PURIFICATION AND PROPERTIES OF *BUNGARUS FASCIATUS* VENOM NAD GLYCOHYDROLASE

by

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Dissertation submitted to the Graduate Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Nutrition

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April 1981

Blacksburg, Virginia

ACKNOWLEDGEMENTS

I would first like to thank Dr. Bruce M. Anderson for allowing me the opportunity to work in his laboratory. I also want to thank him for his guidance, encouragement, and most of all his never ending ability to put up with me for four years. Thanks are also due to members of my Commitee, Dr. E.M. Gregory, Dr. J.F. Wolfe, Dr. R.E. Ebel, and Dr. J.L. Hess for their extreme help during my graduate research.

I would also like to thank for the many scientific discussions we had pertaining to this research and for typing the final draft of this dissertation.

Finally, I would like to express my warmest thanks to my wife, , for without her love and dedication to me and my work I may have never finished. You will never walk alone.

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LIST OF ABBREVIATIONS

AAD - 3-aminopyridine adenine dinucleotide

AcPAD - 3-acetylpyridine adenine dinucleotide

ADP - adenosine diphosphate

ADPR - adenosine diphosphoribose

ADP-ribose - adenosine diphosphoribose

AMP - adenosine monophosphate

ATP - adenosine triphosphate

DTT - dithiothreitol

ε-NAD - nicotinamide $1,N^6$- ethenoadenine dinucleotide

HPLC - high pressure liquid chromatography

Nam - nicotinamide

PAS - periodic acid-Schiff stain

Phospho-ADP-ribose - 2'-monophospho-adenosine 5'-diphosphoribose

SDS - sodium dodecyl sulfate

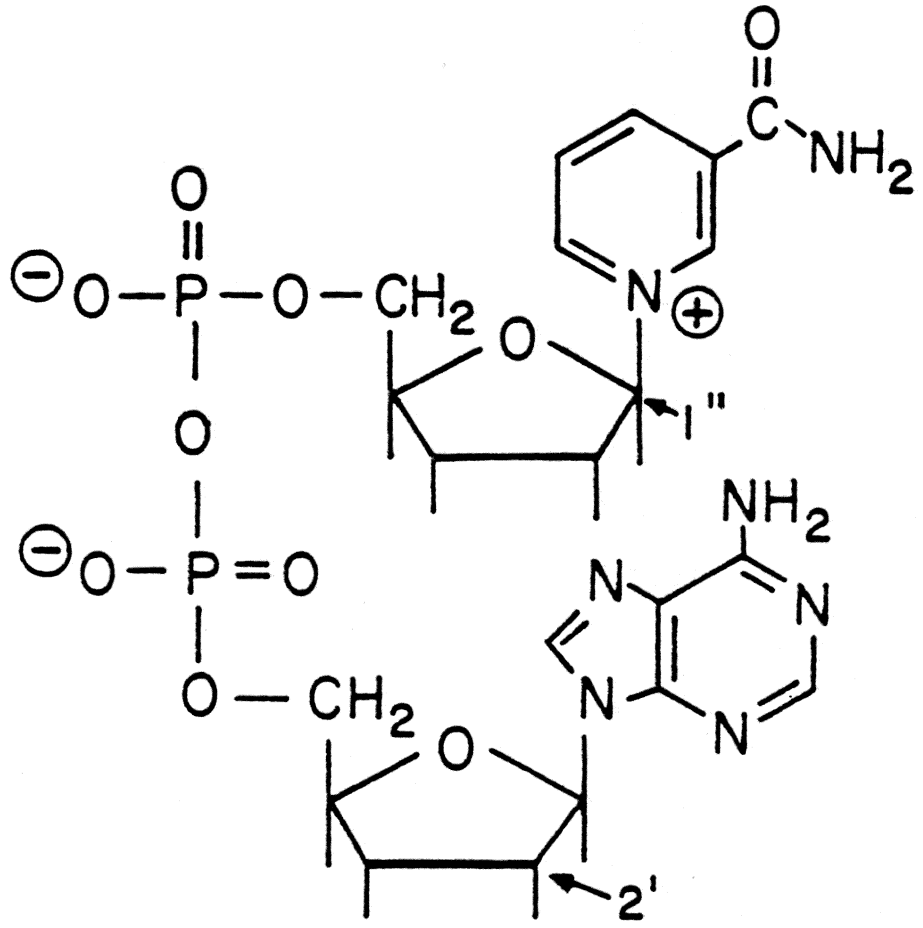
s-NAD - thionicotinamide adenine dinucleotide

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) functions as a coenzyme in many biological oxidation-reduction reactions (1). Hans von Euler et. al (2) purified and determined the structure of the oxidized form of NAD (Figure 1). NAD is the most abundant of the coenzymes which led Gholson to postulate that NAD was important in cellular metabolic processes of unknown function (3). Rechsteiner and coworkers (4) have shown that the cell nucleus is a site of rapid NAD turnover and have proposed a cellular NAD cycle. These investigators found that human D98/AH2 cells growing with a generation time of 24 hours were turning over almost twice as much NAD as Esherichia coli cells having a 30 minute generation time. The net rate of NAD synthesis to maintain an adequate pool size for cellular metabolic reactions for D98/AH2 cells was calculated as 3,900 molecules/sec/cell (4). However, Rechsteiner and coworkers (4) found that approximately 100,000 molecules/sec/cell of NAD were formed. The reason for a 25-fold more rapid rate of NAD synthesis than that required to maintain an adequate pool size in actively growing cells is now thought to be a compensation for the rapid NAD cleavage in cells (4). The rapid turnover of NAD in eukaryotic cells may indicate a more than routine importance for the enzymes involved in the breakdown and synthesis of NAD.

NAD can be hydrolyzed enzymatically at three different bonds; a) the nicotinamide-ribosidic bond, b) the pyrophosphate bond, and c) the adenine-ribosidic bond. The least studied of the enzymes involved in

FIGURE 1 - The structure of β-nicotinamide adenine dinucleotide (NAD).



NICOTINAMIDE ADENINE DINUCLEOTIDE

these hydrolyses is the NAD:purine nucleosidase which catalyzes the hydrolysis of the adenine-ribosidic bond of NAD. Kuwahara and Fugii (5) have purified and characterized the purine nucleosidase from Aspergillus niger. The role of this enzyme in NAD turnover is presently unclear and may be limited to fungal metabolism.

NAD hydrolysis at the pyrophosphate bond, catalyzed by either a phosphodiesterase or nucleotide pyrophosphatase results in the formation of two mononucleotides, nicotinamide mononucleotide (NMN) and adenosine monophosphate (AMP). The phosphodiesterases appear to be relatively non-specific for substrates containing a pyrophosphate linkage. In contrast, nucleotide pyrophosphatases show a definite specificity for nucleotide derivatives containing pyrophosphate linkages (6).

The third class of enzymes, NAD glycohydrolases (NADases) catalyze the hydrolysis of the β-nicotinamide-ribosidic bond of NAD to form nicotinamide and ADP-ribose. NADase activity has been reported in a variety of mammalian cells, microorganisms and to a lesser extent in certain plant tissues.

In addition to the hydrolases described above, there are a number of other enzymes that utilize NAD as a substrate. In general, these enzymes catalyze the transfer of ADP-ribose from NAD to a variety of acceptor molecules with the concommitant release of free nicotinamide. They can be classified as ADP-ribose transferases and include diphthera toxin, cholera toxin, E. coli enterotoxin, turkey erthyrocyte mono ADP-ribosyl transferase, poly(ADP-ribose) synthetase, and certain mammalian NADases that catalyze ADP-transfer to pyridine bases.

Diphtheria toxin (fragment A) has recently been shown to catalyze the mono ADP-ribosylation of a unique amino acid residue within elongation factor 2, leading to cessation of protein biosynthesis (7). Van Ness et al. (8) have determined the structure of this amino acid to be 2-[3-carboxyamido-3-(trimethylammonio)propyl] histidine. These investigators suggest that the ADP-ribose moiety is bound to the histidine derivative through a glycosidic linkage to one of the nitrogen atoms of the imidazole ring.

Cholera toxin and E. coli enterotoxin have likewise been shown to catalyze (9,10) the transfer of an ADP-ribose moiety from NAD to a component of the adenylate cyclase system (9,10). Recent investigations (11-13) have shown the ADP-ribosylated 42,000 MW protein to be the GTP binding protein of the cyclase system.

Moss et al. (14,15) have isolated and purified a mono ADP-ribosyl transferase over 500,000-fold from turkey erythrocytes. The function of mono ADP-ribosylation reactions although not well understood may be involved in a number of complex cellular regulatory processes.

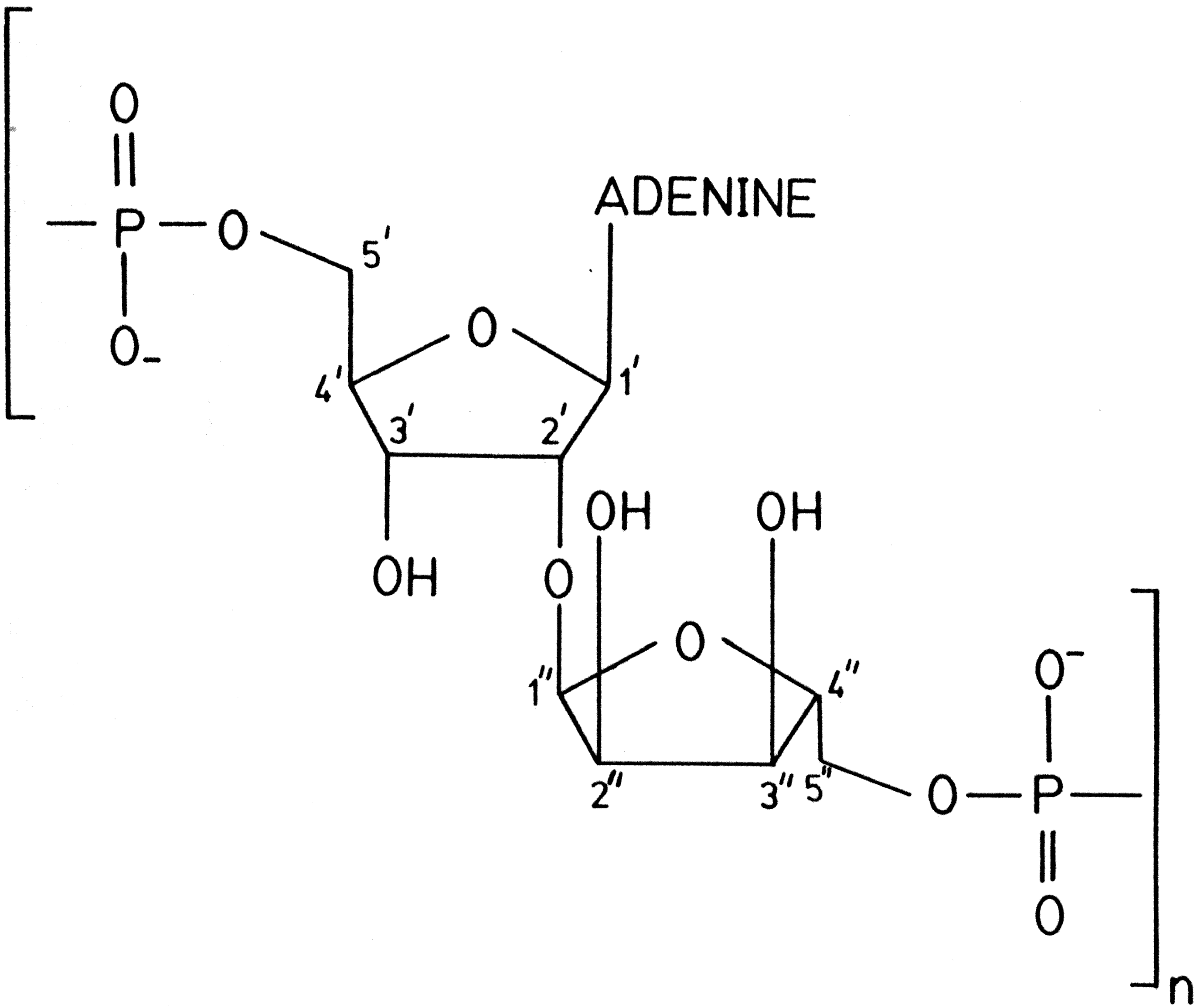
Another type of ADP-ribosyl transferase, poly(ADP-ribose) synthetase differs from those discussed above in that more than one ADP-ribose molecule is transferred to form a chain of repeating ADP-ribosyl units. This enzyme present in the nuclei of all eukaryotes is thought to be responsible for the majority of cellular NAD turnover. Poly(ADP-ribose) synthetase has been found associated with chromatin, and appears to require histone for full activity. Synthesis of poly(ADP-ribose) involves the initial transfer of one ADP-ribose moiety from NAD to some specific

acceptor group on a nuclear protein followed by the transfer of a second ADP-ribose from NAD to the ADP-ribose bound to the protein. Both transfers are thought to be catalyzed by the same enzyme (16).

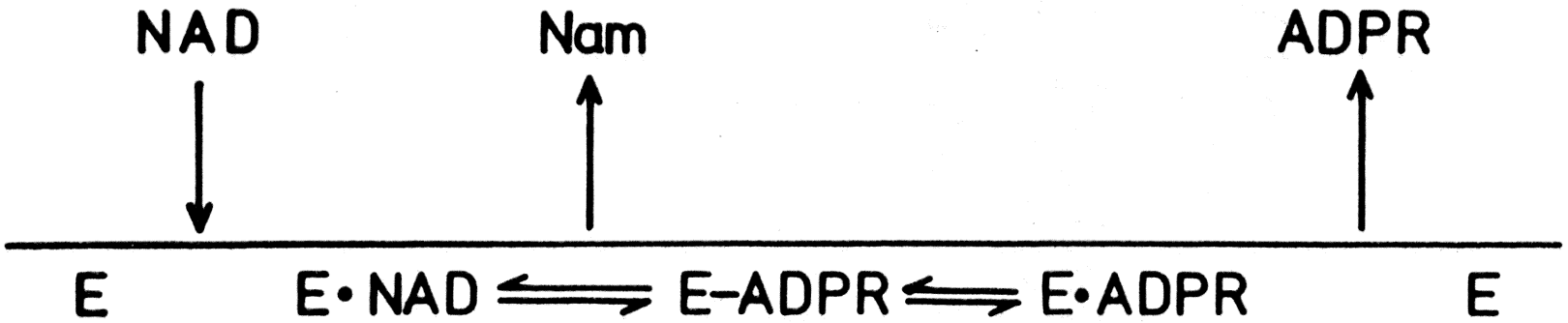
The glycosidic linkage between the 2' and 1" - carbons of the riboses has the α-configuration as shown in Figure 2 (17). This is an apparent inversion of the stereochemistry from the original NAD molecule. The elongation reaction can occur up to 40 times producing a linear chain of ADP-riboses as shown in Figure 2 or as recently shown (17-19) as a branched network of linked ADP-ribose molecules. The function of poly(ADP-ribose) may involve the regulation of cellular DNA synthesis and repair (16,20,26) cell proliferation (16) and cell differentiation (16,27). A summary of some of the known ADP-ribose transfer reactions is shown in Table I.

The precise role of these nonoxidation-reduction reactions of NAD is presently unclear. The rapid NAD turnover suggests that these ADP-ribosyl transfers constitute a major cellular function for NAD. Rechsteiner et al. (32) have found that there is twice as much adenine leaving NAD than is being incorporated into DNA. The amount of poly(ADP-ribose) synthesized in one cell was suggested to be a similar order of magnitude to the amount of DNA synthesized in that cell (32). Both poly(ADP-ribose) synthesis and mono ADP-ribosylation reactions result in the release of free nicotinamide from NAD. Nicotinamide can be salvaged for the resynthesis of NAD or converted to excretory products such as N^1-methylnicotinamide.

FIGURE 2 - The partial structure of poly(ADP-ribose).



ADENINE
O
P
O-
5′
4′
3′
2′
1′
OH
OH
OH
1″
2″
3″
4″
5″
n



Scheme 1 - Cleland Diagram of NADase-Catalyzed Reaction

TABLE I

ENZYMES CATALYZING ADP-RIBOSE TRANSFER

Enzyme	Acceptor	ADP-ribose	Ref.
NAD glycohydrolase	H_2O	monomer	1
NAD transglycosidase	pyridine base	monomer	1
Choleragen	guanidino group	monomer	9,11
E. coli enterotoxin	guanidino group	monomer	10
Mono ADP-ribosyl transferase	guanidino group	monomer	14,15
Diphtheria toxin	elongation factor 2	monomer	7,8
Pseudomonas toxin	elongation factor 2	monomer	28
T_4 phage	RNA polymerase	monomer	29
N_4 phage	*E. coli* proteins	monomer	30
Poly(ADP-ribose) synthetase	chromosomal proteins	polymer	16
Mitochondrial synthetase	mitochondrial proteins	oligomer	31

The biochemical pathways for the metabolism of pyridine nucleotides have been recently reviewed (33-35). Johnson (36) and Bernofsky (37) have suggested that serum nicotinamide which is converted to NAD in the liver is the key element governing the metabolic pool of pyridine nucleotides. During fasting nicotinamide homeostasis is maintained by the NADase catalyzed hydrolysis of NAD.

Excess nicotinamide is toxic to most animal cells. A suppression by nicotinamide of both RNA and DNA synthesis has been reported (37). Excess nicotinamide is methylated at the ring nitrogen in a reaction requiring S-adenosyl methionine to form N^1-methylnicotinamide. This pyridinium derivative not function in NAD synthesis is concentrated and excreted in the urine.

The breakdown and synthesis of NAD appears to be a very tightly controlled cycle. Nicotinamide formed during the enzymatic cleavage of the nicotinamide-ribosidic bond of NAD apparently controls the amount of cellular NAD resyntheses. Intracellular NAD can play an important role in the regulation of metabolic pathways as a substrate for poly(ADP-ribose) synthesis and mono ADP-ribosylation reactions. Mono ADP-ribosylation as a post-translational modification of cellular proteins may prove to have an important regulatory function.

Mechanistic studies of the nonoxidation-reduction reactions of NAD have been hampered by the difficulties encountered in the preparation of sufficient quantities of purified enzymes that catalyze ADP-ribosyl transfer reactions. Membraneous mammalian NADases require harsh treatments for solubilization and purified forms of these enzymes have been

too labile for most mechanistic studies. The observation that certain snake venoms contained soluble NADases that could catalyze trans-glycosidation reactions suggested a new approach to mechanistic studies of ADP-ribosyl transfer reactions. Purification of a stable NADase would allow a mechanistic investigation of ADP-ribosyl transfer reactions. This possibility prompted the present study of Bungarus fasciatus venom NADase.

LITERATURE REVIEW

Enzyme catalyzed hydrolysis of nicotinamide adenine dinucleotide (NAD), resulting in the loss of coenzymatic activity was reported by many investigators (2,38,39). Handler and Klein first demonstrated that this inactivation was due to the hydrolysis of the nicotinamide-ribosidic bond (40). The enzyme responsible was named nicotinamide adenine dinucleotide glycohydrolase (NADase; EC 3.2.2.5). All NADases are characterized as catalyzing the hydrolysis of the nicotinamide-ribosidic bond of NAD to produce nicotinamide and ADP-ribose in equimolar amounts (Figure 3). NADases have been reported in a number of microorganisms (1,41-55), and a variety of animal tissues (1,56-87). There have been only a few reports of NADase activity in higher plants (88-90). A partial listing of these NADases is shown in Table II.

Plant NADases

Masahiro Hori et al. (88) have found the formation of ^{14}C-nicotinamide when rice seedings were fed ^{14}C-nicotinic acid. These authors suggested that ^{14}C-nicotinamide was formed from a direct breakdown of newly synthesized ^{14}C-NAD. Further evidence in support of this is that no enzyme catalyzing amination of nicotinic acid was found. However, in these investigations NADase activity was not assayed directly.

Tumor tissue of Rumex acetosa was reported to contain two different forms of NADase (89), one bound to microsomes the other bound to the nuclear membrane. Although both enzymes exhibit similar pH optima, and hydrolyze both NAD and NADP, they differ in K_m values for NAD. The

FIGURE 3 - The reaction catalyzed by NAD glycohydrolase.

$$NAD^+ + H_2O \xrightarrow{\text{NADase}} \text{Nicotinamide} + \text{ADP-ribose} + H^+$$

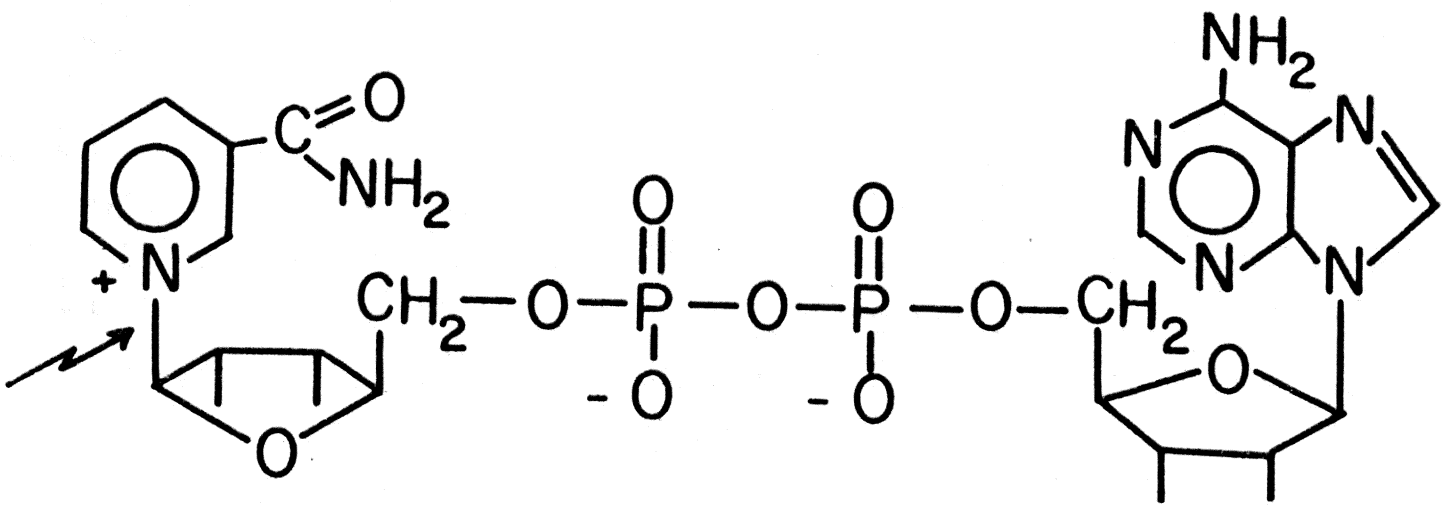


TABLE II

NADases

Plant Source	Molecular Weight	Reference
Rice seedling	N.D.	88
Rumex acetosa	N.D.	89
Lupinus luteus	N.D.	90

Microorganism	Molecular Weight	Reference
Streptococcus pyrogenes C203	55,000	43
Streptococcus CH 46A	70,000	46
Fasarium nivale	27,000	45
Bacillus subtilus	24,000	49
Pseudomonas putida KBI	23,500	41
Mycobacterium butericum	39,000	47
Mycobacterium tuberculosis	N.D.	45
Corynecacterium diphtheria	63,000	7
active peptide	24,000	7
Vibro cholerae	83,000	13
active peptide	23,500	15
Escherichia coli	24,000	10
Neurospora crassa	31,500	49

TABLE II (cont')

Animal Source	Molecular Weight	Reference
Human		
Liver	90,000	84
Synaptic Membrane	N.D.	60
Erythrocyte	N.D.	66
Guinea Pig		
Polymorphonuclear leuckocytes	N.D.	85
Liver	N.D.	86
Cerebal cortex	N.D.	86
Spleen	N.D.	86
Cow/Calf/Bull		
Cow brain	24,000	84
Bull spleen	23,000	84
Calf spleen (membrane)	90,000	75
Calf spleen (hydrosoluble)	24,000	87
Bull seminal plasma	36,000	77
Cow erythrocyte	N.D.	73
Calf liver	23,000	68
Mice		
Ehrlich Ascites	60,000	67
Liver	69,000	68
Kidney	80,000	84
Brain	80,000	84
Spleen	N.D.	84
Macrophage	N.D.	84
Pig		
Brain	26,000	68

TABLE II (cont')

Animal Source	Molecular Weight	Reference
Pig		
Liver	22,000	68
Spleen	25,000	84
Lung	23,000	84
Rabbit		
Heart	N.D.	74
Liver	89,000	68
Erythrocyte	N.D.	73
Rat		
Liver	83,000	64
Small intestine	85,000	70
Brain	N.D.	70
Stomach	N.D.	70
Ascending colon	N.D.	70
Spleen	N.D.	70
Dog		
Liver	43,000	68
Brain	50,000	84
Snake Venom		
Agkistrodon bilineatus	N.D.	63
Agkistrodon halys blomhoffi	100,000	83
Bungarus fasciatus	125,000	57

microsomal enzyme was inhibited by nicotinamide noncompetitively whereas the nuclear enzyme was inhibited competitively. The nuclear NADase has many properties very similar to those reported for other nuclear poly(ADP-ribose) synthetases (17) and therefore may not be a true NADase. The Rumex acetosa microsomal NADase exhibits properties similar to mammalian microsomal NADases.

The only NADase of plant origin to be verified was obtained from Lupinus luteus (90). Direct hydrolysis of the nicotinamide-ribosidic bond of NAD was evidenced by the formation of nicotinamide and confirmed by the cyanide addition assay. The formation of the cyano-adduct requires an intact nicotinamide-ribosidic bond. Neither nicotinamide-riboside or nicotinamide mononucleotide were substrates for the plant enzyme. The NADase exhibited a broad pH profile with an apparent maximum at pH 6.0. Although product inhibition studies were incomplete, nicotinamide was a competitive inhibitor with respect to NAD, exhibiting a K_i = 28 mM. At present this report by Hesse (90) represents the only positively identified NADase from a plant source.

Microbial NADases

Kern and Natale (47) reported that extracts of Mycobacterium butyricum contained a heat-activated NADase tightly associated with a heat-labile protein inhibitor. Similar results were found by Kasarov and Moat (45) when studying Mycobacterium tuberculosis.

Another thermostable NADase was found in extracts of Pseudomonas putida along with a heat-labile inhibitor (41). The P. putida NADase

like other microbial NADases was essentially specific for NAD and NADP. The presence of NAD decreased the affinity of the inhibitor for the NADase by 60-fold. Like most NADases a broad pH profile was observed.

Carlson et al. (46) investigating the metabolic effects of Streptococcus culture filtrates on rabbit myocardium, found NADase activity in these filtrates. The NADase was found to be released into the extracellular medium by certain strains of Streptococcus (42-44,46, 52). This extracellular NADase, unlike the P. putida and M. butyricum enzymes was not associated with a heat-labile inhibitor. Early investigators (42,44) suggested that the Streptococcus enzyme also exhibited hemolytic activity, streptolysin-0. Fehrenbach (42) postulated that the bacterial NADase penetrated the plasma membrane and that the enzymatic hydrolysis of NAD was the key to NADase:streptolysin-0 induced hemolysis of red blood cells. However, Grushoff et al. (43) described a separation of NADase activity and hemolytic activity which argues against the two activities being catalyzed by the same protein. The Streptococcus extracellular NADase was purified over 3500-fold to a final specific activity of 11,200 units/mg protein. The purified enzyme catalyzed the hydrolysis of either NAD or NADP but no pyridine base exchange reaction was reported.

