pyrophosphate bond of NAD or ADPR to adenosine. Neu (87) has demonstrated 5'-nucleotidase activity in Haemophilus influenzae which readily hydrolyzes AMP. This demonstration of pyrophosphatase activity led to efforts to try to extract and purify this enzyme for further characterization.

Release of the Nucleotide Pyrophosphatase Activity

Preliminary experiments designed to produce the nucleotide pyrophosphatase in a soluble form were conducted. It was found that sonication of cells released only limited quantities of the enzyme, as the majority (>90%) of the activity remained in the pellet after low speed

centrifugation. In addition, the osmotic shock procedure of Neu and Heppel (80), used in an attempt to release periplasmic contents, also resulted in limited release of the enzyme. Non-ionic detergents, specifically alkyl-glucosides, were seen to be effective at solubilization of the nucleotide pyrophosphatase. As seen in Table III, initial experiments to screen various alkyl chain lengths revealed that octyl-glucoside was particularly effective at solubilizing the enzyme. Further work, shown in Table IV, demonstrated that the optimal concentration of octyl-glucoside for release of the enzyme was 2.5% (w/v).

It was also determined that the nucleotide pyrophosphatase is released into a soluble form during the conversion of cells of Haemophilus influenzae to spheroplasts by incubation with lysozyme and ethylenediaminetetraacetic acid (EDTA). It has been shown in other laboratories that EDTA alone is capable of disrupting the outer membrane of E. coli (81) and Haemophilus parainfluenzae (82) with the subsequent release of outer membrane components. As can be seen in Table V, release of the nucleotide pyrophosphatase could not be accomplished with EDTA alone and was dependent on the additional presence of lysozyme. The release of the enzyme was proportional to the concentration of EDTA present, and was similar to the

TABLE III

Solubilization of the Nucleotide Pyrophosphatase with Detergents

Detergent	Concentration	Activity	
		Supernatant	Pellet
	%	U/ml	
Hexyl-glucoside	2.2	7.4	32.4
Octyl-glucoside	2.5	40.5	5.1
Decyl-glucoside	0.4	5.5	36.0
Triton X-100	0.5	0.9	49.3
Control	0.0	0.9	37.0

TABLE IV

Solubilization of the Nucleotide Pyrophosphatase with Octyl-glucoside

Concentration	Activity	
	Soluble	Membrane
%	U/ml	
1.0	4.2	43.2
1.6	25.0	16.5
2.2	33.5	11.9
2.5	40.5	5.1
2.8	40.0	8.3

TABLE V

Solubilization of the Nucleotide Pyrophosphatase with Lysozyme and EDTA

Addition	% Activity Released		
	SDH	Cyclic Phosphodiesterase	Nucleotide Pyrophosphatase
No Lysozyme			
1.0 mM EDTA	0.0	0.0	0.0
15 µg/ml Lysozyme			
0.5 mM EDTA	0.0	7.2	8.3
1.0 mM "	0.0	11.1	10.9
4.0 mM "	0.2	16.2	16.3
7.0 mM "	0.2	15.9	27.5

release of the periplasmic marker, cyclic 2',3'-phosphodiesterase. Under the conditions employed, very little of the inner membrane marker, succinate dehydrogenase, was released. In a second experiment, it was determined that approximately 6% of the total glutathione reductase activity was released simultaneous to the release of 18% of the nucleotide pyrophosphatase. These results are consistent with earlier reports estimating that 10% of this cytoplasmic marker were released using similar conditions (75).

Purification of the Nucleotide Pyrophosphatase

Purification was initiated by preparing spheroplasts of frozen *Haemophilus influenzae* cells using the procedure of Malamy and Horecker (73) with slight modifications as described in Experimental Procedures.

Ammonium Sulfate Precipitation

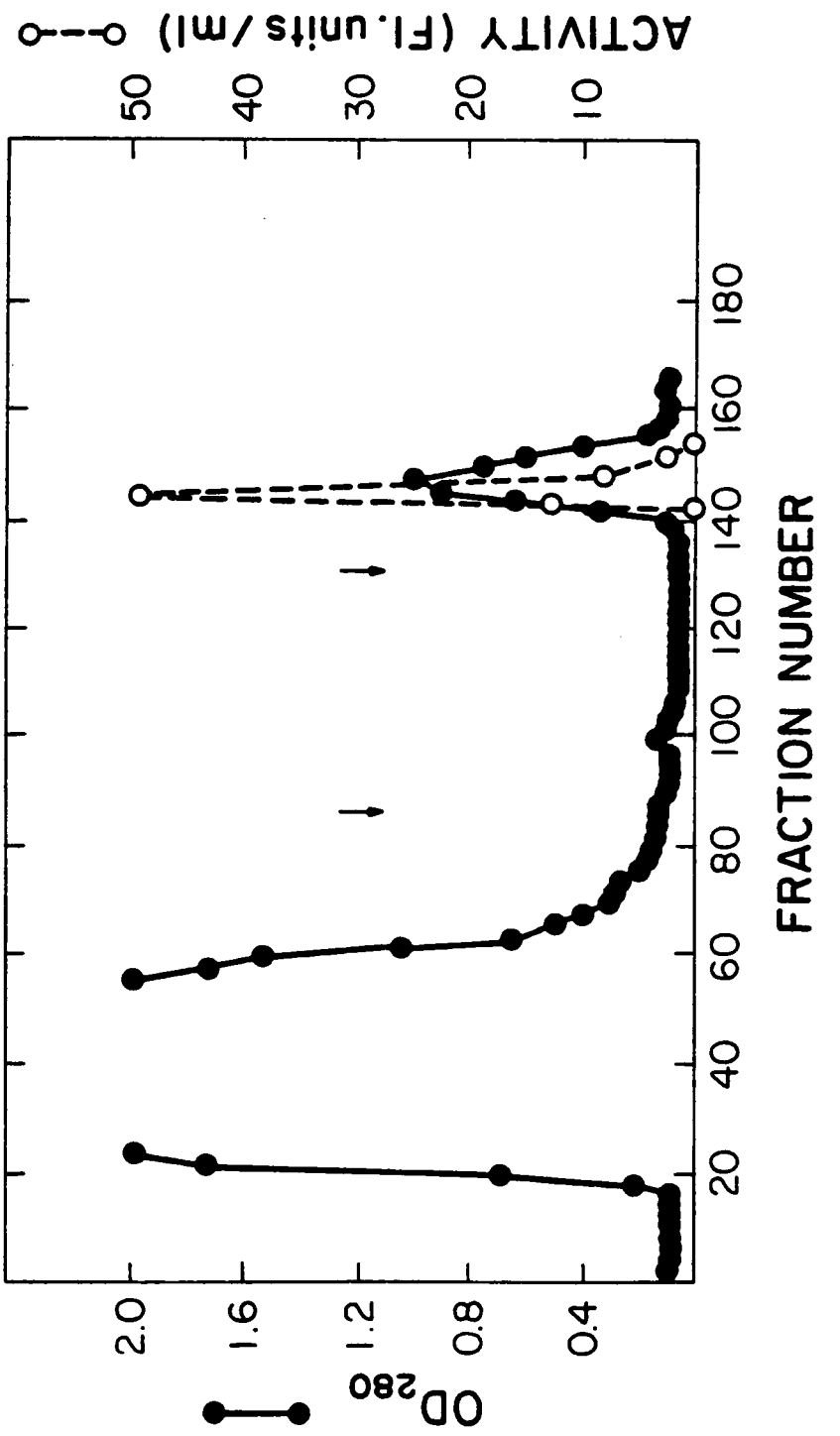
The soluble fraction from the lysozyme digestion was collected by centrifugation of the mixture for 10 min at 19,000 x g. The supernatant was dialyzed overnight against 2 x 4 l of 50 mM Tris-HCl, pH 8.5. The dialysate was then adjusted to 45% saturation of ammonium sulfate by slowly adding the solid salt with continuous stirring at 4°C. This solution was stirred for 15 min and centrifuged for 10 min at 19,000 x g. The supernatants were pooled and adjusted to

65% saturation of ammonium sulfate in a manner similar to that previously described. This solution was stirred for 15 min and was centrifuged for 10 min at 19,000 x g. The supernatants were discarded and the precipitated protein was resuspended in approximately 60 ml of 50 mM Tris-HCl, pH 8.5. This solution was dialyzed overnight against 2 l of 50mM Tris-HCl, pH 8.5, at 4°C.

Phosphocellulose Ion-Exchange Chromatography

The dialysate from above was applied to a column (1.5 x 30 cm) of phosphocellulose that was equilibrated in 50 mM Tris-HCl, pH 8.5. Two column volumes of the equilibration buffer were passed through the column to remove unbound protein. One column volume of 0.2 M NaF in equilibration buffer was then washed through the column in an effort to elute alkaline phosphatase activity from the column. The nucleotide pyrophosphatase was eluted from the column using a linear gradient from 200-1000 mM KCl in the equilibration buffer which was achieved by placing 100 ml of the equilibration buffer with 200 mM KCl in the mixing chamber and 100 ml of equilibration buffer with 1 M KCl in the reservoir of a linear gradient-forming apparatus. Fractions (2.5 ml) were collected and those fractions which contained activity (see Figure 6), determined by the fluorescent assay described in Experimental Procedures, were pooled and

Figure 6. Phosphocelluslose ion-exchange chromatography. The dialyzed, resuspended pellet obtained from the ammonium sulfate precipitaion step was applied to a column of phosphocellulose that had been equilibrated in 50 mM Tris-HCl, pH 8.5. At the first arrow, the eluting buffer used was 50 mM Tris-HCl, pH 8.5 containing 200 mM sodium fluoride. At the second arrow, a linear gradient from 200-1000 mM potassium chloride in equilibrating buffer (2 x 100 ml) was applied. Both the $O.D._{280}$ (●——●) and the enzyme activity (o--o), determined fluorimetrically and reported in fluorescence units/ml are shown.


ACTIVITY (Fl. units/ml)
50
40
30
20
10
OD280
2.0
1.6
1.2
0.8
0.4
20
40
60
80
100
120
140
160
180
FRACTION NUMBER

dialyzed overnight against 2 l of 50 mM Tris-HCl, pH 8.5.

Matrex Green Gel A Affinity Chromatography

The dialysate from above was applied to a column (1.2 x 8.0 cm) of Matrex Green gel A that was equilibrated in 50 mM Tris-HCl, pH 8.5. Two column volumes of equilibration buffer were passed through the column to remove unbound protein. The enzyme was then eluted using a linear gradient of 0-500 mM KCl in the equilibration buffer which was achieved by placing 100 ml of the equilibration buffer in the mixing chamber and 100 ml of 500 mM KCl in equilibration buffer in the reservoir of a linear gradient-forming apparatus. Fractions (2.5 ml) were collected and fractions which contained enzyme activity were pooled and used for enzyme studies (see Figure 7). This preparation rapidly hydrolyzed bis-(paranitrophenyl) phosphate (bis-PNPP), a substrate most frequently used to assay phosphodiesterase activities. Experiments were conducted that clearly demonstrated that the FAD-hydrolyzing activity, observed fluorimetrically, and the bis-PNPP-hydrolyzing activity were the result of two different proteins based on the thermolability of the two activities as shown in Figure 8. An additional step, Matrex Blue gel A affinity chromatography, was therefore added to the purification scheme which resulted in the separation of the two activities. Experiments involving the

Figure 7. Matrex Green Gel A affinity chromatography. The dialyzed pool of activity from the phosphocellulose chromatography step was applied to a column of Matrex Green Gel A that had been equilibrated in 50 mM Tris-HCl, pH 8.5. The position of the arrow in this figure indicates the point of initiation of a linear gradient from 0-500 mM potassium chloride in the equilibration buffer which was used to elute the enzyme from the column. The $O.D._{280}$ (•——•) and the enzyme activity (o--o), determined fluorimetrically and reported as fluorescence units/ml are shown.

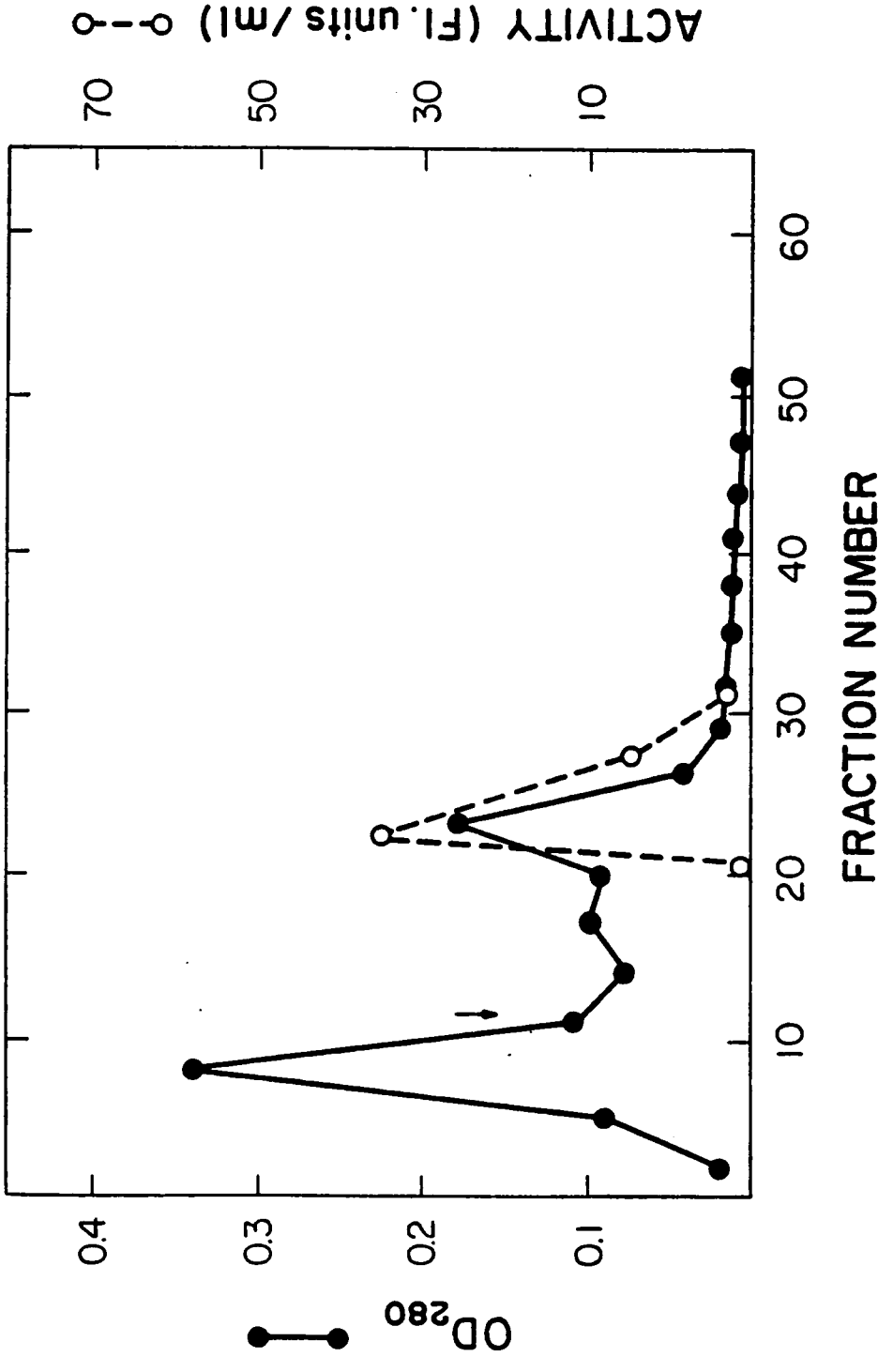
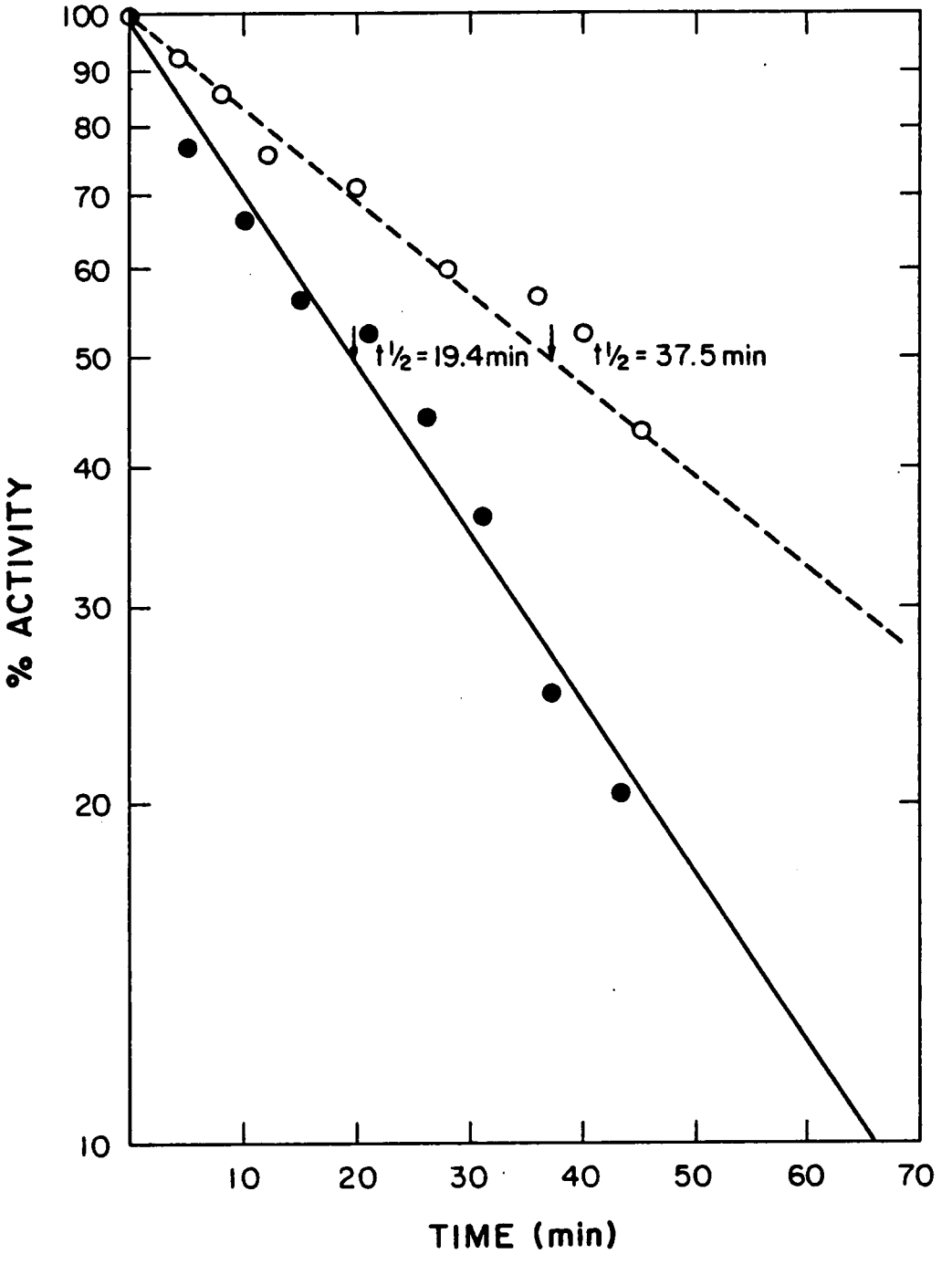

ACTIVITY (Fl. units /ml) o--o
70
50
30
10
OD280
0.4
0.3
0.2
0.1
10
20
30
40
50
60
FRACTION NUMBER
a
b

Figure 8. The thermal denaturation of the nucleotide pyrophosphatase and 2',3'-cyclic phosphodiesterase activities. Two ug of the partially purified nucleotide pyrophosphatase was added to 50 mM Tris-HCl, pH 8.5 in a total volume of 0.5 ml. This solution was incubated at 45°C, and at timed intervals, aliquots were removed and assayed for the nucleotide pyrophosphatase activity (•——•) or the 2',3'-cyclic phosphodiesterase activity (o– –o) using the assay procedures described in Experimental Procedures.


100
90
80
70
60
50
40
30
20
10
% ACTIVITY
t½ = 19.4 min
t½ = 37.5 min
10
20
30
40
50
60
70
TIME (min)

characterization of the phosphodiesterase activity are discussed in a later section. For further purification of the nucleotide pyrophosphatase, the pooled fractions from the Matrex Green gel A step were dialyzed overnight in 2 l of 50 mM Tris-HCl, pH 8.0.

Matrex Blue Gel A Affinity Chromatography

The dialyzed fractions from the Matrex Green gel A step were applied to a column (1.2 x 8.0 cm) of Matrex Blue gel A equilibrated in 50 mM Tris-HCl, pH 8.0. Two column volumes of the equilibration buffer were passed through the column to remove unbound protein. The enzyme was then eluted from the column using a linear gradient of 0-1 M KCl in the equilibration buffer, achieved by mixing 100 ml equilibration buffer with 100 ml equilibration buffer containing 1 M KCl in a gradient-forming apparatus (see Figure 9). Phosphodiesterase activity was seperated from the nucleotide pyrophosphatase and eluted in fractions 46 to 56. Fractions containing nucleotide pyrophosphatase activity were pooled and dialyzed against 4 l of 200 mM KCl twice, in preparation for titrimetric analyses of the characteristics of the enzyme. The pH of the dialysis solution was adjusted to pH 8.0 by the gradual addition of a dilute NaOH solution. Using this procedure, the enzyme was purified 700-fold with a 24% recovery of the initial activity as shown in Table VI.

Figure 9. Matrex Blue Gel A affinity chromatography. The dialyzed pool of activity from the Matrex Green Gel A column was applied to a column of Matrex Blue Gel A equilibrated in 50 mM Tris-HCl, pH 8.0. The position of the arrow indicates the point of initiation of a linear gradient from 0-1 M potassium chloride in the equilibration buffer which was used to elute the nucleotide pyrophosphatase from the column. The $O.D._{280}$ (●——●) and the enzyme activity (o- -o), determined fluorimetrically and reported as fluorescence units/ml are shown.