Everse and coworkers (49) purified the Bacillus subtilis NADase which like most bacterial NADases was shown to be very tightly complexed to an inhibitor. Both the NADase and its inhibitor were shown to contain a large amount of carbohydrate, 55 and 71% respectively. The protein inhibitor:NADase complex was able to bind but not cleave NAD.

Active B. subtilis NADase was also reported not to catalyze the pyridine base exchange reaction.

NADase from Neurospora crassa was also purified and characterized by Everse and coworkers (49,53). The NADase was found in the conidia, and as mycelial growth developed was released into the extracellular medium. Like the B. subtilis enzyme, the N. crassa NADase also contains a large amount of carbohydrate. However, the N. crassa NADase was not associated with an inhibitor and was specific for NAD or NADP as substrates.

With the exception of those from Streptococcus and N. crassa, the microbial NADases can be generally characterized as soluble, heat stable proteins which interact very tightly with heat labile protein inhibitors. All the NADases appear to be specific for NAD or NADP as substrates. The N. crassa and B. subtilis enzymes were found to be soluble glycoproteins. Microbial NADases are futher characterized by their inability to catalyze the pyridine base exchange reaction between the nicotinamide moiety of NAD and other pyridine bases.

Mammalian NADases

Mammalian NADases have been demonstrated in a number of tissues from many different species as shown in Table II. The majority of these NADases are membrane bound (56,58-62,64-69,80-82,84-87,106). Cell fractionation studies indicated the NADases were associated with many subcellular fractions, including the endoplasmic reticulum (62,64,67,95, 97), the plasma membrane (60,64,70,78,79,103-105), the nuclear membrane (80,91-93), secondary lysosomes (71), and more recently, the intermediate

saccules of the golgi apparatus (82). Enzyme activity has also been detected on the surface of intact erythrocytes and erythrocyte ghosts (66,72,73,99-101). The only non-membrane bound mammalian NADase has been isolated from bovine seminal plasma (77).

Waravdekar and Griffin (93) first isolated a nuclear NADase from normal and tumor cells; however, a characterization of the enzyme was not done. Green and Dobrjansky (94) later described the properties of a NADase partially purified from nuclei of mouse Ehrlich ascites tumor cells, the activity of which was found to rapidly decrease during incubation with NAD. The enzymatic hydrolysis of NAD at pH 6.0 by this nuclear NADase was linear up to 1 hour. However, at pH 8.0 the nuclear enzyme was rapidly inactivated. Nuclear NADase inactivation was stereochemically specific for the β-isomer of NAD. Nicotinamide completely protected the enzyme from inactivation. Inactivated NADase could not be reactivated by changes in temperature, pH, or by extensive dialysis.

Two distinct forms of NADase exist in nuclei of Ehrlich ascites tumor cells (95) and rat liver (91). One form was found to be bound to DNA and irreversibly inactivated in the presence of high NaCl concentrations or by treatment with DNase. This enzyme was also shown to contain poly(ADP-ribose) synthetase activity. The other form of NADase was bound to the nuclear membrane. Green and Dobrjansky (95) reported this enzyme had properties very similar to those demonstrated with a number of microsomal NADases. DNase treatment did not affect this NADase activity. These investigators (95) did not detect any NADase activity associated with plasma membranes fractions.

and the impermeability of NAD clearly indicates the presence and catalytic functioning of the NADase on the exterior surface of the erythrocyte plasmalemmal membrane. The erythrocyte NADase can catalyze the hydrolysis of NAD in the surrounding plasma, while the intracellular levels of NAD and NADP would be unaffected.

Bekierkunst (102) reported that suspensions of Ehrlich ascites cells could cleave NAD present in the cell suspension media. The loss of NAD could not be accounted for by the penetration of the dinucleotide into the cells. Sonication of Ehrlich ascites cells did not greatly increase the amount of NADase activity. Bekierkunst suggested that intact Ehrlich ascites cells probably contained a NADase located on the outer cell surface. Alivisatos and coworkers (60) found that sonication of intact rat brain cells did not result in any pronounced increase in the level of NADase activity. Cellular subfractionation of the brain tissue localized 30% of the NADase activity within the crude mitochondrial fraction and another 35% in the microsomal fraction. When the crude mitochondrial portion was further fractionated, 65% of the remaining NADase activity was found in the synaptosomes. Synaptosomes are closed vesicles formed from the synaptic endings of nerve cells during tissue homogenation, and are derived from the plasma membrane. Synaptic membranes were designated as such by the marker enzyme $(Na^{+}-K^{+})$-ATPase. The NADase that was localized within the microsomal fraction was thought to have arisen from vesicle contamination by plasma membranes during cellular disruption. Alivisatos et al. (60) also found

that the NADase activity was distributed in the same cellular subfractions as the serotonin binding sites and concluded that the NADase was pericellular on the plasma membrane.

Muller and Schuber (103) have thoroughly characterized the cellular localization of calf spleen NADase. Using discontinuous density gradient centrifugations, the highest amount of NADase was found associated with microsomes. The distribution pattern of NADase was similar to alkaline phosphodiesterase or 5'nucleotidase, known plasma membrane markers but unlike that of NAD(P)H-cytochrome C reductase, a marker for endoplasmic reticulum. They concluded that the bulk of the NADase was associated with light membrane fractions originating from the plasmalemma, or components derived from it (endocytotic vesicles). Since the vesicles were predominantly right side out, the NADase was considered an ecto enzyme similar to the erythrocyte NADase (72,73). Beaufay and Amar-Costesec obtained similar results showing that rat liver NADase was predominantly found in fractions derived from the plasma membrane (104). Both would be capable of hydrolyzing exogenous NAD.

Further evidence for the localization of mammalian NADases in the plasma membrane was presented by Artman and Seely (79,105). They found that murine macrophages contained higher levels of NADase than any other cells, tissues, and, or, organs of the mouse. Artman and Seely (105) found that the majority of NADase of the macrophages was localized on the external side of the plasma membrane. The nonpenetrating diazonium salt of sulfanilic acid was shown to inactivate the NADase.

Peritoneal macrophages obtained from mice injected with living Mycobacterium bovis or inactivated Corynebacterium parvum bacteria contained no pericellular NADase activity (105). The disappearance of the plasma membrane NADase could result from the interiorization of protein and membrane during endocytosis and subsequent digestion by phagolysomal proteases. The reported association of NADase with rabbit liver secondary lysosomes supports this conclusion (71).

Kakehi et al. (70) using isolated everted small intestine segments, studied the fate of NAD and its penetration into the mucosal membrane. When ^{14}C-NAD exposed to the mucosal side was hydrolyzed to yield ^{14}C-nicotinamide, no radioactive NAD was found on the serosal side. The amount of ^{14}C-nicotinamide on the serosal side increased linearly during the incubation. It was concluded that NAD present in the small intestine was cleaved by a NADase and the nicotinamide produced was absorbed by the small intestine. These observations provide further support that mammalian NADases are localized on the plasma membrane.

The association of mammalian NADases with the external surface of the plasma membrane explains the observation that cells known to contain high NADase activity also have a relatively stable pool of NAD. In addition, the regulation of intracellular levels of NAD as suggested by Kaplan (106) is probably not the major function of these enzymes (103).

Several laboratories (35,70,107-108) have reported that nicotinamide may enter the NAD biosynthetic pathway by way of nicotinamide mononucleotide. This may prove to be the major route for NAD

recycling in mammals since dietary NAD cannot be directly absorbed through the mucosal membrane.

There are several examples of extracellular NADases isolated from a variety of animal sources (57,63,77,83,109,110). The function of these soluble NADases is presently unclear. Yuan and Anderson (107) have purified bull semen NADase to electrophoretic homogeneity. Bull semen NADase did not catalyze the pyridine base exchange reaction; however, it did catalyze a self-inactivation reaction (110) (vide infra).

Snake Venom NADases

Recent studies by Yost and Anderson (57) and others (63,83,111), have demonstrated NADase activity in snake venoms. Bhattacharya (111) first reported the existence of a NAD destroying enzyme in the crude venom of Bungarus fasciatus. Suzuki and coworkers (83) found NADase in 9 of 35 venoms analyzed. Venom NADase apparently was restricted to snakes of the species Crotalidae, Viperidae, and Elapidae. Tatsuki et al. (83) partially purified the NADase from Agkistrodon halys blomhoffii venom. Venom NADases exhibited similar properties to most mammalian membrane bound NADases. These enzymes were shown to be soluble and in some cases catalyzed the pyridine base exchange reaction. The purification of one of these snake venom NADases would provide an experimental system for a mechanistic study of the pyridine base exchange reaction.

Self-Inactivation

In addition to hydrolysis and pyridine base exchange, several mammalian NADases catalyze a self-inactivation reaction. Lieberman

(112) first described the loss of NADase activity in mouse fibroblast cell cultures when incubated with NAD. The loss in NADase activity was time dependent and was not recovered even after extensive cell washing. ADP-ribose, but not nicotinamide protected the enzyme from inactivation.

Zervos et al. (110) described the self-inactivation of the soluble NADase from bovine seminal fluid during NAD hydrolysis. Additional substrate or dilution of the reaction mixtures did not result in further substrate hydrolysis which excluded product inhibition as the cause for loss of enzyme activity.

Pig brain NADase was inactivated during incubation with dithiothreitol and NAD (61). NAD hydrolysis was not a strict prerequisite for inactivation, since similar results were obtained with NADH, a competitive inhibitor of NAD hydrolysis. Cordes and coworkers (61) proposed that a conformational change was responsible for enzyme inactivation.

Green and Dobrjansky (68) described the irreversible inactivation of the membrane associated NADase from Ehrlich ascites cells. At pH 6.0 enzyme activity was linear up to 1 hour, whereas at pH 8.0 a rapid loss in NADase activity was observed. The presence of nicotinamide prevented inactivation.

Recently, Pekala et al. (73) described the self-inactivation of a number of erythrocyte NADases. Inactivation was dependent on NAD hydrolysis and followed pseudo-first order kinetics. NADase-catalyzed self-inactivation may be an internal ADP-ribosylation of these enzymes. An alternate suggestion is the breakdown of a oxocarbonium ion intermediate proposed for NADase-catalyzed reactions to a stable tetrahedral complex between the enzyme and ADP-ribose.

Mechanisms of NADase Catalyzed Reactions

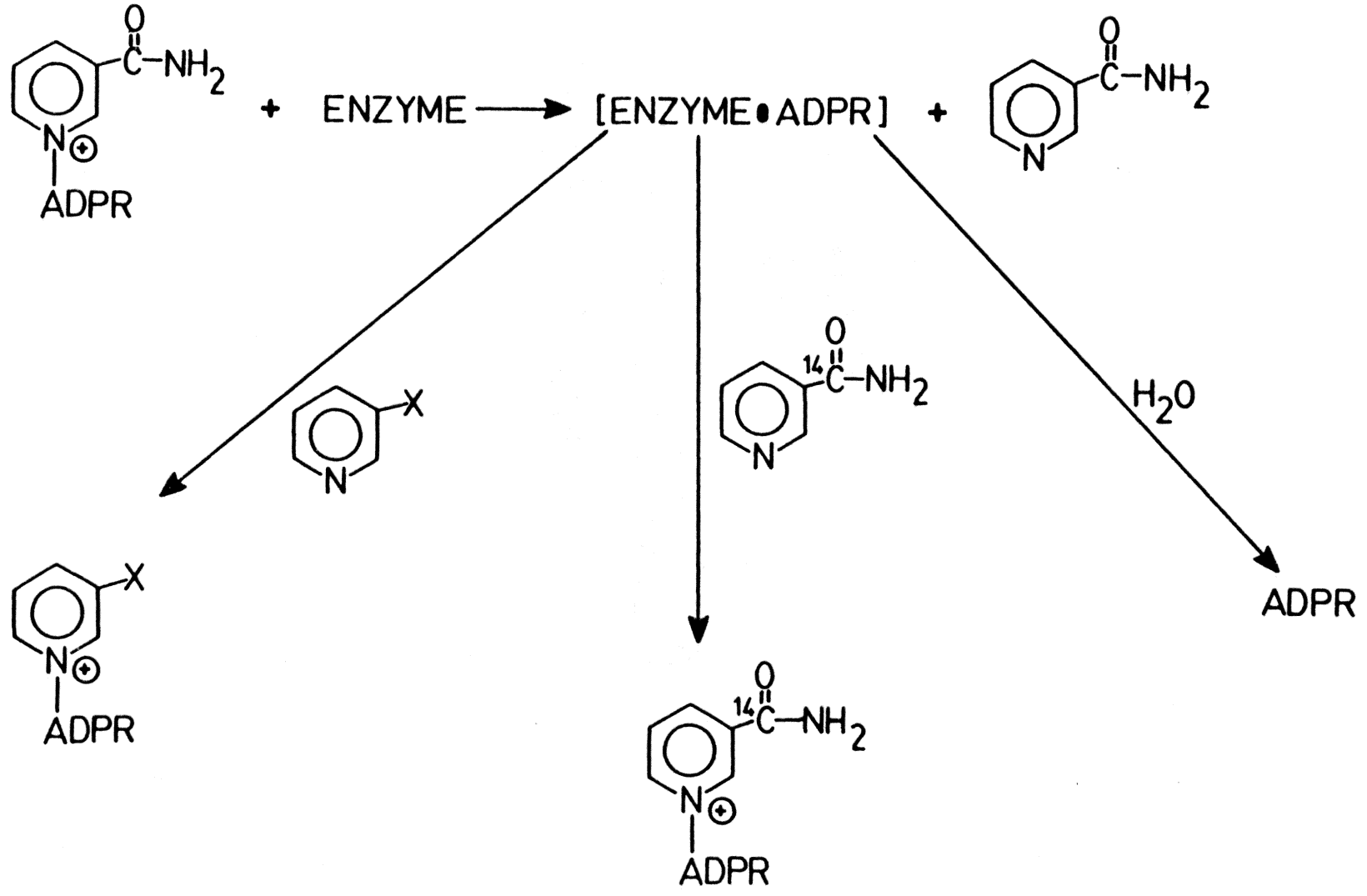
The mechanism of action of the mammalian membrane bound NADases was first described by Zatman et al. (56). In studies of bovine spleen NADase, nicotinamide was demonstrated to be a noncompetitive inhibitor of NAD hydrolysis. When the enzyme and NAD were incubated in the presence of ^{14}C-nicotinamide, ^{14}C-NAD was obtained. These investigators suggested that the hydrolysis of NAD proceeded through an enzyme-ADP-ribose intermediate. The competition of water or nicotinamide with this complex was offered as an explanation for hydrolysis, noncompetitive inhibition by nicotinamide, and the formation of labeled NAD (Figure 4).

Pyridine bases other then nicotinamide are substrates for the pyridine base exchange reaction. By utilizing this reaction, more then 70 NAD analogs have been synthesized (113).

Alivisatos (114) reported using beef spleen NADase, the exchange of 5-amino-4-carboxamido imidazole for the nicotinamide moiety of NAD. This was the first demonstration of a transglycosidation reaction between NAD and a non-pyridine base. Alivisatos further suggested (114) that the NADase catalyzed reaction proceeds by a double-displacement reaction, in which the ADP-ribose moiety is first transferred to a histidiyl residue later to be displaced by water. This mechanism requires a double Walden inversion.

Anderson et al. (115) found that 3-acetylpyridine adenine dinucleotide (AcPAD) was a less effective ADP-ribose donor than the corresponding thionicotinamide adenine dinucleotide (s-NAD). Pig brain NADase-catalyzed formation of NAD from nicotinamide and s-NAD was approximately

FIGURE 4 - The Kaplan model of the pyridine base exchange reaction.


ADPR
+ ENZYME
[ENZYME • ADPR]
+
X
14
H_2O
ADPR
ADPR
ADPR

10-fold faster than from nicotinamide and AcPAD. However, s-NAD was hydrolyzed by the NADase only 2-fold faster than AcPAD. These investigators concluded that these data were inconsistent with the mechanism proposed by Zatman et al. (56). Since the pyridine moieties of these ADP-ribose donors exerted an effect on the rate limiting step in these transglycosidation reactions (115) a common enzyme-ADP-ribose intermediate would not fully explain these results. The direct displacement of the pyridine moiety of the enzyme-bound dinucleotide by another pyridine base or water was proposed (115).

Cordes and associates (61) were the first to describe a detailed kinetic analysis of the NADase-catalyzed hydrolysis of NAD. Since the leaving group, nicotinamide, bears a full positive charge in the substrate, specific or general acid catalysis can be excluded. The unshared electron pair of the ribose ring oxygen atom could provide the driving force necessary for the expulsion of the pyridine group and thus eliminate the need for nucleophilic catalysis. It was concluded that catalysis proceeded through a unimolecular decomposition of NAD (61). The resulting oxocarbonium ion could be stabilized by amino acid side chains at the enzyme active site.

Schuber and Travo (116-118) studying calf spleen NADase concluded that the minimum kinetic mechanism is an ordered bi-bi ping pong. If water is not considered the kinetic pattern would reduce to ordered uni-bi with nicotinamide being the first product released followed by ADP-ribose. These investigators demonstrated that methanol could replace water as the ADP-ribose acceptor. These results, along with those of

Kaplan and coworkers (56), support the involvement of an activated intermediate in the NADase mechanism.

Cordes and Bull (119) found the kinetic alpha secondary deuterium isotope effect (k_H/k_D) for the non-enzymatic pH-independent hydrolysis of NAD to be 1.11. An isotope effect of this magnitude is consistent with a positively charged transition state in which the charge is equally divided between the nicotinamide leaving group and the developing oxocarbonium ion. Pig brain NADase-catalyzed hydrolysis of NAD however, occurred without any detectable secondary deuterium isotope effect. One can question whether or not these observations reflect a nucleophilic displacement of nicotinamide or the proceeding of the reaction through a carbonium-like pathway where bond-cleavage is masked, resulting in no observable secondary isotope effects. Cordes and coworkers (119) indicated that the absence of a detectable secondary isotope effect of the enzyme-catalyzed hydrolysis of NAD occurs via a oxocarbonium ion pathway and that the rate determining step does not involve carbon-nitrogen bond cleavage.

The secondary isotope effects (119,120) are consistent with product inhibition patterns observed for calf spleen and bovine seminal plasma NADases (116,121). Further support for this mechanism was the capacity of certain mammalian NADases to catalyze the pyridine base exchange reaction and a nucleophilic competition of hydrolysis with methanol. These reactions proceed with retention of configuration about the N-ribosidic bond. Pascal and Schuber (122) using calf spleen NADase

concluded that 1) nicotinamide was the first product released 2) an enzyme-ADP-ribose complex was formed subsequent to the rate determining step, and 3) substrate binding to the enzyme was reversible.

If a unimolecular decomposition of NAD is assumed then kinetic data (118,122) eliminate the secondary isotope effect of unity as resulting from an irreversible substrate binding or that product release was rate determining. If carbon-nitrogen bond cleavage is not rate determining (120) then all one is left to account for the absence of a detectable secondary isotope effect would be a rate determining conformational change within the enzyme. Cordes and coworkers (61) indicated that the pig brain NADase proceeded through an enzyme conformational change upon substrate binding.

Although the enzyme-catalyzed hydrolysis of NAD has been extensively studied, the mechanism involved in enzyme-catalyzed pyridine base exchange are only poorly understood. The absence of kinetic data for the pyridine base exchange reaction prompted the present studies. The objective was to purify and characterize a soluble NADase capable of catalyzing the pyridine base exchange reaction.

EXPERIMENTAL PROCEDURES

Materials - All mono and dinucleotides were purchased from Sigma Chemical Company except, ε-NAD and thionicotinamide-, 3-aminopyridine-, 3-aminomethylpyridine-, 3-pyridinealdehyde-, pyridine-, 3-methylpyridine-, 3-pyridylacetonitrile-, 3-pyridylcarbinol-, 4-aminopyridine-, isonicotinic acid hydrazide-, and isonicotinamide analogs of NAD which were prepared by published procedures (123-125). Molecular weight standards were purchased from Sigma Chemical Company, except glutathione reductase, which was purchased from Calbiochem. All snake venoms were purchased from Sigma Chemical Company as lyopholized powders. Pyridine bases and Procion Blue HB were obtained from Aldrich Chemical Company. Matrix gel Blue A was purchased from Amicon. Phosphocellulose P-11 was obtained from Whatman. Concanavalin A-agarose and Woodward's Reagent K were obtained from Sigma Chemical Company. High pressure liquid chromatography products were purchased from Alltech. All other reagents used were Baker chemical or of equivalent grade. Bull semen NADase was purified by the published procedure (126).

Methods

Activation of phosphocellulose - Whatman phosphocellulose P-11 was stirred in a three-fold volume of 0.5 N NaOH for 15 min; after settling, the fines were removed by decanting. The resin was then washed with 10 ℓ of water through a fritted glass funnel afterwhich the pH was 8.0-8.5. The resin was then stirred in a three-fold volume of 0.5 N HCl for 15 min and brought to pH 6.0 by washing with water as before. Phosphocellulose was stirred in a five-fold volume of 0.5 M Tris HCl, pH 8.2.

The resin was titrated to pH 8.2 with 6 N NaOH. Before use, phosphocellulose columns were equilibrated with 0.05 M Tris-Hcl, pH 8.2.

Protein Determinations - All protein determinations were performed according to Lowry et al. (127) or Bradford (128). Crystalline bovine serum albumin was used as the protein standard.

Native polyacrylamide gel electrophoresis - Polyacrylamide disc gel electrophoresis was performed by the method of Davis (129). A 9% separating gel with a 3% stacking gel was used. Electrophoresis was carried out at 3.5 mA per gel at 4°. After electrophoresis, proteins were stained with Coomassie Blue or periodic acid-Schiff reagent for glycoproteins (130). Gels were scanned using a Gilford 250 spectrophotometer. Periodic acid-Schiff stained gels were scanned at 560 nm and Coomassie Blue stained gels at 550 nm.

SDS Polyacrylamide Gel Electrophoresis - Purity and molecular weights were determined by SDS gel electrophoresis by the method of Laemmli (131) modified for vertical slabs rather than tubes. Slab gel electrophoresis was carried out in a Bio-Rad Model 220 unit at 25 mA per slab. Molecular weight of the purified NADase from Bungarus fasciatus venom was determined by measuring its mobility relative to other protein standards.

Spectrophotometric Measurements - Ultraviolet or visible absorbance measurements were performed on a Beckman Acta MVI recording spectrophotometer.

pH Measurements - Measurements of pH were made with a Radiometer digital pH meter PHM 52.

Ultrafiltration - Concentration of protein solutions were carried out by ultrafiltration using Amicon YM-10 membranes.

Assays of NADase Activity - Bungarus fasciatus venom NADase activity was determined utilizing four different assay systems. The first method employed was the cyanide addition reaction originally described by Kaplan (1) and modified by Yuan and Anderson (126). Reaction mixtures at 37°C contained 1 mM NAD, 50 mM potassium phosphate buffer, pH 7.5, and NADase in a final volume of 3.0 ml. At timed intervals 0.2 ml aliquots were transferred from the reaction mixture to 2.8 ml 1 M potassium cyanide. The absorbance at 327 nm was read against a blank of 0.2 ml 50 mM potassium phosphate buffer, pH 7.5, plus 2.8 ml 1 M potassium cyanide. A millimolar absorption coefficient of 5.9 $mM^{-1}cm^{-1}$ for the cyanide adduct of NAD was used for concentration determinations. A unit of NADase was defined as the amount of enzyme required to hydrolyze one μmole NAD per minute.

In a second assay, reaction rates were determined titrimetrically by the consumption of 1.0 mM NaOH titrant needed to maintain the reaction mixture at a constant pH, 7.5. Kinetic measurements were made in 4 ml reaction mixtures at 37°C using a Radiometer type TTTII titrator, type SBR 2C titragraph, PHM 26 C pH meter, type ABU 12 automatic burette unit, 0.25 ml burette, and type TTA 31 microtitration assembly equipped with a GK 2320C combination electrode. Aqueous solutions were degassed by boiling. Rates determined by this assay were expressed as micro-equivalents H^+ released per minute, which equaled micromoles of NAD cleaved per minute.

A third, fluorimetric assay employing ε-NAD as substrate was used predominantly to monitor NADase activity in column fractions during purification. This assay was 100 to 1000 times more sensitive than the cyanide assay and was a modification of the method described by Pekala and Anderson (72). Reaction mixtures contained 0.05 mM ε-NAD, 50 mM potassium phosphate buffer pH 7.5, and NADase, in a final volume of 1.0 ml. All fluorimetric assays were carried out at room temperature on a Perkin-Elmer 650-40 spectrophotofluorometer. Fluorescence emission was monitored at 410 nm with excitation at 300 nm. NADase activity was expressed as relative fluorescence increase/min.

A high performance liquid chromatographic assay was developed to measure product formation during NADase catalyzed reactions. Reaction mixtures were maintained at 37°C and contained 1.5 mM NAD, 50 mM potassium phosphate buffer, pH 7.5 and NADase, in a final volume of 1.0 ml. At predetermined times 0.1 ml aliquots from the reaction mixture were withdrawn and immediately added to 0.5 ml 50 mM potassium phosphate buffer pH 3.2. These samples were frozen quickly at -60°C and stored on ice until later use. High performance liquid chromatography of products were analyzed on a Spectra-Physics SP8000 HPLC, equipped with a SP Model 770 UV spectrophotometric detector. The column routinely used was an Alltech 5 micron RSiL AN anion exchange. The mobile phase contained 5 mM potassium phosphate, pH 3.5, and 500 mM potassium phosphate, pH 3.5, 75 and 25 percent, respectively. Sample size was 10 µl. Product concentrations were calculated by the SP8000 integrator, previously calibrated with known concentrations of products to be analyzed.

Enzyme Modification With Group-Specific Reagents - Inactivation mixtures were studied in a total volume of 1 ml containing 50 mM potassium phosphate buffer, pH 7.5, 2 units of purified venom NADase, and varying concentrations of the different chemical reagents to be investigated. Inactivation mixtures were preincubated at 37°C for 5 minutes prior to the addition of the enzyme. At timed intervals an aliquot of the inactivation mixture was transferred to a solution containing 1.5 mM NAD and 50 mM potassium phosphate buffer, pH 7.5. The assay mixture had a final volume of 1.5 ml. NADase activity remaining after incubation with chemical modification reagents was measured by the potassium cyanide addition assay, described above.

Procion Blue Purification - The dye as purchased from Aldrich, was only about 80% pure. Purification was accomplished by streaking a 50 mg/ml dye solution in water on Whatman 3 MM chromatography paper and developing the chromatagram in a solvent system of n-butanol, water and ethanol (40:32:28) for six hours. The major blue band was cut from the chromatagram and dye concentration was determined by the absorbance at 610 nm using a millimolar extinction coefficient of 13.6.

RESULTS

Occurrence of NADase Activity in Various Snake Venoms - Lyophilized venoms were dissolved to a final concentration of 5 to 10 mg/ml in 50 mM Tris-HCl potassium phosphate buffer, pH 8.2. NADase activity was measured by the cyanide addition assay and the results are shown in Table III. Venom from the genus Agkistodron or Causus contained up to 0.082 units/mg NADase activity. No NADase activity could be detected in venom from the genus Cerastes, Naja, or Crotalus. In comparison, a relatively high amount of NADase activity was found in the snake venoms from the genus Bungarus. Bungarus caeruleus demonstrated the highest NADase activity per milligram of crude venom; however, Bungarus fasciatus venom was chosen as the source for NADase purification due to lower cost and greater availability.

Purification of Bungarus Fasciatus Venom NADase

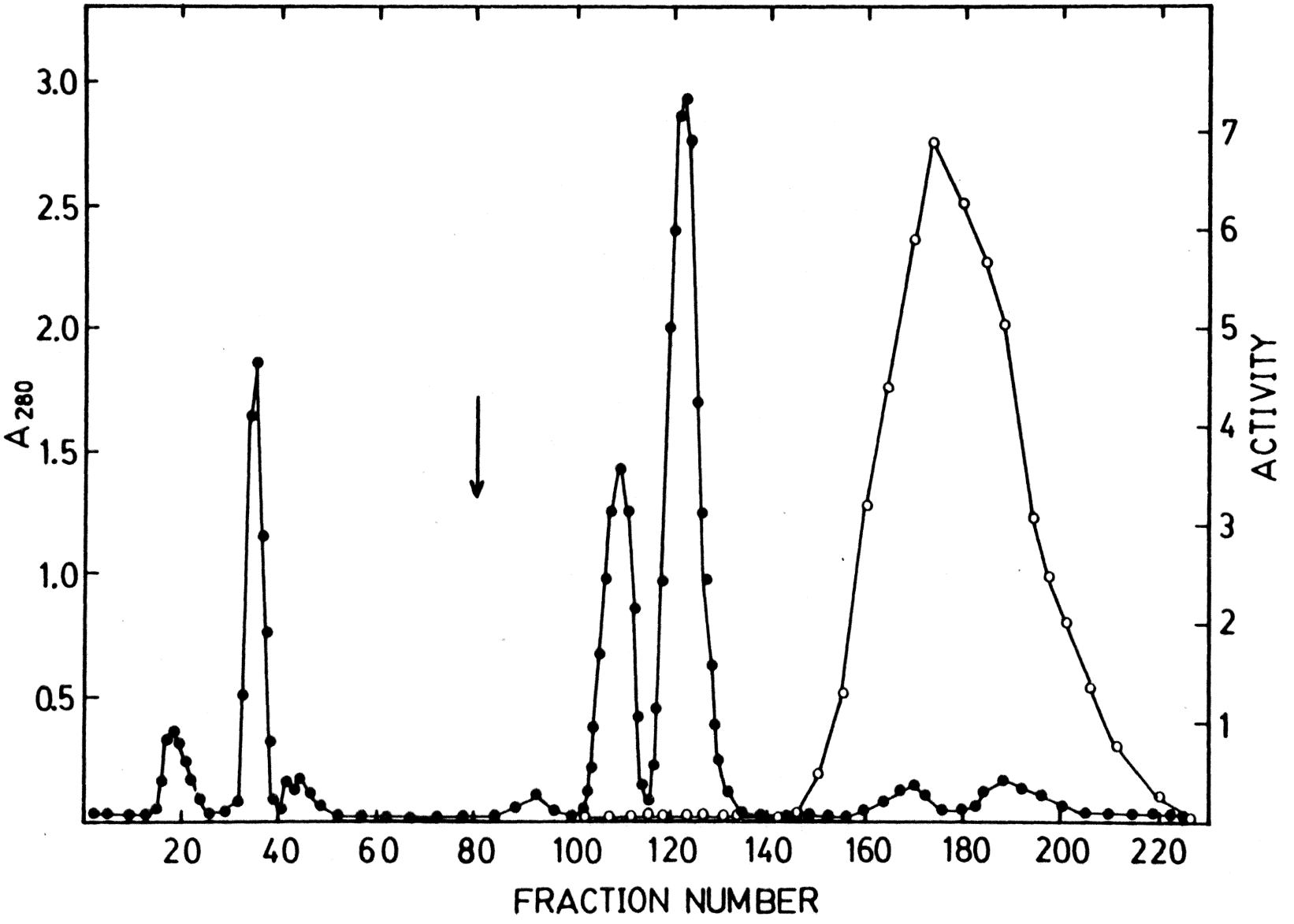
Phosphocellulose Chromatography - Two hundred milligrams of crude lyophilized Bungarus fasciatus venom were dissolved in 10 ml of 0.05 M Tris-HCl buffer, pH 8.2 at 4°, (Fraction 1), and applied to a phosphocellulose column (1.5 x 40 cm) previously equilibrated with 0.05 M Tris-HCl, pH 8.2. The column was washed with this buffer until the absorbance at 280 nm was essentially zero. A linear gradient of 0.05 M Tris-HCl, pH 8.2 and 0.05 M Tris-HCl, pH 8.2, 0.2 M potassium chloride (2 x 600 ml) was then applied. Fractions of 5 ml were collected. Protein concentration was monitored at 280 nm, and NADase activity was detected by the fluorimetric assay. A typical elution profile is shown in Figure

TABLE III

OCCURRENCE OF NADase ACTIVITY IN SNAKE VENOMS

Species	NADase Activity units/mg Venom
Agkistrodon piscivorus leukostoma (Western Cottonmouth Moccasin)	0.082
Agkistrodon bilineatus (Tropical Moccasin)	0.074
Agkistrodon piscivorus piscivorus (Eastern Cottonmouth Moccasin)	0.035
Agkistrodon halys (Mamushi)	0.027
Agkistrodon contortrix mokason (Northern Copperhead)	0.024
Agkistrodon rhodostoma (Malayan Pit Viper)	0.021
Cerastes cerastes (Desert Horned Viper)	---
Causus rhombeatus (Rhombic Night Adder)	0.072
Crotalus adamanteus (Eastern Diamondback Rattlesnake)	---
Naja naja atra (Formosan Cobra)	---
Bungarus fasciatus (Banded Krait)	1.5
Bungarus caeruleus (Indian Krait)	4.9
Bungarus multicinctus (Formosan Banded Krait)	0.42

FIGURE 5 - Phosphocellulose column chromatography of crude Bungarus fasciatus venom. The resuspended lyophilized snake venom (200 mg) was applied to a phosphocellulose column previously equilibrated with 50 mM Tris-HCl, pH 8.2. A linear gradient 0.0 to 0.2 M potassium chloride (2 x 600 ml) was applied, as indicated by the arrow. Fractions were assayed for absorbance (●—●) and NADase activity (o—o) was measured as discussed in the experimental procedures. NADase activity is reported as relative fluorence increase/min/ml.


3.0
2.5
2.0
1.5
1.0
0.5
A280
7
6
5
4
3
2
1
ACTIVITY
20
40
60
80
100
120
140
160
180
200
220
FRACTION NUMBER

5. Fractions containing NADase activity were pooled and concentrated by ultrafiltration (Fraction 2).

Gel Filtration - A column (4 x 100 cm) was packed with Sephadex G-100 and equilibrated at 4° with 0.05 M sodium pyrophosphate buffer, pH 7.0. Fraction 2 was applied to the column and eluted with the same buffer at a flow rate of 0.6 ml/min. Fractions of 5 ml were collected. The elution profile is shown in Figure 6. Protein and NADase activity were monitored as described above. Fractions containing NADase activity were pooled and concentrated by ultrafiltration (Fraction 3).

Affinity Chromatography - A column (0.9 x 5 cm) packed with Amicon Matrex gel Blue A and equilibrated with 0.05 M sodium pyrophosphate buffer, pH 7.0 was used as an affinity column for the NADase purification. Fraction 3 was applied and eluted with 0.05 M sodium pyrophosphate buffer, pH 7.0. After the majority of the 280 nm absorbing material was removed, the column was eluted with 0.05 M sodium pyrophsphate buffer, pH 7.0, containing 0.5 M potassium chloride. The column was then washed with 20 to 30 column volumes of the above buffer, after which a buffer containing 0.005 M potassium phosphate, pH 8.0, plus 2.0 M potassium chloride was applied. Protein concentration and NADase activity were determined as previously described. A typical elution profile is shown in Figure 7. Fractions containing NADase activity were pooled, concentrated, and washed free of remaining salt by ultrafiltration. A summary of the Bungarus fasciatus venom NADase purification procedure is shown in Table IV.

FIGURE 6 - Gel filtration of Fraction 2. Concentrated fractions from the phosphocellulose column were applied to a Sephadex G-100 column (4 x 100 cm) previously equilibrated with 0.05 M sodium pyrophosphate buffer, pH 7.0. Fractions of 5 ml were collected. Eluted fractions were monitored at 280 nm (●—●) and NADase activity measured (o—o) as described in the experimental procedures. NADase activity is expressed as relative fluorescence increase/min/ml.

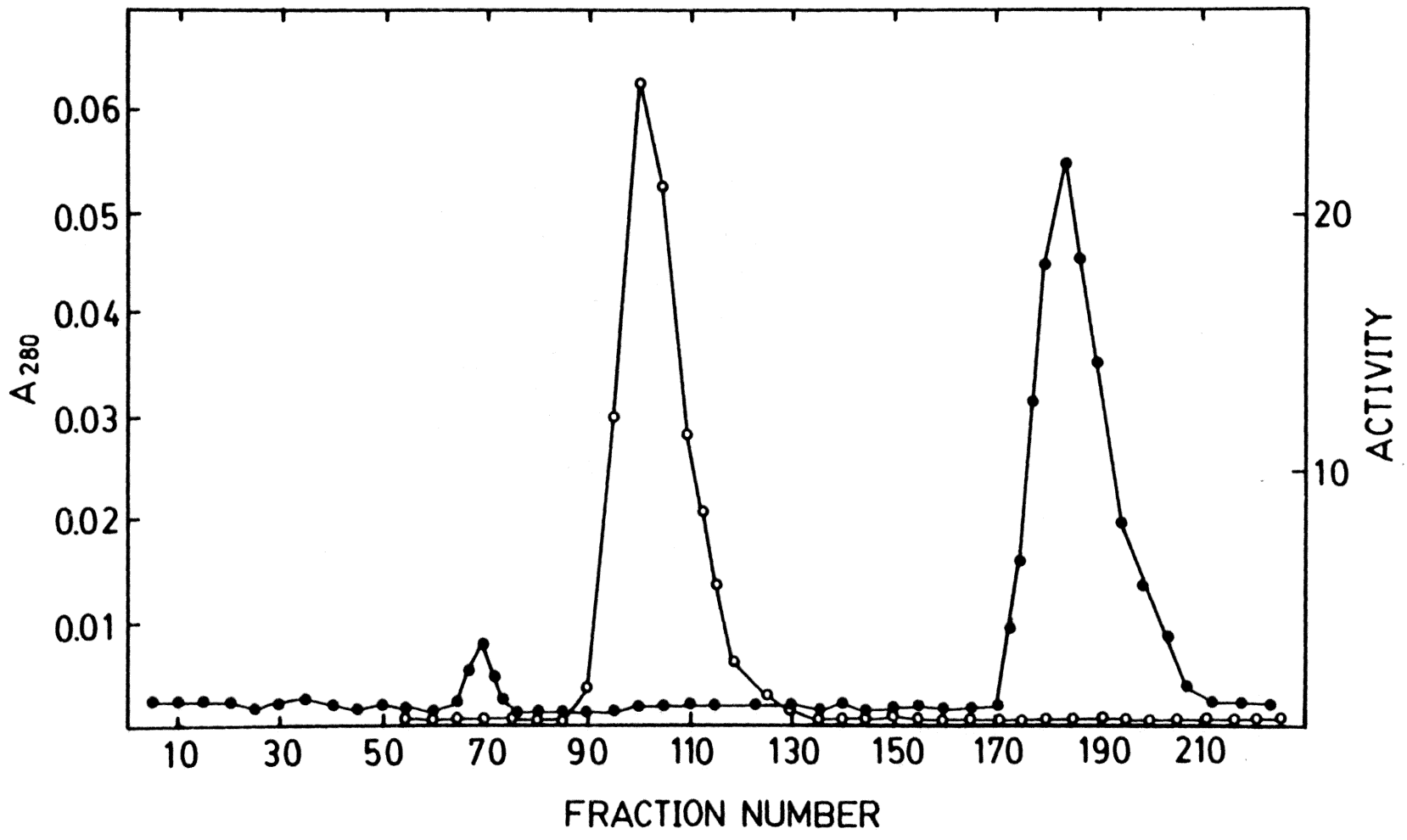

0.06
0.05
0.04
0.03
0.02
0.01
A280
20
10
ACTIVITY
10
30
50
70
90
110
130
150
170
190
210
FRACTION NUMBER

FIGURE 7 - Affinity chromatrography on Amicon Matrex Gel Blue A. Fraction 3 was applied to a previously washed and equilibrated Amicon Matrex Gel Blue A column (0.9 x 5 cm). The eluting buffer contained 0.05 M sodium pyrophosphate, pH 7.0. The arrow labeled A designated the point at which 0.5 M potassium chloride in 0.05 M sodium pyrophosphate, pH 7.0 was started; 2 M potassium chloride in 0.005 M potassium phosphate buffer, pH 8.0, was started at the arrow labeled B. Fractions of 2.0 ml were collected. Absorbances at 280 nm were monitored (o--o) and NADase activity determined (o--o) as discussed in the experimental methods. NADase activity is reported as relative fluorescence/min/ml.

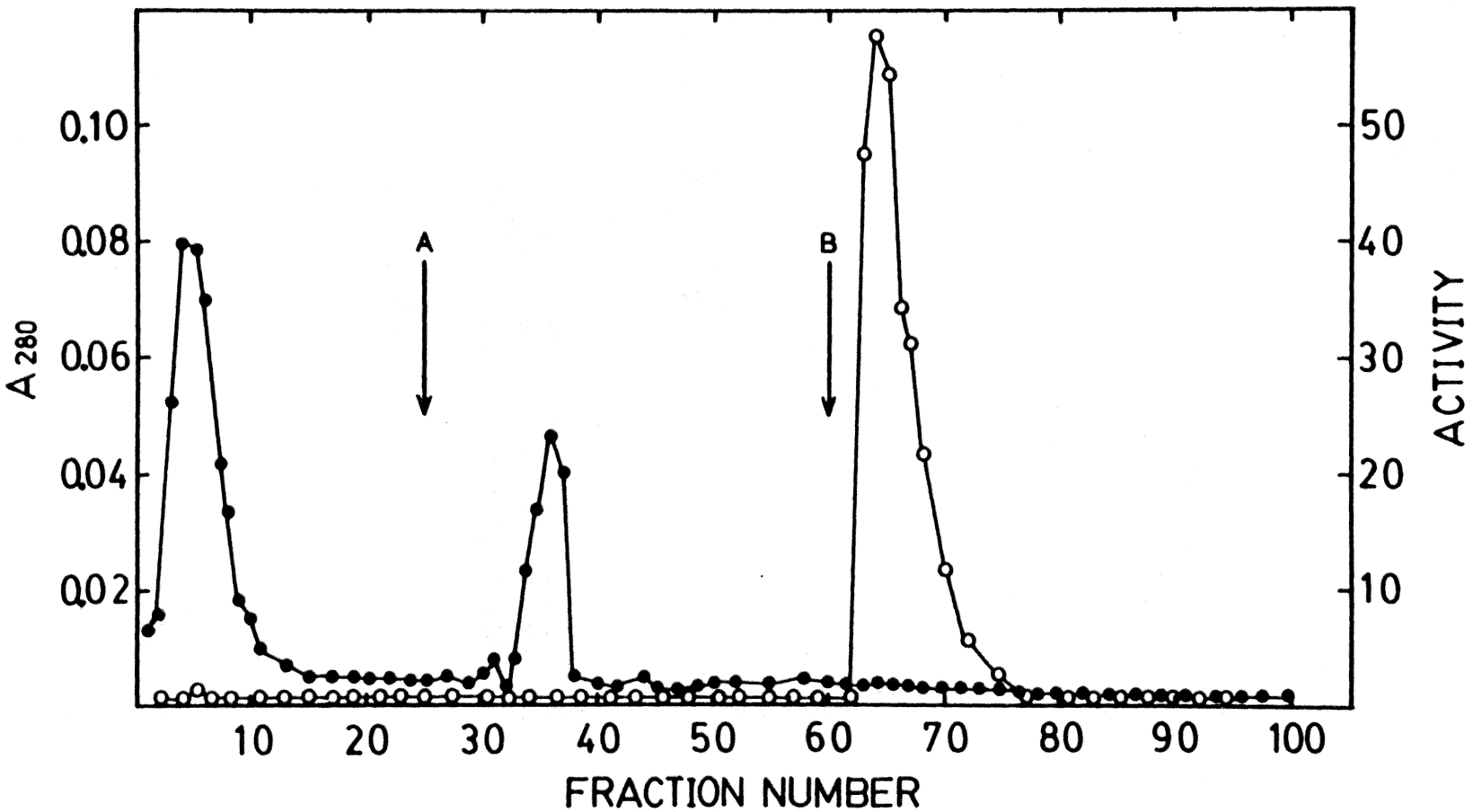

0.10
0.08
0.06
0.04
0.02
A280
A
B
50
40
30
20
10
ACTIVITY
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

TABLE IV

PURIFICATION OF NADASE FROM BUNGARUS FASCIATUS

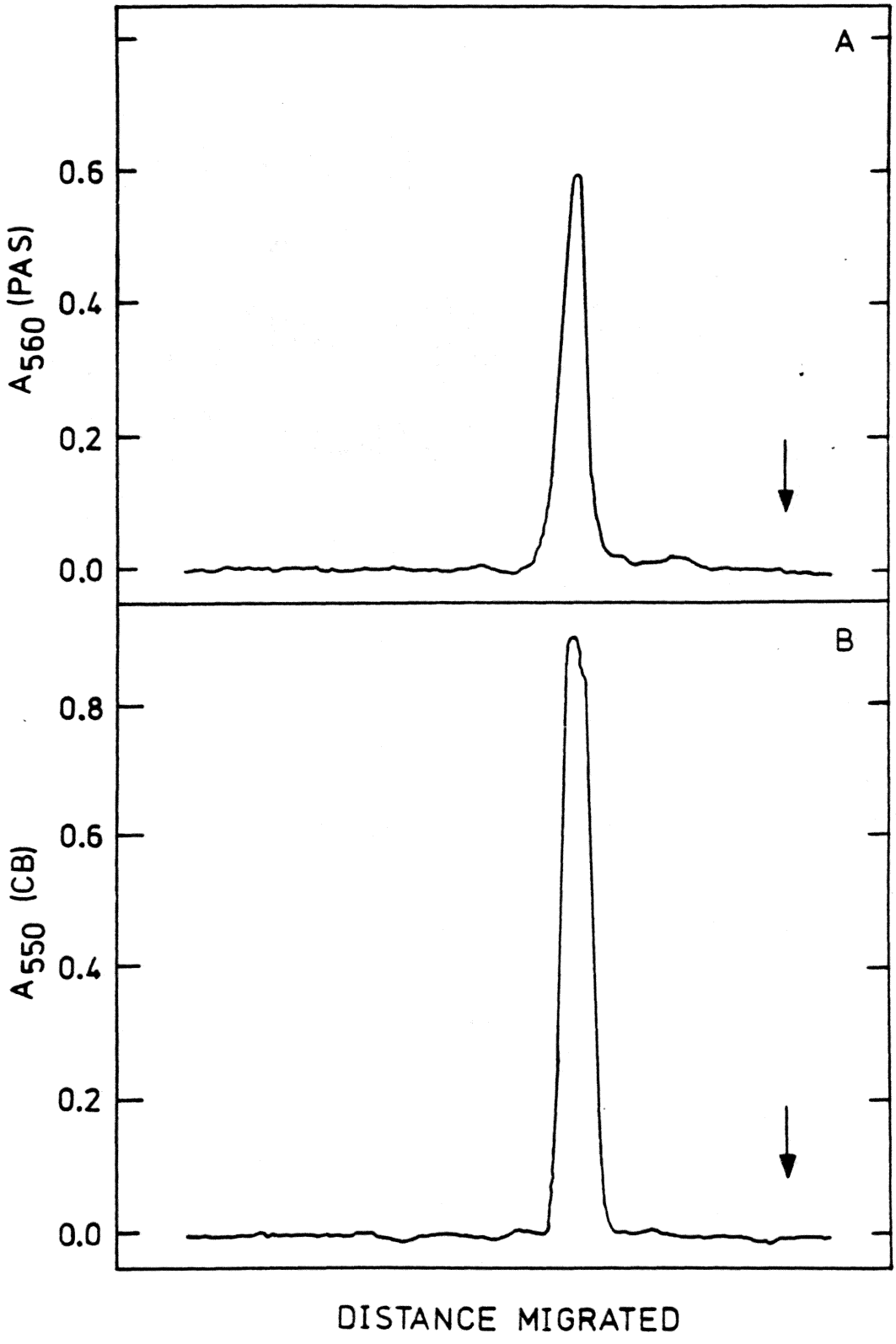
Fraction	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	Purification (fold)
1. Crude	200	260	1.3	100	1
2. Phosphocellulose	8.1	280	35	108	27
3. Sephadex G-100	0.65	260	400	100	308
4. Amicon Matrex™ Gel Blue A	0.19	250	1320	96	1030

Properties of the Purified NADase

Estimation of Purity - When 40 µg of NADase from the Amicon Matrex gel Blue A affinity column was analyzed by either polyacrylamide disc gel electrophoresis or SDS polyacrylamide gel electrophoresis and stained with Comassie blue, only one protein staining band was observed. When gels run simultaneously under identical conditions were stained with periodic acid-Schiff reagent, a distinctly positive pink band was observed, which migrated the same distance as the Comassie blue staining band. Electrophoresis results are shown in Figure 8.

Determination of Molecular Weight - The molecular weight of the purified Bungarus fasciatus venom NADase was first determined by gel filtration at 4°C on a Sephacryl S-200 column (1.5 x 85 cm). The column was equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, 0.1 M potassium chloride. Column standardization was accomplished with 5 mgs of each of the following proteins: cytochrome c, chymotrypsinogen, ovalbumin, bovine serum albumin, horse liver alcohol dehydrogenase, yeast alcohol dehydrogenase, and catalase. The elution volume for each of the standard proteins was determined by protein absorbance at 280 nm. Results of the molecular weight determinations are shown in Figure 9, line A. The logarithms of the molecular weights of the standard proteins were plotted against the average distribution coefficients, K_d*. The snake venom enzyme exhibited an apparent molecular weight of 125,000.

FIGURE 8 - Results of electrophoresis of purified Bungarus fasciatus venom NADase. Gels were scanned in a Gilford Model 250 spectrophotometer. The upper scan is the periodic acid-Schiff reagent stained gel scanned at 560 nm. The lower scan is the Coomassie blue stained gel scanned at 550 nm.


A
B
0.6
0.4
0.2
0.0
0.8
0.6
0.4
0.2
0.0
A_{560} (PAS)
A_{550} (CB)
DISTANCE MIGRATED

The molecular weight of the purified NADase was also determined by the zone sedimentation density gradient centrifugation method of Martin and Ames (132). Catalase and yeast glutathione reductase were used as standards. The molecular weight of the snake venom enzyme determined by this technique was 130,000.

The molecular weight of the B. fasciatus venom NADase was also determined under the denaturing conditions of SDS polyacrylamide gel electrophoresis. Molecular weight standards, yeast alcohol dehydrogenase, bovine serum albumin, ovalbumin, phosphorylase a, and yeast glutathione reductase were used. Results are shown in Figure 9, line B. Migration of proteins was reported as relative mobility, which is defined as the distance a particular protein migrated in the gel divided by the distance the tracking dye migrated. Under denaturing conditions the NADase migrated with an apparent molecular weight of 62,000. B. fasciatus venom NADase migrated the same distance during SDS polyacrylamide gel electrophoresis with or without dithiothreitol.

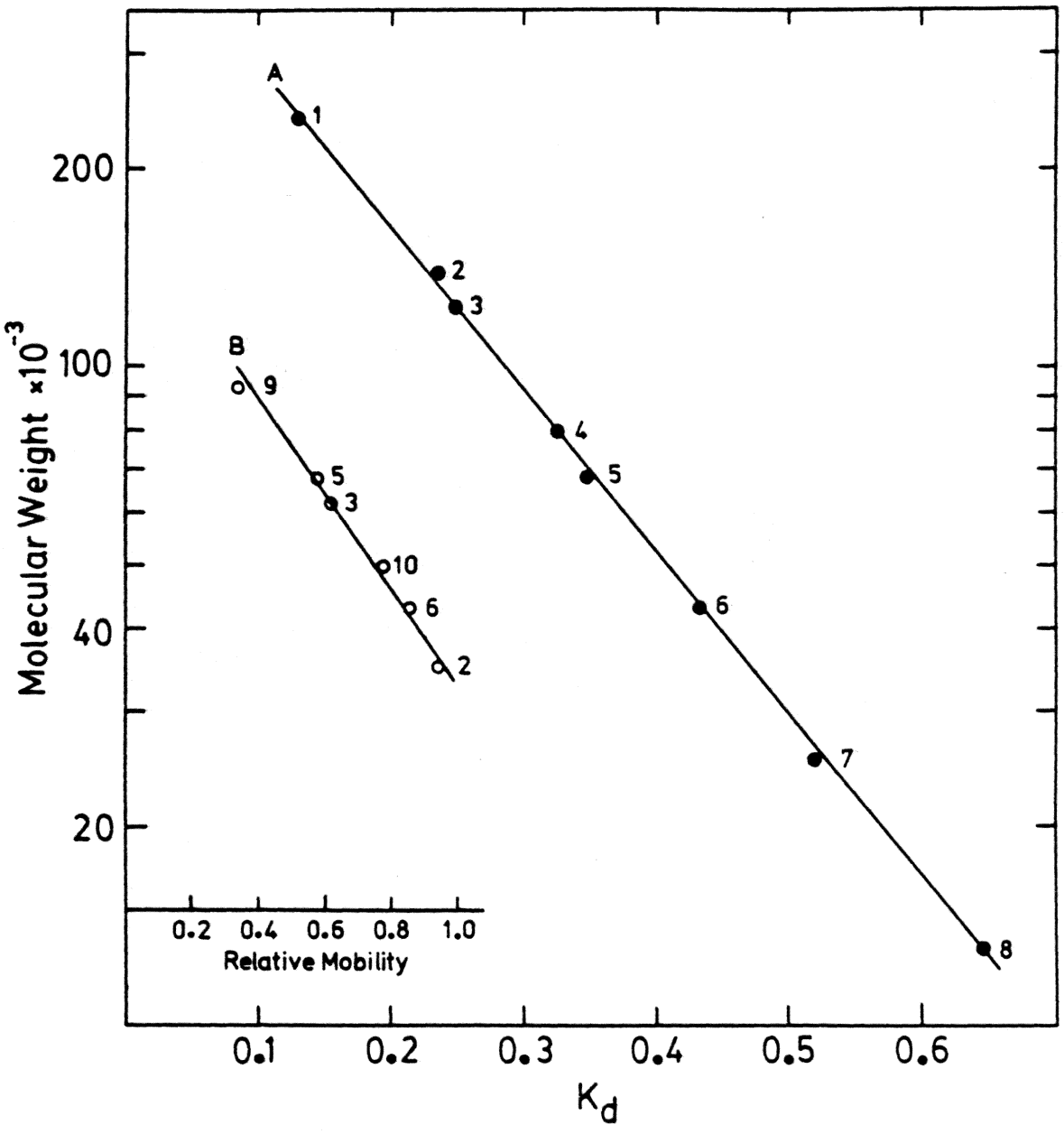
$$*K_d = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = protein elution volume

V_o = void volume of column

V_t = total volume of the column

FIGURE 9 - Molecular weight of Bungarus fasciatus venom NADase. (A) (•—•) Gel filtration on Sephacryl S-200. Molecular weight standards were 1) catalase, 2) yeast alcohol dehydrogenase, 3) Bungarus fasciatus venom NADase, 4) horse liver alcohol dehydrogenase, 5) bovine serum albumin, 6) ovalbumin, 7) chymotrypsinogen, and 8) cytochrome c. (B) (o—o) Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Molecular weight standards were 2) yeast alcohol dehydrogenase, 3) Bungarus fasciatus venom NADase, 5) bovine serum albumin, 6) ovalbumin, 9) phosphorylase a, and 10) yeast glutathione reductase. Electrophoresis was carried out as described in the experimental procedures.