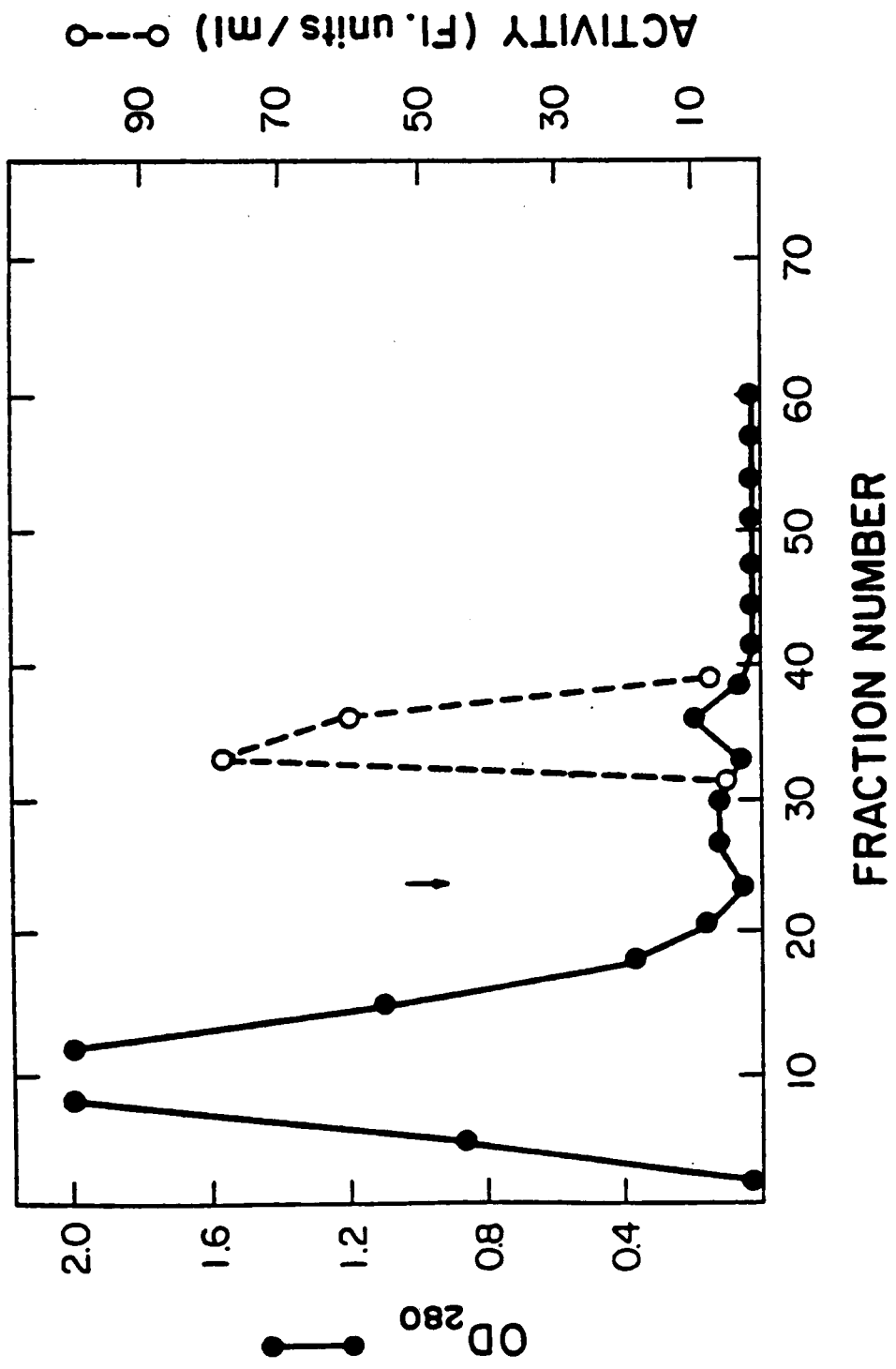

ACTIVITY (Fl. units / ml) o---o
90
70
50
30
10
OD280
2.0
1.6
1.2
0.8
0.4
10
20
30
40
50
60
70
FRACTION NUMBER

TABLE VI

Purification of the Haemophilus influenzae Nucleotide Pyrophosphatase

Fraction	Total Protein	Total Activity	Specific Activity	Yield	Purification
	mg	Units	U/mg	%	-fold
1. Lysozyme digest	731.4	3823	5	100	-
2. Ammonium sulfate	282.9	1814	6	47	1
3. Phosphocellulose	15.5	1694	109	44	21
4. Matrex Green gel A	1.0	1007	1007	26	201
5. Matrex Blue gel A	0.3	928	3500	24	700

Properties of the Purified Enzyme

Estimation of Purity

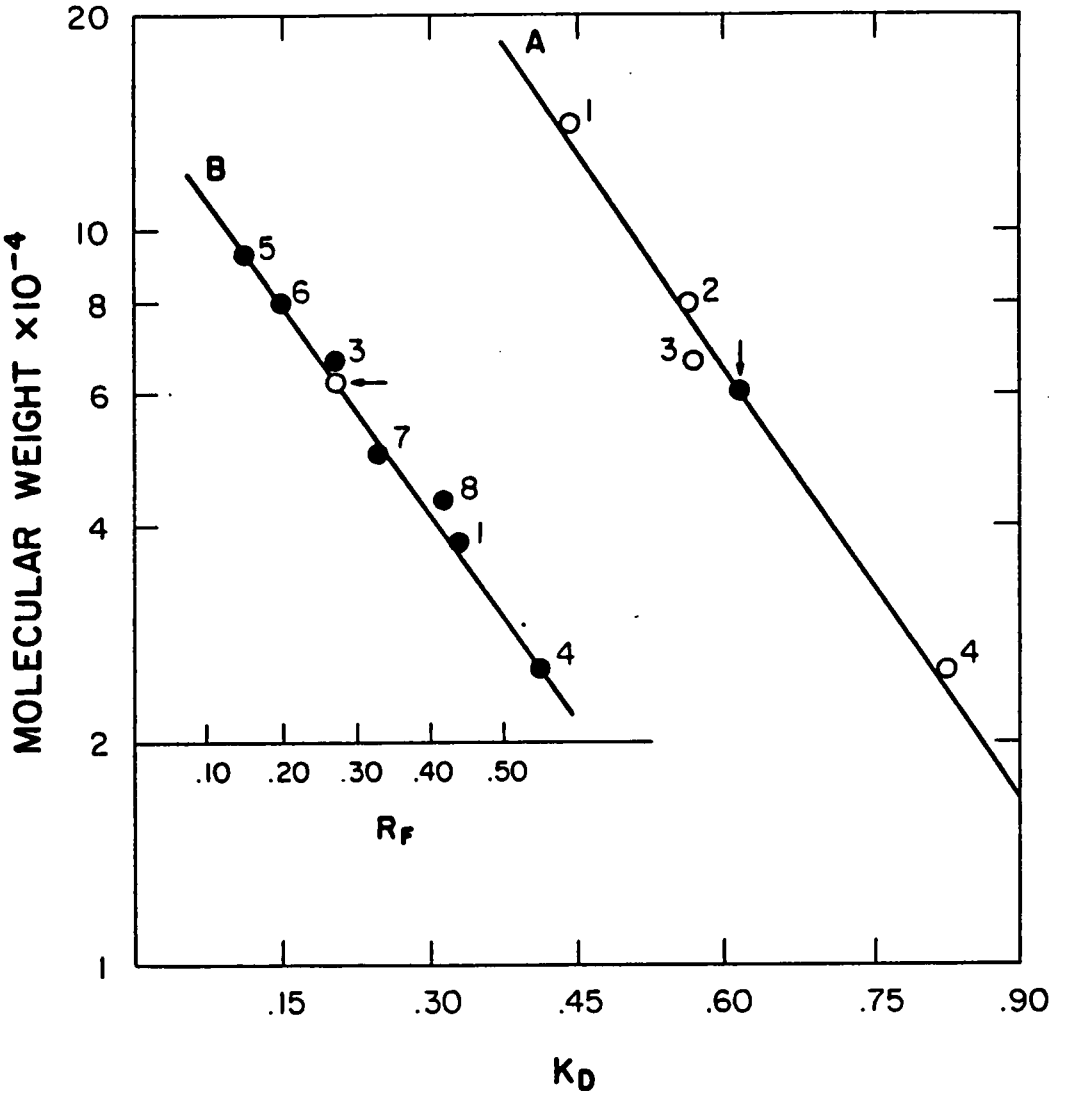
The purified enzyme migrated as a single band when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein was visualized on the gel by staining with Coomassie Brilliant Blue R as described in Experimental Procedures.

Molecular Weight Determination

The native molecular weight of the enzyme was determined using a column (1.5 x 80 cm) of Sephacryl S-200 equilibrated in 50 mM Tris-HCl, pH 8.0, 200 mM KCl. The column was calibrated using standard proteins of known molecular weight as shown in Figure 10. The molecular weight of the nucleotide pyrophosphatase was determined by comparing the partition coefficient, K_D , for the enzyme with those of the standard proteins. The elution volumes of the proteins were determined either by assay of enzyme activity (YADH, Horse liver ADH) or by observing the absorbance of the fractions at 280 nm (chymotrypsinogen, BSA). The average K_D value for the nucleotide pyrophosphatase from two experiments was 0.61, which extrapolated to an apparent M_r =62,500.

The molecular weight was also determined under the denaturing conditions of sodium dodecyl sulfate

Figure 10. Molecular weight determination of the nucleotide pyrophosphatase. A. Gel filtration on a Sephacryl S-200 column equilibrated with 200 mM potassium chloride, 50 mM Tris-HCL, pH 8.0. Molecular weight standards were (1) yeast-alcohol dehydrogenase, (2) horse liver-alcohol dehydrogenase (3) bovine serum albumin and (4) alpha-chymotrysinogen A. B.Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Molecular weight standards were (1) yeast-alcohol dehydrogenase,(3) bovine serum albumin,(4) alpha-chymotrypsinogen A,(5) rabbit muscle phosphorylase a,(6) glucose oxidase, (7) heavy chain-human IgG and (8) ovalbumin. The arrows in A and B indicate the positions of the Haemophilus influenzae nucleotide pyrophosphatase when analyzed by these procedures.


MOLECULAR WEIGHT X10⁻⁴
20
10
8
6
4
2
1
A
B
1
2
3
4
5
6
7
8
.15
.30
.45
.60
.75
.90
K_D
.10
.20
.30
.40
.50
R_F

polyacrylamide gel electrophoresis. The molecular weight was estimated by comparison of the nucleotide pyrophosphatase relative mobility (R_f) to that of several protein standards. Using this method, the average denatured molecular weight of the enzyme was determined to be 65,800 as shown in Figure 10. The nucleotide pyrophosphatase therefore appears to consist of a single polypeptide chain with an approximate M_r =64,000.

Amino Acid Analysis

The amino acid composition of the purified nucleotide pyrophosphatase is shown in Table VII. The molecular weight of the enzyme, based on the amino acid analysis, was equal to 61,600.

Carbohydrate Content

Samples of the purified nucleotide pyrophosphatase were analyzed for carbohydrate content by a modified phenol-sulfuric acid procedure as described by Lee and Montgomery (86). Beta-D-(+) glucose was used as a standard for construction of a standard curve. The enzyme was observed to contain 16% carbohydrate by weight.

Spectral Properties

The purified enzyme produced a typical ultraviolet-visible absorption spectrum with a single absorption maximum at 275 nm. The absorption of a 1% (w/v)

TABLE VII

Amino Acid Analysis

Amino Acid	moles/mole enzyme
Lysine	44
Arginine	13
Histidine	14
Aspartate	72
Glutamate	58
Serine	40
Threonine	34
Proline	24
Cysteic acid	1
Methionine	10
Glycine	49
Alanine	50
Valine	41
Leucine	47
Isoleucine	30
Tyrosine	20
Phenylalanine	22
	569

solution of the protein at 280 nm was calculated to be 47.8.

The fluorescence spectrum of the purified enzyme is shown in Figure 11. The enzyme was seen to possess an excitation maximum at 286 nm and an emission maximum at 337 nm. This fluorescence profile is characteristic of the presence of tryptophan residues in proteins.

Product Analysis

The activity of the purified enzyme was confirmed to be that of a nucleotide pyrophosphatase by product analysis. Chromatographs obtained from ion-pair, reverse phase HPLC analysis are shown in Figure 12. An aliquot of the purified enzyme was incubated with FAD at a final concentration of 2 mM as described in Figure 12. Hydrolysis of the FAD was observed by HPLC analysis and was confirmed by monitoring the increase in fluorescence produced on hydrolysis in a manner similar to the fluorimetric assay described in Experimental Procedures. Flavin mononucleotide (FMN) and AMP were observed to be the sole products of the FAD breakdown. These products arise as the result of cleavage of FAD at the pyrophosphate bond.

Thermostability

The purified enzyme in 0.2 M KCl was stable at -10^{o}C for one month, while the activity gradually decreased thereafter. Experiments were conducted to determine the

Figure 11. The fluorescence spectrum of the nucleotide pyrophosphatase. The excitation spectrum was determined with an emission wavelength of 337 nm and the emission spectrum was determined at an excitation wavelength of 286 nm.

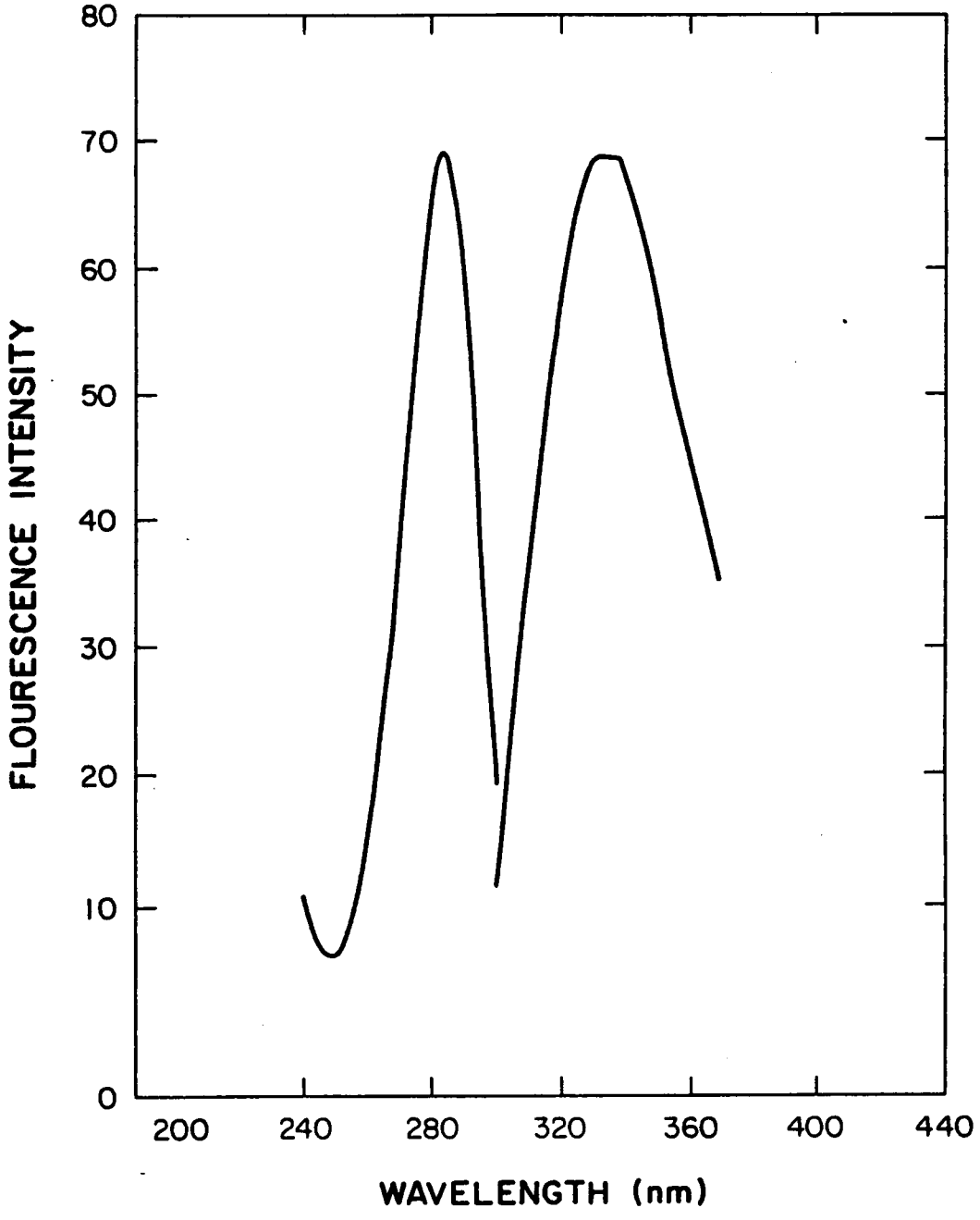
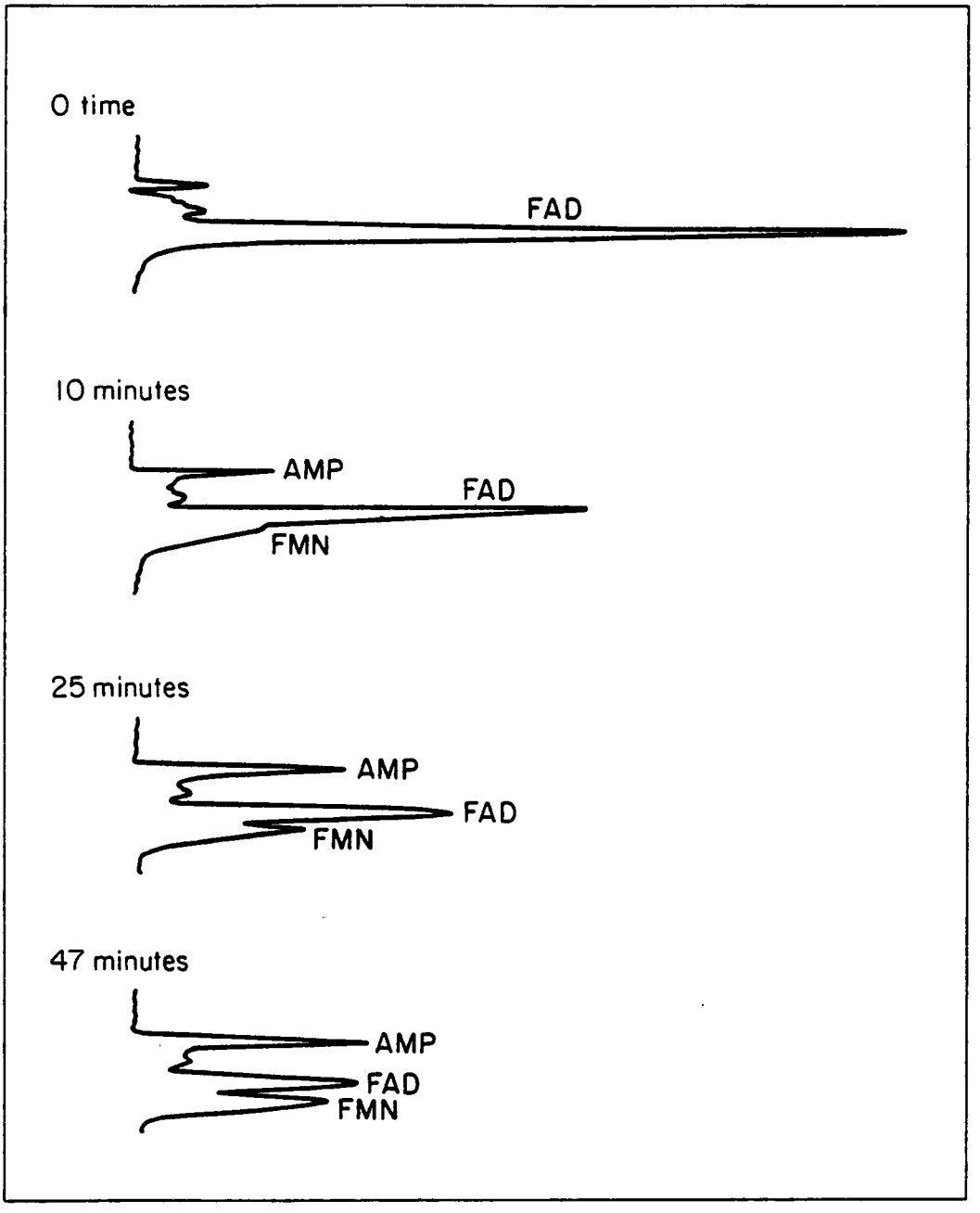

80
70
60
50
40
30
20
10
0
FLOURESCENCE INTENSITY
200
240
280
320
360
400
440
WAVELENGTH (nm)

Figure 12. Product analysis of the nucleotide pyrophosphatase-catalyzed hydrolysis of FAD by reverse-phase, ion-pair HPLC. Incubation mixtures which contained 25 mM Tris-HCl, pH 8.5, 1 mM magnesium chloride, 333 μM FAD and 2 μg of the nucleotide pyrophosphatase in a total volume of three ml at 37°C were analyzed by reverse-phase, ion-pair, HPLC as described in Experimental Procedures.


0 time
FAD
10 minutes
AMP
FAD
FMN
25 minutes
AMP
FAD
FMN
47 minutes
AMP
FAD
FMN

thermostability of the purified preparation over the range 15-50°C. The results are shown in Figure 13. The loss in activity was determined using the fluorimetric assay. At each temperature investigated, activity was lost as a first-order rate process. Half-lives of the loss of enzyme activity at each temperature are presented in Table VIII.

Effect of Temperature on Activity

The effect of temperature on the rate of the enzyme-catalyzed reaction was investigated over the range from 5.4-45.8°C using the titrimetric assay described in Experimental Procedures. These data are presented in Figure 14 as an Arrhenius plot. The data were linear over the entire temperature range observed and from the slope of the line an activation energy of 8.2 Kcal/mole for the enzyme-catalyzed reaction was calculated. In addition, from the velocities at 25 and 35°C, a Q_{10} value of 1.6 was determined.

The Proportionality of the Reaction Rate to the Enzyme Concentration

The rate of the enzyme-catalyzed NAD hydrolysis was determined to be directly proportional to the amount of enzyme added as shown in Figure 15.

Effect of Various Compounds on the Enzyme Activity

At an initial stage of the investigation into the

Figure 13. Thermal denaturation of the nucleotide pyrophosphatase. Incubation mixtures were constructed that contained 1 μg of the nucleotide pyrophosphatase and 50 mM Tris-HCl, pH 8.0 in a total volume of one ml. At timed intervals, aliquots were removed and assayed for activity by the fluorimetric assay described in Experimental Procedures. The temperature of the incubation mixtures were: line 1, 15°C; line 2, 35°C; line 3, 40°C; line 4, 50°C.