A
1
2
3
4
5
6
7
8
B
9
5
3
10
6
2
200
100
40
20
Molecular Weight ×10⁻³
0.2
0.4
0.6
0.8
1.0
Relative Mobility
0.1
0.2
0.3
0.4
0.5
0.6
Kd

A summary of the molecular weight determinations observed under the various conditions studied is shown in Table V.

Stability - The purified NADase at a concentration of 400 to 500 µg/ml in 0.005 M potassium phosphate buffer, pH 7.5, lost less then 10 percent of its enzyme activity after 4 weeks when stored at 4°C and was completely stable at -15°C for over a period of 4 months. Therefore, the purified NADase was stored at -15°C in 200 µl or less aliquots until needed. It was of interest that the NADase did not lose any enzymatic activity when incubated at 37°C, pH 7.5, for 24 hours. The importance of this result will become apparent later when the synthesis of NAD analogs is discussed.

TABLE V

SUMMARY OF MOLECULAR WEIGHT DETERMINATIONS OF BUNGARUS FASCIATUS VENOM NADASE

Method	Molecular Weight Observed
1. Gel Filtration on Sephacryl S-200	125,000
2. Sucrose Density Gradient Cenrifugation	130,000
3. SDS-Disc Polyacrylamide Gel Electrophoresis*	62,000

*With or without dithiothreitol

Effect of pH on V_{max} and K_m - The enzyme activity of the purified Bungarus fasciatus venom NADase was measured at 6 pHs between pH 6.0 and pH 8.5. At each pH value, five concentrations of substrate were employed. Reactions were carried out at 37°, and initial velocities were measured by the titrimetric assay. The kinetic parameters of NAD hydrolysis were determined by the method of Cleland (133) and plotted according to Lineweaver and Burk (134). The effect of pH on the kinetic parameters is shown in Table VI. From pH 6.0 to 8.5 there was little change in any of the kinetic parameters for the snake venom enzyme. B. fasciatus venom NADase catalytic activity exhibited a broad pH profile with a pH optimum for hydrolysis between pH 7.5 and pH 8.0. The calculated K_m values varied little over the pH range studied. As indicated on Table VI, the V_{max}/K_m ratio for NAD hydrolysis varied no more than 20 percent.

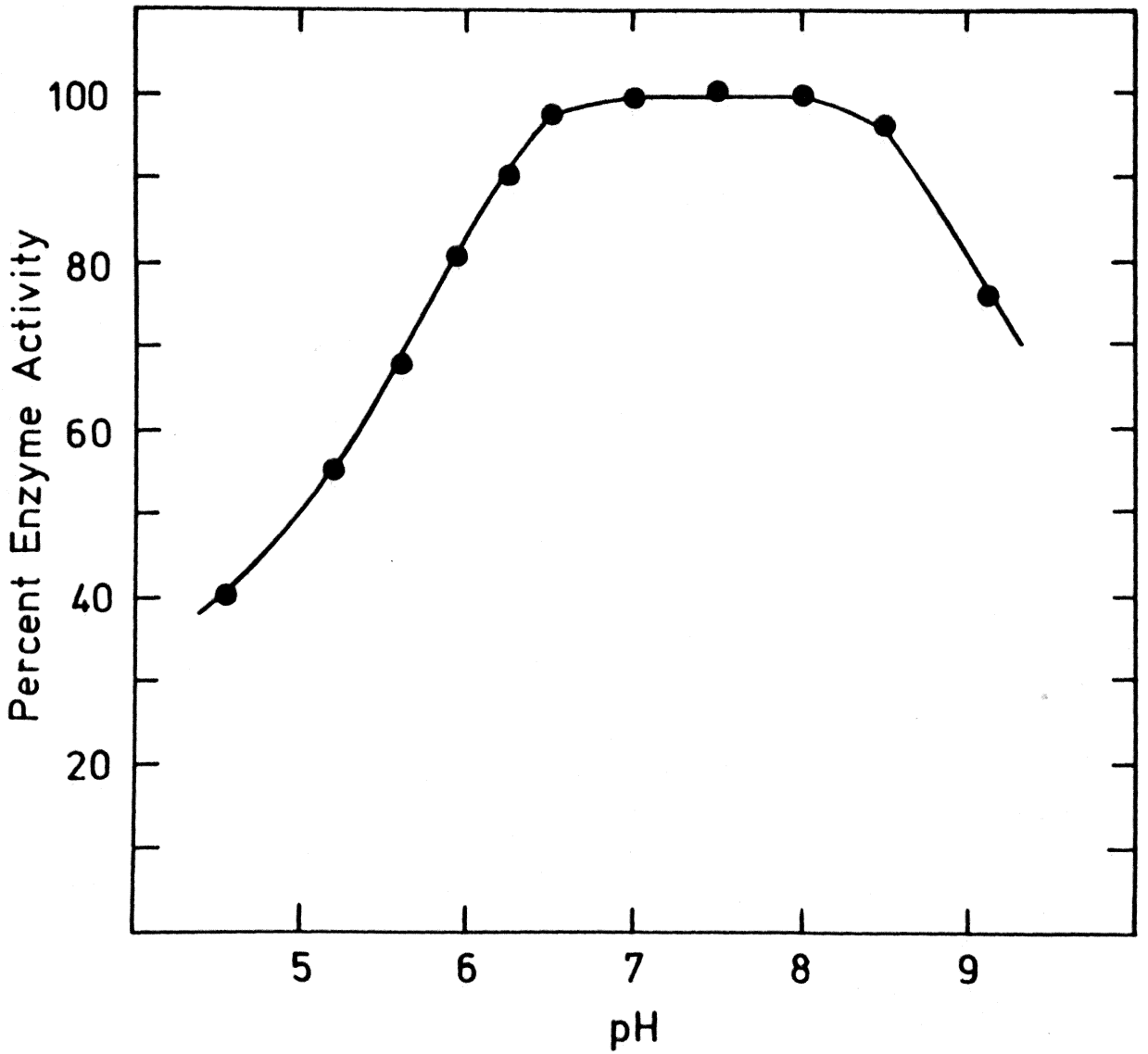
Effect of pH on Stability of the Enzyme - The stability of the enzyme was measured between pH 4.5 and pH 9.0. The purified NADase was incubated for 10 minutes at 37° in the absence of NAD at each of the desired pH values. The reaction mixtures were then adjusted to pH 7.5 and saturating amounts of NAD were added. The enzyme activity was measured by the titrimetric assay at 37°. The results of the pH stability studies are shown in Figure 10 and indicate that the enzyme was stable at neutral pHs for at least 10 minutes. A rapid loss of enzyme activity was observed below pH 6.0 and above pH 8.5. At pH 5.0 and pH 9.0 the enzyme lost approximately 50 percent of the catalytic activity in 10 minutes.

TABLE VI

EFFECT OF pH ON V_{max} AND K_m

pH	K_m	V_{max}	V_{max}/K_m
	(μM)	(μmol/min/mg)	
6.0	16	1140	71
6.5	16	1290	81
7.0	14	1290	92
7.5	15	1350	90
8.0	15	1350	97
8.5	14	1290	92

FIGURE 10 - The effect of pH on the stability of Bungarus fasciatus venom NADase. The pH stability of the enzyme was measured at 11 pHs between pH 4.5 and pH 9.0. The purified NADase (74 ng) was incubated for 10 minutes at the desired pH at 37° C. The remaining enzyme activity was measured by the previously described titrimetric assay after adjusting the pH of the reaction mixture to 7.5 with 100 mM NaOH. The reaction was started by the addition on 1 mM NAD.


Percent Enzyme Activity
100
80
60
40
20
5
6
7
8
9
pH

Effect of Ionic Strength on Kinetic Parameters - The effect of varying concentrations of potassium chloride on the kinetic parameters of the NADase was investigated. Initial rates of hydrolysis of NAD were determined titrimetrically. The ionic strength was varied by adding different amounts of a stock solution of potassium chloride to the reaction mixtures. The effect of ionic strength on the kinetic parameters is shown in Table VII. Increasing the ionic strength from 10 to 87 mM resulted in a slight increase in the maximum velocity; however, there was no significant effect on the determined K_m value. An ionic strength of 87 mM was therefore chosen for all future kinetic studies of the snake venom enzyme. At higher ionic strengths, 500 mM, an increase in the K_m and a decrease in the V_{max} was observed.

K_m and V_{max} Under Optimal Assay Conditions - Using the optimal assay conditions found for ionic strength and pH, kinetic constants for the enzyme catalyzed reaction were redetermined. The concentration of NAD was varied from 10 to 400 µM. The initial velocities determined by the method of Cleland (133) were plotted according to Lineweaver and Burk (134) as shown in Figure 11. From these data, a K_m for NAD of 14 µM and a V_{max} of 1300 and 1380 µmoles NAD cleaved/min/mg protein were obtained when 34 and 68 ng of purified NADase were used, respectively. NAD hydrolysis was directly proportional to the amount of NADase added at saturating substrate concentrations.

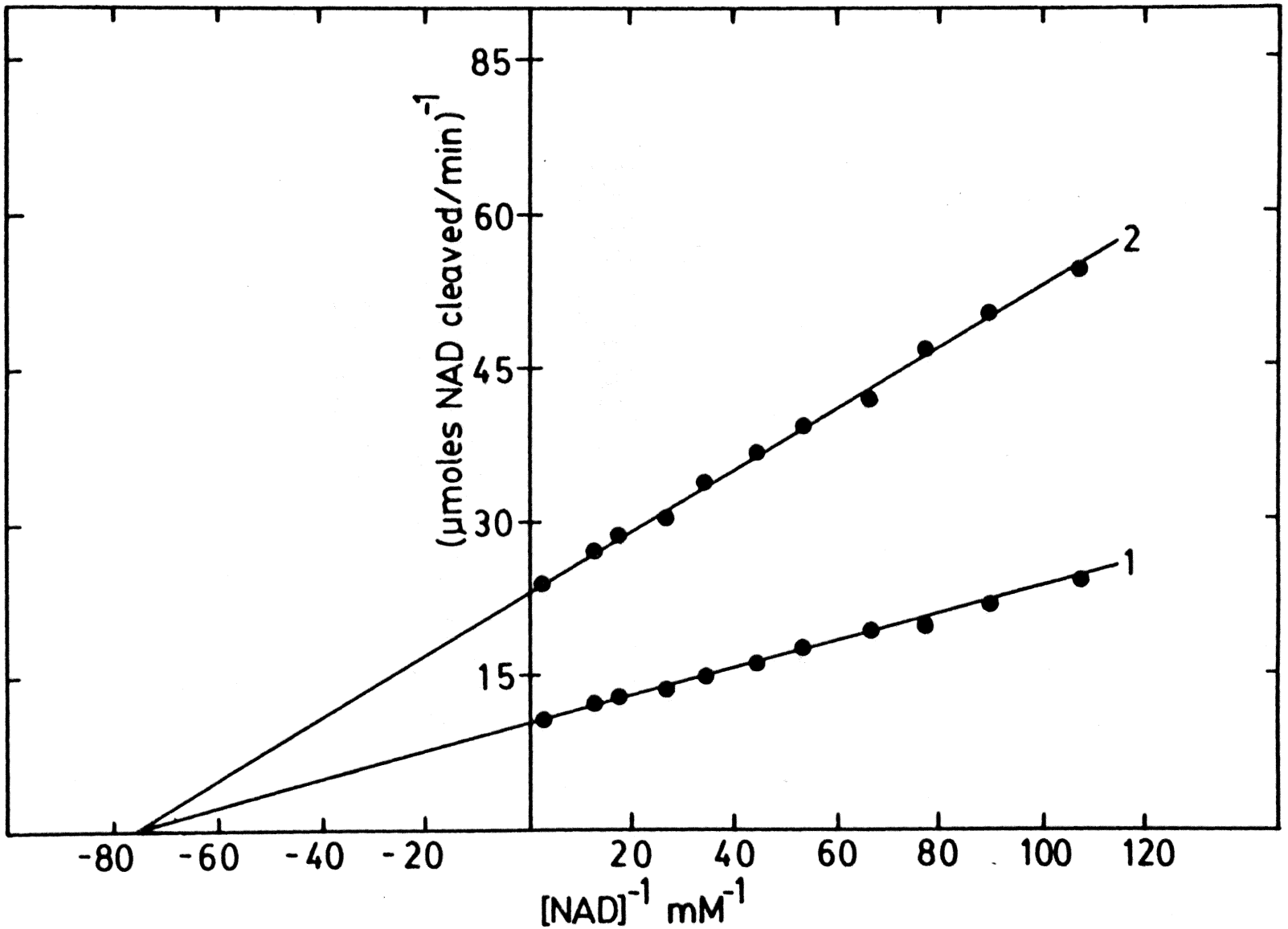
High Pressure Liquid Chromatography of Products of the NADase Reaction - In the NADase catalyzed hydrolysis of NAD, identical rates

TABLE VII

SUMMARY OF IONIC STRENGTH DATA

Ionic Strength	V_{max}	K_m
(KCl M)	(μmoles NAD cleaved/min/mg)	(μM)
0.010	850	15
0.050	1120	14
0.087	1300	14
0.500	940	37

FIGURE 11 - The effect of NAD concentration on the rates of hydrolysis catalyzed by the purified Bungarus fasciatus venom NADase. The reaction mixtures contained 87 mM potassium chloride, NAD concentrations were varied from 10 to 400 μM. Reaction mixtures were adjusted to pH 7.5 and 37° C and reactions were initiated by the addition of NADase, either 68 or 34 ng, Lines 1 and 2 respectively. The rate of consumption of 1 mM NaOH necessary to maintain the pH at 7.5 was determined titrimetrically.


85
60
45
30
15
(µmoles NAD cleaved/min)$^{-1}$
2
1
-80
-60
-40
-20
20
40
60
80
100
120
[NAD]$^{-1}$ mM^{-1}

of reaction were obtained using either the cyanide addition assay or the titrimetric method indicating the absence of nucleotide pyrophosphatase or phosphodiesterase activity. To confirm this, high pressure liquid chromatography (HPLC) of the products formed during the hydrolytic reaction was carried out. Reaction mixtures contained 1.4 mM NAD, 100 mM Tris-HCl, pH 7.8, 0.3 units of B. fasciatus venom NADase, in a final volume of 2.0 ml and were preincubated for 5 minutes at 37° before NADase was added. At 0 time and 20 minutes, a 0.1 ml aliquot was transferred to 0.9 ml of 50 mM potassium phosphate buffer, pH 3.2. HPLC analysis was performed as described in the experimental procedures. The concentrations of NAD, nicotinamide, and ADP-ribose were determined. To further confirm the concentration of NAD remaining in the incubation mixtures, two alternate methods were used. The first method used was the cyanide addition assay and the second was the yeast alcohol dehydrogenase assay. In the latter assay, NAD concentration was determined by reducing the remaining pyridine dinucleotide with yeast alcohol dehyrogenase and measuring the absorbance at 340 nm using a millimolar extinction coefficient of 6.22 OD/mM. Routinely a 0.2 ml aliquot was transferred from the reaction mixture to 2.8 ml unbuffered alcoholic-Tris solution (0.5 M ethanol, 90 mM Tris). The absorbance at 340 nm was read before and after the addition of 2 mg of yeast alcohol dehydrogenase.

A comparison of values for the concentration of NAD remaining in the reaction mixtures determined by HPLC, cyanide addition assay, or by

reduction with yeast alcohol dehydrogenase is shown in Table VIII. The results of product analysis by HPLC are shown in Table IX. The only products formed during NAD hydrolysis in the presence of purified B. fasciatus venom NADase were ADP-ribose and nicotinamide which were produced in a 1:1 stoichiometry.

Effect of Temperature on NADase Activity - Rates of hydrolysis of NAD were determined titrimetrically at 15 temperatures in the range of 13 to 45°C. An Arrhenius plot of the results obtained is shown in Figure 12. Linearity was observed throughout the temperature range studied. An activation energy of 15.7 kcal/mol was determined from these data.

The purified B. Fasciatus venom NADase when incubated at 50°C was observed to be unstable and lost enzymatic activity with a $T_{1/2}$ of 8 minutes and a k_{obs} of 0.087 min^{-1}. At higher incubation temperatures the rate of inactivation of the enzyme was too rapid to measure by the technique employed.

TABLE VIII

COMPARISON OF ASSAY PROCEDURES

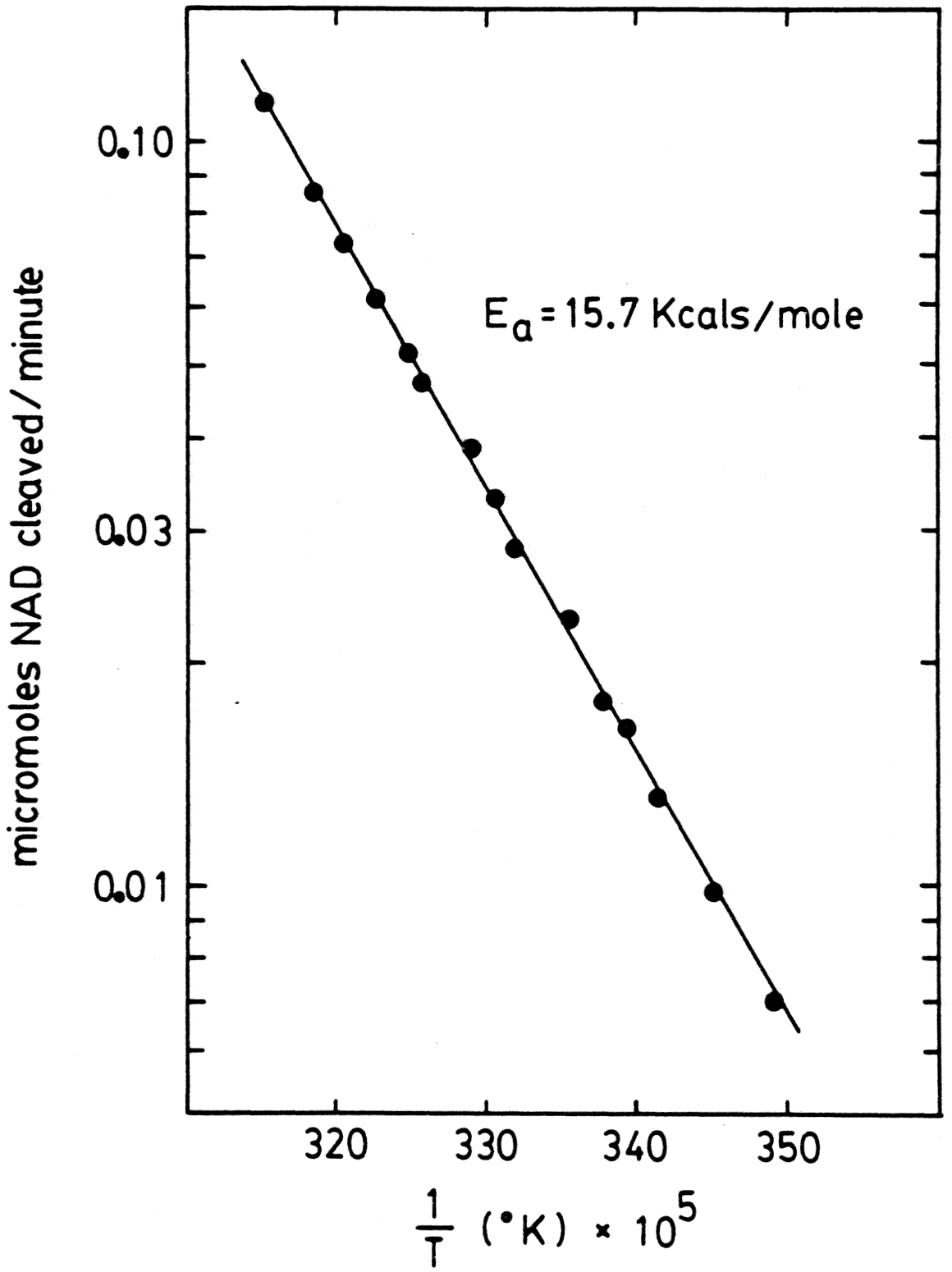
Assay Technique	[NAD] mM	
	0 minutes	20 minutes
Cyanide-addition assay	1.26	0.20
Yeast alcohol dehydrogenase assay	1.20	0.21
High pressure liquid chromatography	1.30	0.22

TABLE IX

HPLC PRODUCT ANALYSIS OF NADASE-CATALYZED REACTION

Incubation Time	Product Concentrations (mM)		
minutes	[NAD]	[Nicotinamide]	[ADP-ribose]
0	1.30	0	0
20	0.22	1.08	1.10

FIGURE 12 - Arrhenius plot of the effect of temperature on purified Bungarus fasciatus venom NADase-catalyzed hydrolysis of NAD. The reaction mixtures contained 87 mM potassium chloride, 56 ng of purified B. fasciatus venom NADase and 400 μM NAD. Initial velocities were measured titrimetrically at pH 7.5 at the temperature indicated.


0.10
0.03
0.01
micromoles NAD cleaved/ minute
E_a = 15.7 Kcals/mole
320
330
340
350
$\frac{1}{T}$ (°K) × 10^5

Substrate Specificity and Substrate Binding Properties - The structural requirements necessary for substrate activity were investigated through the use of various NAD analogs. The ability of the B. fasciatus venom NADase to catalyze the hydrolysis of NAD analogs was studied by the titrimetric assay. Reaction mixtures were prepared and assayed as described in the legend of Table X. Relative rates of the hydrolysis of these pyridine nucleotide derivatives are shown in Table X. Of the 25 NAD analogs tested only NAD, NADP, nicotinamide 1,N^6-ethenoadenine dinucleotide, thionicotinamide adenine dinucleotide, thionicotinamide adenine dinucleotide phosphate, 3-acetylpyridine adenine dinucleotide, 3-acetylpyridine hypoxanthine dinucleotide, nicotinamide hypoxanthine dinucleotide, and nicotinamide guanine dinucleotide served as substrates for the purified snake venom NADase.

Those pyridine dinucleotides which served as substrates were further studied to determine the kinetic constants for their hydrolysis. Each NAD analog was studied at five different concentrations between 5 and 400 μM. Initial velocities were measured by the titrimetric assay and K_m and V_{max} values were determined by the method of Cleland (133). A summary of these results are listed in Table XI. Of the analogs tested, NAD was shown to exhibit the largest V_{max}, while s-NADP was hydrolyzed the slowest. NADP and nicotinamide hypoxanthine dinucleotide both had identical K_m values, 5 μM. Nicotinamide guanine dinucleotide demonstrated the highest K_m of the analogs hydrolyzed. The V_{max}/K_m ratio, a measure of the substrate specificity (135), indicated that NADP was a better substrate for the snake venom enzyme than

TABLE X

SUBSTRATE SPECIFICITY OF BUNGARUS FASCIATUS NADASE

Substrate	Relative Activity
	%
Nicotinamide adenine dinucleotide	100
Nicotinamide adenine dinucleotide phosphate	80
Nicotinamide $1,N^6$-ethenoadenine dinucleotide	88
Nicotinamide hypoxanthine dinucleotide	75
Nicotinamide guanine dinucleotide	87
Thionicotinamide adenine dinucleotide	92
3-Acetylpyridine adenine dinucleotide	50
3-Acetylpyridine hypoxanthine dinucleotide	43
Thionicotinamide adenine dinucleotide phosphate	28
3-Pyridinealdehyde adenine dinucleotide	0
3-Pyridinealdehyde hypoxanthine dinucleotide	0
α-Nicotinamide adenine dinucleotide	0
3-Aminopyridine adenine dinucleotide	0
4-Aminopyridine adenine dinucleotide	0
Pyridine adenine dinucleotide	0
3-Methylpyridine adenine dinucleotide	0
3-Pyridylcarbinol adenine dinucleotide	0
3-Pyridylacetonitrile adenine dinucleotide	0
Isonicotinic acid hydrazide adenine dinucleotide	0
3-Acetylpyridine adenine dinucleotide phosphate	0
Nicotinic acid adenine dinucleotide	0
3-Aminomethylpyridine adenine dinucleotide	0
Nicotinamide mononucleotide	0
1,4 Dihydronicotinamide adenine dinucleotide	0
1,4 Dihydronicotinamide adenine dinucleotide phosphate	0

TABLE XI

MICHAELIS CONSTANTS AND MAXIMUM VELOCITIES FOR SUBSTRATES

Substrate	K_m	V_{max}
	μM	(μmol/min/mg)
Nicotinamide adenine dinucleotide	14	1321
Nicotinamide adenine dinucleotide phosphate	5	1036
Nicotinamide 1,N^6-ethenoadenine dinucleotide	20	1160
Nicotinamide hypoxanthine dinucleotide	5	982
Thionicotinamide adenine dinucleotide	11	1214
3-Acetylpyridine adenine dinucleotide	27	661
3-Acetylpyridine hypoxanthine dinucleotide	7	571
Thionicotinamide adenine dinucleotide phosphate	10	375
Nicotinamide guanine dinucleotide	32	1143

was NAD. NAD analogs which were modified in either the nicotinamide or adenine moieties of NAD resulted in a 6-fold variance in K_m and a 3.5 fold variance in V_{max}. Substrate specificity varied almost 10 fold from 3-acetylpyridine adenine dinucleotide to NADP. Although the enzyme catalyzed the hydrolysis of both NAD and NADP, and exhibited a higher V_{max} for NAD than NADP, the enzyme will still be considered a NADase although NAD(P)ase would also be appropriate.

Inhibitor Studies

The binding of substrates with the complexity of NAD to an enzyme most probably results from a combination of many specific interactions between the amino acid side chains of the enzyme and certain functional groups within the NAD molecule. To probe these possible interactions, a number of specific analogs were studied as possible inhibitors of the B. fasciatus venom NADase. The inhibitors used can be divided into three structurally different groups: (1) nicotinamide analogs, (2) adenine analogs, and (3) dinucleotides which were not hydrolyzed by the purified B. fasciatus venom NADase.

Inhibition of Bungarus Fasciatus NADase by Nicotinamide - The B. fasciatus venom NADase catalyzed hydrolysis of NAD was inhibited by nicotinamide and the inhibition was observed to be noncompetitive with respect to NAD. Inhibitor constants were determined from double reciprocal plots where the initial velocities were measured at six different concentrations of NAD, while the nicotinamide concentration was kept constant. Kinetic parameters for each of the inhibitor concentrations were determined by the method of Cleland (133) and plotted

according to Lineweaver and Burk (134). Results of nicotinamide inhibition are shown in Figure 13. The kinetic parameters, slopes and intercepts from the double reciprocal plots (Figure 13) were replotted against nicotinamide concentration. These secondary plots are shown in Figure 14. Inhibitor constants calculated from replots of slopes and intercepts were found to yield equivalent K_i values, 1.5 mM.

When nicotinamide inhibition was studied under the conditions of varying nicotinamide concentrations at constant substrate concentration, noncompetitive inhibition was also observed in plots of the reciprocal of the initial velocities versus nicotinamide concentration (136). A K_i of 1.4 mM for nicotinamide was determined under these experimental conditions.

Inhibition of NADase by Nicotinamide Analogs - Inhibition of the enzymatic hydrolysis of NAD by 21 nicotinamide analogs was investigated. The type of inhibition and the inhibitor constants were determined for each of the nicotinamide analogs as described above for nicotinamide inhibition. All of the nicotinamide analogs which inhibited the NADase reaction were shown to be linear noncompetitive inhibitors with respect to NAD. K_i values for the nicotinamide analogs are shown in Table XII. The measured K_i values ranged from 19.5 mM for 3-acetylpyridine to 0.009 mM for isonicotinic acid hydrazide. Pyrazinamide, which can be envisioned as nicotinamide with a nitrogen in place of carbon number 4 of the pyridine ring, showed no effect on the rate of the NADase catalyzed hydrolysis of NAD. Negative results were also obtained for pyridazine, pyrimidine, aniline, and benzene. The results in Table XII also indicate there is no direct relationship between the pK_a of the

FIGURE 13 - Noncompetitive inhibition of NADase by nicotinamide. The reaction mixtures contained 87 mM potassium chloride, NAD concentrations were varied from 10 to 400 μM, nicotinamide concentrations were as indicated, and 55 ng of purified snake venom NADase. Initial velocities were measured by the consumption of 1 mM NaOH at 37° C, and pH 7.5. The concentrations of nicotinamide used were as follows: Line 1, 0; Line 2, 0.45 mM; Line 3, 0.9 mM; Line 4, 1.4 mM.

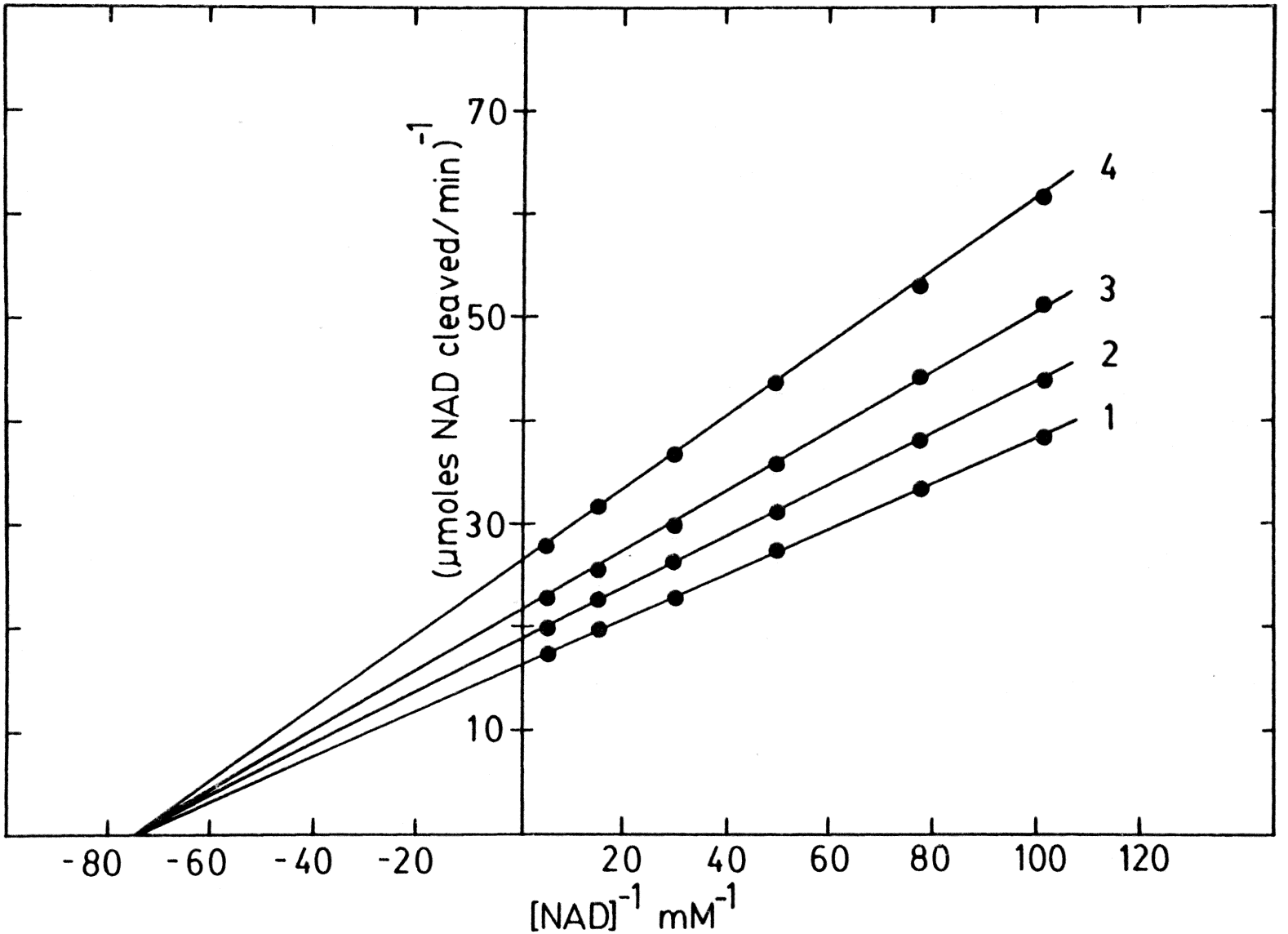

70
50
30
10
(μmoles NAD cleaved/min)$^{-1}$
4
3
2
1
-80
-60
-40
-20
20
40
60
80
100
120
[NAD]$^{-1}$ mM^{-1}

FIGURE 14 - A replot of the slopes, designated by Line 1 (open circles) and intercepts by Line 2 (closed circles), obtained from Figure 13, as a function of nicotinamide concentration.

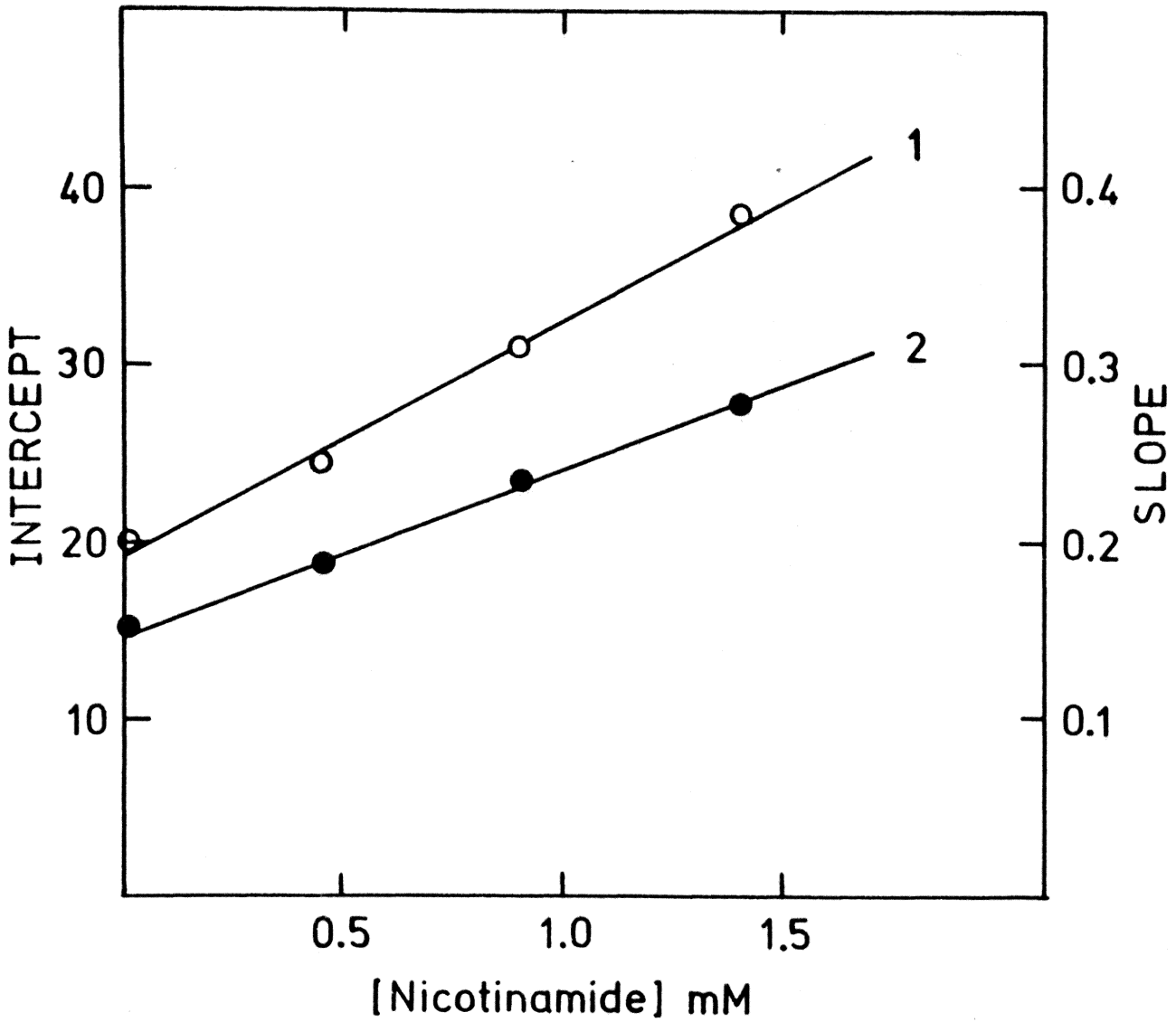

INTERCEPT
SLOPE
40
30
20
10
0.4
0.3
0.2
0.1
0.5
1.0
1.5
[Nicotinamide] mM
1
2

TABLE XII

INHIBITION BY NICOTINAMIDE AND NICOTINAMIDE ANALOGS

Compound*	K_i (μM)	pK_a
Nicotinamide	1,400	3.33
Isonicotinamide	15	3.87
3-Acetylpyridine	19,500	3.13
2-Acetylpyridine	13,500	2.64
4-Acetylpyridine	51	3.51
Thionicotinamide	1,150	3.35
3-Pyridinecarboxyaldehyde	1,440	3.80
3-Methylpyridine	218	5.64
4-Methylpyridine	80	6.00
3-Aminopyridine	66	5.98
4-Aminopyridine	41	9.87
3-Pyridylacetonitrile	127	4.87
3-Pyridylcarbinol	457	4.92
Nicotinic acid	7,900	4.77
Isonicotinic acid	250	4.86
Nicotinic acid hydrazide	500	3.58
Isonicotinic acid hydrazide	9	3.58
6-Aminonicotinamide	4,000	N.D
Pyridine	32	5.23
Pyrimidine	-	N.D.
Pyrazinamide	-	N.D.
Pyridazine	-	N.D.
Aniline	-	N.D.
Benzene	-	-

*All substituted pyridine bases were noncompetitive inhibitors

substituted pyridine ring nitrogen and the ability to inhibit the hydrolytic reaction catalyzed by the snake venom NADase.

Another interesting result obtained from these experiments can be seen in Table XII, when comparing the K_i values of 3-substituted to those of 4-substituted pyridine bases. For any given substituent group, the K_i value obtained for the 4-substituted derivative was always lower than that observed with the corresponding 3-substituted compound. In the case of 3-amino and 4-aminopyridine, the difference in K_i values was small, 66 μM versus 41 μM. This effect was more pronounced when one compared 3-acetylpyridine to 4-acetylpyridine. A 380-fold increase in the binding of 4-acetylpyridine compared to 3-acetylpyridine was observed. Surprisingly, pyridine exhibited a much lower K_i than most of the substituted pyridine bases tested as inhibitors of the NADase catalyzed hydrolysis of NAD.

Inhibition of NADase by ADP-ribose - Inhibition by ADP-ribose was found to be competitive with respect to NAD, as indicated in Figure 15. Using fixed concentrations of ADP-ribose at varying concentrations of NAD, inhibition by ADP-ribose was shown to be of the linear competitive type, as illustrated in the replots of the double reciprocal plots, Figure 16. An inhibitor constant, K_i = 380 μM was calculated from these secondary plots. A similar value of K_i = 360 μM was found when the inhibition was analyzed by the method of Dixon (136).

Inhibition by Other Adenosine Derivatives - Inhibition by adenosine, AMP, ADP, ATP, and phospho-ADP-ribose was studied by the methods previously described in the ADP-ribose experiments. K_i values

FIGURE 15 - Competitive inhibition of NADase by ADP-ribose. The reaction mixtures contained 87 mM potassium chloride, ADP-ribose as indicated, 55 ng of purified snake venom NADase, and NAD concentrations were varied from 10 to 400 μM. Initial velocities were measured by the consumption of 1 mM NaOH at 37° C, and pH 7.5. The concentrations of ADP-ribose used were as follows: Line 1, 0; Line 2, 0.4 mM; Line 3, 0.9 mM; and Line 4, 1.9 mM.

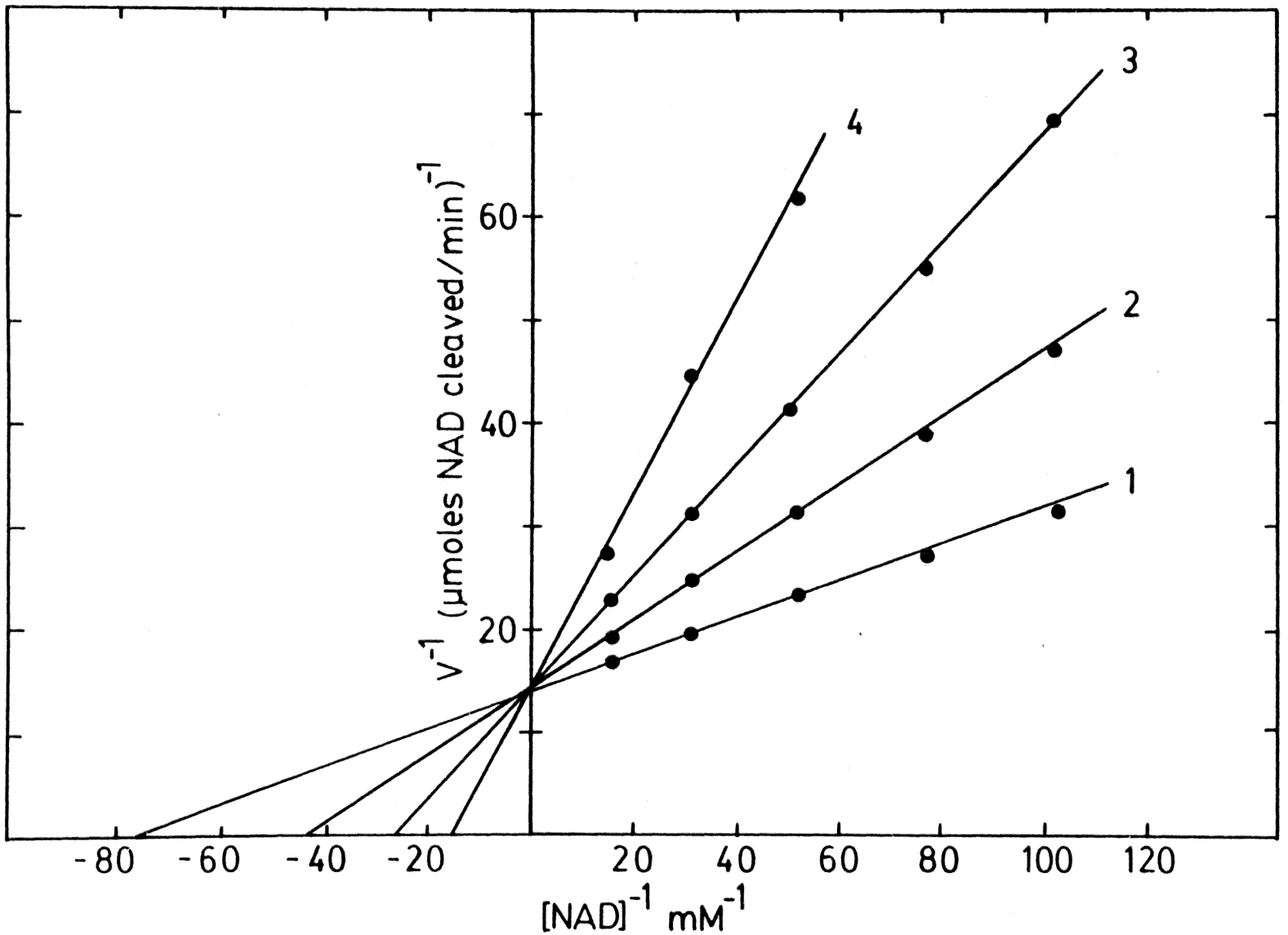
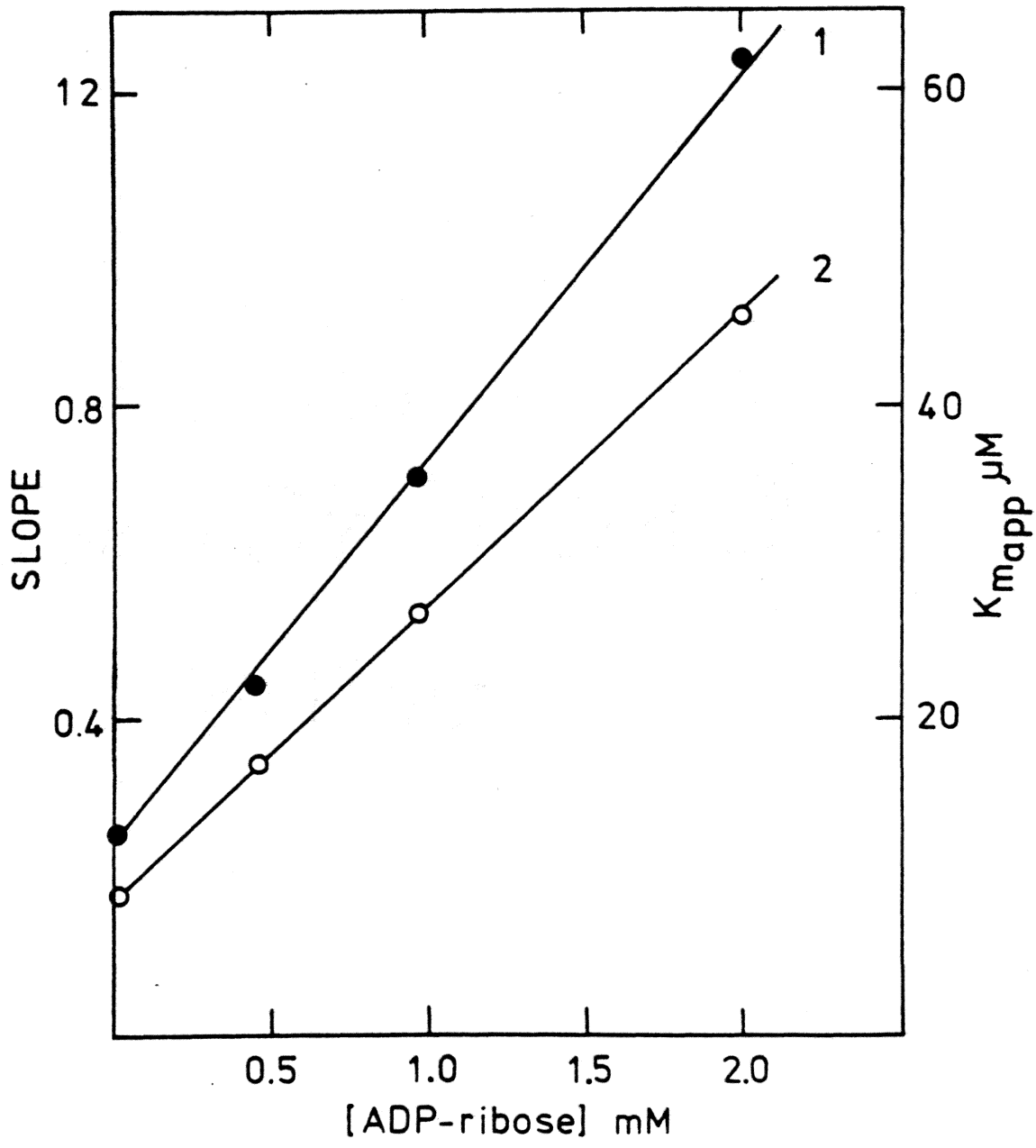

4
3
2
1
60
40
20
v^{-1} (µmoles NAD cleaved/min)$^{-1}$
-80
-60
-40
-20
20
40
60
80
100
120
[NAD]$^{-1}$ mM^{-1}

FIGURE 16 - Replot of the apparent K_m values designated by Line 1 (closed circles) and slopes designated by Line 2 (open circles), obtained from Figure 15, as a function of ADP-ribose concentration.


1
2
12
0.8
0.4
SLOPE
60
40
20
$K_{m_{app}}$ µM
0.5
1.0
1.5
2.0
[ADP-ribose] mM

adenosine derivatives are shown in Table XIII. Adenosine did not inhibit the NADase activity at concentrations up to 20 mM. ADP and ADP-ribose had essentially the same K_i values, whereas, AMP was not as effective. Phospho-ADP-ribose was shown to have a K_i about 2 times lower than that for ADP-ribose.

Inhibition of Bungarus fasciatus NADase by NAD analogs - NAD analogs which were not utilized as substrates by the Bungarus fasciatus venom NADase (Table X), were investigated as possible inhibitors of the enzymatic hydrolysis of NAD. The type of inhibition and values for inhibitor constants were determined from replots of Lineweaver-Burk plots. All NAD analogs tested were found to be competitive inhibitors with respect to NAD as represented in Figure 17, which illustrates the inhibition of NAD hydrolysis by 3-aminopyridine adenine dinucleotide, AAD. The inhibition of NADase by AAD was of the linear competitive type as shown from replots of these double reciprocal plots, Figure 18. An inhibitor constant of 0.32 μM was calculated from both apparent K_m and slope replots. The low K_i value for AAD inhibition of NADase was further verified when inhibition was studied at varying concentrations of AAD at constant concentrations of NAD. These results were plotted according to Dixon (136), Figure 19. Under these experimental conditions a K_i = 0.30 μM was determined. Table XIV lists the inhibitor constants determined for the NAD analogs studied.

Inhibition of NADase by Cibacron Blue F3GA - During purification procedures Bungarus fasciatus venom NADase was shown to bind very tightly to the affinity column, Amicon Matrex™ gel Blue A (Figure 7).

TABLE XIII

INHIBITION BY ADENOSINE ANALOGS

Adenosine Derivative*	K_i
	(mM)
Adenosine	-
AMP	2.1
ADP	0.5
ATP	0.9
ADP-ribose	0.4
Phospho-ADP-ribose	0.2

*All derivatives were linear competitive inhibitors

FIGURE 17 - Competitive inhibition of NADase by AAD. The reaction mixtures contained 87 mM potassium chloride, AAD as indicated, 55 ng of purified snake venom NADase, and NAD concentrations were varied from 10 to 400 µM. Initial velocities were measured by the consumption of 1 mM NaOH at 37° C, pH 7.5. The concentrations of AAD used were as follows: Line 1, 0; Line 2, 0.24 µM; Line 3, 0.48 µM; Line 4, 0.72 µM; Line 5, 0.96 µM.

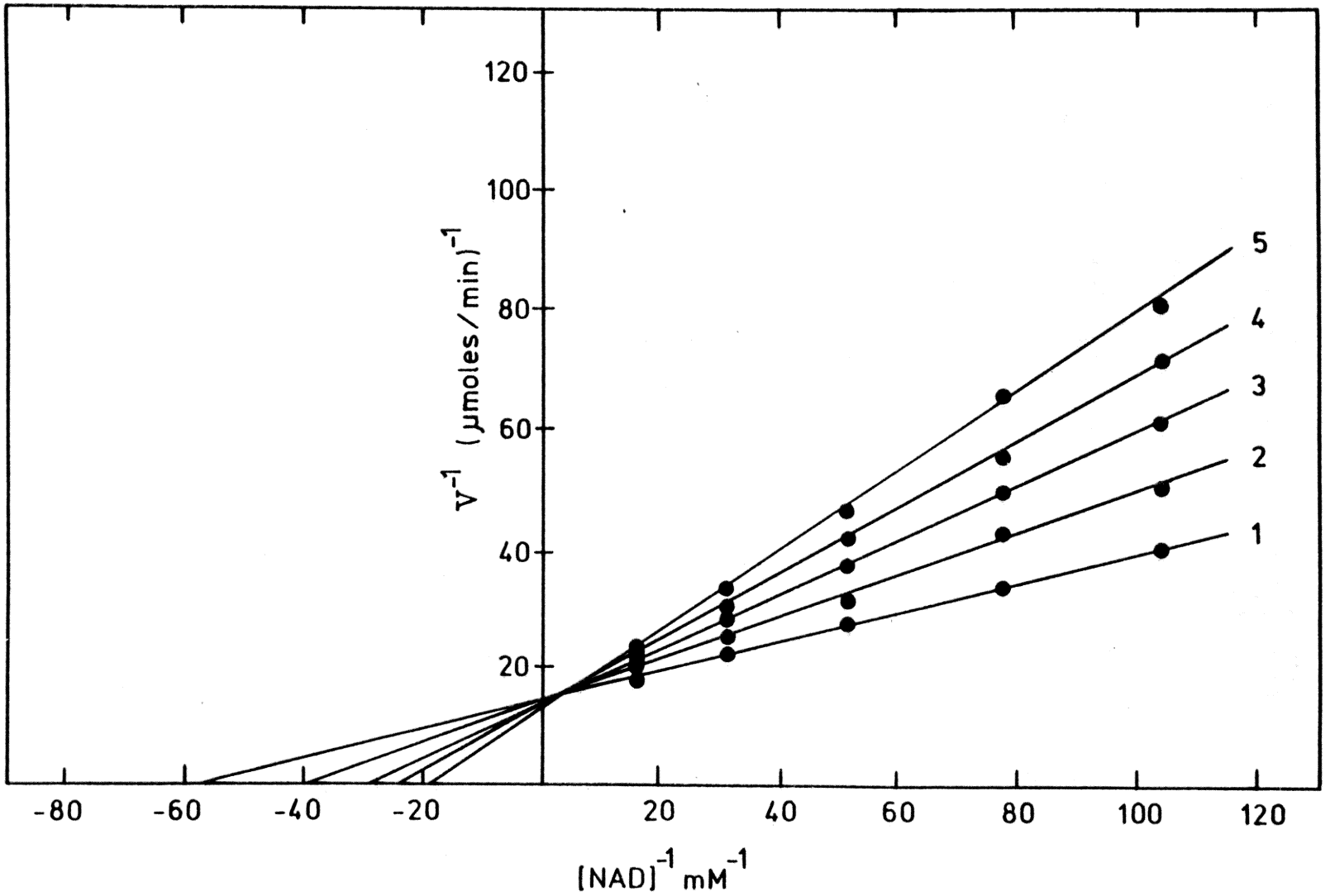

120
100
80
60
40
20
v^{-1} (µmoles/min)$^{-1}$
5
4
3
2
1
-80
-60
-40
-20
20
40
60
80
100
120
[NAD]$^{-1}$ mM^{-1}

FIGURE 18 - A replot of the apparent K_m values designated by Line 1 (closed circles) and slopes designated by Line 2 (open circles) obtained in Figure 17, as a function of AAD concentration.