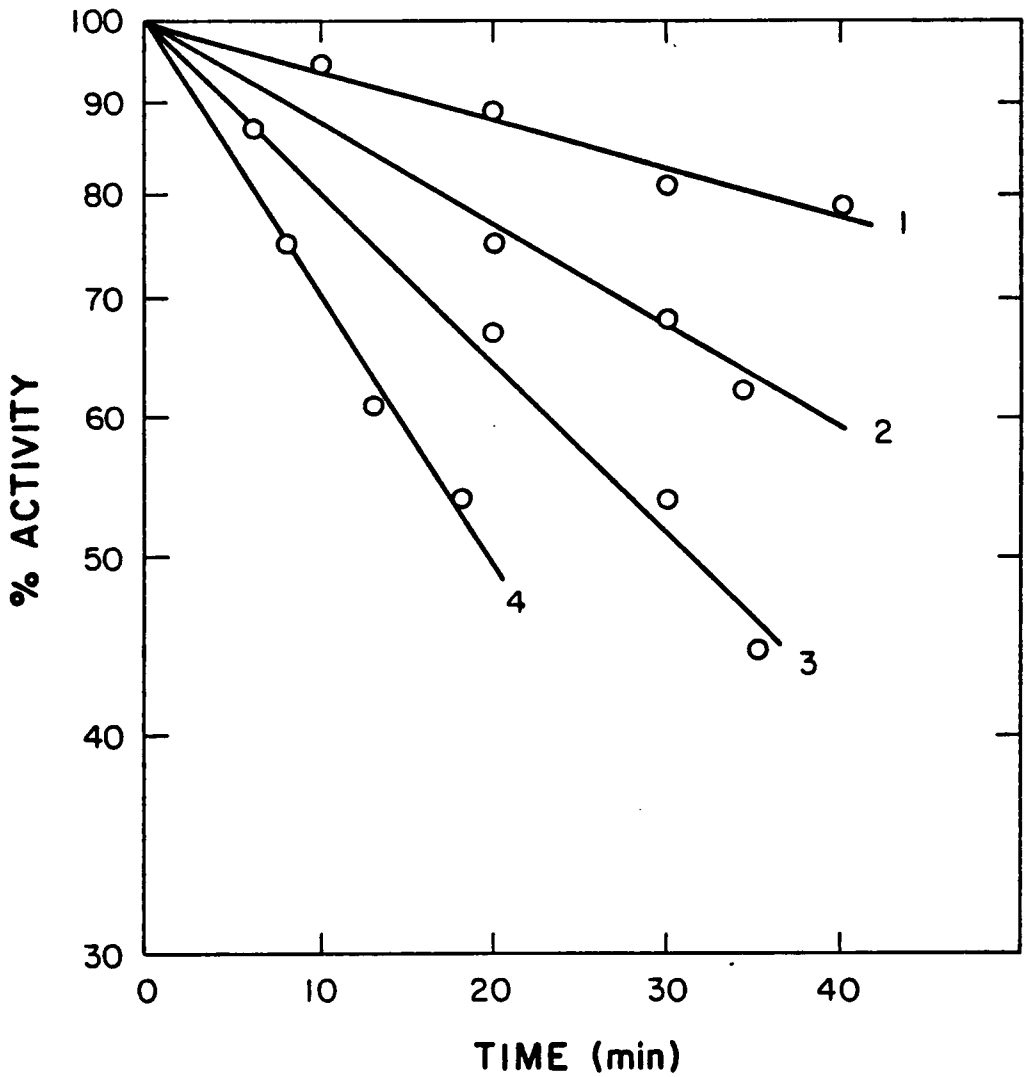

100
90
80
70
60
50
40
30
% ACTIVITY
0
10
20
30
40
TIME (min)
1
2
3
4

TABLE VIII

Thermal Denaturation of the Nucleotide Pyrophosphatase

Temperature	Half-life
degrees Celsius	min
15	110
35	53
40	31
50	19

Figure 14. Arrhenius plot of the effect of temperature on the nucleotide pyrophosphatase-catalyzed hydrolysis of NAD at pH 8.0. Activity was determined using the titrimetric assay described in Experimental Procedures using an NAD concentration of 188 μM.

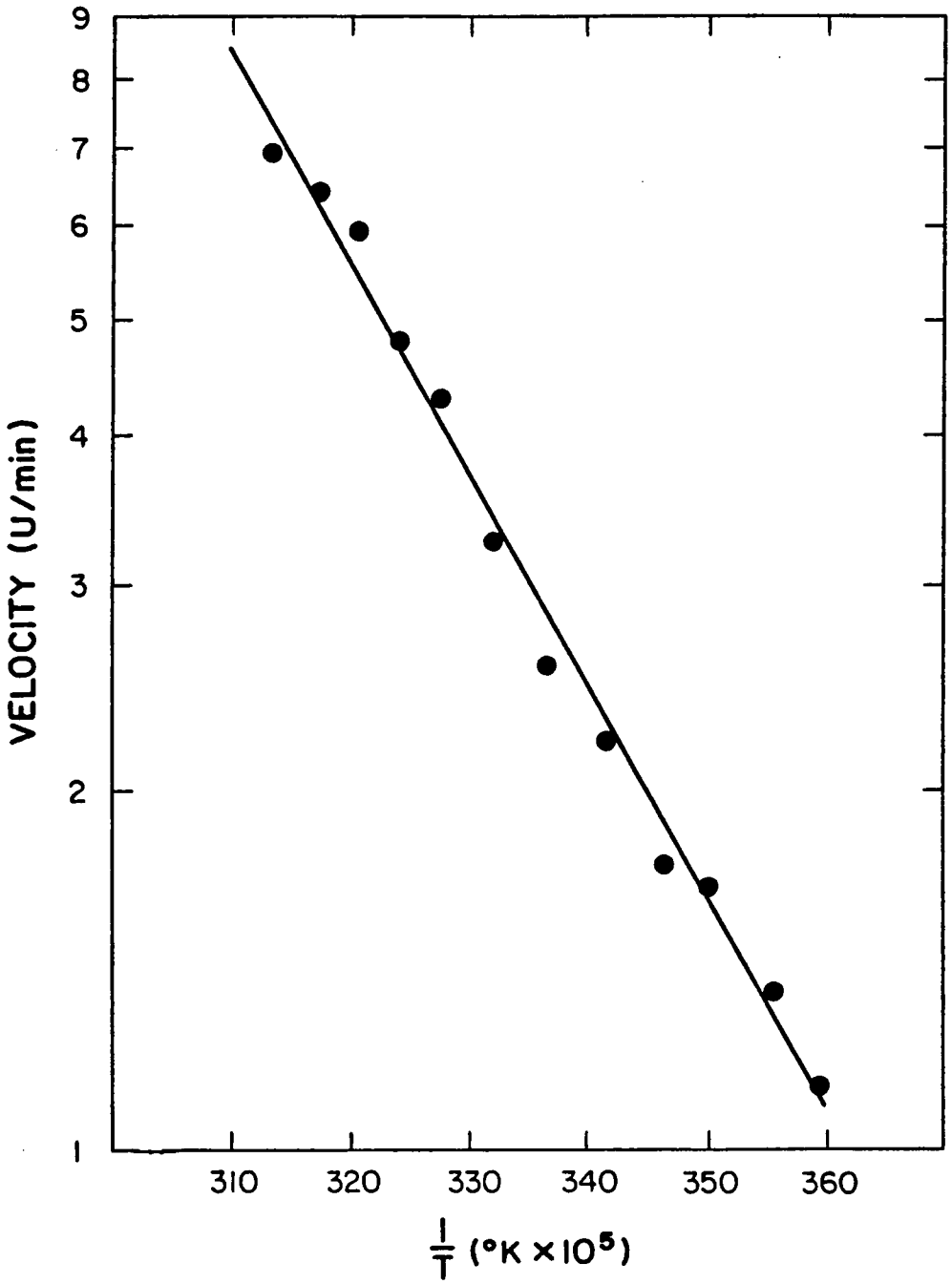
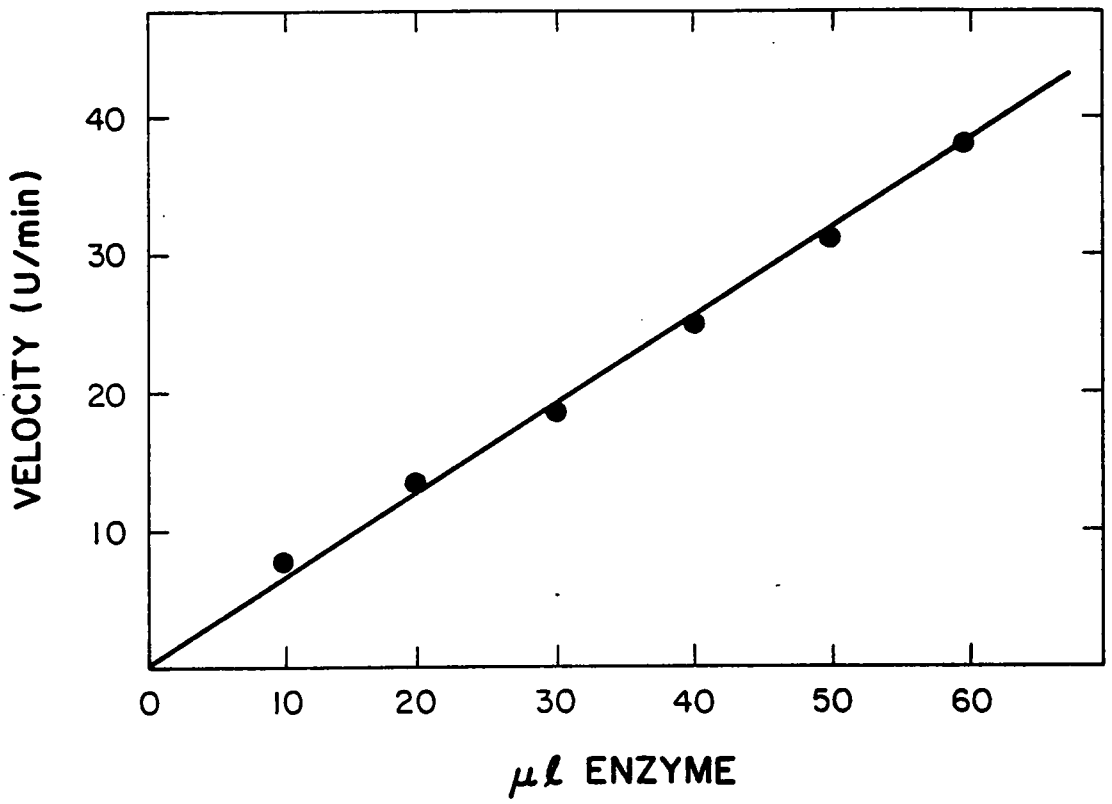

9
8
7
6
5
4
3
2
1
VELOCITY (U/min)
310
320
330
340
350
360
1/T (°K × 10⁵)

Figure 15. The proportionality of the hydrolysis of NAD to the amount of nucleotide pyrophosphatase present. Reaction mixtures contained 50 mM potassium chloride, 149 μM NAD and the indicated amounts of nucleotide pyrophosphatase in a total volume of 3.0 ml at 37° C. Activity was determined using the titrimetric assay described in Experimental Procedures with an endpoint of 8.0. The protein concentration of the enzyme solution was 18.5 μg/ml.


VELOCITY (U/min)
40
30
20
10
0
10
20
30
40
50
60
μl ENZYME

properties of the pyrophosphatase, several compounds were screened for their effects on enzyme activity. Reaction mixtures were prepared and the yeast-ADH assay conducted as described in Experimental Procedures. As shown in Table IX, the enzyme was inhibited by phosphate and pyrophosphate buffers, as well as by AMP and ADP. Enzyme activity was stimulated only modestly by high concentrations of KCl and NaCl. The results of more extensive investigations into the inhibitory effects of the adenine nucleotides are presented in a later section.

Effect of Various Cations on Activity

The effect of several mono- and divalent cations on the rate of enzyme activity at two concentrations of substrate (NAD) was investigated titrimetrically. As seen in Figure 16, large changes in ionic strength affected enzyme activity only slightly. In addition, the enzyme activity was not significantly affected by the presence of sodium or magnesium ions. Calcium ions appeared to cause a slight inhibition of the enzyme activity. The enzyme was not affected by including 5 mM EDTA, rather than a cation, in the reaction mixture.

Effect of pH on Enzyme Activity

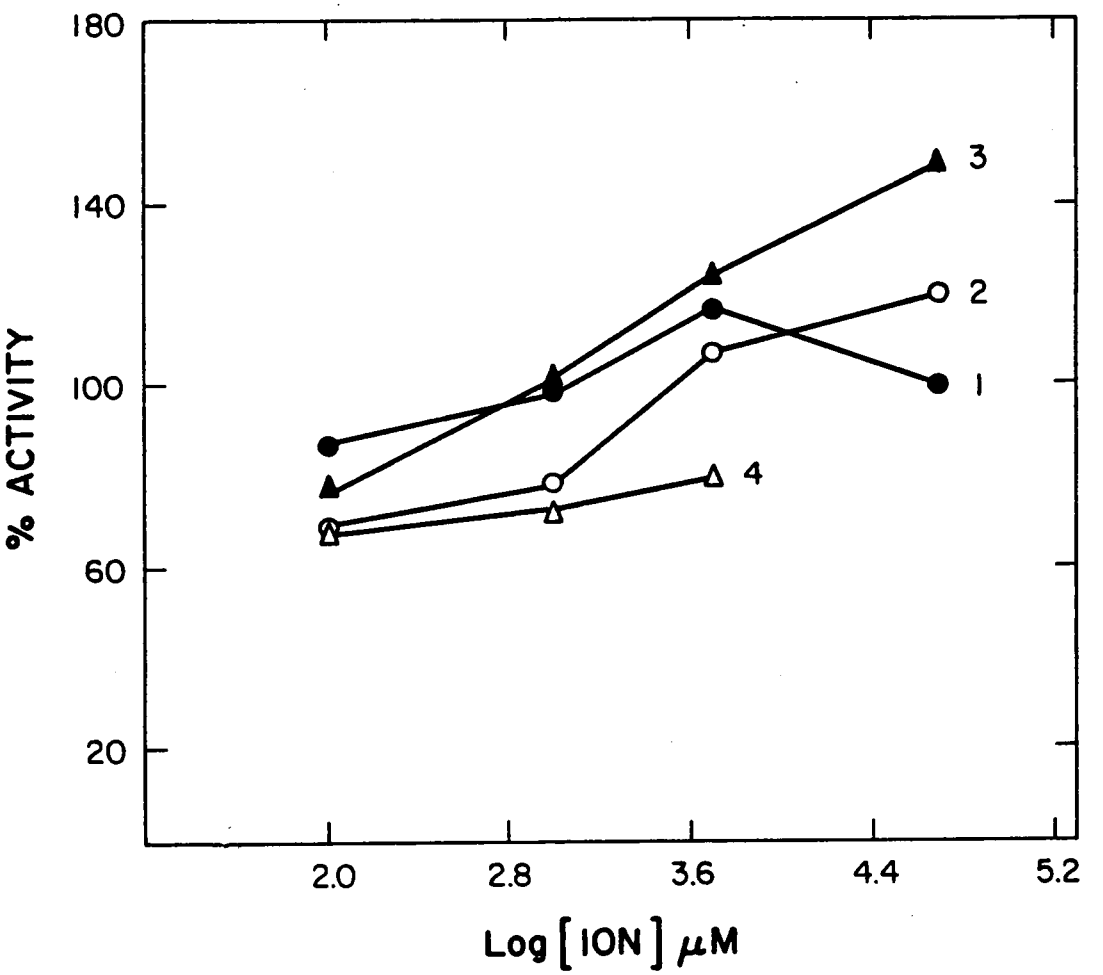
The effect of pH on enzyme activity was investigated using the titrimetric assay. Due to the unusual kinetic

TABLE IX

The Effect of Various Compounds
on the Nucleotide Pyrophosphatase Activity

Compound	% Activity
Control	100
400 mM Potassium phosphate	48
200 mM Sodium pyrophosphate	28
2 mM Adenosine 5'-monophosphate	34
2 mM Adenosine 5'-diphosphate	39
2 M Potassium chloride	121
2 M Sodium chloride	104

Figure 16. The effect of several cations on the nucleotide pyrophosphatase activity. Reaction mixtures contained 74 μM NAD, 1 μg of enzyme and the indicated amounts of cation, added as the chloride salt, in a total volume of 3.0 ml. Activity was determined using the titrimetric assay described in Experimental Procedures. The cations used were: line 1, potassium; line 2, sodium; line 3 magnesium; line 4, calcium. All reactions were maintained at 37°C.


180
140
100
60
20
% ACTIVITY
2.0
2.8
3.6
4.4
5.2
Log [ION] μM
1
2
3
4

properties of the enzyme, which are described in another section, the effect of pH on the reaction rate was observed at two different NAD concentrations. The enzyme displayed a large decrease in activity as the pH was lowered below 8.0, as seen in Figure 17. Activity was constant and was optimal over the range from 8.0 to 9.0. The profiles obtained at both low and high NAD concentrations were essentially identical.

Substrate Specificity

The ability of the nucleotide pyrophosphatase to catalyze hydrolysis of NAD, as well as other potential substrates was investigated using the titrimetric assay described earlier. The outstanding feature in these studies was the fact that the majority of substrates that were able to function with the enzyme did so in a non-hyperbolic fashion. This property is seen in Figures 18 and 19. NAD and the nicotinic acid analog of NAD were both seen to display biphasic kinetics which is indicative of negatively cooperative interactions in the functioning of the enzyme. The only substrates analyzed that did not appear to function in this way were compounds which contained modifications of the adenine ring. For compounds which displayed biphasic kinetics, apparent high and low kinetic constants were obtained by extrapolation to x- and y-axis intercepts. As

Figure 17. The effect of pH on the rate of the nucleotide pyrophosphatase-catalyzed hydrolysis of NAD at high and low concentrations of substrate. Activity was determined using the titrimetric assay described in Experimental Procedures. Reaction mixtures contained 50 mM potassium chloride, 1 μg enzyme and 150 μM (line 1) and 15 μM (line 2) NAD.

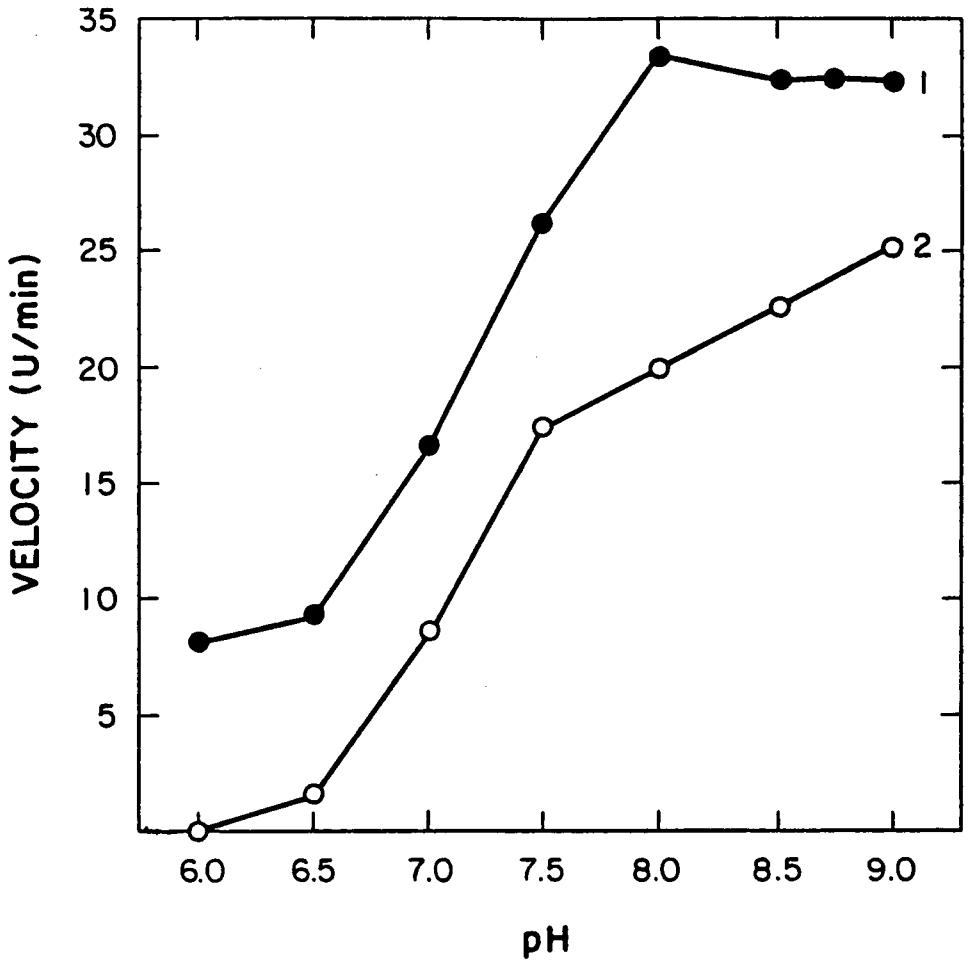

35
30
25
20
15
10
5
VELOCITY (U/min)
1
2
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

Figure 18. The effect of NAD concentration on the initial velocity of the nucleotide pyrophosphatase-catalyzed hydrolysis of NAD. The initial velocities were determined using the titrimetric assay described in Experimental Procedures. Reactions contained 50 mM potassium chloride, 2 μg enzyme and the indicated amounts of NAD at 37°C in a total volume of 3.0 ml.