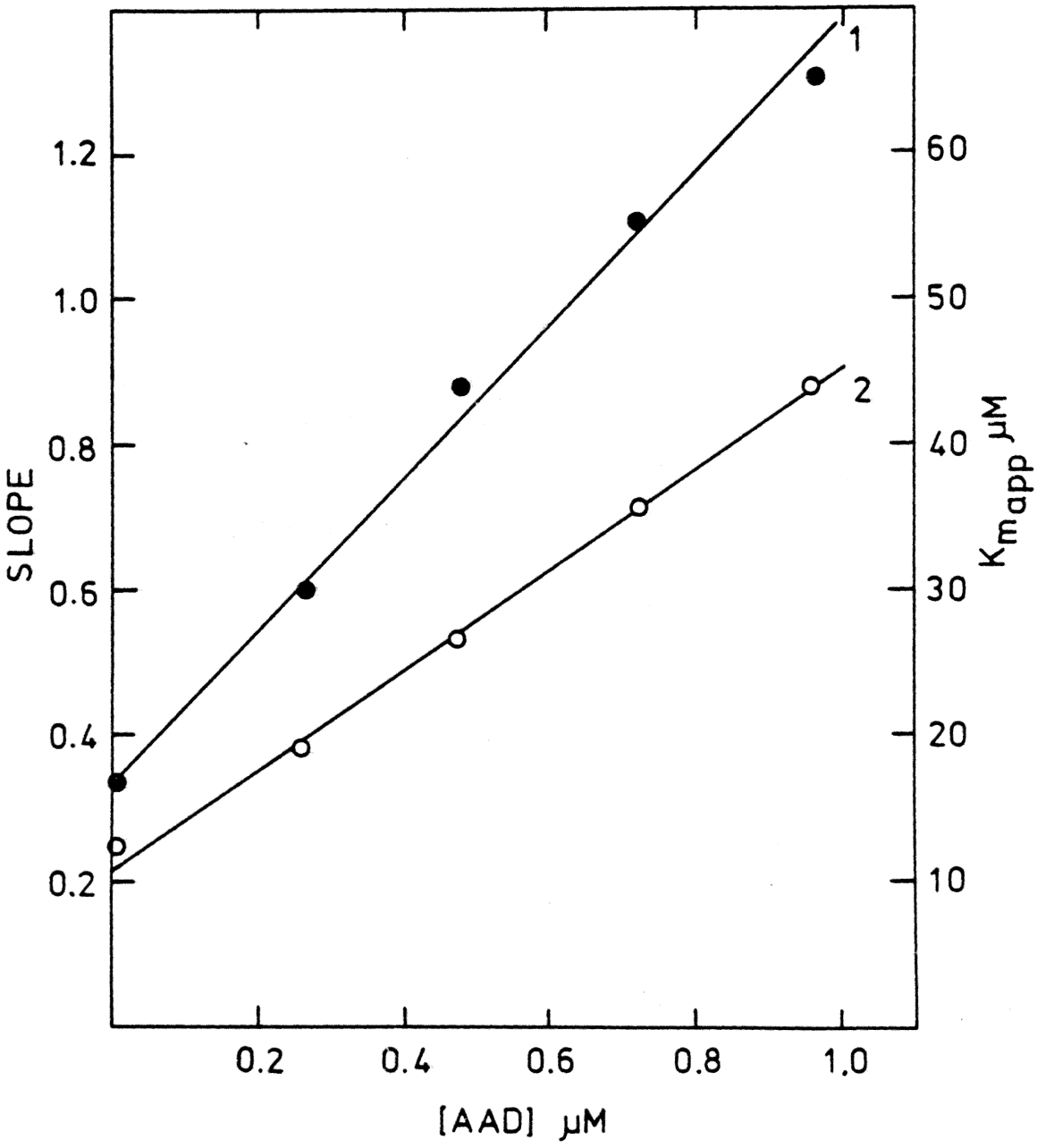

SLOPE
1.2
1.0
0.8
0.6
0.4
0.2
0.2
0.4
0.6
0.8
1.0
[AAD] µM
$K_{m_{app}}$ µM
60
50
40
30
20
10
1
2

FIGURE 19 - Dixon plot of AAD inhibition of B. fasciatus venom NADase. Reaction mixtures were prepared as described in the legend of Figure 17. Inhibitor concentrations were as indicated. NAD concentrations were 16 µM designated by Line 1 and 32 µM designated by Line 2.

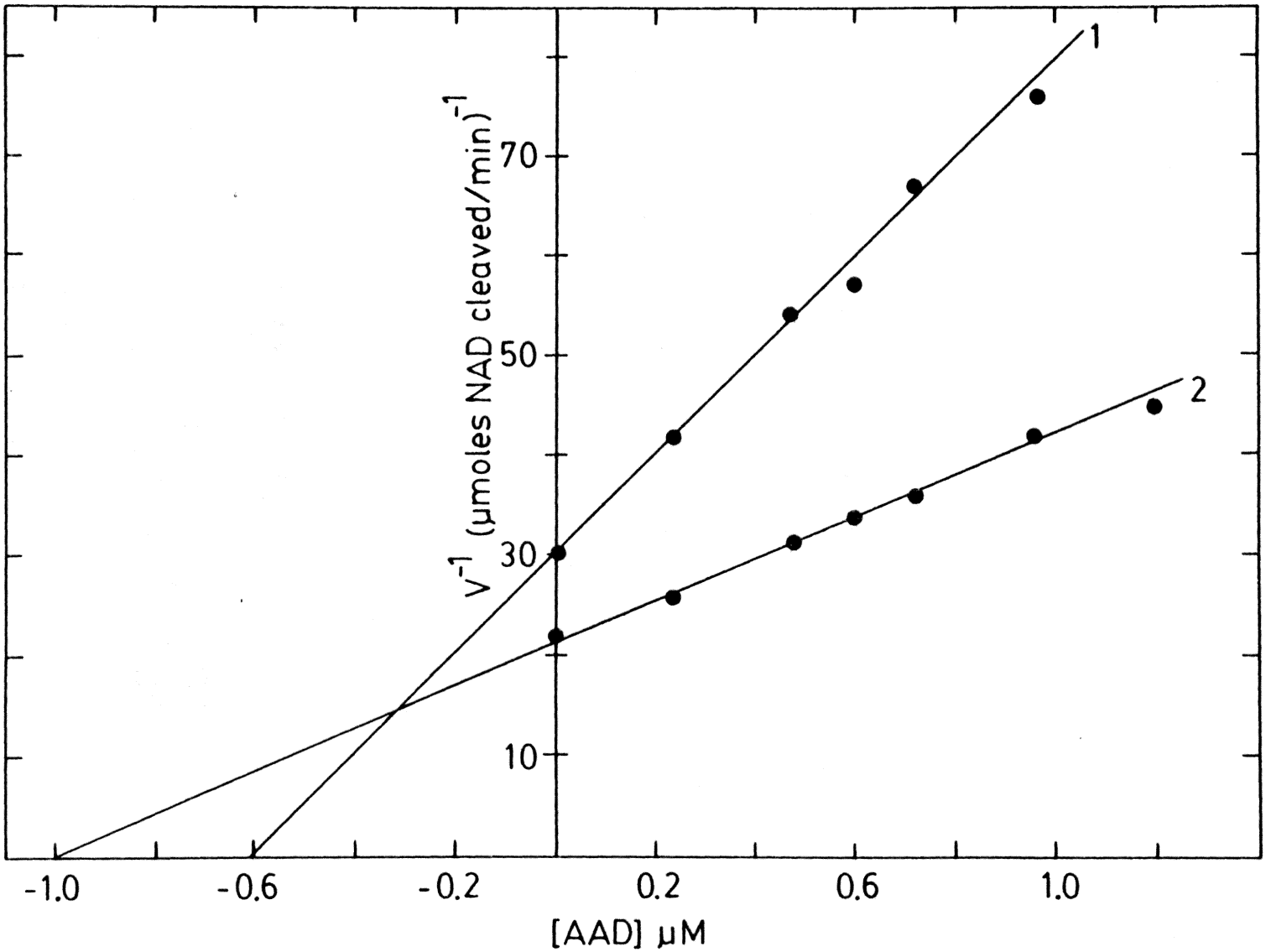

1
2
70
50
30
10
V^{-1} (μmoles NAD cleaved/min)$^{-1}$
-1.0
-0.6
-0.2
0.2
0.6
1.0
[AAD] μM

TABLE XIV

SUMMARY OF INHIBITION BY NAD ANALOGS

Compound*	K_i
	(μM)
Nicotinamide mononucleotide	860
1,4-Dihydronicotinamide adenine dinucleotide	440
Adenosine diphosphoribose	380
α-Nicotinamide adenine dinucleotide	85
Nicotinic acid adenine dinucleotide	76
3-Pyridinecarboxyaldehyde adenine dinucleotide	6
3-Pyridylcarbinol adenine dinucleotide	4
3-Methylpyridine adenine dinucleotide	2
3-Aminopyridine adenine dinucleotide	0.30
Pyridine adenine dinucleotide	0.22
Isonicotinic acid hydrazide adenine dinucleotide	0.18
Procion Blue HB (Cibacron Blue F3GA)	0.033

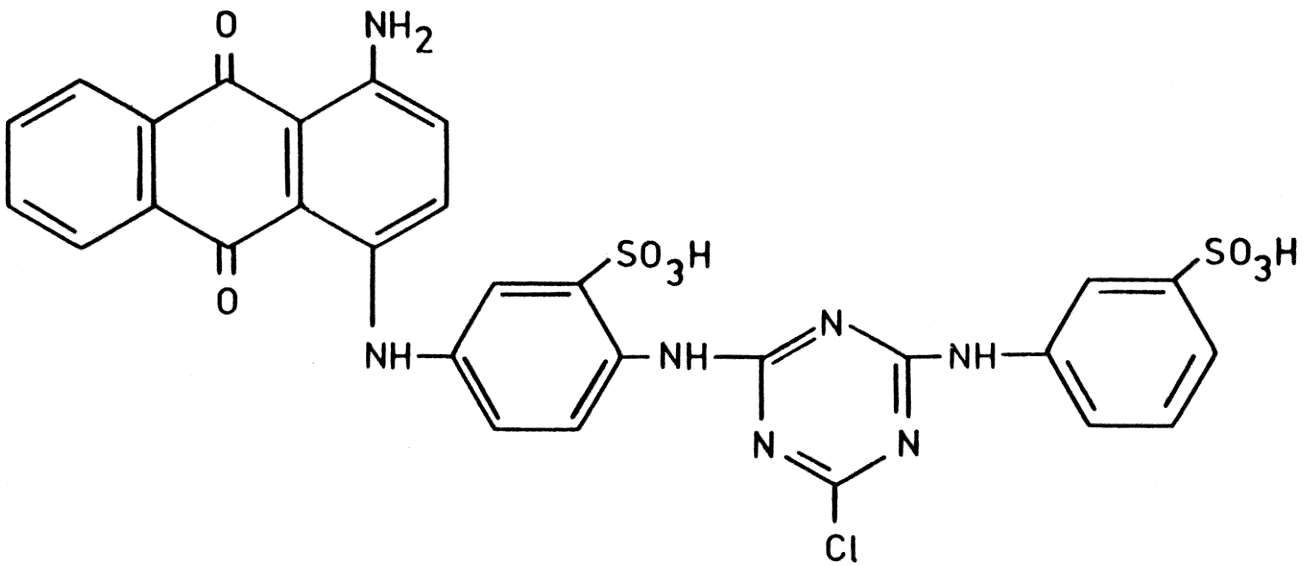
*All NAD analogs studied exhibited competitive inhibition.

This result prompted investigation of the mode of inhibition and inhibitor constant for the free ligand, Cibacron Blue (Procion Blue HB). The structure of this triazine dye is shown in Figure 20 and inhibition by the dye was studied by varying the substrate concentration at five constant inhibitor concentrations. The results were plotted according to Lineweaver and Burk (Figure 21). Secondary plots of the slopes and apparent K_m values are shown in Figure 22. Procion Blue HB inhibition of NADase was observed to be of the linear competitive type, with a K_i = 0.033 μM. In comparison to all other inhibitors studied, Cibracon Blue was by far the most effective inhibitor of the enzyme.

Amino Acid Modification - The chemical modification of Bungarus fasciatus venom NADase with specific chemical reagents was investigated to identify possible amino acid residues essential for catalytic activity. When the purified snake venom enzyme was incubated with various sulfhydryl reagents, no loss of enzyme activity was observed. Sulfhydryl reagents tested included N-ethylmaleimide, iodoacetamide, p-chloromercuribenzoate, and diazotized 3-aminopyridine adenine dinucleotide.

Inhibitor studies indicated the importance of the pyrophosphate group in the binding of the adenosine derivatives to the B. fasciatus venom NADase. Phenylglyoxal and 2,3-butanedione have been shown to modify specific guanidino groups and to a much lesser extent the ε-amino group of lysine residues (137). Incubation of the snake venom enzyme with up to 500 mM of either reagent resulted in no observable loss of enzyme activity after incubations of up to 4 hours at 37°C.

FIGURE 20 - The stucture of Cibacron Blue F3GA (Procion Blue HB).



Cibacron Blue F3GA
(Procion Blue HB)

FIGURE 21 - The effect of Procion Blue HB on the rates of hydrolysis catalyzed by the purified Bungarus fasciatus venom NADase. Reaction mixtures contained 87 mM potassium chloride, NAD concentrations were varied from 11 to 200 µM. Reaction mixtures were adjusted to pH 7.5 and 37° C, reactions were initiated by the addition of 34 ng purified snake venom NADase and the initial rates were monitored titrimetrically. The concentrations of Procion Blue HB used were as follows: Line 1, 0; Line 2, 25 nM; Line 3, 48 nM; Line 4, 73 mM; and Line 5, 97 nM.

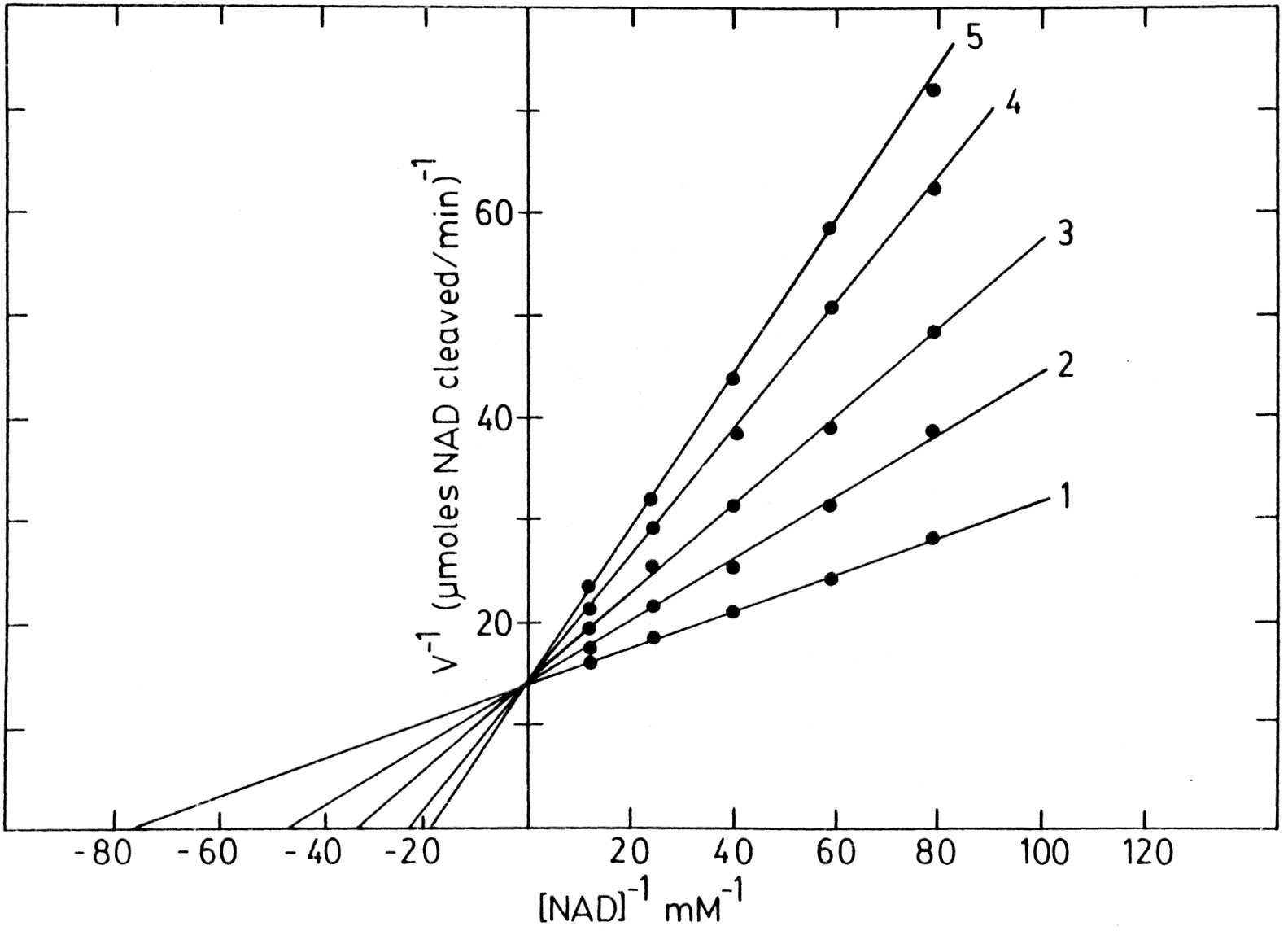
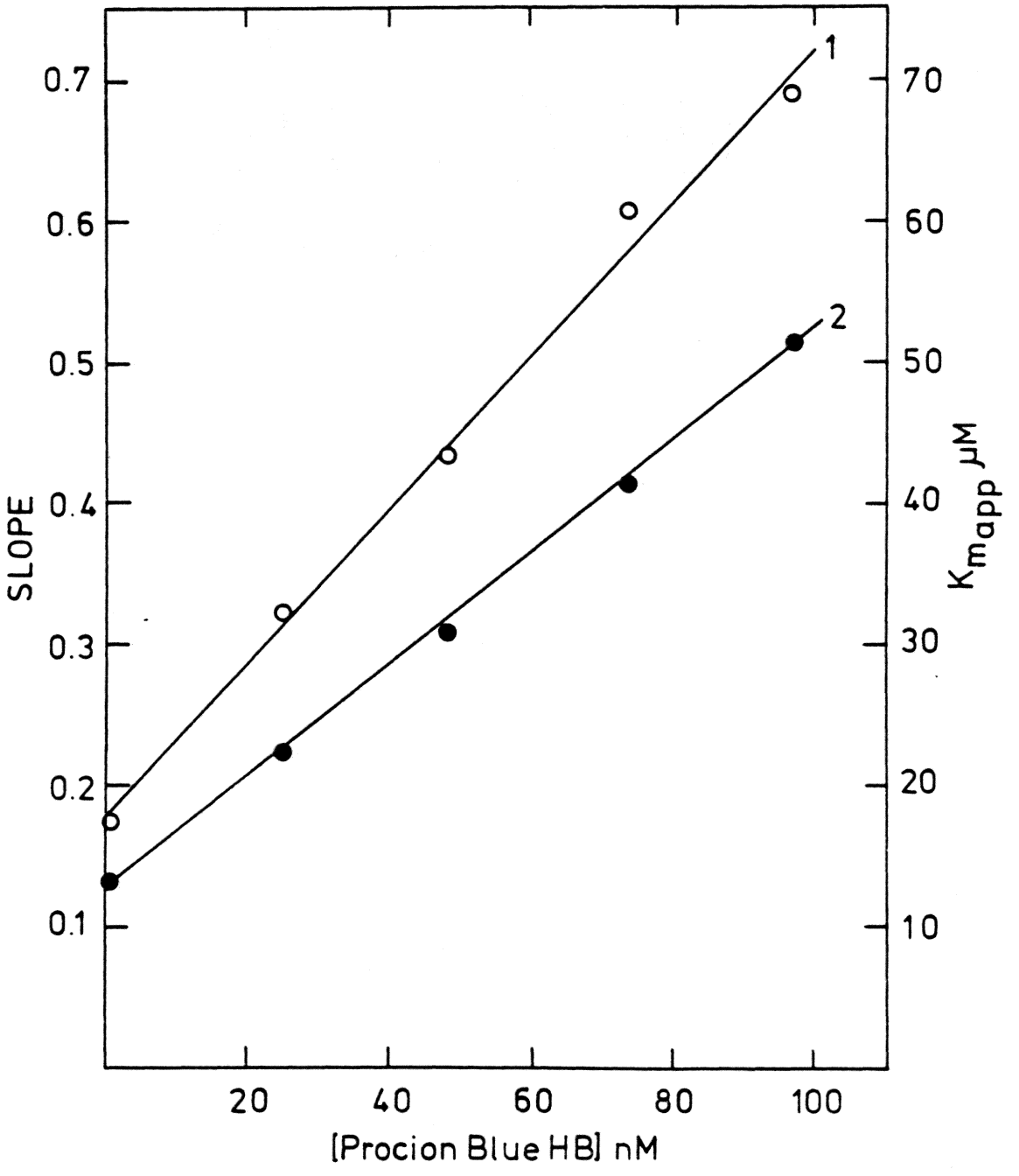

5
4
3
2
1
60
40
20
V^{-1} (μmoles NAD cleaved/min)$^{-1}$
-80
-60
-40
-20
20
40
60
80
100
120
[NAD]$^{-1}$ mM^{-1}

FIGURE 22 - A replot of slopes, designated by Line 1 (open circles) and apparent K_m values (closed circles) designated by Line 2, obtained in Figure 21, as a function of Procion Blue HB concentration.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
SLOPE
70
60
50
40
30
20
10
$K_{m_{app}}$ µM
1
2
20
40
60
80
100
[Procion Blue HB] nM

Gilbert and O'Leary (138) have shown that 2,4-pentanedione at pH 7, almost exclusively modified the ε-amino groups of lysine residues rather than modification of arginine residues. The B. fasciatus venom NADase incubated with 200 mM 2,4-pentanedione lost almost 40 percent of its catalytic activity after 1 hour. The results obtained with two different concentrations of 2,4-pentanedione are shown in Figure 23. Enzyme inactivation followed pseudo-first order kinetics. Incubation of the NADase with 400 mM 2,4-pentanedione resulted in complete inactivation after 2.5 hours. Rate constants for inactivation of 0.0071 and 0.0128 min^{-1} were determined for 200 and 400 mM 2,4-pentanedione, respectively. An apparent second order rate constant of 0.34 M^{-1} min^{-1} for the inactivation of the NADase with 2,4-pentanedione was calculated.

The presence of the competitive inhibitor, 3-aminopyridine adenine dinucleotide, AAD, protected the enzyme from inactivation by 2,4-pentanedione. Results of these experiments are shown in Figure 24 where line 1 represents the control experiment containing 400 mM 2,4-pentanedione and the pseudo-first order rate constant for inactivation was 0.0128 min^{-1}. Line 2, the protection experiment, containing 2.1 μM AAD indicated a k_1 = 0.0023 min^{-1}. The presence of 2.1 μM AAD in the inactivation mixture provided 82% protection of the venom NADase from 2,4-pentanedione inactivation.

2,4-pentanedione modified NADase inactivated to less than 10% of its original activity when treated with 0.1 M neutral hydroxylamine resulted in a reactivation of enzyme activity after a one hour incubation. Only 70% of the original activity could be recovered, even after

FIGURE 23 - Time-dependent inactivation of B. fasciatus venom NADase with 2,4-pentanedione. Reaction mixtures were prepared as described in the experimental procedures. The concentrations of 2,4-pentanedione used were as follows: Line 1, 0; Line 2, 200 mM; and Line 3, 400 mM.

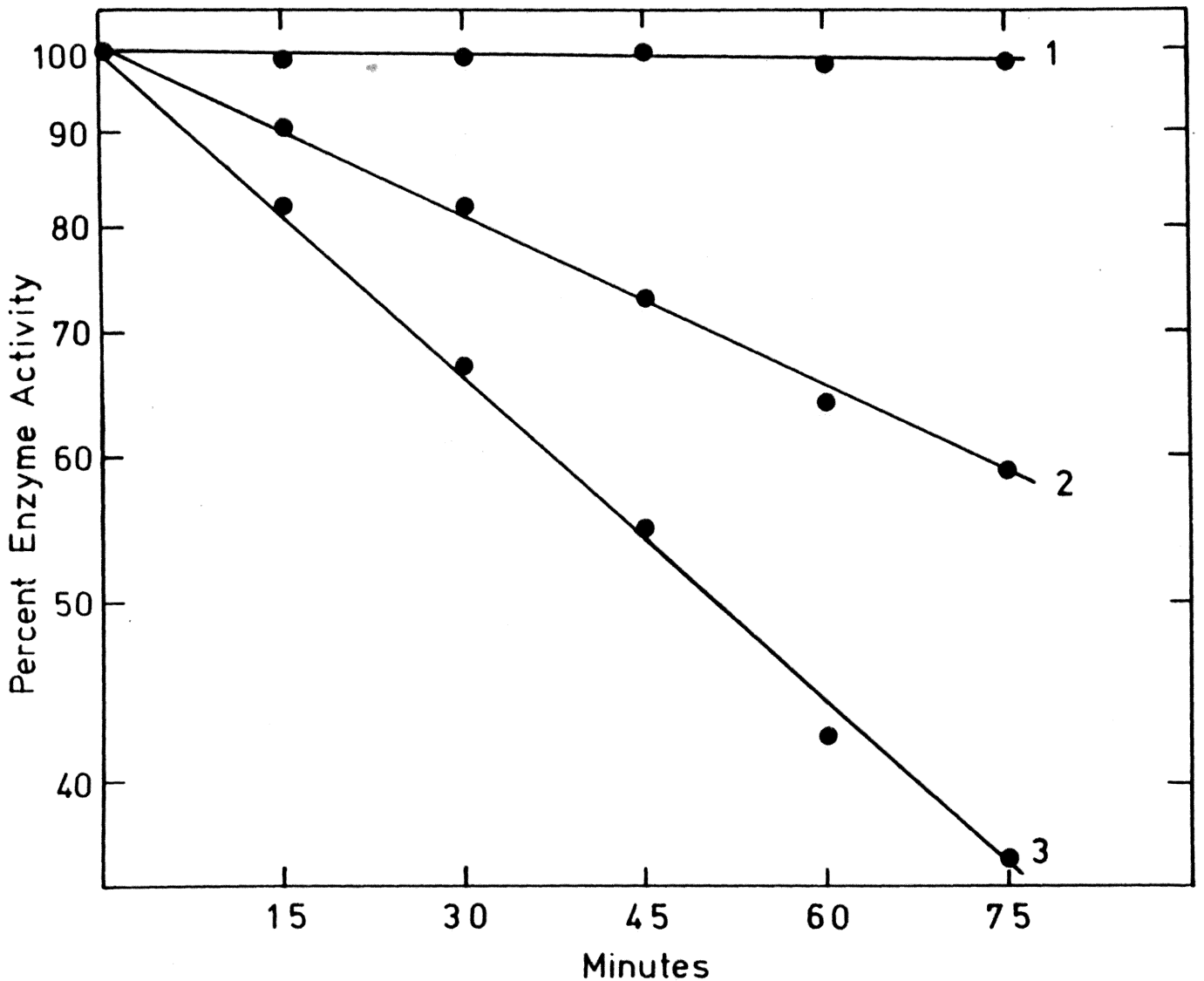
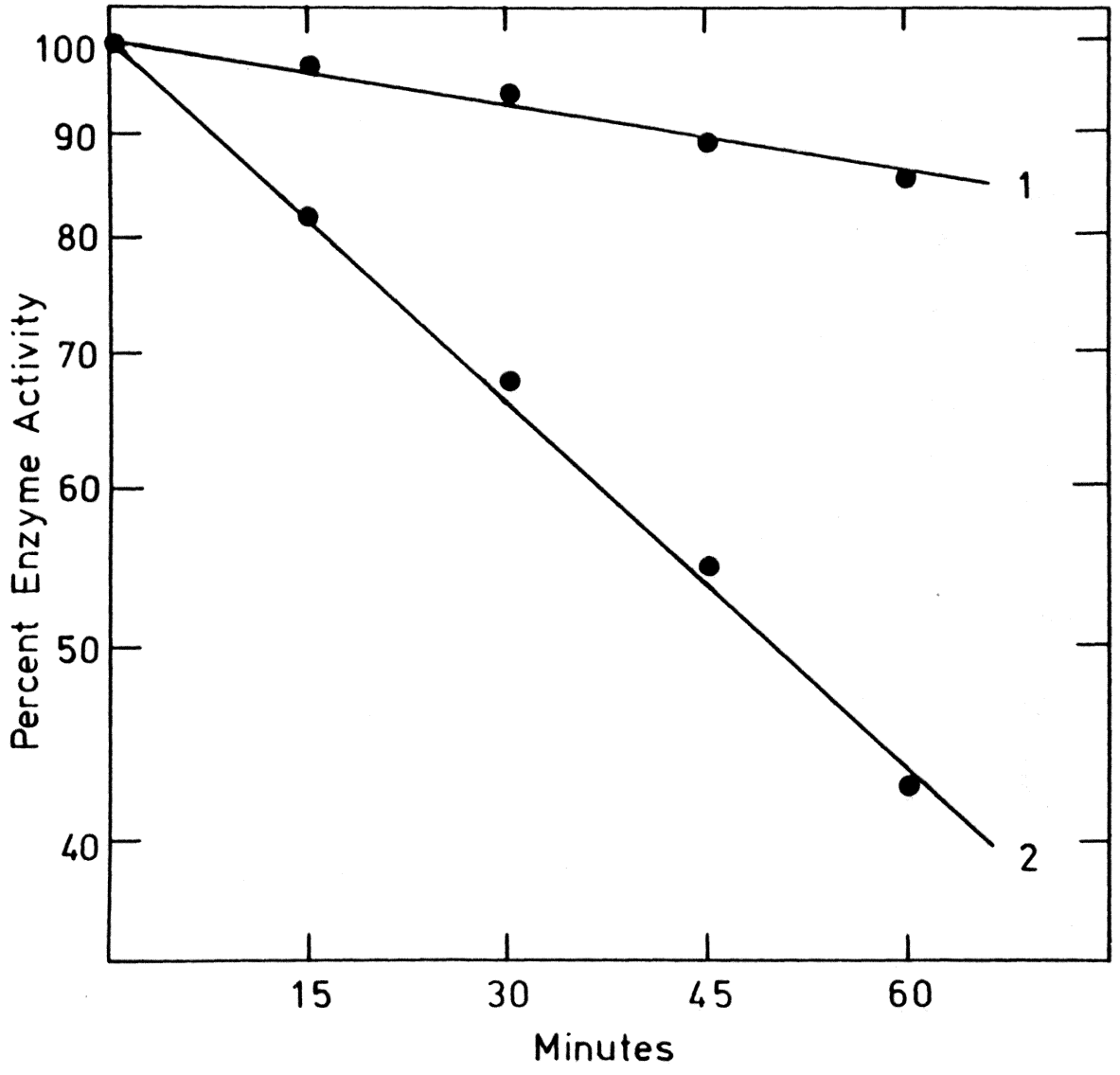

100
90
80
70
60
50
40
Percent Enzyme Activity
15
30
45
60
75
Minutes
1
2
3

FIGURE 24 - AAD protection of NADase activity from inactivation with 2,4-pentanedione. Reactions were done as described in Figure legend 23. Conditions used were as follows: Line 1, 400 mM 2,4-pentanedione, plus 2.1 μM AAD and Line 2 represents enzyme activity in the presence of 400 mM 2,4-pentanedione.

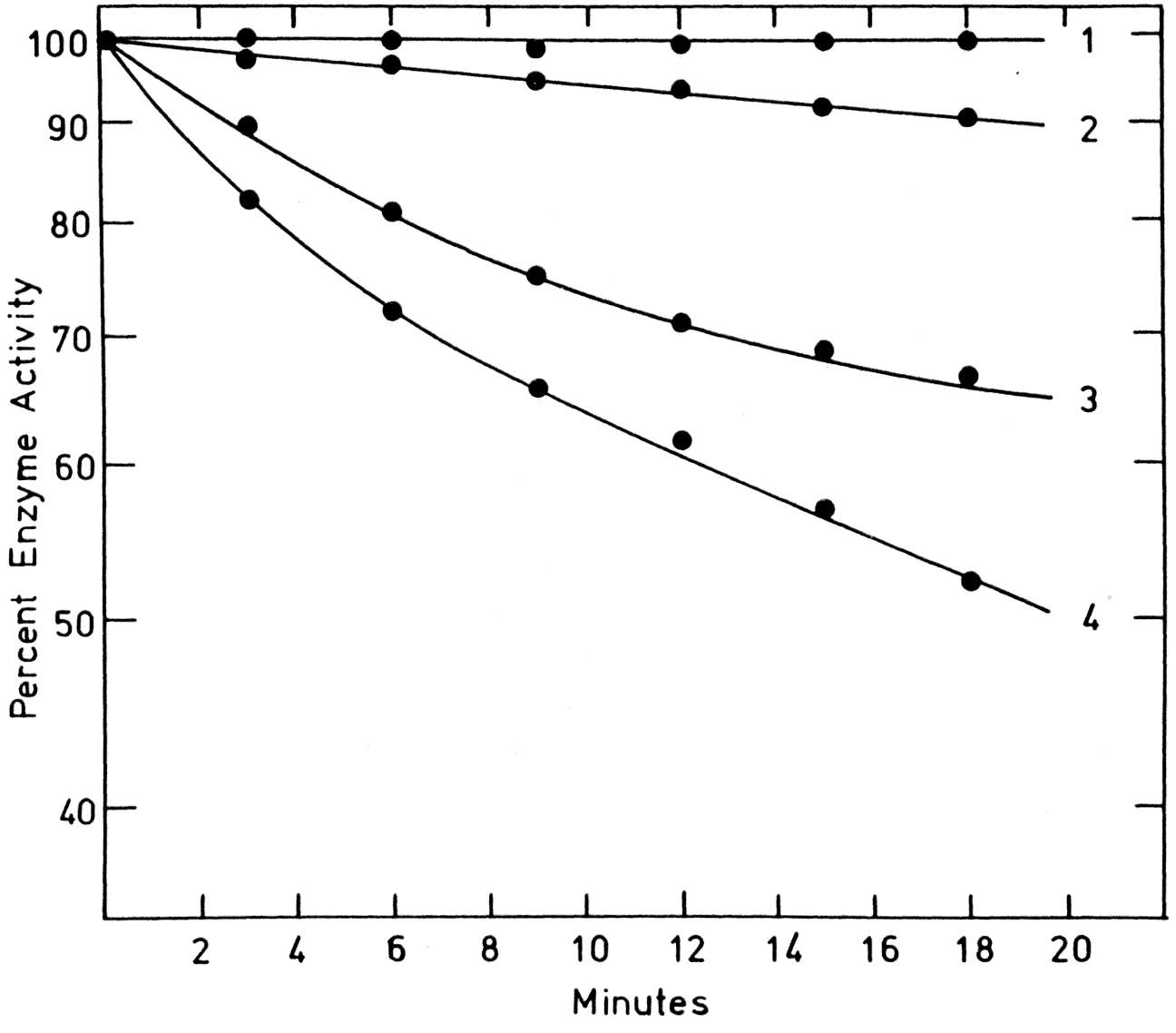

100
90
80
70
60
50
40
Percent Enzyme Activity
15
30
45
60
Minutes
1
2

extensive dialysis at 4°C against 5 mM sodium phosphate buffer pH 7.5. The results of these experiments are consistent with the essential involvement of a lysine residue(s) at the active site of B. fasciatus venom NADase.

Another ionic interaction to be expected in the binding of NAD to the snake venom enzyme would occur between the positively-charged pyridinium ring nitrogen and a negatively-charged glutamate or aspartate residue of the enzyme. The most commonly employed method for modification of protein carboxyl groups involves a coupling with nucleophiles mediated by a water soluble carbodiimide (137). 1-Ethyl-3-(dimethylaminopropyl)-carbodiimide plus n-butylamine when incubated with the snake venom enzyme resulted in no observable loss of catalytic activity. Woodward's Reagent K, N-ethyl-5-phenyl isoxazolium-3'-sulfonate also shown to modify protein carboxyl groups (139,140) caused a rapid loss of enzyme activity (Figure 25). Pseudo-first order kinetics were not observed. Protection from inactivation was observed in the presence of the competitive inhibitor, AAD. These results are indicated by Line 3, Figure 25. Schuber (141) has suggested that the reason enzyme inactivation by Woodward's Reagent K does not follow pseudo-first order kinetics is probably due to the instability of the Woodward's Reagent K. This reagent was shown by Dunn and Anfinsen (139) to hydrolyze at pHs above 3.

Alivisatos suggested NADases may function through an enzyme-bound intermediate presumed to be an ADP-ribosylated histidine residue (58). In an attempt to further characterize the active site of B. fasciatus

FIGURE 25 - The effect of Woodward's Reagent K (WK) on B. fasciatus venom NADase activity. Reactions were prepared and assayed as described in Figure legend 23. Reaction conditions used were as follows: Line 1, control, no WK; Line 2, 5 mM WK, plus 2.1 μM AAD; Line 3, 2.5 mM WK; Line 4, 5 mM WK.


Percent Enzyme Activity
100
90
80
70
60
50
40
2
4
6
8
10
12
14
16
18
20
Minutes
1
2
3
4

venom NADase, modification of enzyme histidine residues was investigated. Diethyl pyrocarbonate a specific reagent for the modification of histidine residues in proteins (142,143). When incubated at 4 mM with the B. fasciatus venom NADase and no enzyme inactivation was detected after 2 hours.

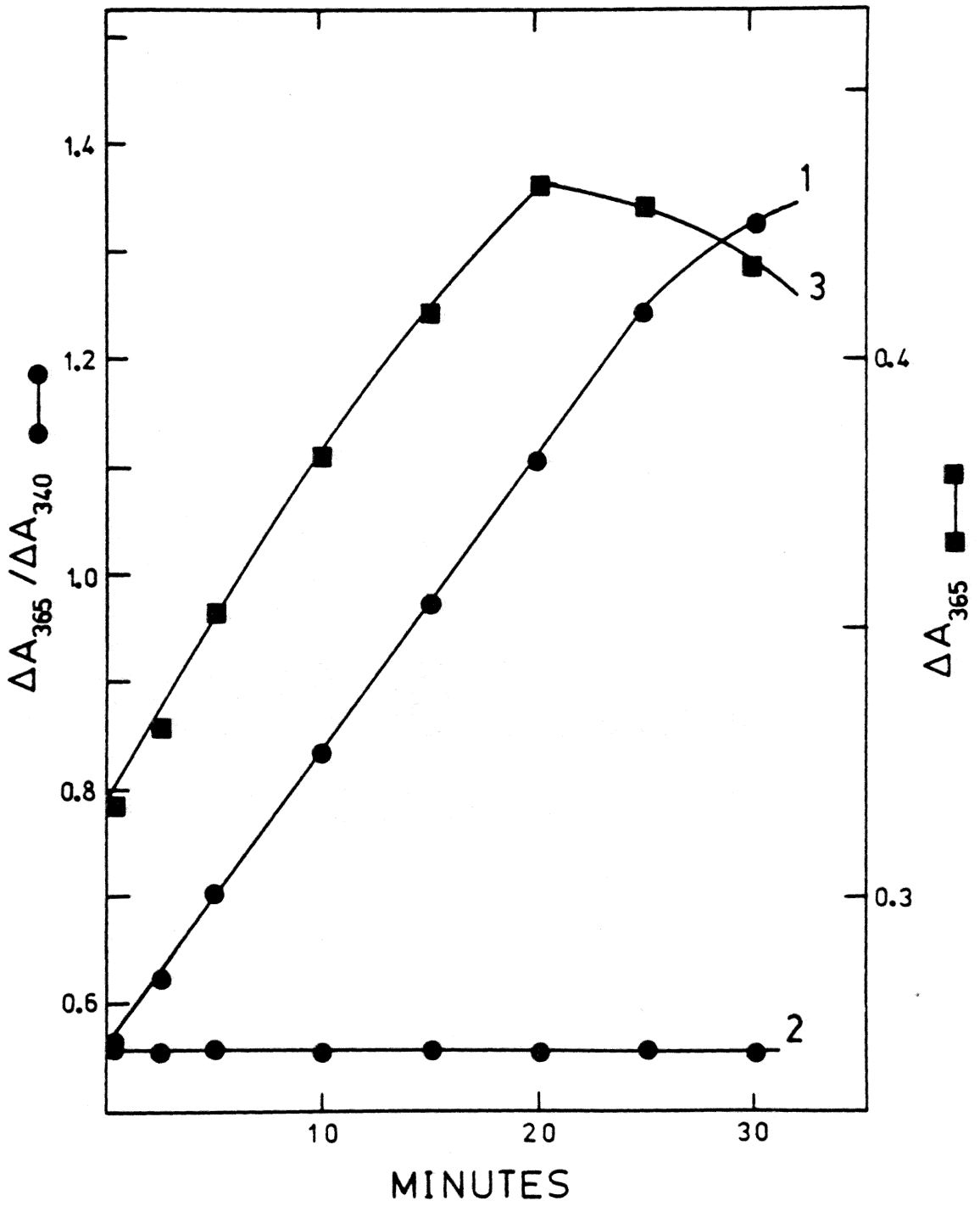
Studies of the Pyridine Base Exchange Reaction

Zatman et al. (56) and Schuber et al. (116) have suggested that the noncompetitive inhibition of certain NADases by nicotinamide is a result of an enzyme-catalyzed pyridine base exchange reaction facilitated by the existence of an intermediate enzyme-ADP-ribose (E-ADP-ribose). When water is the nucleophile reacting with the proposed intermediate, free-ADP-ribose is formed. However, if an alternate nucleophile (i.e. pyridine base) competes with water for the intermediate, base exchange occurs. This proposed model is shown in Figure 4. The ability of the purified B. fasciatus venom NADase to catalyze the pyridine base exchange reaction was investigated. Transglycosidase activity was measured by the formation of 3-acetylpyridine adenine dinucleotide from NAD and the free base 3-acetylpyridine. As a control reaction, purified bovine seminal plasma NADase, an enzyme which does not catalyze the pyridine base exchange reaction was tested under similar conditions. Reaction mixtures contained 50 mM sodium phosphate buffer, pH 7.5, 1 unit of either NADase, 100 mM 3-acetylpyridine, and 3 mM NAD in a total volume of 3 ml. At timed intervals, 0.2 ml aliquots from the reaction mixture were transferred to 0.2 ml of ice-cold 15 percent trichloroacetic acid. Dinucleotide concentration was determined in a yeast

alcohol dehydrogenase assay mixture. The 3-acetylpyridine adenine dinucleotide on reduction exhibits a new absorbance maximum at 365 nm, while reduced NAD has the characteristic 340 absorbance maximum. Therefore, analog formation results in an increase in the 365/340 ratio. Yeast alcohol dehydrogenase-catalyzed reductions were assayed as follows; 0.2 ml trichloroacetic acid-reaction mixture were transferred into 2.8 ml of 90 mM Tris (unbuffered) containing 0.5 M ethanol. Absorbances at 365 and 340 nm were recorded before and after the addition of 0.2 ml (10 mg/ml) yeast alcohol dehydrogenase. The difference in the absorbances at each wavelength was plotted as $\Delta 365/\Delta 340$ versus time. An increase in this ratio was observed with respect to time in reaction mixtures containing B. fasciatus venom NADase, (Figure 26, line 1) indicating the catalysis of a pyridine base exchange reaction. The change in the absorbance at 365 nm (Figure 26, line 3) is directly related to the formation of 3-acetylpyridine adenine dinucleotide. Maximum formation of the 3-acetylpyridine analog occurred in 20 minutes, after which a decrease in the 365 absorbance was observed. This decrease was due to the utilization of newly formed 3-acetylpyridine adenine dinucleotide as a substrate by the NADase (Table XI). Purified bovine seminal plasma NADase on the other hand, did not catalyze the pyridine base exchange reaction (Figure 26, line 2). This observation was previously reported by Yuan and Anderson (77).

Proportionality Between Transglycosidase and Glycohydrolase Activity - The relationship between transglycosidase and glycohydrolase activity was studied as described in the legend of Figure 26, except

FIGURE 26 - Pyridine base exchange reaction with NAD and 3-acetylpyridine. Reaction mixtures contained 3 mM NAD, 100 mM 3-acetylpyridine, 1 unit of either snake venom NADase or bovine seminal plasma NADase, 50 mM sodium phosphate buffer, pH 7.5, in a total volume of 3 ml. Dinucleotide concentrations were determined as described in text. Line 1, $\Delta A_{365}/\Delta A_{340}$ obtained with B. fasciatus venom NADase; Line 2, $\Delta A_{365}/\Delta A_{340}$ of purified bovine seminal plasma NADase; Line 3, ΔA_{365} with B. fasciatus venom NADase.


1.4
1.2
1.0
0.8
0.6
0.4
0.3
10
20
30
MINUTES
1
2
3
ΔA365 /ΔA340
ΔA365

that varying concentrations of purified B. fasciatus venom NADase were used. Rates of pyridine base exchange were reported as the increase in Δ365/Δ340 ratio with respect to time. These results are shown in Figure 27. A linear relationship between transglycosidase and glycohydrolase activities was observed, Figure 28.

Kinetic Parameters for 3-acetylpyridine Adenine Dinucleotide Formation - Reaction mixtures were prepared as described in the legend of Figure 26, except 3-acetylpyridine concentration was varied. Initial velocities were calculated from tangents to plots of Δ365/Δ340 ratios versus time. A double reciprocal plot of these initial velocities versus 3-acetylpyridine concentration is shown in Figure 29. The calculated K_m was 21 mM for 3-acetylpyridine in the pyridine base exchange reaction. The V_{max} could not be readily calculated under these experimental conditions.

HPLC Assay for the Pyridine Base Exchange Reaction - Analysis of the pyridine base exchange reaction yielding NAD analogs which can not be enzymatically reduced is limited by the lack of methods of detection. An alternate assay was developed to analyze for the formation of such NAD analogs. Product formation was monitored by high pressure liquid chromatography (HPLC). A standard reaction mixture contained; 1.5 mM NAD, 50 mM potassium phosphate buffer, pH 7.5, 1 unit of snake venom NADase (740 ng), and varying concentrations of the free pyridine base to be studied, all in a total volume of 1 ml, at 37°C. At predetermined times 0.1 ml aliquots were transferred to 0.5 ml of 50 mM potassium phosphate buffer, pH 3.2 and rapidly frozen at -60°C. Product analysis

FIGURE 27 - Proportionality between transglycosidase activity and the amount of NADase present. Transglycosidase activity was measured as described in the legend of Figure 26. Line 1 is the transglycosidase rate when 185 ng of snake venom NADase was added, Line 2, 340 ng: Line 3, 555 ng; and Line 4, 740 ng.

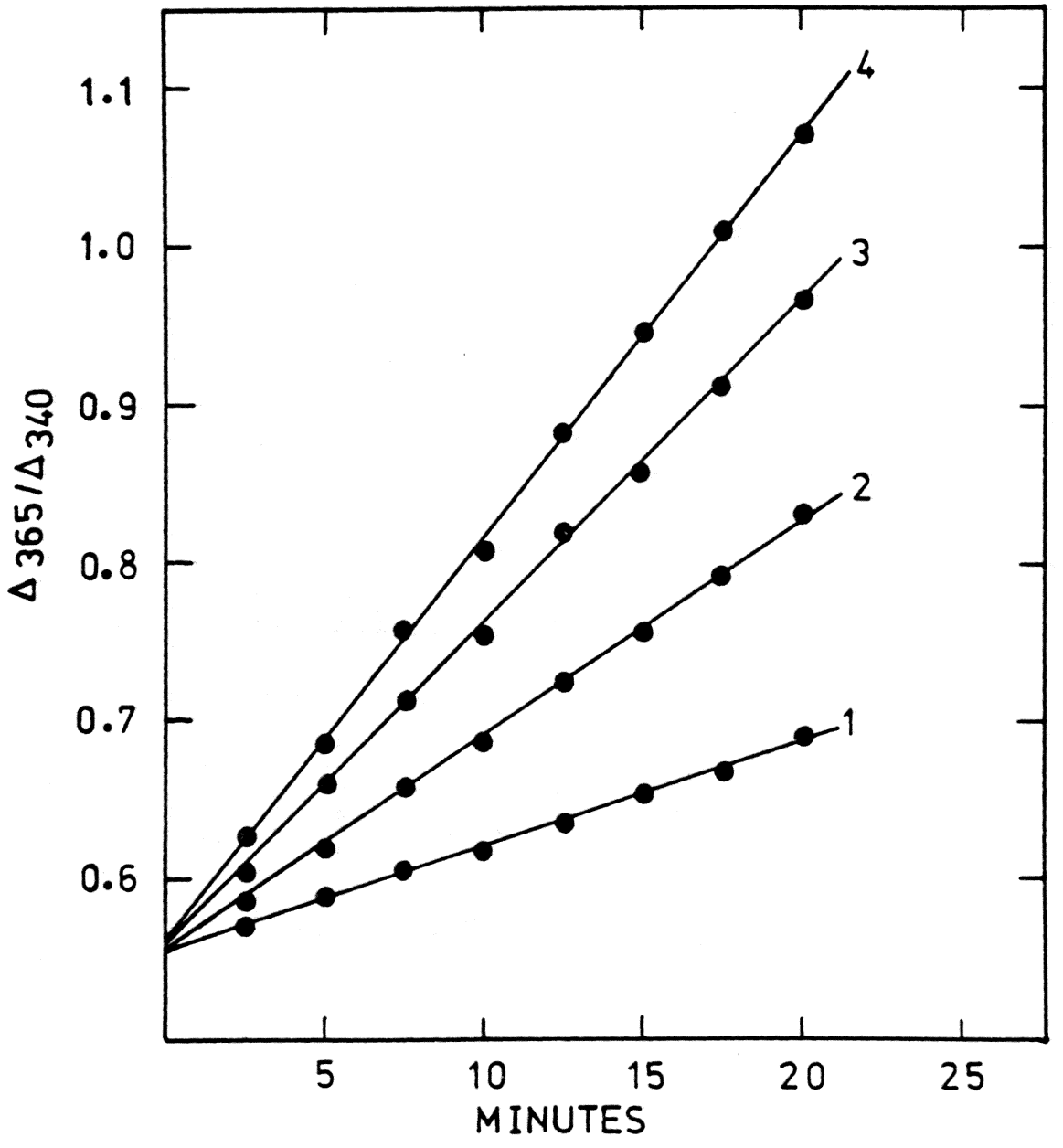

1.1
1.0
0.9
0.8
0.7
0.6
Δ365/Δ340
5
10
15
20
25
MINUTES
4
3
2
1

FIGURE 28 - Linear relationship between transglycosidase activity and NAD glycohydrolase (NADase) activity. Slopes from lines in Figure 27 were replotted against the units of NADase added, (740 ng NADase equals 1 hydrolytic unit).