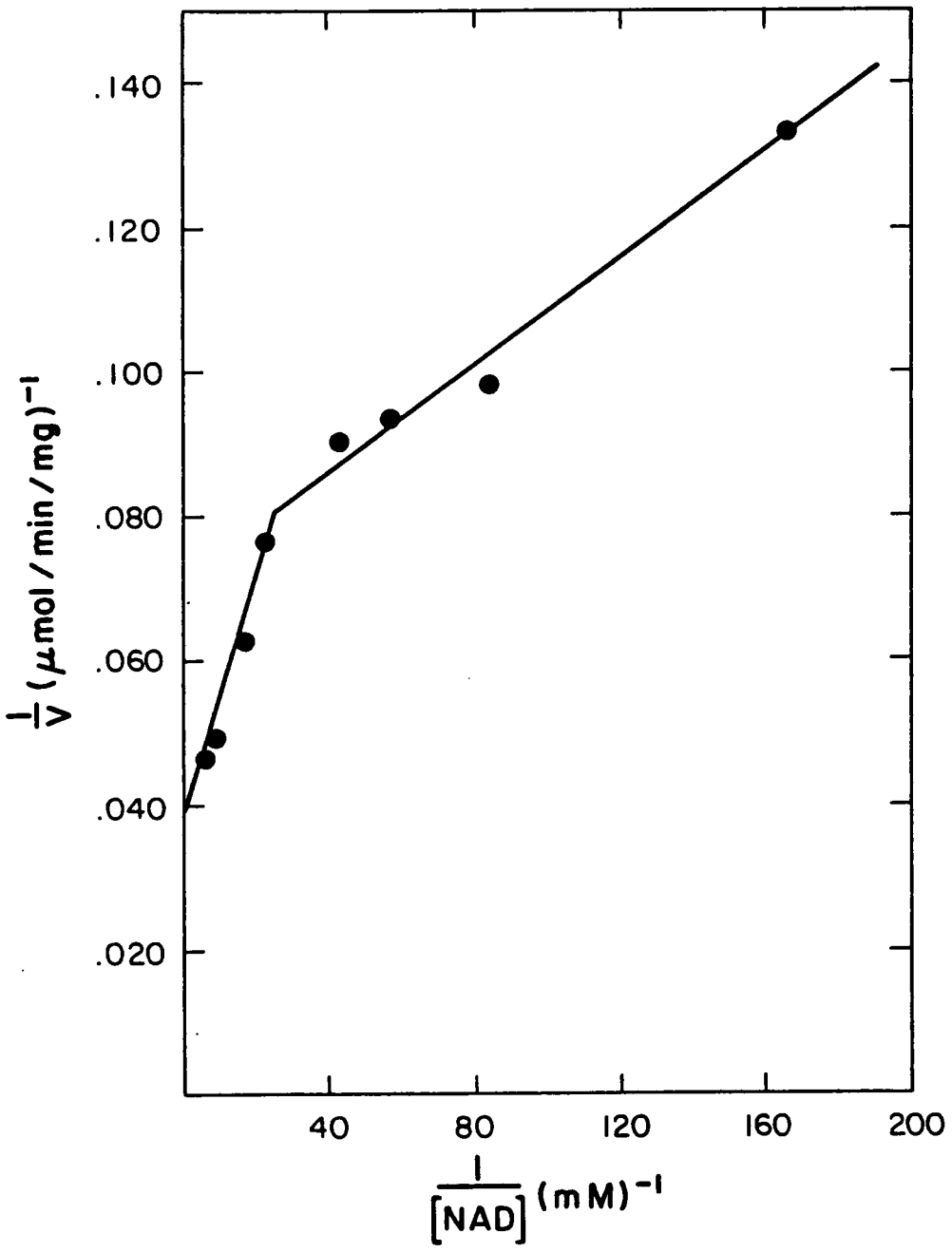
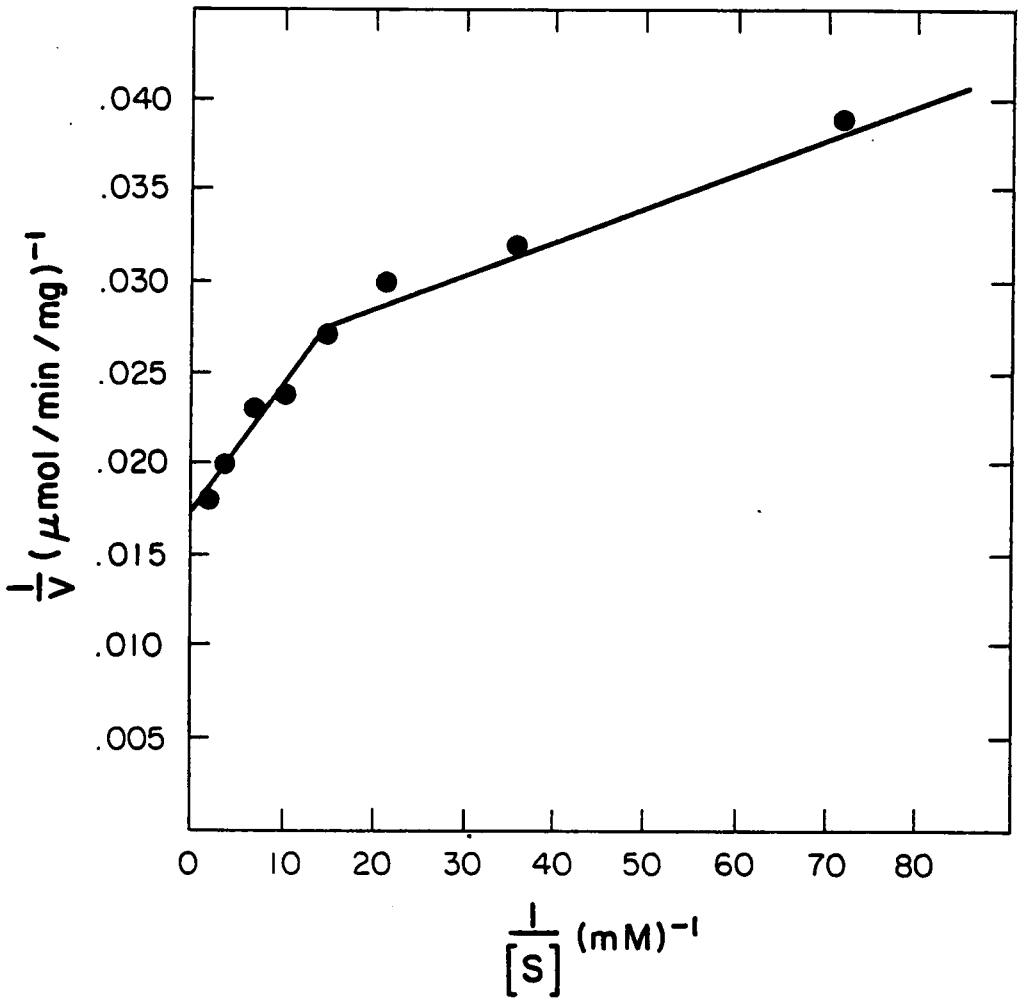

.140
.120
.100
.080
.060
.040
.020
1/V (μmol/min/mg)⁻¹
40
80
120
160
200
1/[NAD] (mM)⁻¹

Figure 19. The effect of nicotinic acid adenine dinucleotide concentration on the initial velocity of the nucleotide pyrophosphatase-catalyzed hydrolysis of the dinucleotide. Reaction mixtures were constructed as described in Figure 18.


.040
.035
.030
.025
.020
.015
.010
.005
0
10
20
30
40
50
60
70
80
1/V (μmol/min/mg)⁻¹
1/[S] (mM)⁻¹

shown in Table X, both minor (e.g. 3-acetylpyridine and nicotinic acid analogs) and major (e.g. NADH, FAD) changes in the nicotinamide portion of NAD appeared to affect binding and turnover only modestly. The relative importance of the carboxamide group at the 3-position of the pyridine ring to the ability to serve as a substrate for the enzyme is uncertain as 3-aminopyridine adenine dinucleotide (AAD) was a very poor substrate while the analog which lacked the group altogether, pyridine adenine dinucleotide (PAD), was as efficient as NAD. Use of substrates lacking a second heterocyclic base (i.e. ADPR and UDPG) also resulted in kinetic constants similar to those obtained with NAD. The purified enzyme showed no phosphatase or phosphodiesterase activity as shown by the lack of activity towards AMP, NMN and bis-(paranitrophenyl) phosphate. It is also apparent that in addition to the requirement for a pyrophosphate region in a potential substrate, both phosphates must be in a diester linkage as shown by the lack of activity towards ADP. Substitution of other purine bases for adenine altered the manner in which the enzyme functioned with these substrates. While substitution of hypoxanthine for adenine resulted in less favorable kinetic constants relative to NAD, substitution of guanine for adenine eliminated the biphasic kinetics observed with the other dinucleotides.

TABLE X

Substrate Specificity

Substrate	Low K_m	Low V_m	High K_m	High V_m
	μM	U/mg	μM	U/mg
Nicotinamide adenine dinucleotide	5.4	2.7	72.6	5.5
3-Acetylpyridine adenine dinucleotide	6.2	1.3	22.3	1.9
3-Aminopyridine adenine dinucleotide	56.1	1.6	127.4	2.3
Pyridine adenine dinucleotide	3.2	1.7	35.7	3.6
Nicotinic acid adenine dinucleotide	7.9	3.6	39.8	5.1
1,4 Dihydronicotinamide adenine dinucleotide	10.9	1.5	32.2	2.4
Flavin adenine dinucleotide	20.0	2.3	67.8	4.2
Nicotinamide hypoxanthine dinucleotide	14.8	1.7	114.6	3.3
Nicotinamide guanine dinucleotide	25.5	3.7	-	-
Nicotinamide 1,N^6 ethenoadenine dinucleotide	632.0	3.6	-	-
3-Aminopyridine 1,N^6 ethenoadenine dinucleotide	1290	2.2	-	-
Nicotinamide mononucleotide	*			
Adenosine 5'-monophosphate	*			
Adenosine 5'-diphosphate	*			
Adenosine diphosphoribose	9.4	4.7	32.4	6.3
Uridine diphosphoglucose	5.9	2.0	49.8	4.6
bis-(para-nitrophenyl) phosphate	*			

* did not serve as substrate at 1 mM

Using substrates containing ethenoadenine altered the functioning of the enzyme extensively. Both ethenoadenine-containing compounds analyzed were acted upon by the enzyme in a non-cooperative manner. In addition, as shown in Table X, both compounds displayed higher Michaelis constants over their adenine-containing parent compounds, NAD and AAD. The Lineweaver-Burk plot for etheno-NAD is shown in Figure 20.

The apparent negative cooperativity displayed by the enzyme when acting on the majority of the substrates analyzed was confirmed by the use of the Hill plotting method (106). A Hill plot of the data obtained with NAD as substrate is shown in Figure 21. In cooperative systems, Hill plots will have a slope of one at very high and very low substrate concentrations because these ends represent the binding of the first and last ligand to the enzyme. The slope of the data from intermediate concentrations, which is equal to the Hill coefficient (n), is greater than, equal to, or less than one in positively cooperative, Michaelis-Menton, and negatively cooperative enzymes, respectively. It can be seen in Figure 21 that the Hill coefficient at low substrate concentrations is equal to 0.26, while at the higher concentrations, the Hill coefficient is 1.16. For negatively cooperative enzymes,

Figure 20. The effect of etheno-NAD concentration on the initial velocity of the nucleotide pyrophosphatase-catalyzed hydrolysis of the dinucleotide. Reaction mixtures, and the x- and y-axes are as described in Figure 18.

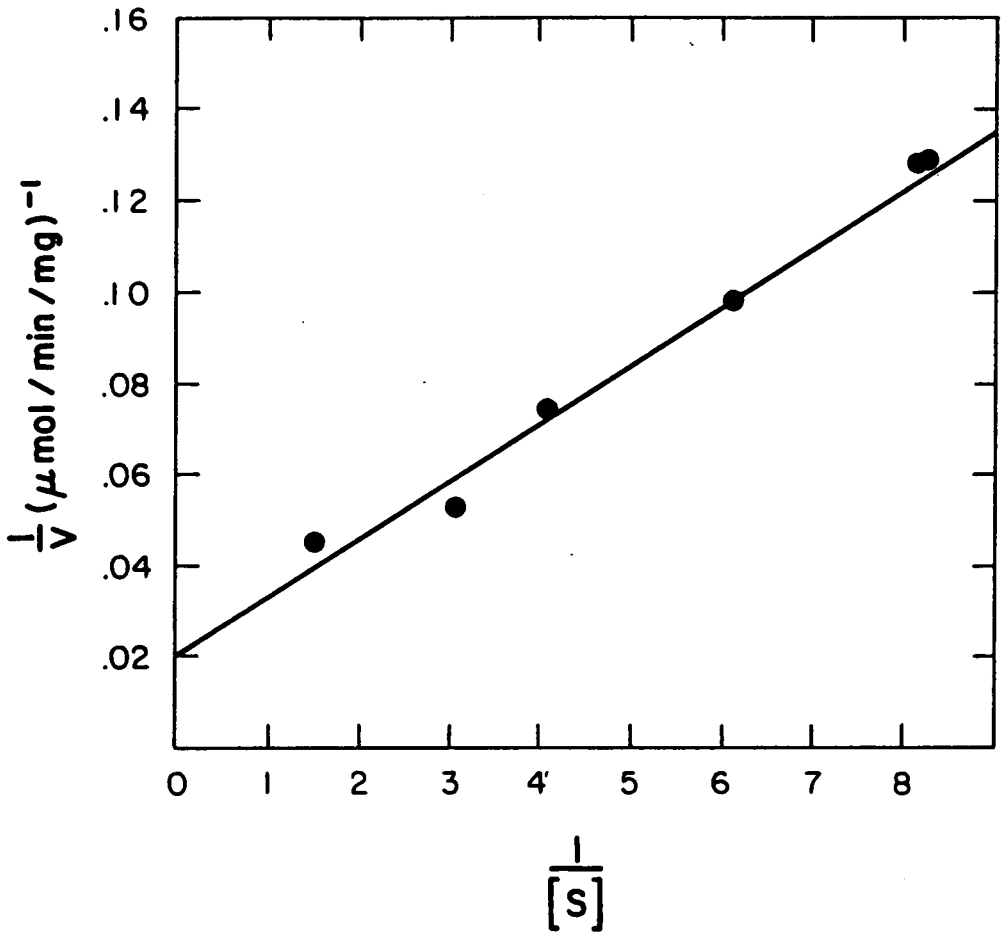
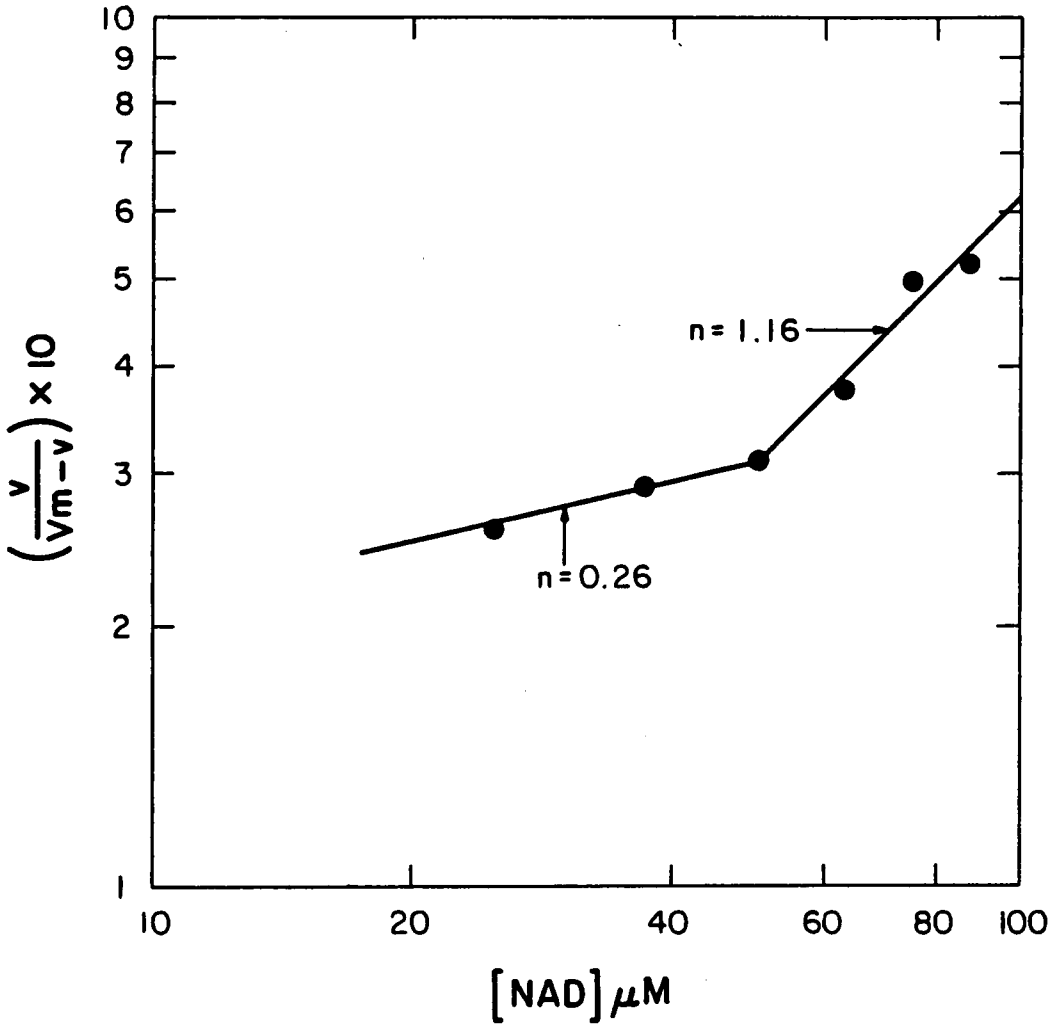

.16
.14
.12
.10
.08
.06
.04
.02
0
1
2
3
4
5
6
7
8
$\frac{1}{V}$ (μmol / min / mg)$^{-1}$
$\frac{1}{[S]}$

Figure 21. Hill plot of the effect of NAD concentration on the rate of the nucleotide pyrophosphatase-catalyzed hydrolysis of NAD.


10
9
8
7
6
5
4
3
2
1
$\left(\frac{v}{Vm-v}\right) \times 10$
n = 1.16
n = 0.26
10
20
40
60
80
100
[NAD] μM

there are several methods for determining the degree of cooperativity displayed by the enzyme in its action on various substrates. One of these is the Hill coefficient, in which smaller values reflect greater cooperativity. Ainslie et al. have proposed that the ratio of the extrapolated maximal velocity at high substrate concentrations to that which is calculated at low substrate concentrations may serve as an index of cooperativity, wherein a larger value for the ratio is indicative of greater cooperativity (107). Table XI depicts the ratios of high and low values for both kinetic constants and the Hill coefficients for all the substrates analyzed. NAD had the highest ratios of kinetic constants and the lowest Hill coefficient of all the substrates analyzed. As seen in Table XI, substrates that did not display biphasic kinetics had Hill coefficients approximately equal to one.

Inhibition of the Pyrophosphatase Activity

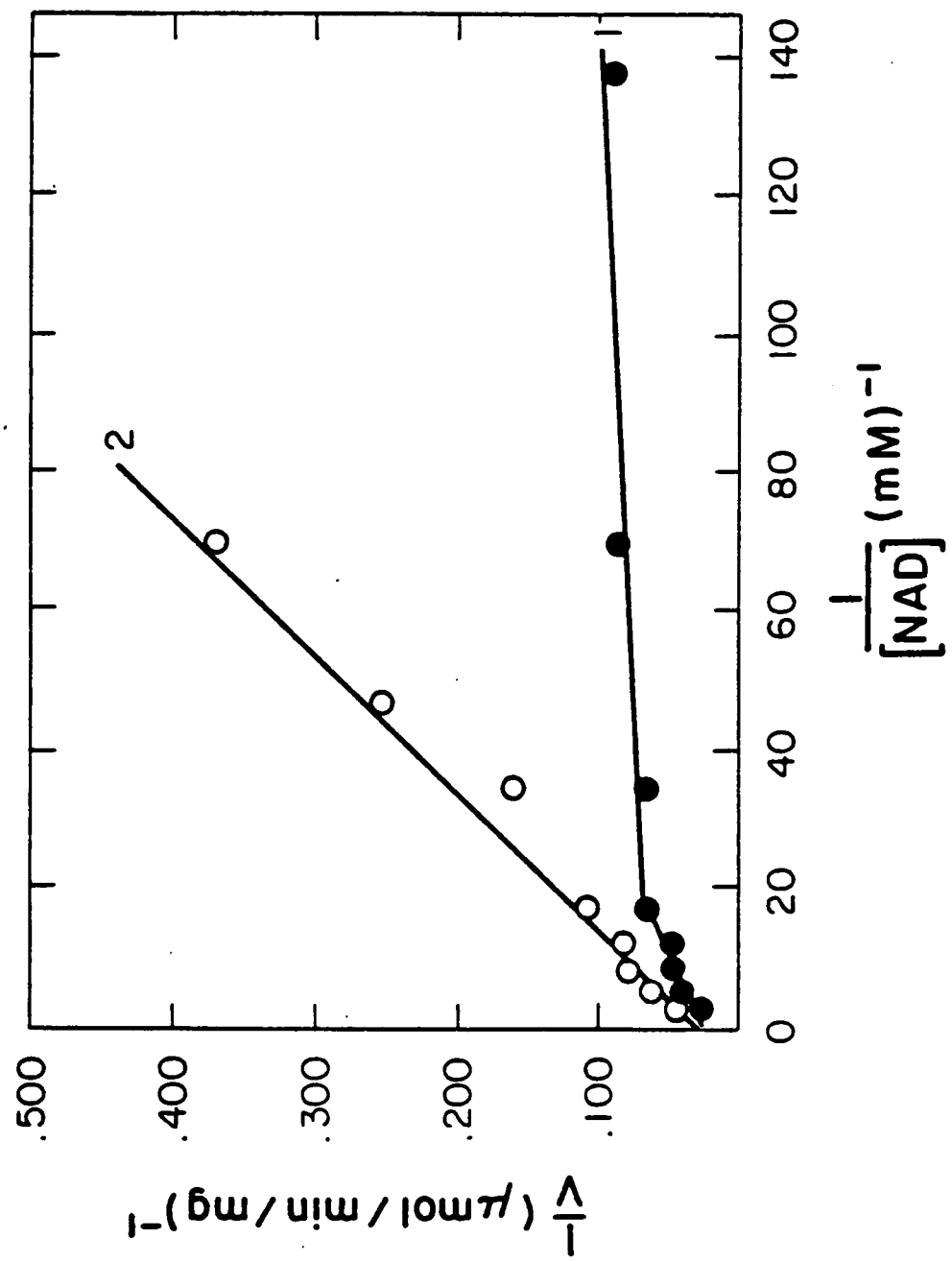
Several compounds were investigated for their ability to inhibit the enzyme-catalyzed hydrolysis of NAD. Inhibition was analyzed by the Lineweaver-Burk method. The inhibition of activity by AMP is shown in Figure 22. At 20 μM AMP, the biphasic kinetics observed in the absence of AMP was abolished, and simple, hyperbolic kinetics were observed. The Hill coefficient for the hydrolysis of NAD in the

TABLE XI

Negative Cooperativity in the
Functioning of the Nucleotide Pyrophosphatase

Substrate	K_m ratio	V_m ratio	Hill coefficient (n_H)
Nicotinamide adenine dinucleotide	13.4	2.0	0.26
Pyridine adenine dinucleotide	11.1	2.1	0.49
Uridine diphosphoglucose	8.4	2.3	0.61
Nicotinamide hypoxanthine dinucleotide	7.7	2.2	0.61
Nicotinic acid adenine dinucleotide	5.0	1.4	0.51
3-Acetylpyridine adenine dinucleotide	3.6	1.5	0.42
Flavin adenine dinucleotide	3.4	1.8	0.62
Adenosine diphosphoribose	3.0	1.6	0.69
1,4 Dihydronicotinamide adenine dinucleotide	3.0	1.6	0.73
3-Aminopyridine adenine dinucleotide	2.3	1.4	0.93
Nicotinamide guanine dinucleotide	-	-	0.91
Nicotinamide $1,N^6$ ethenoadenine dinucleotide	-	-	0.91
3-Aminopyridine $1,N^6$ ethenoadenine dinucleotide	-	-	1.12

Figure 22. The effect of AMP on the rates of hydrolysis of NAD catalyzed by the nucleotide pyrophosphatase. Reaction mixtures were constructed and assayed as described in Figure 18. Line 1 and line 2 are reactions in the absence and presence of 20 μM AMP, respectively.


1
2
0
20
40
60
80
100
120
140
.100
.200
.300
.400
.500
$\frac{1}{[NAD]}$ (mM)$^{-1}$
$\frac{1}{v}$ (μmol / min / mg)$^{-1}$

presence of 20 μM AMP was 0.87. The binding constants for other inhibitors are shown in Table XII. The importance of the pyrophosphate region in binding of molecules to the enzyme was again observed as the affinity for binding of adenine compounds increased in the order: ADP>AMP>Adenosine. The affinity of the enzyme for guanosine monophosphate (GMP) was essentially identical to that of AMP. In addition, the affinity of the enzyme for nicotinamide mononucleotide was significantly less than its affinity for the other half of NAD, AMP.

Fluorescence Studies

Numerous studies of the binding of ligands to proteins which possess intrinsic fluorescence have been conducted (83-85). These studies have yielded information about the affinity and number of ligands bound by the protein.

The fluorescence spectrum of the purified nucleotide pyrophosphatase showed strong maxima for excitation at 286 nm and for emission at 337 nm. This profile is characteristic of the presence of the amino acid tryptophan. On addition of compounds determined to be inhibitors of the enzyme, the fluorescence intensity was quenched. By addition of small aliquots of these compounds, the fluorescence intensity was quenched in a concentration-dependent process. Data obtained were plotted as a double reciprocal plot to

TABLE XII

Inhibition of the
Nucleotide Pyrophosphatase

Inhibitor	K_i
	μM
Adenosine	*
Adenosine 5'-monophosphate	15.1
Adenosine 5'-diphosphate	1.6
Guanosine 5'-monophosphate	21.9
Nicotinamide mononucleotide	*

* no inhibition observed at 20 μM

Figure 23. The quenching of the intrinsic fluorescence of the nucleotide pyrophosphatase with adenosine. Cuvettes containing 25 mM Tris-HCl, pH 8.0 and 9.0 µg of enzyme in a total volume of 1.0 ml were monitored for fluorescence intensity at an excitation wavelength of 286 nm and an emission wavelength of 337 nm. Titrations were performed by addition of 5.0 µl aliquots of 780 µM adenosine. Titrations were performed at room temperature.

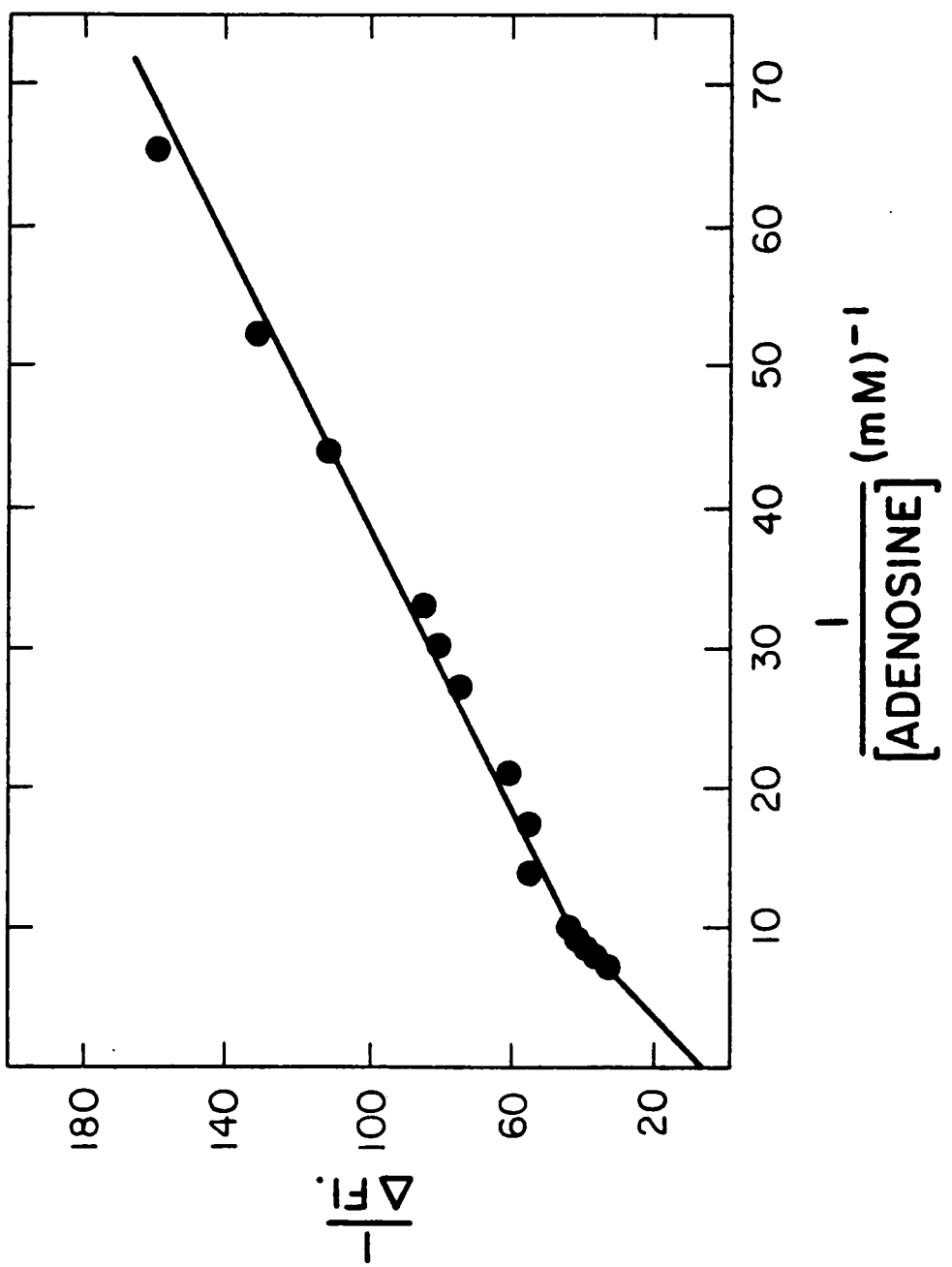

180
140
100
60
20
$\frac{1}{\Delta F_i}$
10
20
30
40
50
60
70
$\frac{1}{[\text{ADENOSINE}]}$ (mM)$^{-1}$

Figure 24. The quenching of the intrinsic fluorescence of the nucleotide pyrophosphatase with 5'-AMP. Titrations were performed in an identical manner to those described in Figure 23 with the exception that the enzyme was titrated with 10.0 µl aliquots of 500 µM AMP.

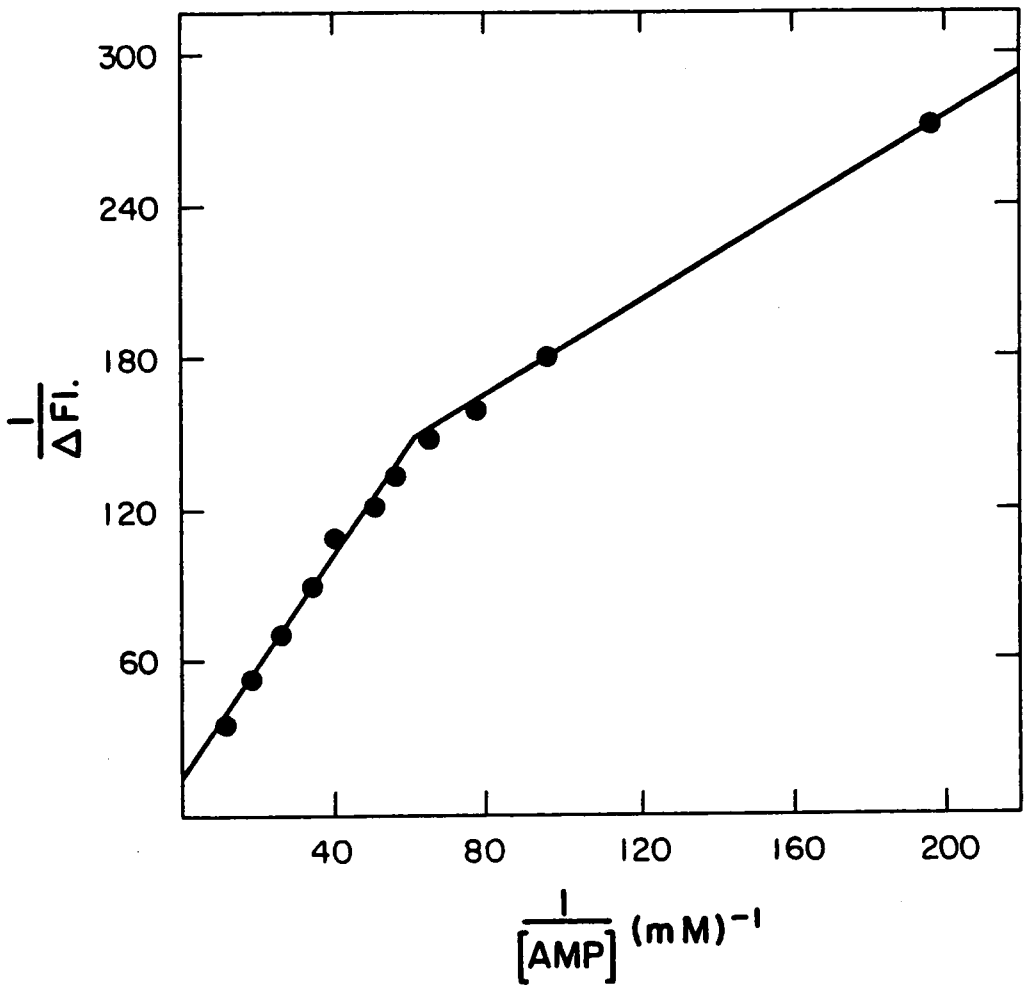

300
240
180
120
60
1/ΔFl.
40
80
120
160
200
1/[AMP] (mM)⁻¹

TABLE XIII

Titration of the Intrinsic Fluorescence of the Nucleotide Pyrophosphatase

Compound	Low K_d	High K_d
	μM	μM
Adenosine	97.6	526.1
Adenosine 5'-monophosphate	17.0	420.0
Adenosine 5'-diphosphate	1.9	79.7

obtain the dissociation constants, K_d . Dissociation constants for the adenine-containing compounds were obtained by taking the negative reciprocal of the extrapolated x-intercepts. Figures 23 and 24 depict the data obtained when adenosine and AMP were used to quench the enzyme fluorescence. It is clear that for both of these compounds, the process of fluorescence quenching proceeded in a biphasic manner. Similar results were obtained with ADP. The biphasic nature of these data indicated that the binding of these compounds to the enzyme occurred in at least two modes. Apparent high and low dissociation constants are shown in Table XIII.

The Effect of NAD on the Apparent Molecular Weight of the Enzyme

The effect of NAD on the apparent molecular weight of the enzyme was determined using gel filtration HPLC (Table XIV). In the absence of NAD, the nucleotide pyrophosphatase exhibited an apparent molecular weight of 67,800, consistent with the values obtained with both Sephacryl S-200 column chromatography and SDS gel electrophoresis. When 100 or 400 μM NAD was present in the elution buffer, the enzyme exhibited apparent molecular weights of 104,800 and 108,100, respectively. This observation has important implications which will be discussed later.

TABLE XIV

The Effect of NAD on the Molecular Weight of the Nucleotide Pyrophosphatase

Concentration (NAD)	Molecular Weight
μM	
0.0	67,800
100	104,800
400	108,100

Chemical Modification of the Purified Enzyme

Experiments were conducted in order to identify specific functional groups essential to the function of the enzyme. Studies of substrate specificity and inhibition indicated a preference for compounds which contained a pyrophosphate moiety. It may be inferred that this highly polar and negatively-charged region of potential substrates might be complemented by positively-charged groups in or near the catalytic site(s). The enzyme was inactivated in a time-dependent, first-order manner by 2,3-butanedione (Figure 25). The calculated half-lives of inactivation at various 2,3-butanedione concentrations are shown in Table XV. The enzyme was also inactivated by 2,4-pentanedione; however, 2,3-butanedione, a reagent which shows preference for arginine residues, was much more effective at inactivating the enzyme as much lower concentrations of the reagent were required to inactivate the enzyme, relative to 2,4 pentanedione. The apparent second-order rate constant for 2,3-butanedione was 3.8 times that of 2,4-pentanedione. ADP, a potent inhibitor of the enzyme, was capable of limited protection of the enzyme from inactivation by 2,3-butanedione (Figure 25, Table XV). Other specific modification reagents were tested. The inactivation of the enzyme with N-ethyl-5-phenylisoxazolium-3-sulfonate

Figure 25. Inactivation of the nucleotide pyrophosphatase with 2,3-butanedione. Reaction mixtures contained 25 mM Tris-HCl, pH 8.5, the indicated amount of 2,3-butanedione and 1 µg of the nucleotide pyrophosphatase in a total volume of 1.0 ml. The reaction mixtures were maintained at 15°C. At timed intervals, aliquots were removed and assayed for activity using the fluorimetric assay described in Experimental Procedures. The reactions contained: line 1, 2 mM BD; line 2, 20 mM BD; line 3, 100 mM BD + 20 µM ADP; line 4, 100 mM BD.

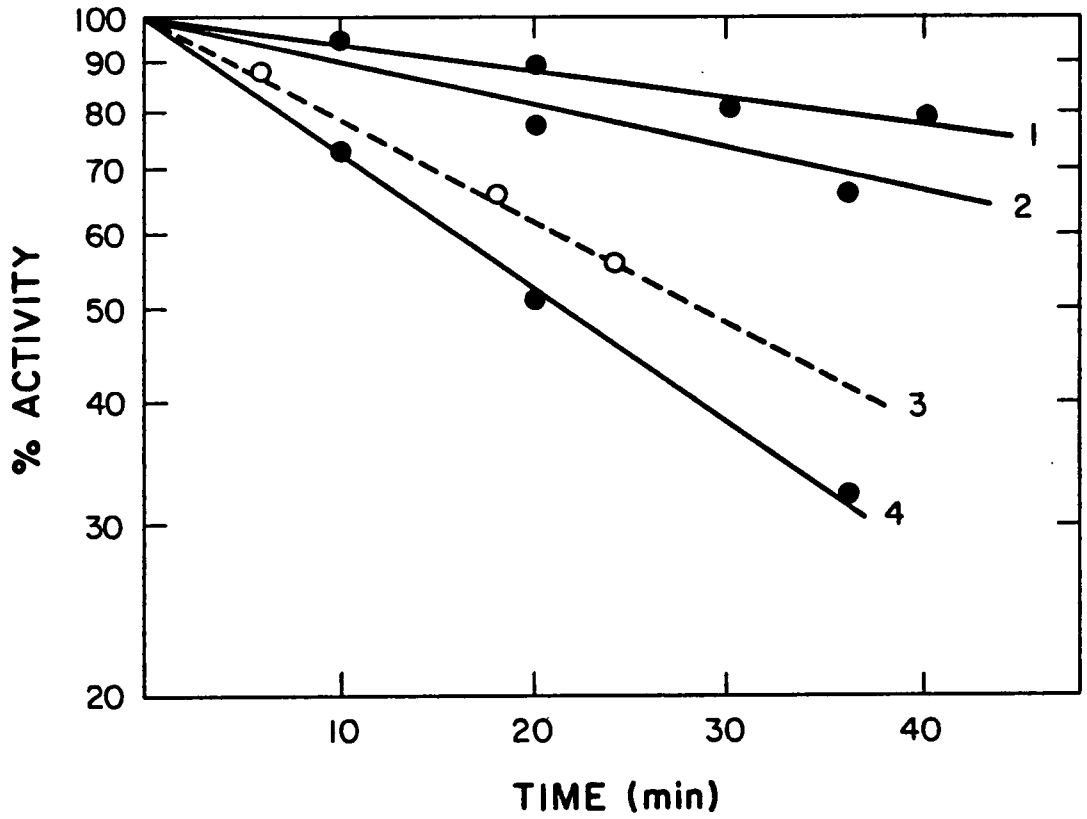

100
90
80
70
60
50
40
30
20
% ACTIVITY
10
20
30
40
TIME (min)
1
2
3
4

TABLE XV

Inactivation of the Nucleotide Pyrophosphatase with 2,3-Butanedione

Concentration	Half-life
mM	min
2.0	69.0
20.0	32.9
100.0	21.8
100.0 + 20 μM ADP	29.0

(Woodward's Reagent K), a compound which covalently modifies reactive carboxyl groups, is shown in Figure 26. The inactivation process was time dependent and occurred as a first order process. Half-lives of inactivation were extrapolated from the data and are presented in Table XVI. In the presence of 20 μM ADP, the inactivation process was enhanced, as shown by the lower half-life of inactivation.

Iodoacetamide and N-ethylmaleimide, reagents specific for nucleophilic functional groups, especially sulfhydryl groups, did not affect the enzyme activity when incubated with the enzyme at a concentration of 20 mM.

Growth Studies

As a complement to the investigations of the *Haemophilus influenzae* nucleotide pyrophosphatase, extensive work was done to probe the ability of numerous compounds to either: 1) serve as V-factor or 2) inhibit growth of the organism, presumably by interfering with the pyridine nucleotide metabolism of the organism. The growth of the organism, measured turbidimetrically, with various concentrations of NAD is shown in Figure 27. Concentrations of NAD >0.1 μg/ml readily supported growth. At maximal concentrations of NAD, the growth of the bacteria appeared to pass through a 2-3 hour lag phase followed by 7-8 hours of linear growth before entering stationary phase. The

Figure 26. Inactivation of the nucleotide pyrophosphatase with Woodward's Reagent K (WR-K). Incubations and assay of the nucleotide pyrophosphatase activity were identical to the procedures described in Figure 25. The reactions contained: line 1, 2 mM WR-K; line 2, 10 mM WR-K; line 3, 50 mM WR-K; line 4 50 mM WR-K + 20 μM ADP.

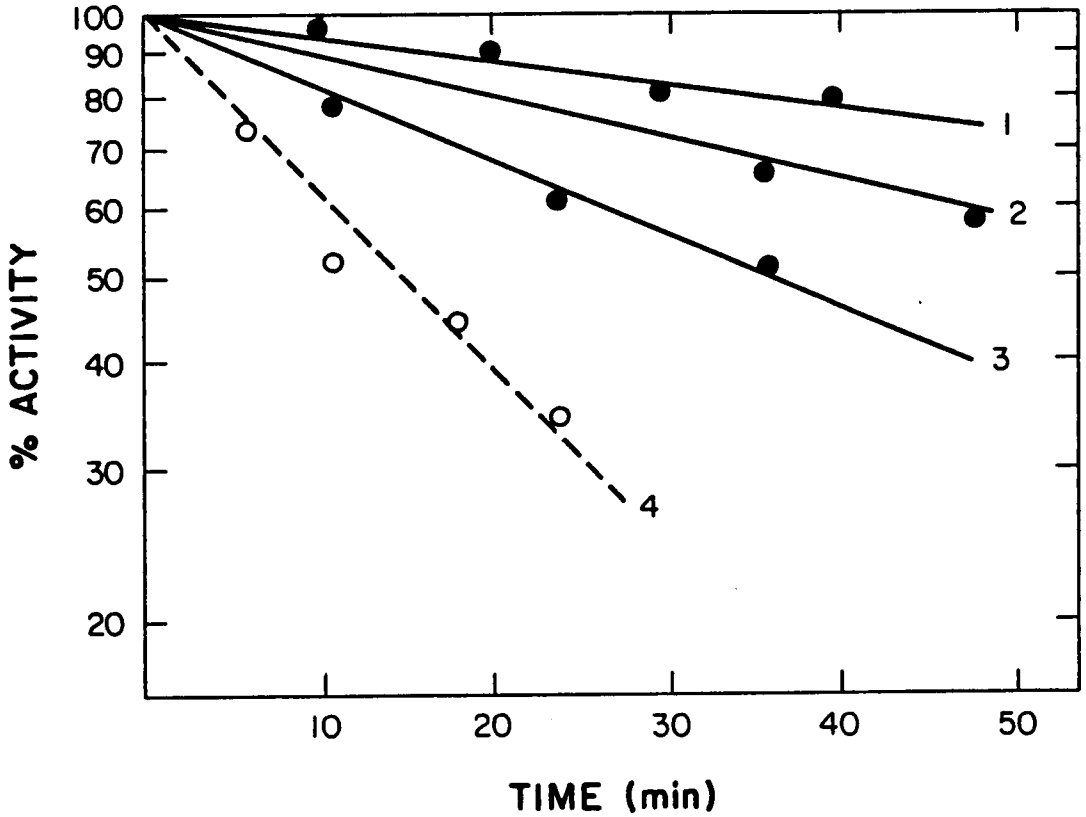
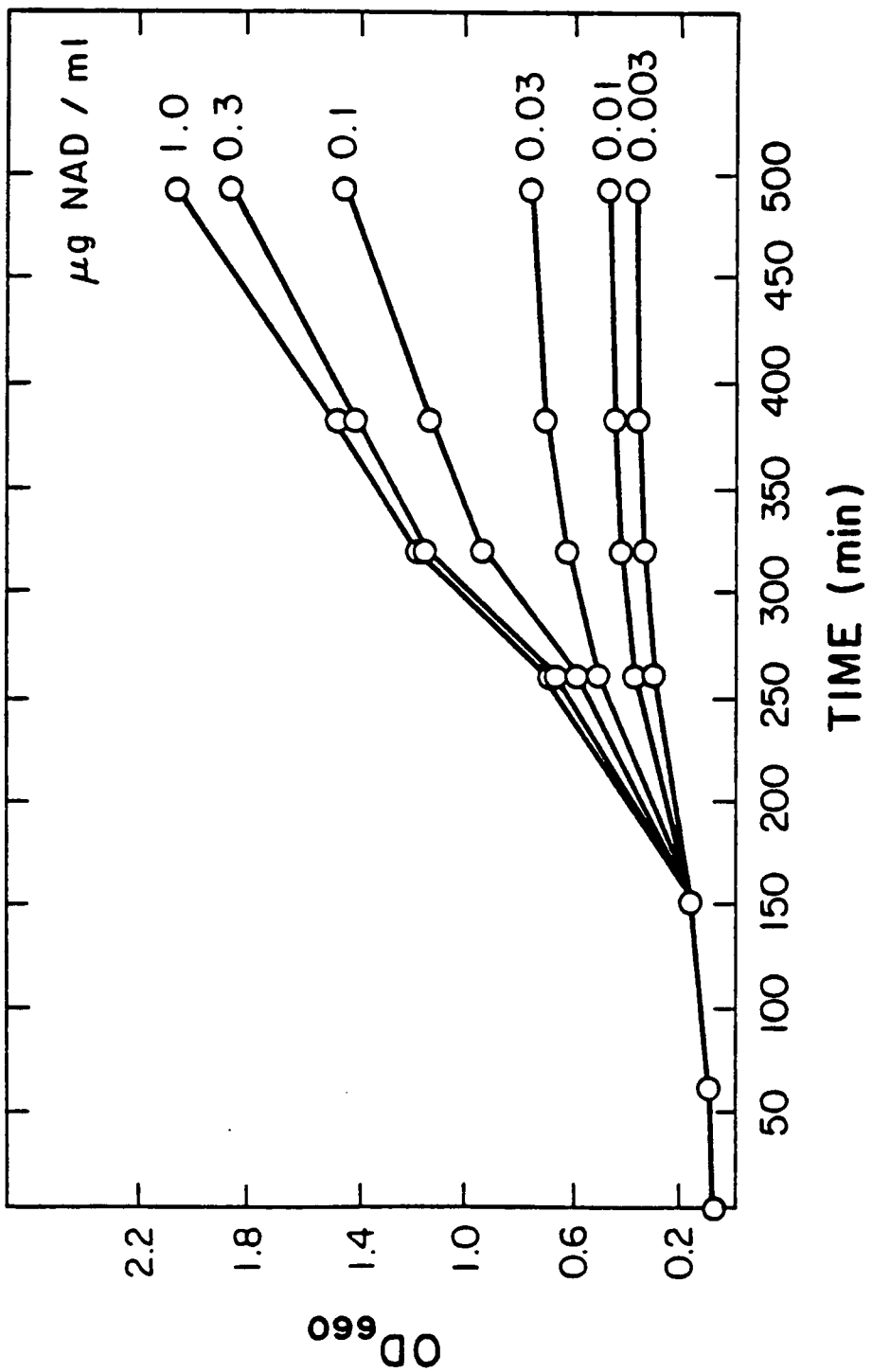

100
90
80
70
60
50
40
30
20
% ACTIVITY
10
20
30
40
50
TIME (min)
1
2
3
4

TABLE XVI

Inactivation of the Nucleotide Pyrophosphatase with Woodward's Reagent K

Concentration	Half-life
mM	min
0.05	83.6
1.0	63.3
50.0	36.0
50.0 + 20 μM ADP	14.7

Figure 27. Growth of Haemophilus influenzae with NAD as V-factor. The growth of the organism was conducted as described in Experimental Procedures.


μg NAD / ml
1.0
0.3
0.1
0.03
0.01
0.003
OD660
2.2
1.8
1.4
1.0
0.6
0.2
50
100
150
200
250
300
350
400
450
500
TIME (min)

organism also grew readily with NMN substituted for NAD as V-factor. Growth of the organism with NMN as V-factor is shown in Figure 28. Higher concentrations of NMN were required to obtain growth rates and levels comparable to those observed with NAD as V-factor. The rate of growth, expressed as the doubling time of the organism, was determined as the amount of time needed for the optical density at 660 nm to rise from a value of 0.2 to 0.4. The rates of growth provided by various compounds that were able to serve as V-factor are presented in Table XVII. Nicotinamide riboside (NR) was synthesized as described in Experimental Procedures and tested for its ability to serve as V-factor. The riboside supported growth at a rate more closely resembling NMN than NAD. Both NMN and NR were less efficient (slower doubling times at equivalent concentrations) than NAD and both compounds, at maximal concentrations, were not able to produce growth rates comparable to those at saturating concentrations of NAD. As expected, neither nicotinamide nor nicotinic acid served as V-factor at concentrations as high as 1 mg/ml.

Several NAD analogs which served as substrates for the nucleotide pyrophosphatase were tested for their ability to act as V-factor. Nicotinamide hypoxanthine dinucleotide (NHD) and NADH functioned very well, producing doubling

Figure 28. Growth of Haemophilus influenzae with NMN as V-factor. The growth of the organism was conducted as described in Experimental Procedures.

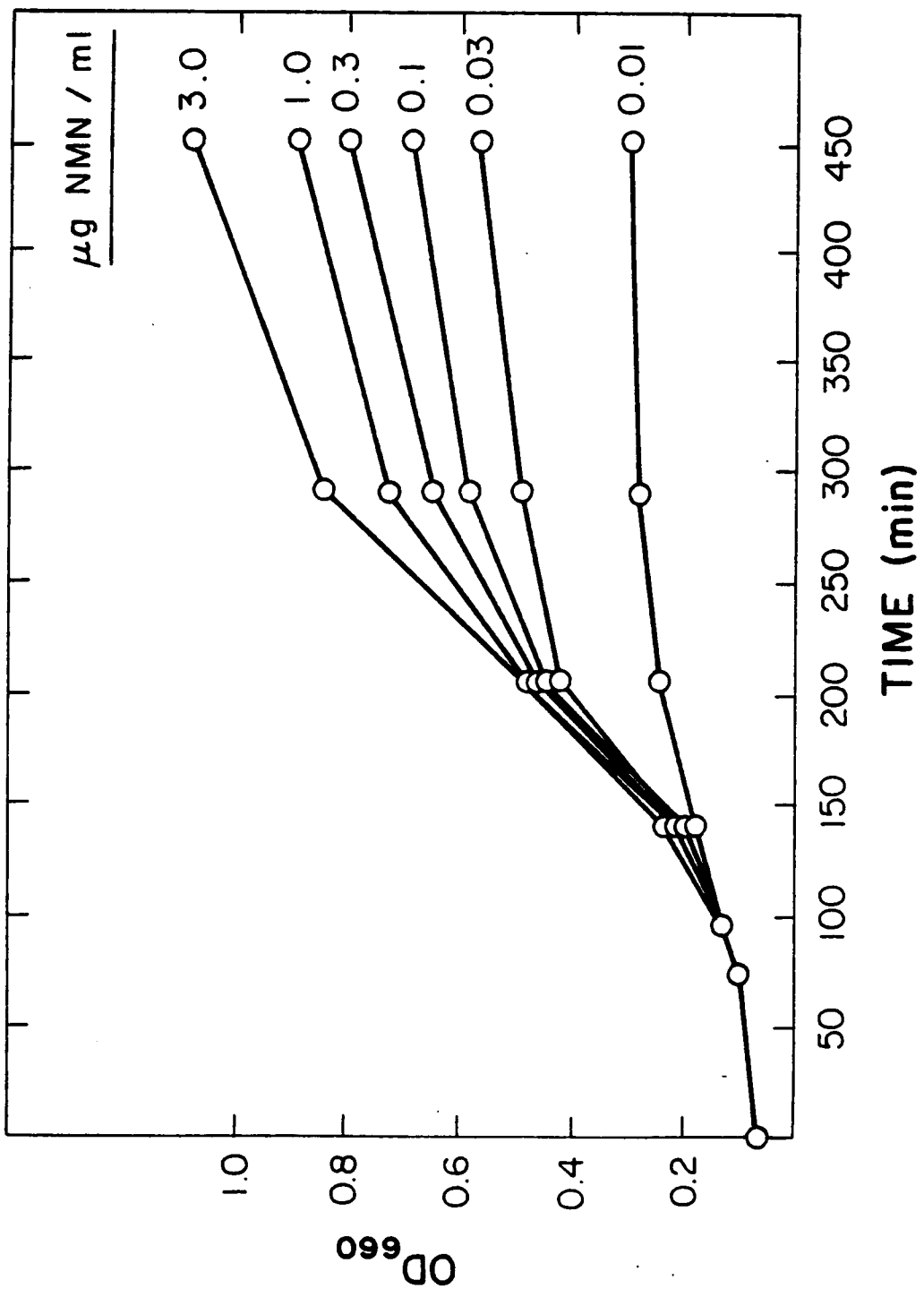

μg NMN / ml
3.0
1.0
0.3
0.1
0.03
0.01
OD660
1.0
0.8
0.6
0.4
0.2
50
100
150
200
250
300
350
400
450
TIME (min)

TABLE XVII

The Ability of Various Compounds to Serve as V-factor

Concentration	Doubling Times (min)								
µg/ml	NR	NMN	NAD	3-Acetylpyridine adenine dinucleotide	Nicotinamide hypoxanthine dinucleotide	Nicotinic acid adenine dinucleotide	NADH	Thionicotinamide adenine dinucleotide	Etheno-NAD
0.003	-	-	143.5	-	-	-	-	-	-
0.01	-	206.3	94.1	-	-	-	-	-	-
0.03	-	62.5	61.5	-	-	-	-	-	-
0.1	87.7	57.9	49.1	-	55.2	-	54.2	-	-
0.3	60.1	56.1	42.3	-	-	-	-	-	-
1.0	55.6	53.8	38.6	266.7	42.4	-	44.3	225.8	406.2
3.0	53.4	50.4	-	-	-	-	-	-	-
10.0	-	-	-	54.4	40.2	-	44.8	128.6	75.8
100.0	-	-	-	49.7	35.0	-	47.8	128.6	48.2

times very similar to NAD. 3-acetylpyridine adenine dinucleotide (APAD), a NAD analog known to undergo enzyme-catalyzed oxidation-reduction, also served as V-factor. Nicotinic acid adenine dinucleotide did not fulfill the V-factor requirement. These data are consistent with the inability of Haemophilus influenzae to metabolize deamidated derivatives of NAD. The ethenoadenine analog of NAD, a poor substrate for the nucleotide pyrophosphatase, was also very poor at serving as V-factor. This compound, an analog of NAD modified at the adenine ring, would, subsequent to hydrolysis by the nucleotide pyrophosphatase, produce NMN, a compound which readily serves as V-factor.

Several compounds which did not serve as V-factor were analyzed for their ability to inhibit the growth of Haemophilus influenzae in the presence of NAD. Figure 29 shows the inhibition of growth of the organism by addition of the indicated amounts of 3-aminopyridine adenine dinucleotide (AAD). This compound was one of the most effective inhibitors, completely inhibiting growth at a concentration of 1 μg/ml. 4-AAD was clearly not as inhibitory as 3-AAD (Table XVIII). Adenine nucleotides were effective as growth inhibitors, with ADP being more effective than AMP. Adenosine was ineffective and ADPR, a substrate for the nucleotide pyrophosphatase, was only

Figure 29. Inhibition of growth of Haemophilus influenzae with NAD as V-factor by AAD . The growth inhibition experimental procedures are described in Experimental Procedures.

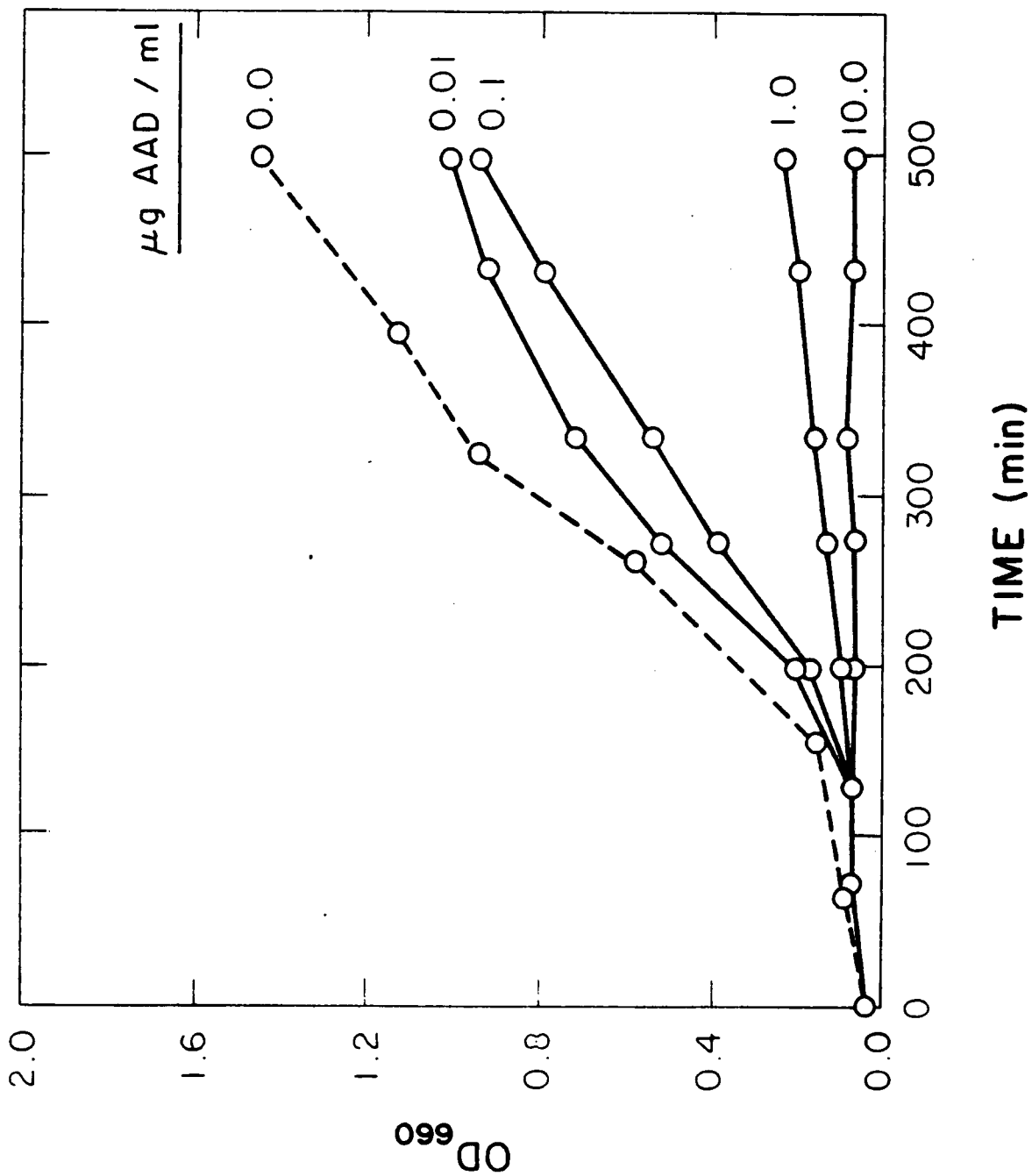

μg AAD / ml
0.0
0.01
0.1
1.0
10.0
OD660
2.0
1.6
1.2
0.8
0.4
0.0
0
100
200
300
400
500
TIME (min)

TABLE XVIII

Inhibition of the Growth of Haemophilus influenzae
With NAD as V-factor

Concentration	Doubling Times (min)									
µg/ml	Adenosine	AMP	ADP	ADPR	GMP	UMP	3-Aminopyridine adenine dinucleotide	Pyridine adenine dinucleotide	3-Methylpyridine adenine dinucleotide	3-Pyridine-aldehyde adenine dinucleotide
100.0	-	N.G.	-	-	-	-	N.G.	-	173.8	-
10.0	-	95.7	N.G.	69.9	59.0	57.6	N.G.	88.6	97.4	71.9
1.0	41.8	66.2	60.6	55.2	61.0	59.6	559.7	58.0	58.5	71.4
0.1	-	59.2	55.1	56.6	60.6	59.6	84.6	53.8	58.7	70.3
0.01	-	58.4	56.6	54.8	61.4	61.7	57.8	54.0	56.4	69.0

N.G.= no growth observed

TABLE XVIII (cont.)

Concentration µg/ml	Doubling Times (min)							
	3-Pyridyl carbinol adenine dinucleotide	Nicotinic acid adenine dinucleotide	3-Pyridyl acetonitrile adenine dinucleotide	4-Aminopyridine adenine dinucleotide	FAD	Ethyl-nicotinamide chloride	Decyl-nicotinamide chloride	Etheno-AAD
100.0	-	-	51.3	85.7	70.9	128.1	N.G.	-
10.0	95.5	65.2	53.4	56.6	57.5	79.3	N.G.	156.0
1.0	58.5	61.7	51.7	54.2	53.8	64.0	65.7	54.6
0.1	61.4	62.9	49.6	52.6	54.9	65.3	64.5	53.2
0.01	59.9	59.2	51.2	54.0	-	-	-	51.0

N.G.= no growth observed

slightly inhibitory. Other mononucleotides, GMP and UMP, were analyzed and were not effective as inhibitors. Several analogs of NAD which are known to be incapable of undergoing enzyme-catalyzed oxidation-reduction were also used and all acted as inhibitors of growth, with varying degrees of effectiveness. Particularly effective were the 3-methylpyridine and 3-pyridylcarbinol analogs. Two N^1-alkylnicotinamide chlorides, analogs of the pyridinium portion of NAD, were used and observed to be capable of growth inhibition. The ethenoadenine derivative of AAD was capable of inhibiting growth, but at a much decreased level of effectiveness relative to AAD itself.

Growth studies in which the ability of NMN and NAD to serve as V-factor indicated that the two compounds functioned as V-factor, but that acquisition of these compounds was through two different routes, as increasing concentrations of both compounds produced different profiles of growth efficiency. The doubling times observed for NMN decreased to a minimal time (maximum growth rate) at an intermediate concentration of NMN. Also the rate of growth at saturating concentrations of NMN produced doubling times which were significantly longer than those produced by maximal concentrations of NAD. In order to probe further this apparent difference between the functioning of these

two compounds as V-factor, the effect of AMP on the growth of Haemophilus influenzae with NMN or NAD as V-factor was investigated (Table XIX). AMP acted as an inhibitor of growth, but only when NAD was used as V-factor. The growth of the organism at various concentrations of NMN was the same in the presence and absence of AMP. These data indicate that the processes in which NAD and NMN meet the V-factor requirement are distinctly different. With NAD as V-factor, the dinucleotide was internalized in a manner which is inhibited by AMP. NMN can be utilized by the organism in a manner which is unaffected by the presence of extracellular AMP.

Demonstration of NAD Pyrophosphorylase Activity

The ability of Haemophilus influenzae to use NMN as V-factor implies the existence of one or more pathways that lead to the incorporation of this compound into NAD. The inability of this organism to use deamidated derivatives of NAD is consistent with the ability to synthesize NAD directly from NMN rather than through nicotinic acid mononucleotide. In addition, the demonstration of an externally-directed nucleotide pyrophosphatase, capable of the rapid degradation of external NAD to NMN places greater importance on the fate of NMN in Haemophilus influenzae . It was therefore of interest to determine whether Haemophilus

TABLE XIX

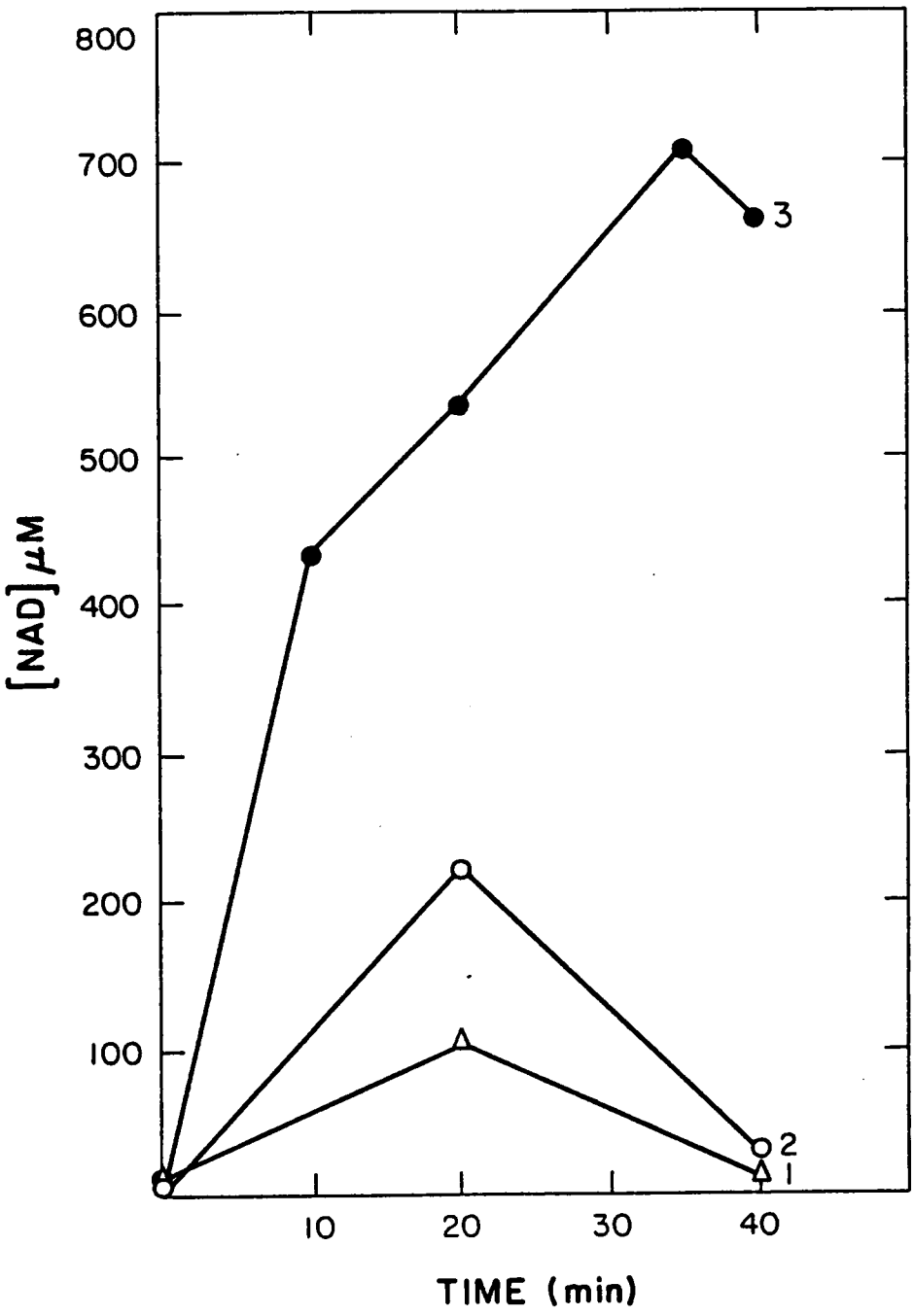
The Effect of AMP on the growth of
of Haemophilus influenzae with NMN or NAD as V-factor

V-factor Concentration µg/ml	Doubling Times (min) NAD No AMP	NAD + 3.6 µg/ml AMP	NMN No AMP	NMN + 10.0 µg/ml AMP
0.003	143.5	-	-	-
0.01	94.1	N.G.	184.2	140.0
0.03	61.5	72.0	62.7	57.6
0.10	49.1	60.0	56.3	52.6
0.30	42.3	48.7	54.1	51.9
1.0	38.6	49.8	53.5	49.6

N.G.= no growth observed

influenzae has the ability to catalyze synthesis of NAD from NMN. A soluble fraction from sonicated Haemophilus influenzae cells was prepared as described in Experimental Procedures. This fraction was then assessed for the ability to synthesize NAD from NMN and ATP, a reaction catalyzed by NAD pyrophosphorylase. An incubation mixture containing NMN and ATP as described in Experimental Procedures was prepared, and NAD was determined using the yeast-ADH assay. As shown in Figure 30, under standard conditions, the soluble fraction catalyzed NAD synthesis. A maximum level of synthesis at approximately 20 min after initiation of the reaction was followed by rapid degradation over the next 20 minutes of incubation. Degradation was due to the additional presence of the nucleotide pyrophosphatase in the soluble fraction. An identical incubation was prepared which contained 10 μM AMP, a known inhibitor of the nucleotide pyrophosphatase. The incubation mixture was adjusted at 10 min and 25 min to apparent AMP concentrations of 20 and 30 μM, respectively to offset alkaline phosphatase activity. Under these conditions the net synthesis of NAD was increased three-fold over the incubation not containing AMP (Figure 30). In addition, there was very little degradation of the newly synthesized NAD. Therefore, Haemophilus influenzae possesses a soluble enzyme capable of catalyzing

Figure 30. NAD pyrophosphorylase activity in sonicates of Haemophilus influenzae. The soluble fraction from sonicated cells of Haemophilus influenzae was assayed for NAD pyrophosphorylase activity using the assay described in Experimental Procedures. The enzyme source for the reactions were: line 1, 0.6 ml of the supernatant from a low-speed centrifugation step which followed sonication; line 2, 0.6 ml of the supernatant from a 100,000 x g centrifugation step which followed the low-speed centrifugation step; line 3, identical to line 2 with 10 µl aliquots of 10 mM AMP added at 0, 10 and 25 minutes.


800
700
600
500
400
300
200
100
[NAD] μM
10
20
30
40
TIME (min)
3
2
1

the synthesis of NAD from NMN and ATP, i e a NAD pyrophosphorylase.

Characterization of the Cyclic Phosphodiesterase Activity

As described earlier, it became apparent in studies of the nucleotide pyrophosphatase, that a co-purifying phosphodiesterase activity was present in preparations of the partially-purified enzyme. Studies were therefore conducted in order to characterize this enzyme activity, especially with regards to the potential participation of this enzyme in the NAD metabolism of the organism. NAD, at a concentration of 700 μM, was not hydrolyzed. The enzyme also did not hydrolyze 5'-nucleotides, nor did it catalyze the hydrolysis of 3',5'-cyclic AMP. 2',3'-cyclic AMP was readily hydrolyzed to adenosine, as determined by reverse phase, ion-pair HPLC. 3'-Nucleotides were rapidly hydrolyzed to the free nucleosides. In a review of the literature, it became apparent that the enzyme activity was the 2',3'-phosphodiesterase described by Rodden and Scocca (75). Further characterization of the enzyme revealed that the bis-PNPP was a substrate with a Michealis constant of 22 μM and that 5'-nucleotides were competitive inhibitors in this reaction. The dissociation constants for several of these inhibitors are given in Table XX.

TABLE XX

Competitive Inhibition of the
2',3'-Cyclic Phosphodiesterase

Inhibitor	K_i
	μM
Adenosine 5'-monophosphate	6.5
Guanosine 5'-monophosphate	2.9
Cytidine 5'-monophosphate	8.7
Uridine 5'-monophosphate	11.0
Adenosine 5'-diphosphate	2.7
Adenosine 5'-triphosphate	2.8
Adenosine diphosphoribose	13.6
3',5'-cyclic AMP	5.6
Adenosine 2'-monophosphate	2.4
Ribose 5'-phosphate	*
Adenosine	3.9
Guanosine	5.3
Cytidine	5.3
Uridine	3.0

* no inhibition at 1 mM

DISCUSSION

Haemophilus influenzae, and several other members of the genus *Haemophilus*, have an absolute growth requirement for intact NAD. This growth requirement is entirely unique to this genus. Most microorganisms biosynthesize NAD by using the enzyme-catalyzed condensation of dihydroxyacetone phosphate and aspartate to form quinolinic acid which is readily converted to nicotinic acid mononucleotide. This mononucleotide is converted to the NAD via the Preiss-Handler pathway. The other common method of acquiring NAD in microorganisms is through the use of pyridine nucleotide cycles IV and VI. The critical difference between these two prokaryotic cycles is that in the PNC IV, NMN is converted directly to nicotinic acid mononucleotide, avoiding steps used in the PNC VI involving nicotinamide and nicotinic acid as intermediates.

Previous investigations into the nature of the V-factor requirement of *Haemophilus influenzae* were primarily growth studies in which likely precursors of NAD were assessed for their ability to serve as V-factor. Nicotinamide and nicotinic acid, as well as the normal precursors of *de novo* NAD biosynthesis, do not serve as V-factor, while nicotinamide riboside, NMN, NAD, NADP, and the reduced

pyridine nucleotides are known to satisfy this growth requirement (49-52, 122). The ability of Haemophilus influenzae to use precursors of NAD as V-factor implies the existence of biochemical processes capable of producing the dinucleotide from these compounds. The inability of Haemophilus influenzae to use nicotinic acid mononucleotide or dinucleotide as V-factor (122) distinguishes the organism from those which have been genetically manipulated to rely totally on the PNC IV for a supply of intracellular pyridine nucleotides (45). Such organisms, unlike Haemophilus influenzae , are able to use nicotinic acid mononucleotide for NAD biosynthesis. Haemophilus influenzae may therefore acquire NAD by use of a pyridine nucleotide cycle other than the PNC IV or PNC VI. The investigation into the existence of a functional pyridine nucleotide cycle was initiated with the knowledge that many microorganisms which possess pyridine nucleotide cycles initiate them by the external cleavage of NAD. Salmonella typhimurium and Escherichia coli degrade extracellular NAD at the pyrophosphate bond, a reaction catalyzed by NAD pyrophosphatase (45,66). Experiments conducted to determine the fate of extracellular NAD with Haemophilus influenzae showed a rapid breakdown of the NAD to NMN and adenosine (Figure 5). The production of adenosine from the clevage of the pyrophosphate bond of NAD

was the result of either alkaline phosphatase or 5'-nucleotidase activity acting on the 5'-AMP produced directly from the hydrolysis of NAD. Comparison of the predicted stoichiometry to that which was actually observed showed quantitative conversion of NAD to adenosine. The amount of NMN produced extracellularly from the hydrolysis of NAD reached only 65% that of the predicted value and, unlike adenosine, rapidly fell to 48% of the predicted extracellular level on continued incubation. During this time period, there was no accumulation in the external medium of other products, such as NR or nicotinamide, that could be produced from the hydrolysis of NMN. This suggested that two of the essential components of the NAD metabolism of the organism were the hydrolysis of NAD to NMN and the internalization of NMN or a derivative of it.

The S. typhimurium nucleotide pyrophosphatase was found to be associated exclusively with the inner membrane of the organism (65) while the E. coli enzyme was released into a soluble form by grinding frozen cells of the organism with glass beads. Further characterization of the location of this enzyme has not been described in the literature. The release of the Haemophilus influenzae nucleotide pyrophosphatase was accomplished using either detergent-solubilization or enzymatic procedures (Tables

III-V). Alkyl-glucosides are a class of non-ionic detergents which have been shown to be very effective at membrane solubilization with minimal damage to the proteins released (90,91). Their use is also advantageous due to the fact that they have high critical micelle concentrations which aids in their removal by dialysis (92). Several alkyl-glucosides were effective at solubilizing the enzyme; however, on removal of the detergents by dialysis, the enzyme formed insoluble aggregates.

The most effective method of releasing the enzyme was accomplished by conversion of the cells to spheroplasts by exposure to lysozyme and EDTA. Higher concentrations of both EDTA and lysozyme were required for effective recovery of the enzyme than originally recommended by Malamy and Horecker (71). The release of the nucleotide pyrophosphatase paralleled the release of the periplasmic marker 2',3'-cyclic phosphodiesterase and minimal quantities of the cytoplasmic and plasma membrane markers, glutathione reductase and succinate dehydrogenase, were released. Various outer membrane components of several gram-negative organisms are released after incubation with EDTA alone (81,82). The release of the nucleotide pyrophosphatase, however, was lysozyme-dependent. The nucleotide pyrophosphatase therefore probably exists as a soluble

enzyme located in the periplasmic space of the organism.

After establishing a successful method of releasing the nucleotide pyrophosphatase, experiments were conducted to establish a reproducible purification scheme to isolate the enzyme. The enzyme was bound with very high affinity to concanavalin-A sepharose, phenyl sepharose and Sephadex G-100 columns; however, elution of the enzyme from these columns could not be accomplished. A purification scheme was developed which involved the sequential use of ammonium sulfate precipitation, ion-exchange chromatography and affinity chromatography. The enzyme was purified 700-fold with a 24% recovery of the units of activity initially released by the lysozyme digestion (Table VI). The purified enzyme was demonstrated to be electrophoretically homogenous. The specific activity of the purified enzyme in catalyzing the hydrolysis of NAD was 5.5 μmol/min/mg protein. The purified nucleotide pyrophosphatases from bovine seminal fluid (96), yeast (102), and Proteus vulgaris (98) had specific activities of 2.2, 0.09, and 3.7 μmol/min/mg, respectively.

The properties and characteristics of the purified enzyme were elucidated. The native molecular weight was determined by gel-filtration chromatography using Sephacryl S-200. The enzyme eluted from this column with an apparent

molecular weight of 62,500. The native molecular weight as determined by gel-filtration HPLC was 67,800. The denatured protein, analyzed by SDS gel electrophoresis, migrated as a single band with an apparent molecular weight of 65,800. The molecular weight determined from amino acid composition was 61,600. This value would not account for the presence of tryptophan or carbohydrate residues. The nucleotide pyrophosphatase therefore exists as a single polypeptide with an approximate molecular weight of 64,000. The nucleotide pyrophosphatase from yeast was also determined to consist of a single polypeptide with a molecular weight of 65,000 (102) while those obtained from mammalian sources are considerably larger (141).

The purified enzyme consists of 16% carbohydrate by weight. This fact may serve as an explanation for the unusually high affinity that the enzyme displayed for concanavalin-A sepharose columns and the difficulties encountered in trying to elute the protein from the column.

Thermolability above physiological temperatures is a property of most isolated proteins. Several bacterial nucleotide pyrophosphatases,however, are extremely heat-stable, and are activated on exposure to high temperatures for short periods of time (98,142). The Haemophilus influenzae nucleotide pyrophosphatase activity

was lost as a first-order process at all temperatures investigated with half-lives from 111-19 min over the temperature range from 15-50°C (Figure 13). No other nucleotide pyrophosphatase on which similar studies have been published was as succeptible to thermal denaturation. The effect of temperature on the rate of the enzyme-catalyzed reaction was also investigated over the range of 5.4-45.8°C (Figure 14). From these data, the activation energy of 8.2 kcal/mole for the enzyme-catalyzed hydrolysis of NAD indicated a more efficient catalysis than that observed with the nucleotide pyrophosphatase from bovine seminal fluid which exhibited an activation energy of 14.7 kcal/mole (96).

The effect of several mono- and divalent cations on the nucleotide pyrophosphatase was investigated. The enzyme was affected in a similar manner by the presence of equivalent concentrations of potassium, sodium and magnesium cations. Relative to these cations, the enzyme activity appeared to be slightly decreased in the presence of comparable concentrations of calcium ions. The enzyme activity increased only slightly over a range of ion concentrations and was due to an ionic strength effect rather than specific metal activation. The activity was not affected by inclusion of 5 mM EDTA in the incubation mixture. A survey of other

nucleotide pyrophosphatases shows that these properties are fairly unusual for this type of enzyme. Most nucleotide pyrophosphatases show a requirement for divalent cations for activity and are strongly inhibited by chelating agents such as EDTA (93-95); however, there are known exceptions such as the bovine seminal fluid and rat liver lysosomal nucleotide pyrophosphatases (96,97). The Haemophilus influenzae nucleotide pyrophosphatase may be devoid of a metal requirement or it may possess tightly-bound metal that is inaccessible to EDTA.

The pH v. activity profiles, shown in Figure 17, were identical at both low and high concentrations of NAD. As the pH was lowered below 8.0, a large decrease in activity was observed. The activity from pH 8.0-9.0 was constant and maximal. This profile suggests that one or more functional group(s) exist on the enzyme that possess a pK_a of 7-7.5 and are essential to the catalytic activity of the enzyme. This property is typical of most purified nucleotide pyrophosphatases (96,99,101-104).

The enzyme was observed to be capable of utilizing a large number of substrates with relatively the same level of efficiency (Table X). Substrates with modifications at the three-position of the pyridine ring, such as the 3-methylpyridine and nicotinic acid analogues of NAD were

readily hydrolyzed. 3-Aminopyridine adenine dinucleotide (AAD) was the least effective substrate of the compounds that were modified at the three-position. NADH, which lacks the positive charge on the pyridinium ring, and FAD, which possesses an isoalloxazine ring instead of the pyridine ring of NAD, were readily hydrolyzed. Substrates which lacked a second heterocyclic base, such as ADPR and UDPG, were also hydrolyzed. The pyridinium portion of NAD appears to be of minor importance in the functioning of the enzyme with substrates.

Compounds with modifications of the adenine ring, as in the ethenoadenine analogs of NAD and AAD, were very poor substrates for the enzyme. Conversion of adenine to ethenoadenine in these compounds would be expected to have very little effect on the chemistry of the pyrophosphate bond. The conversion involves the addition of a two-carbon segment to adenine with the subsequent formation of a five-membered ring adjacent to the purine ring. Since the guanosine analog of NAD was a suitable substrate for the enzyme, the inefficiency of the ethenoadenine derivatives is most likely due to steric factors rather than the loss of a hydrogen-bonding amino group which occurs on formation of the new ring system. The bovine seminal fluid nucleotide pyrophosphatase catalyzed the hydrolysis of etheno-NAD with

kinetic constants almost identical to those obtained with NAD (96).

The Haemophilus influenzae nucleotide pyrophosphatase also displayed specificity in its requirement for a pyrophosphate region in potential substrates as bis-PNPP, a compound which consists of a diesterified phosphate group was not hydrolyzed in the presence of the enzyme. The enzyme is therefore a true nucleotide pyrophosphatase and lacks phosphodiesterase activity. The enzyme acts only on pyrophosphate moieties which are diesterified, since ADP was not a suitable substrate. In conclusion, the enzyme displays relatively few requirements of potential substrates. This property is characteristic of most nucleotide pyrophosphatases which have been characterized to date (93,96,98-104).

A property displayed by the enzyme when acting on all the substrates with the exception of a few which possessed modifications of the adenine ring, was non-Michaelian kinetics. Lineweaver-Burk plots of the functioning of the enzyme with various substrates were biphasic, which is indicative of negative cooperativity (Figure 18,19).

Cooperativity in enzyme action is a property that has become apparent in an increasing number of enzymes. Hill et al. estimate that a minimum of 22% of all known enzymes

deviate from simple Michaelis-Menton kinetics (105). Negative cooperativity was first predicted in 1966 from the allosteric model of Koshland (108) and was first observed experimentally in 1968 (109). The demonstration of the existence of negative cooperativity in enzyme action was highly significant in that it could not be accounted for by the models of allosterism proffered by Monod et al. (110). Several enzymes, including the nucleotide pyrophosphatase from sheep liver (104), are now known to act in a negatively cooperative fashion (111-116).

The Haemophilus influenzae nucleotide pyrophosphatase showed negative cooperativity in its functioning with many substrates (Figures 18 and 19). Hill plots of these data enabled the assessment of the degree of cooperativity for each of the substrates (Table XI). Hill coefficients ranged from 0.26 for NAD to values close to unity for several compounds which displayed little or no cooperativity. The extremely low value of the Hill coefficient for NAD indicates that it is the strongest inducer of negative cooperativity of all the substrates analyzed. It has been proposed that the interaction of the adenine ring of NAD with the NAD binding site of sturgeon muscle glyceraldehyde 3-phosphate dehydrogenase is critical to the induction of negative cooperativity in this enzyme (117,118). A similar

conclusion may be made on observation of the kinetic data for the nucleotide pyrophosphatase. All substrates functioned in a biphasic manner except etheno-NAD, etheno-AAD, and nicotinamide guanine dinucleotide, compounds which have been modified in the adenine ring, relative to NAD. Substrates which lacked the pyridine ring of NAD were capable of inducing negative cooperativity but the nature of the substituents on the ring, when present, was also influential on the interaction of the substrate with the enzyme. AAD not only had much poorer binding affinity for the enzyme relative to NAD, but was almost devoid of the ability to induce cooperative interactions as indicated by its Hill coefficient of 0.93. Other analogs with various minor changes in the pyridine ring produced a wide spectrum of Hill coefficients, indicative of widely different capabilities for induction of negative cooperativity in the functioning of the enzyme. Gloggler et al. have proposed that the nicotinamide moiety of NAD is critical in orienting the adenine ring in the NAD binding site of glyceraldehyde 3-phosphate dehydrogenase and is therefore influential on the induction of negative cooperativity in this enzyme (118). AMP, when present at a concentration of 20 μM, abolished the negative cooperativity of the enzyme with NAD as substrate (Figure 22). It may be that the AMP is capable

of producing the changes in the enzyme that occur when the enzyme functions with dinucleotides at high concentrations. By definition of negative homotropic cooperativity, increasing concentrations of substrate induce a different mode of enzyme function which requires higher concentrations of substrate for efficient turnover. The AMP might therefore be acting as a negative effector which disrupts the ability of the enzyme to function at low concentrations of dinucleotide by inducing the enzyme to function in a "high concentration mode." Alternatively, AMP might be acting as a competitive inhibitor at a single catalytic site; however, evidence from fluorimetric studies indicate that AMP is capable of binding to the enzyme in at least two different modes.

Other compounds were analyzed for inhibition of the enzyme. On the basis of apparent dissociation constants (Table XII), the enzyme showed a higher affinity for the "adenine-half" of NAD than for the "nicotinamide-half". AMP was a much more effective inhibitor than NMN. NMN, when present at 20 μM, had no apparent effect on activity. The apparent recognition by the enzyme of pyrophosphate moieties is manifested in the binding affinities for the adenine-containing compounds, i.e. ADP>AMP>adenosine. GMP was bound to the enzyme with a dissociation constant

comparable to AMP but at 20 μM was not capable of abolishing the biphasicity observed in double reciprocal plots. This observation is in accord with the observed functioning of the nicotinamide guanine dinucleotide with the nucleotide pyrophosphatase. This NAD analog had a binding affinity similar to that of NAD, but was incapable of inducing negative cooperativity in the action of the enzyme.

The binding of adenosine, AMP, and ADP were also observed fluorimetrically. It was found that the intrinsic fluorescence of the nucleotide pyrophosphatase could be quenched by the addition of low concentrations of these compounds. Over the concentration range investigated, from 0-150 μM, double-reciprocal plots of the quenching of enzyme fluorescence v. concentration of ligand were biphasic for adenosine, AMP and ADP (Figures 23 and 24). This indicates that the binding of these compounds to the enzyme occurs in two distinctly different modes. Apparent dissociation constants were calculated from these data, and the binding affinity for these compounds was also in the order ADP>AMP>adenosine (Table XIII). The "low" dissociation constants for ADP and AMP correlated well with the binding constants that were determined from kinetic data.

The nucleotide pyrophosphatase exists as a single polypeptide under native and denaturing conditions. Because

most, but not all, enzymes which display negative cooperativity consist of multiple subunits which are assumed to be responsible for conferring the ability to act in a cooperative fashion on the enzyme (130), alternative models for the nucleotide pyrophosphatase were considered. The nucleotide pyrophosphatase from Phaseolus aureus exists as a dimer with a molecular weight of 65,000, but in the presence of AMP is converted to a tetramer (119). Experiments were conducted, using gel-filtration HPLC, to investigate the effect of NAD on the apparent molecular weight of the enzyme. In the absence of NAD, the enzyme migrated through the gel-filtration column with a molecular weight of 67,800. In the presence of 400 μM NAD in the elution buffer, the enzyme migrated with an appparent molecular weight of 108,100 (Table XIV). It is possible that on exposure to these concentrations of substrate that the enzyme undergoes conformational changes, such as a change in the axial ratio, which could affect the mobility of the enzyme through the column. Future work might be aimed at more clearly defining what changes are occurring; however, assuming that the change in mobility is the result of changes in quaternary structure, a simple model for the negative cooperativity of the enzyme can be constructed. The negative cooperativity of the nucleotide pyrophosphatase might reflect the transition

of the enzyme from a single monomer to a dimer with concomitant conformational changes affecting enzyme function. A similar model has been proposed for the negative cooperativity displayed by orotidylate decarboxylase (112).

An alternate explanation for the negative cooperativity displayed by the nucleotide pyrophosphatase is that the single polypeptide may contain one or more effector sites capable of allosteric interaction with the catalytic site(s). This mechanism is rare, but examples, such as chymotrypsin, are known (132). In this system it has been determined that certain azobenzene compounds are able to bind to "sites", presumably at the surface of the enzyme, which induces the enzyme to catalyze hydrolysis of synthetic dipeptides with greater maximal velocity. Due to the fact that high concentrations of the effectors, and effector-like molecules, do not compete for the substrate binding site, this system has been classified as being positively cooperative. There is no observed effect on the Michaelis constants and this system therefore differs from the Haemophilus influenzae nucleotide pyrophosphatase.

Several groups have addressed the question of what selective advantage may be conveyed to the enzyme by negative cooperativity. Conway et al. have suggested that the benefit is that, exactly opposite to the case of

positive cooperativity, the flux through negatively cooperative enzymes is relatively insensitive to large changes in substrate concentration (109). Engel and Dalziel (120) proposed that the conferred ability to make the reaction rate continuously responsive to changes in concentration over several concentration ranges is highly advantageous. Friedrich postulated that enzymes which are negatively cooperative are ideally suited for the vectorial flow of metabolites down a certain pathway (121). All of these theories would seem to be applicable to the Haemophilus influenzae nucleotide pyrophosphatase. The constant turnover of NAD, leading to a fairly constant internal supply of this dinucleotide, in the presence of the uncertain and fluctuating amounts of extracellular NAD most likely encountered by this parasitic organism, would be beneficial. The vectorial flow of pyridine nucleotides through this enzyme, the first in a series of steps involved in the acquisition of NAD, would also be of benefit.

Arginine and lysine residues are prime candidates for participation in binding the negatively charged pyrophosphate region of substrates to the nucleotide pyrophosphatase. The enzyme lost activity as a first-order process in the presence of 2,3-butanedione, a reagent that shows preference for the guanidino moieties of arginyl

residues (128). ADP, at 20 μM, partially protected the enzyme from the inactivation process (Figure 25). The enzyme was also inactivated by 2,4-pentanedione, but much higher concentrations were required relative to the 2,3-butanedione.

A time-dependent inactivation of the enzyme was also accomplished by exposing the enzyme to Woodward's Reagent K (Figure 26). This compound has been shown to covalently modify reactive carboxyl groups (131). In the presence of 20 μM ADP, the inactivation process was accelerated relative to an identical reaction in the absence of ADP. Binding of ADP to the enzyme therefore induces a conformational change which makes the essential carboxyl group more susceptible to covalent modification. This observation is consistent with the observed recognition by the nucleotide pyrophosphatase of adenyl moieties for the induction of functional changes in the action of the enzyme which are manifested as negative cooperativity.

The results of studies of the Haemophilus influenzae NAD metabolism at the molecular level produced a framework for investigations at the cellular level. Numerous growth studies were conducted to investigate the ability of various compounds to 1) serve as V-factor or 2) inhibit growth of the organism, presumably by interference with the pyridine

nucleotide metabolism.

Several growth experiments with NAD as V-factor were conducted. At all concentrations of NAD that supported growth, the organism grew with a 2-3 hour lag phase, followed by 6-8 hours of linear growth before entering stationary phase. NAD served as V-factor and, as shown in Figure 27, allowed growth of the organism on brain heart infusion at concentrations greater than or equal to 0.1 µg/ml. Evans et al. determined that numerous strains of Haemophilus influenzae grew well in the presence of 0.2-1.0 µg of NAD (143) and Parker and Hoeprich (144) obtained maximal growth of the organism at 0.5 µg/ml. NMN also served as V-factor but appeared to function in a different manner relative to NAD (Figure 28). Greater concentrations of the mononucleotide were required for comparable levels of growth and the efficiency of growth, as quantified by the observed doubling times, formed a different pattern of values at increasing concentrations of the nucleotide. The doubling times that were obtained with increasing amounts of NMN approached a saturation value of approximately 50 min at high concentrations of NMN. At high concentrations of NAD, the growth of the organism was more rapid, with doubling times under 40 min. The lack of obvious saturation effects with NAD as V-factor is consistent with the negative

cooperativity of the nucleotide pyrophosphatase, which allows for the functioning of the enzyme over a wide range of concentrations of substrate.

Nicotinamide riboside also served as V-factor and appeared to function in the same fashion as NMN (Table XVII). NADH was also seen to function as V-factor. The ability of NADH to function as V-factor could be the result of the presence of NADH oxidase, an enzyme which is known to be present in the inner, plasma membrane of Haemophilus influenzae (19,129); however, NADH was shown to serve as a substrate for the nucleotide pyrophosphatase so the reduced mononucleotide could therefore be transported into the cell and re-oxidized internally as the resynthesized dinucleotide. Nicotinamide, nicotinic acid and nicotinic acid adenine dinucleotide were all ineffective at fulfilling the organism's pyridine nucleotide requirement, which is consistent with previous reports (49-52,122). The inability of nicotinic acid adenine dinucleotide, a substrate for the nucleotide pyrophosphatase, to serve as V-factor is reflective of the organism's inability to metabolize deamidated derivatives of nicotinamide-containing compounds.

The 3-acetylpyridine, thionicotinamide and hypoxanthine analogs of NAD, compounds which have been observed to function with the nucleotide pyrophosphatase, all served as

V-factor. On hydrolysis of the nicotinamide hypoxanthine dinucleotide, as catalyzed by the nucleotide pyrophosphatase, NMN, a compound which readily supports growth, would be produced. The 3-acetylpyridine analog was much more effective at promoting growth than the thionicotinamide analog. Both of these compounds are known to undergo enzyme-catalyzed oxidation-reduction. However, dehydrogenases which are able to utilize both compounds have shown a greater ability to use the 3-acetylpyridine analog (123). It is unlikely that the efficiency of the 3-acetylpyridine analog to serve as V-factor is a result of its conversion to NAD by the organism, since the likely oxidative intermediate, nicotinic acid adenine dinucleotide, is not able to serve as V-factor. The 3-acetylpyridine analog therefore most likely serves as V-factor by undergoing the same processes used in the acquisition of NAD, with the analog itself functioning with the cytosolic dehydrogenases.

The functioning of the etheno-NAD analog as V-factor was of particular interest. This analog of NAD which is modified in the adenine ring would, subsequent to hydrolysis at the pyrophosphate bond, produce NMN, in a manner similar to the use of the nicotinamide hypoxanthine dinucleotide as V-factor. Unlike the nicotinamide hypoxanthine dinucleotide

analog, the etheno-NAD analog was observed to be very poor at functioning as V-factor. It is probable that the decreased ability of etheno-NAD to serve as V-factor is due to the poor ability of this compound to serve as a substrate for the nucleotide pyrophosphatase. Nicotinamide hypoxanthine dinucleotide is a good substrate for the nucleotide pyrophosphatase and also readily serves as V-factor.

Several compounds which did not substitute for NAD as V-factor were analyzed as inhibitors of growth of the organism with NAD as V-factor. These compounds which inhibit growth are most likely doing so at one or more of the following sites of action:

1) at the nucleotide pyrophosphatase, where compounds could compete with NAD for the dinucleotide binding site(s),

2) at the NAD pyrophosphorylase, where a mononucleotide analog of NMN could compete with the internal pool of NMN for synthesis into dinucleotides,

3) at the NAD kinase used to convert NAD to NADP or

4) at the pyridine nucleotide-requiring enzymes themselves (e.g. the dehydrogenases). Each of these locations represent progressively further steps along the pathway proposed as a result of this work, by which Haemophilus influenzae acquires pyridine nucleotide.

The growth of the organism was inhibited by adenine nucleotides (Table XVIII). While adenosine was not effective as an inhibitor, AMP and ADP, compounds which inhibit the functioning of the nucleotide pyrophosphatase, were very effective. In accord with the pattern of inhibition of the nucleotide pyrophosphatase, ADP was observed to be a more effective inhibitor of growth as compared to AMP. It is likely that these ubiquitous biochemicals are capable of inhibiting the growth of Haemophilus influenzae by selective inhibition of the nucleotide pyrophosphatase.

The most effective inhibitor that was studied was the 3-aminopyridine analog of NAD, AAD. In contrast, the 4-aminopyridine analog was only mildly inhibitory to the organism. Several other analogs were effective at inhibiting growth. These include the 3-methylpyridine, 3-pyridylcarbinol and the pyridine analogs of NAD. N^1-alkylnicotinamide chlorides have been used to study the pyridinium subsites of several NAD-requiring enzymes (124,125). Both the ethyl- and decylnicotinamide chlorides were growth inhibitors. In accord with previous observations, etheno-AAD was a less effective growth inhibitor than the parent compound, AAD. The decreased ability of etheno-AAD to function as a growth inhibitor, relative to AAD, is a result of the conversion of the

adenine ring of AAD to ethenoadenine. This modification also greatly decreased the ability of the compound to serve as a substrate for the nucleotide pyrophosphatase, relative to AAD. In all the growth studies conducted, the ability of compounds to serve as V-factor or to act as a growth inhibitor correlated very well with the ability of the compound to either function with, or inhibit, the nucleotide pyrophosphatase.

The ability to selectively inhibit the growth of Haemophilus influenzae has important clinical implications. In recent years, an increasing percentage of Haemophilus influenzae type b infections have been found to be resistant to the use of antibiotics such as ampicillin and chloramphenicol. Alternative methods of treatment have focused on the use of other antibiotics, which might temporarily serve a useful purpose, but does not address the problem of transfer of resistance to growing populations of the microorganism. The selective inhibition of growth of Haemophilus organisms by specifically targeting the unique NAD metabolism of these organisms for inhibition could potentially provide an alternative method of treatment of infections caused by members of the genus which require V-factor. While AAD was the most effective growth inhibitor investigated, its use as a therapeutic agent would be

problematic due to the neurotoxicity of aminopyridines (133,134). Toxicity of N^1-alkylnicotinamide chlorides has also been assessed and it was determined that the amounts of compound causing death in 50% of treated mice was >100 and 5.6 mg/kg body weight for the ethyl- and decyl-nicotinamide chlorides, respectively (135). The adenine nucleotides may be the most applicable compounds to be used therapeutically. While questions may arise regarding the rapid turnover of these biochemicals, use of these compounds is feasible as shown by their effectiveness in treatment of Herpes zoster infections (136). Clinical trials of these compounds, as well as other competitive inhibitors of the nucleotide pyrophosphatase, as specific inhibitors of Haemophilus growth need to be conducted in the near future.

Growth studies in which the ability of NAD and NMN to function as V-factor indicated differences in the manner in which these compounds are used to fulfill the V-factor requirement (Table XVII). Growth of the organism with NAD as V-factor was much more efficient, as comparable levels of NMN could not produce comparable levels of growth. In addition, with increasing concentrations of NMN, the doubling rates gradually decreased and appeared to reach a saturated level of efficiency. This level of efficiency was far below the growth rates which could be obtained with NAD

as well as with high concentrations of other dinucleotides such as the nicotinamide hypoxanthine dinucleotide analog. The apparent difference in the mechanisms by which the organism acquires internal pyridine nucleotide when using either NAD or NMN was investigated further by observing the effect of AMP on the growth of the organism with NAD or NMN as V-factor. The results of this experiment, as shown in Table XIX, provided evidence for two distinct mechanisms by which the organism may acquire pyridine nucleotides. Growth of the organism with various concentrations of NAD as V-factor was strongly inhibited by AMP relative to control incubations in the absence of AMP, while growth of the organism with various concentrations of NMN as V-factor was unaffected by the presence of AMP.

The ability of Haemophilus influenzae to use NMN as V-factor implies the existence of some mechanism by which the organism is able to convert the mononucleotide to NAD. In most microorganisms, the recycling of NMN to NAD is initiated either by deamidation of the mononucleotide or by hydrolysis of the mononucleotide to nicotinamide. In either case, conversion of nicotinic acid mononucleotide to nicotinic acid adenine dinucleotide is a critical step in the salvage pathway. This reaction is catalyzed by a NAD pyrophosphorylase. This enzyme typically has a preference

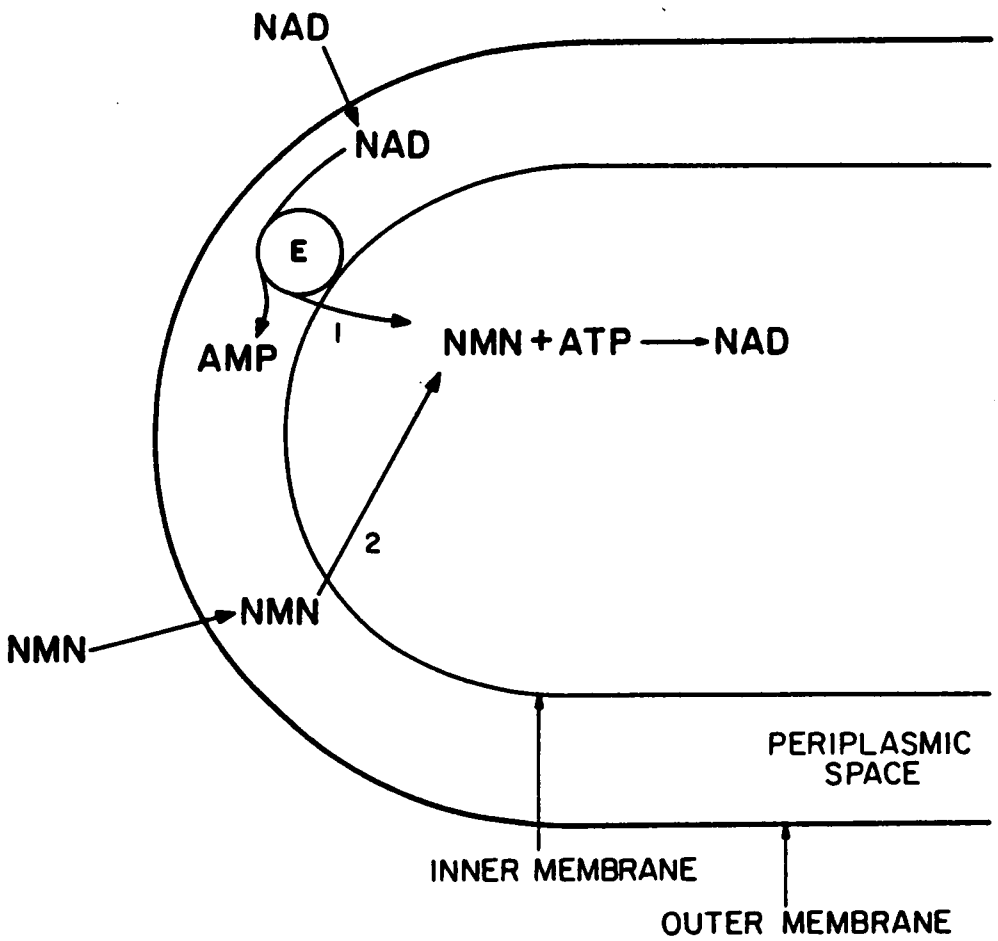
for nicotinic acid mononucleotide over NMN (66), however, organisms which possess a PNC III, such as Haemophilus haemoglobinophilus , utilize a NAD pyrophosphorylase that is capable of catalyzing the synthesis of NAD directly from NMN (126). Due to the inability of Haemophilus influenzae to utilize deamidated derivatives of nicotinamide as V-factor, a search for a similar activity was conducted. A soluble enzyme activity that was released on sonication of cells of Haemophilus influenzae was seen to catalyze the synthesis of NAD from NMN and ATP (Figure 30).

The translocation of molecules into bacterial cells is divided into three categories according to the mode of energization used. In the phosphotransferase system, hydrolysis of an energy-rich phosphate bond in the form of phosphoenolpyrivate is coupled to the translocation of a certain group with the group being phosphorylated as it is transported into the cell (138). These systems are complex and consist of multiple-protein systems. Other transport systems, such as the lactose permease and dicarboxylic acid transport systems, consist of a single protein transporter associated with the inner membrane and utilize a proton-motive force to facilitate transport of these compounds (139), although recent evidence has implicated sodium ion gradients in the transport of melibiose (145).

The third system, the most complex and least understood, involves the use of periplasmic binding proteins. These systems typically are sensitive to osmotic shock and energization is proposed to be derived from the hydrolysis of high energy bonds, most likely ATP (88,140).

Based on the observations described above, a model of the NAD metabolism of Haemophilus influenzae was constructed and is presented in Figure 31. It has been concluded from analysis of the data that two mechanisms exist by which Haemophilus influenzae may acquire pyridine nucleotides. With NMN as V-factor, the mononucleotide would diffuse to the inner membrane where it encounters a transporter protein which facilitates the internalization of the polar nucleotide across the hydrophobic lipid bilayer. This process would be saturable, and would therefore account for the pattern of rates of growth observed with increasing concentrations of NMN. This mechanism would be unaffected by the presence of AMP. A transport protein, specific for NMN, has been observed in Salmonella typhimurium (51,127). In the presence of NAD, the dinucleotide would diffuse into the periplasmic space of the organism where it would encounter the nucleotide pyrophosphatase. The nucleotide pyrophosphatase would cleave the NAD at the pyrophosphate bond and function in a manner that would facilitate the

Figure 31. Model of the NAD metabolism of Haemophilus influenzae.


NAD
NAD
E
AMP
1
NMN + ATP ⟶ NAD
2
NMN
NMN
PERIPLASMIC SPACE
INNER MEMBRANE
OUTER MEMBRANE

internalization of NMN through a mechanism that does not involve the NMN transporter used with free NMN, since growth of the organism with NAD as V-factor is more efficient than with NMN as V-factor. It may be that the nucleotide pyrophosphatase retains the NMN as a covalently-bound, high energy, phosphoryl-enzyme intermediate. The energy released on hydrolysis of the NMN from the enzyme could be coupled to the second process of internalization of NMN. This mechanism would be susceptible to inhibition by AMP which would act by inhibiting the action of the nucleotide pyrophosphatase. This process would therefore be categorized as a form of binding protein transport system, where the nucleotide pyrophosphatase acts as the periplasmic binding protein and the energy of the pyrophosphate bond of NAD, preserved in the enzyme-NMN intermediate, is used to drive transport of NMN into the cell. An alternative model for the facilitated uptake of NMN obtained from NAD could involve a protein-protein interaction between the nucleotide pyrophosphatase and the NMN transporter. The nucleotide pyrophosphatase would not only have a catalytic role but would also act as a periplasmic binding protein interacting with a transporter associated with the inner membrane. The interaction of the protein (with bound NMN) with the membrane-bound transporter would be a more favorable event

than the interaction of the transporter with free, periplasmic, NMN. At the present time, the data are not sufficient to support one model over the other so that this is a question that needs to be addressed in future experimentation. The internalized NMN, acquired by either of these two mechanisms, would be utilized by the NAD pyrophosphorylase to synthesize internal NAD. It is proposed, therefore, that in utilizing NAD as V-factor, Haemophilus influenzae acquires internal pyridine nucleotide by use of a PNC II.

In the future, research might be directed at areas previously mentioned such as questions with regards to the effect of NAD on the structure of the nucleotide pyrophosphatase, understanding in greater depth the negative cooperativity of the enzyme, as well as probing further the apparent difference between growth with NAD or NMN as V-factor in order to more fully understand the transport process. Other enzymes involving NAD might be purified and characterized in order to increase the number of compounds that might be useful clinically. Other potential targets for inhibiting the growth of Haemophilus organisms by selective inhibition of pyridine nucleotide metabolism include the NAD pyrophosphorylase, NAD kinase and cytosolic dehydrogenases.

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STUDIES OF THE NAD METABOLISM OF *HAEMOPHILUS INFLUENZAE*

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(ABSTRACT)

Haemophilus influenzae, as well as other members of the genus which require V-factor, display a unique growth requirement for intact NAD. This organism, the primary cause of bacterial meningitis, is incapable of synthesis of pyridine nucleotides from the usual precursors.

An externally directed nucleotide pyrophosphatase was extracted from the organism and purified 700-fold using ammonium sulfate precipitation, ion-exchange and affinity chromatography. The enzyme was determined to be a periplasmic glycoprotein consisting of a single polypeptide of M_r= 65,000. The enzyme had a pH optimum over the range pH 8.0-9.0 and was not activated by the addition of mono- or divalent cations, nor was it inhibited by EDTA. The enzyme was observed to have a broad substrate specificity and functioned in a manner indicative of negative cooperativity with all substrates except several modified in the adenine ring. The most effective inducer of negative

cooperativity was NAD as indicated by its Hill coefficient of 0.26. The enzyme was inhibited by adenine nucleotides and 5'-AMP, at 20 μM, abolished the negative cooperativity of the enzyme. The enzyme was determined to possess excitation and emission maxima at 286 and 337 nm, respectively, indicative of the presence of tryptophan. The fluorescence of the enzyme was quenched by addition of aliquots of adenine nucleotides. The quenching occurred in a biphasic manner. The enzyme was inactivated by 2,3-butanedione and by Woodward's Reagent K.

Studies of the ability of compounds to serve as V-factor revealed that nicotinamide mononucleotide (NMN), NAD, as well as analogs of NAD, served as V-factor. The ability of compounds to inhibit growth was also accessed, and the growth of the organism was seen to be inhibited by adenine nucleotides as well as other compounds. The inhibition of growth of Haemophilus influenzae has important clinical implications which are discussed, as well as a model of the NAD metabolism of the organism which is presented.