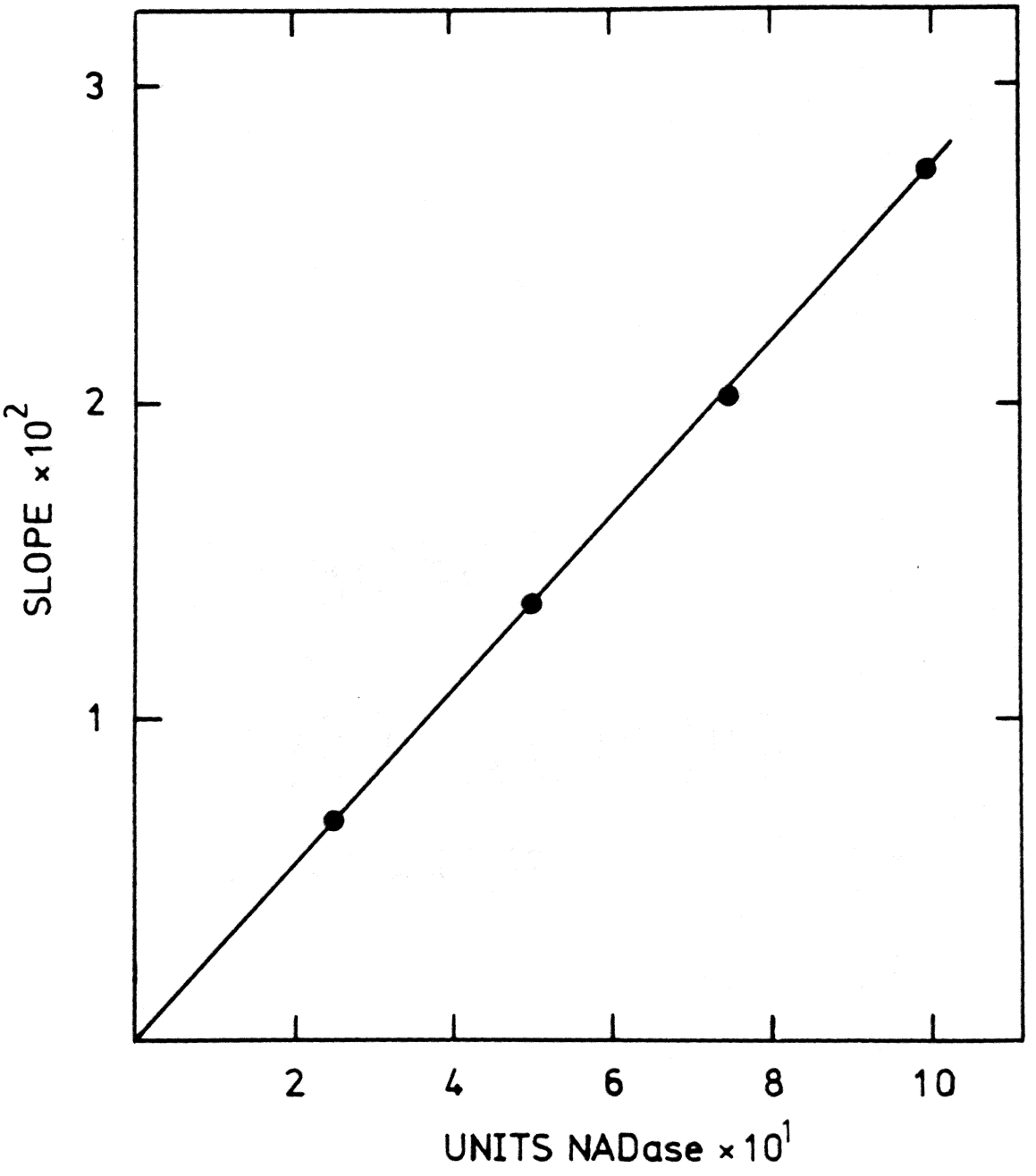
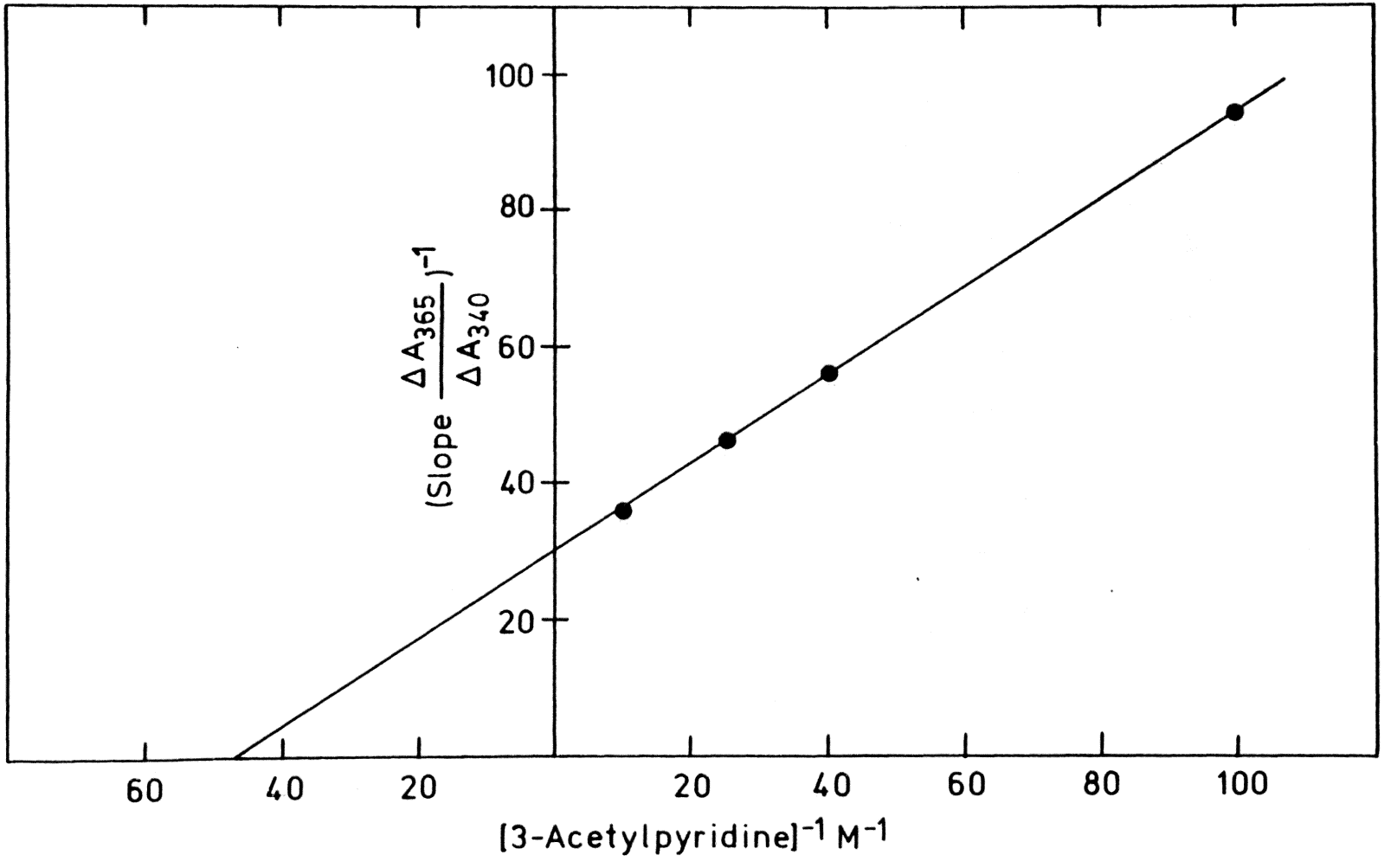

3
2
1
SLOPE ×10²
2
4
6
8
10
UNITS NADase ×10¹

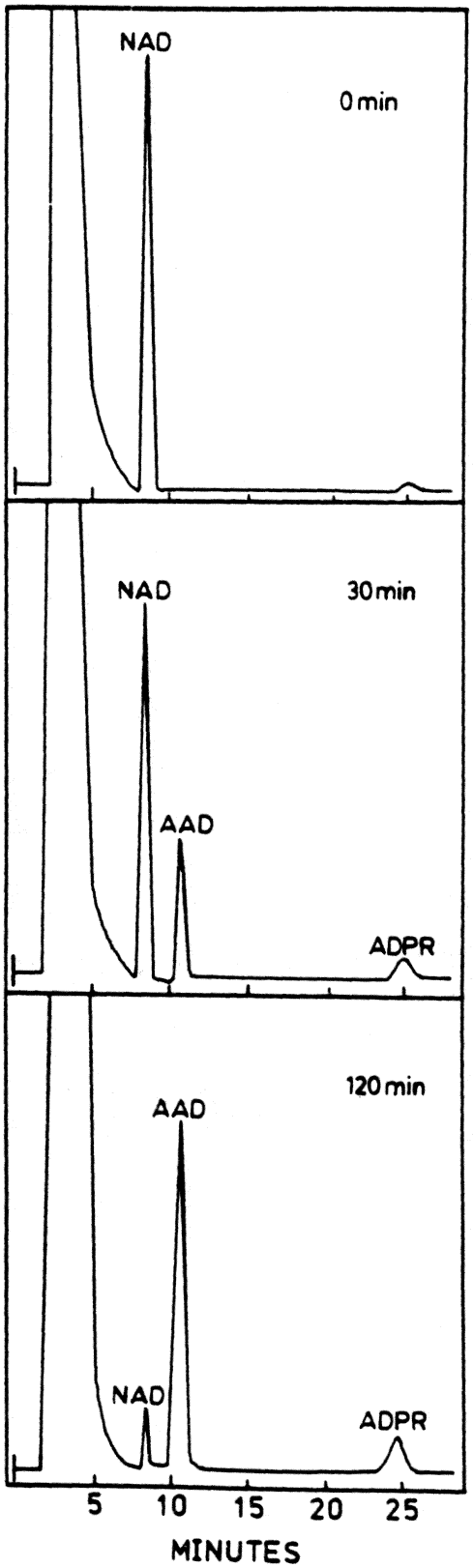
FIGURE 29 - The effect of 3-acetylpyridine concentration on the rates of the pyridine base exchange reaction. Reaction mixtures contained 3 mM NAD, 1 unit of NADase, 500 mM sodium phosphate buffer, pH 7.5, and 3-acetylpyridine concentrations varying from 10 to 100 mM in a final volume of 3 ml. Rates of transglycosidation were determined as described in Figure 26.


100
80
60
40
20
(Slope $\frac{\Delta A_{365}}{\Delta A_{340}}$)$^{-1}$
60
40
20
20
40
60
80
100
[3-Acetylpyridine]$^{-1}$ M^{-1}

was carried out as described in the experimental procedures. Depending on the compounds to be analyzed, chromatography was accomplished by either anion exchange using an Alltech 5 micron RSiL AN or by reverse phase on Alltech 5 micron RSiL ODS HL. Mobile phases were varied to yield optimal separations of the products formed during the pyridine base exchange reaction.

A representative sample of the pyridine base exchange reaction is shown in Figure 30 which indicates the formation of AAD, from NAD and 3-aminopyridine. The initial NAD concentration was 1.5 mM, 3-aminopyridine concentration was 50 mM, and 1.0 unit (740 ng) of snake venom NADase was used. As indicated in the top panel of Figure 30, only NAD and the free pyridine base were detected before the addition of NADase. A small amount of ADP-ribose was also detected, with a retention time of 25 minutes. Routine analysis of NAD purchased from Sigma Chemicals always contained between 3 to 8 percent contamination with ADP-ribose. Thirty minutes after the addition of the snake venom NADase, the formation of AAD could be observed with a concurrent loss of NAD. The progress of pyridine base exchange and hydrolysis, could be observed by this assay. After 120 minutes almost all of the NAD had been converted to AAD. By measuring the amount of AAD formed and comparing it to the amount of free ADP-ribose formed, the extent of the pyridine base exchange could be calculated. When 50 mM 3-aminopyridine was used, a ratio of 7.3 for base exchange over hydrolysis was observed indicating very little of the original NAD was hydrolyzed while all was cleaved.

FIGURE 30 - Formation of AAD monitored by the HPLC. The pyridine base exchange reaction was performed by incubating 1.5 mM NAD, 50 mM 3-aminopyridine, 1 unit of snake venom NADase (740 ng), and 50 mM potassium phosphate buffer, pH 7.5. The total reaction mixture volume was 1 ml. HPLC assays were performed as described in the experimental procedures. Top panel, 0 minutes; Middle panel, 30 minutes after the addition of the NADase; and the Lower panel, was 120 minutes after the addition of the NADase.


NAD
0 min
NAD
30 min
AAD
ADPR
120 min
AAD
NAD
ADPR
5
10
15
20
25
MINUTES

NAD partitioning to either base exchange or hydrolysis was further studied by measuring the amount of analog formed compared to the amount of free ADP-ribose formed at varying concentrations of the free pyridine base. Results are shown in Figure 31. In this figure the base exchange to hydrolysis ratio was measured as a function of 3-aminopyridine concentration. From the slope of this plot it was calculated that 3-aminopyridine reacts 11,000 times faster (assuming $[H_2O]$ = 55 M) at the partitioning step. This value will be referred to as the partitioning index, $K_{partition}$. This parameter is a measure of the reactivity of the competing nucleophile compared to that of water, if equimolar amounts of each were used. Similar results were obtained when other pyridine bases were used instead of 3-aminopyridine. The $K_{partition}$ values for various pyridine bases are shown in Table XV. Varying the amount of venom NADase did not affect the measured partitioning index. Increasing the amount of NADase only increased the rate of both hydrolysis and base exchange to the same extent, therefore, the $K_{partition}$ did not vary with NADase concentration.

A plot of the logarithms of the $K_{partition}$ values versus the pK_a of the ring nitrogen of the pyridine bases indicated a linear relationship (Figure 32) suggesting the existence of a relationship between the ring nitrogen nucleophilicity and its reactivity as compared to water in the base exchange reaction. A Bronsted slope of 0.43 was obtained from these results. Comparing the results from Table XV to Table XII indicated that there was no relationship between the $K_{partition}$ value and the binding of the free base as a noncompetitive inhibitor.

FIGURE 31 - Determination of $K_{partition}$ value for 3-aminopyridine. The formation of AAD was assayed as described in Figure legend 28 except varying concentrations of 3-aminopyridine were used. Each point is an average of the concentration of AAD formed divided by the concentration of ADP-ribose formed at 4 different time points with varying concentrations of 3-aminopyridine.

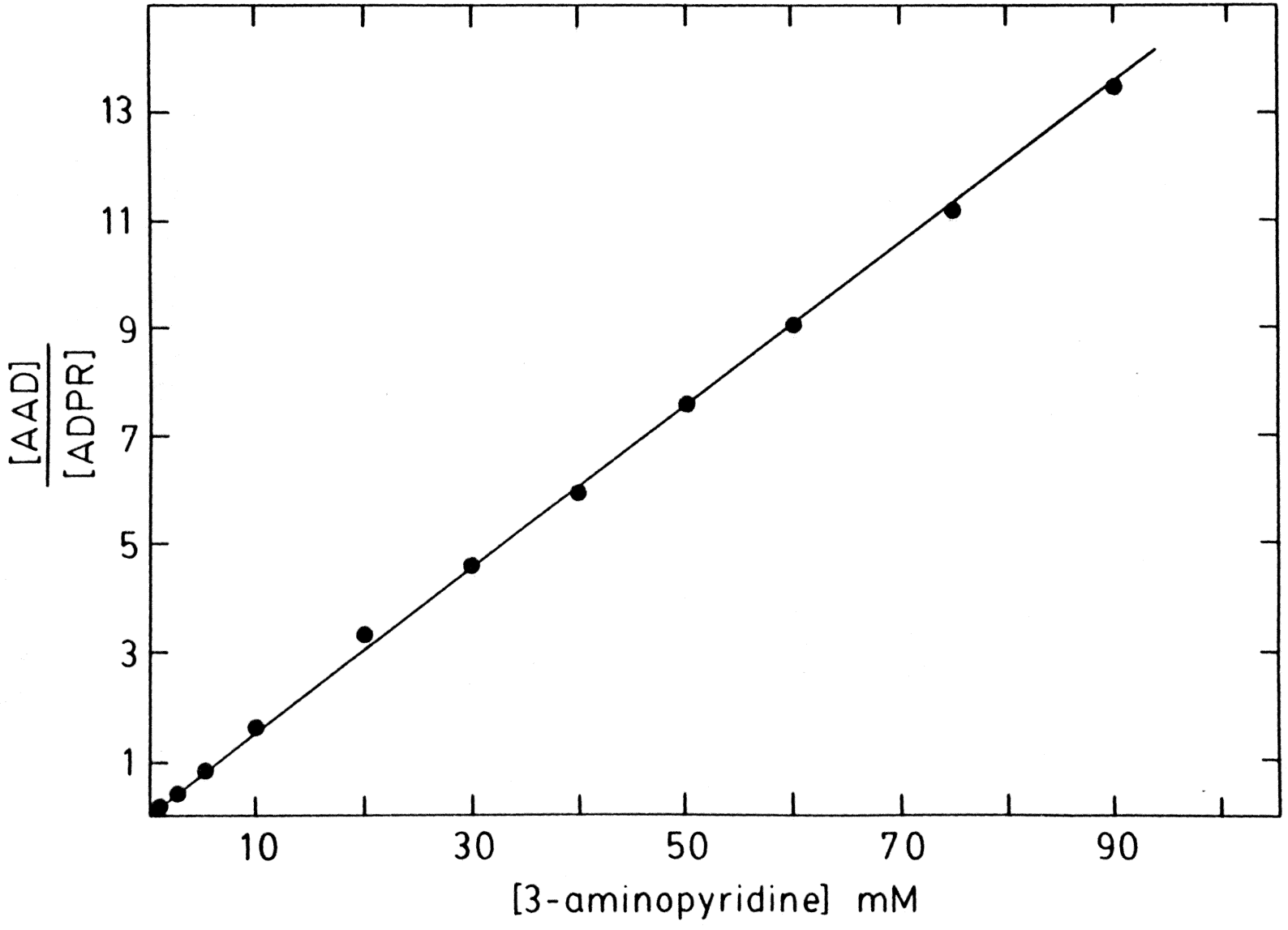
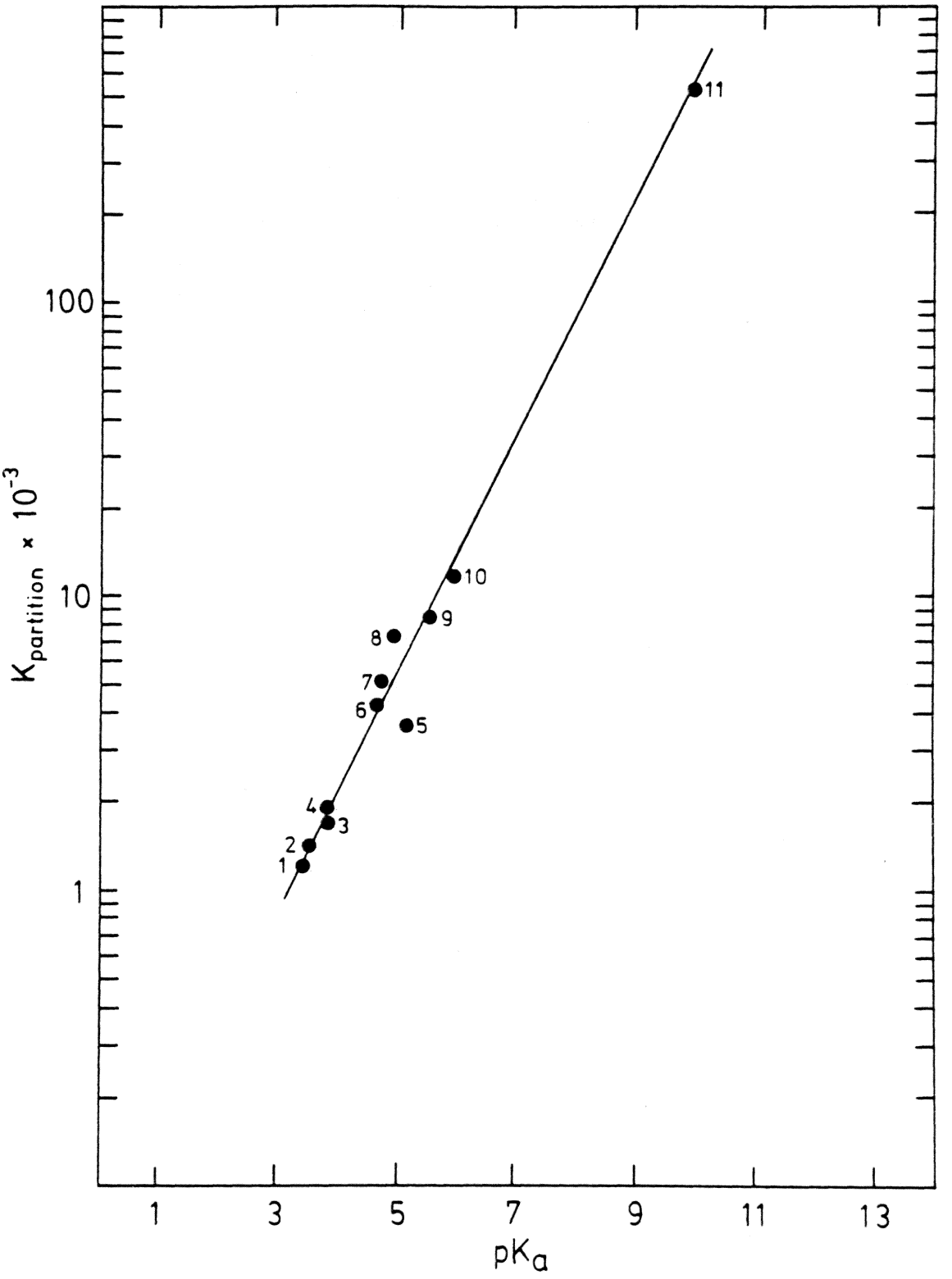

13
11
9
7
5
3
1
[AAD]/[ADPR]
10
30
50
70
90
[3-aminopyridine] mM

TABLE XV

PARTITION INDEX OF PYRIDINE BASES

Pyridine Base	$K_{partition}$	pK_a
4-Aminopyridine	520,000	9.78
3-Aminopyridine	11,000	5.98
3-Methylpyridine	8,550	5.64
3-Pyridylcarbinol	7,400	4.92
3-Pyridylacetonitrile	5,200	4.87
Isonicotinic Acid	4,400	4.86
Pyridine	3,600	5.23
Isonicotinic Acid Hydrazide	1,950	3.51
3-Pyridinecarboxyaldehyde	1,800	3.80
Nicotinic Acid Hydrazide	1,600	3.58

FIGURE 32 - Bronsted plot of the logarithm of the $K_{partition}$ verus the pK_a of the ring nitrogen of different pyridine bases. The pyridine bases used were as follows: 1) 3-acetylpyridine; 2) nicotinic acid hydrazide; 3) 3-pyridinecarboxyaldehyde; 4) isonicotinic acid hydrazide; 5) pyridine; 6) isonicotinic acid; 7) 3-pyridylacetonitrile; 8) 3-pyridylcarbinol; 9) 3-methylpyridine; 10) 3-aminopyridine; and 11) 4-aminopyridine.

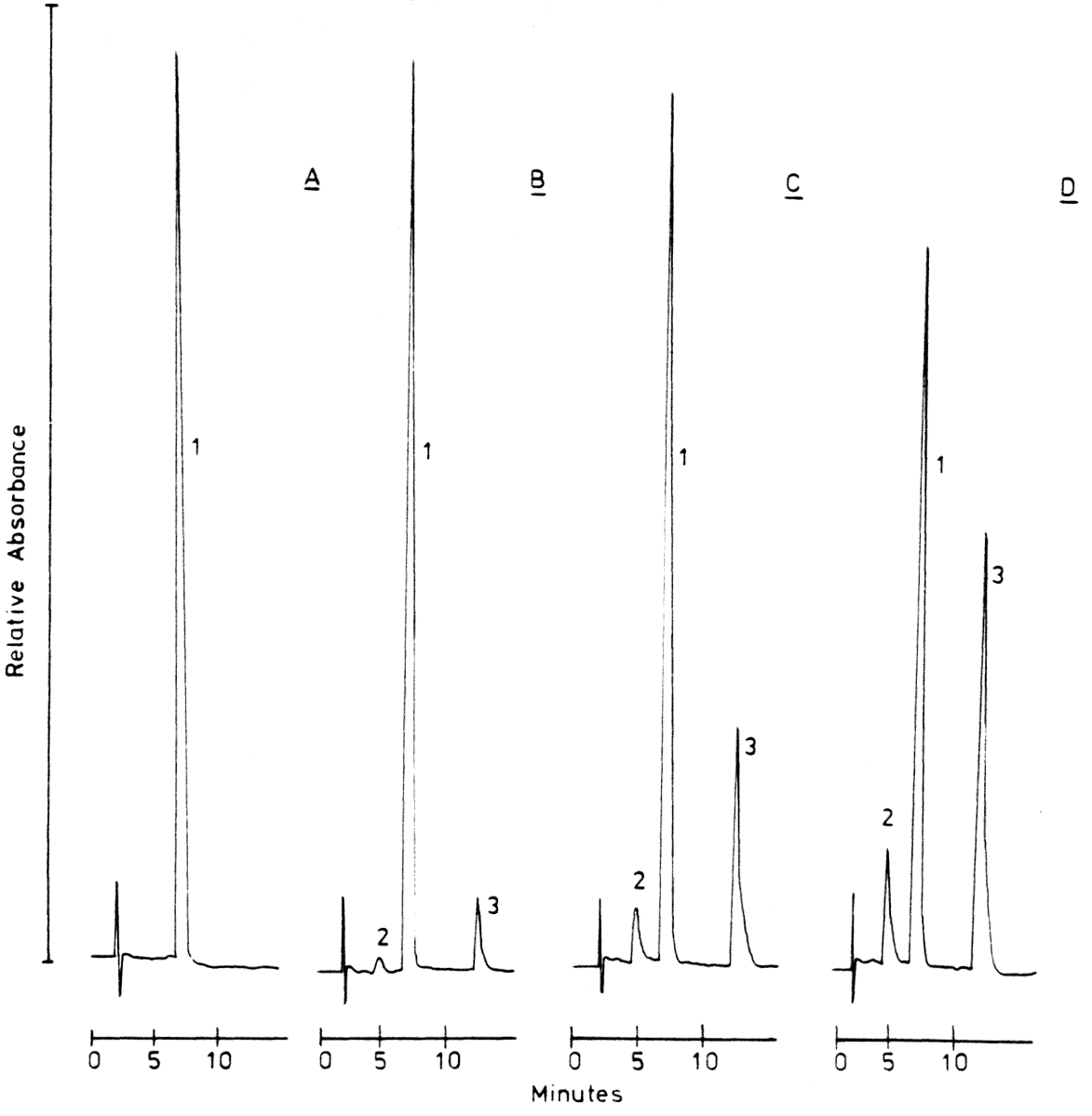

100
10
1
$K_{partition} \times 10^{-3}$
1
3
5
7
9
11
13
pK_a
1
2
3
4
5
6
7
8
9
10
11

Another interesting result was observed during the synthesis of 3-pyridylacetonitrile adenine dinucleotide by the pyridine base exchange reaction. During the prolonged incubation period required to synthesize this analog, a loss in the newly formed analog was observed. To further investigate this observation, 1 mM 3-pyridylacetonitrile adenine dinucleotide was incubated with 1 unit of the snake venom NADase in 50 mM potassium phosphate buffer, pH 7.5, in a total volume of 1 ml. Using the previously described HPLC assay, the reaction products, ADP-ribose and 3-pyridylacetonitrile were observed after a 1 hour incubation (Figure 33). After 3 hours less than 30% of the analog had been hydrolyzed. None of the kinetic parameters could be determined because the rate of hydrolysis was too slow to measure titrimetrically.

Kinetic Parameters of the Pyridine Base Exchange Reaction - Several results suggested that the pyridine base exchange reaction was more complicated then just a process of a pyridine base binding to the postulated E-ADP-ribose intermediate thus forming NAD analog. The inhibitor constant for 3-aminopyridine was 66 μM, but attempts to carry out the pyridine base exchange reaction with this concentration of base proved unsuccessful. As results in Figure 31 indicate, at least 1 mm 3-aminopyridine was required to observe base exchange. These results prompted the following studies.

The kinetic parameters, K_m and V_{max} for pyridine bases in the pyridine base exchange reaction were investigated. Rates of the formation of a given NAD analog at varying concentrations of free pyridine base were monitored by the HPLC assay. Initial rates were

FIGURE 33 - Hydrolysis of 3-pyridylacetonitrile adenine dinucleotide. Reaction mixtures contained 1 mM 3-pyridylacetonitrile adenine dinucleotide, 740 ng purified B. fasciatus venom NADase, 50 mM potassium phosphate buffer, pH 7.5, in a total volume of 1 ml at 37° C. Reaction progress was monitored by the previously described HPLC assay. Panel A was 0 minutes; Panel B was 60 minutes after the addition of the NADase; Panel C was 180 minutes; and Panel D was 360 minutes. Peaks were labeled 1) 3-pyridylacetonitrile adenine dinucleotide; 2) 3-pyridylacetonitrile; and 3) ADP-ribose.

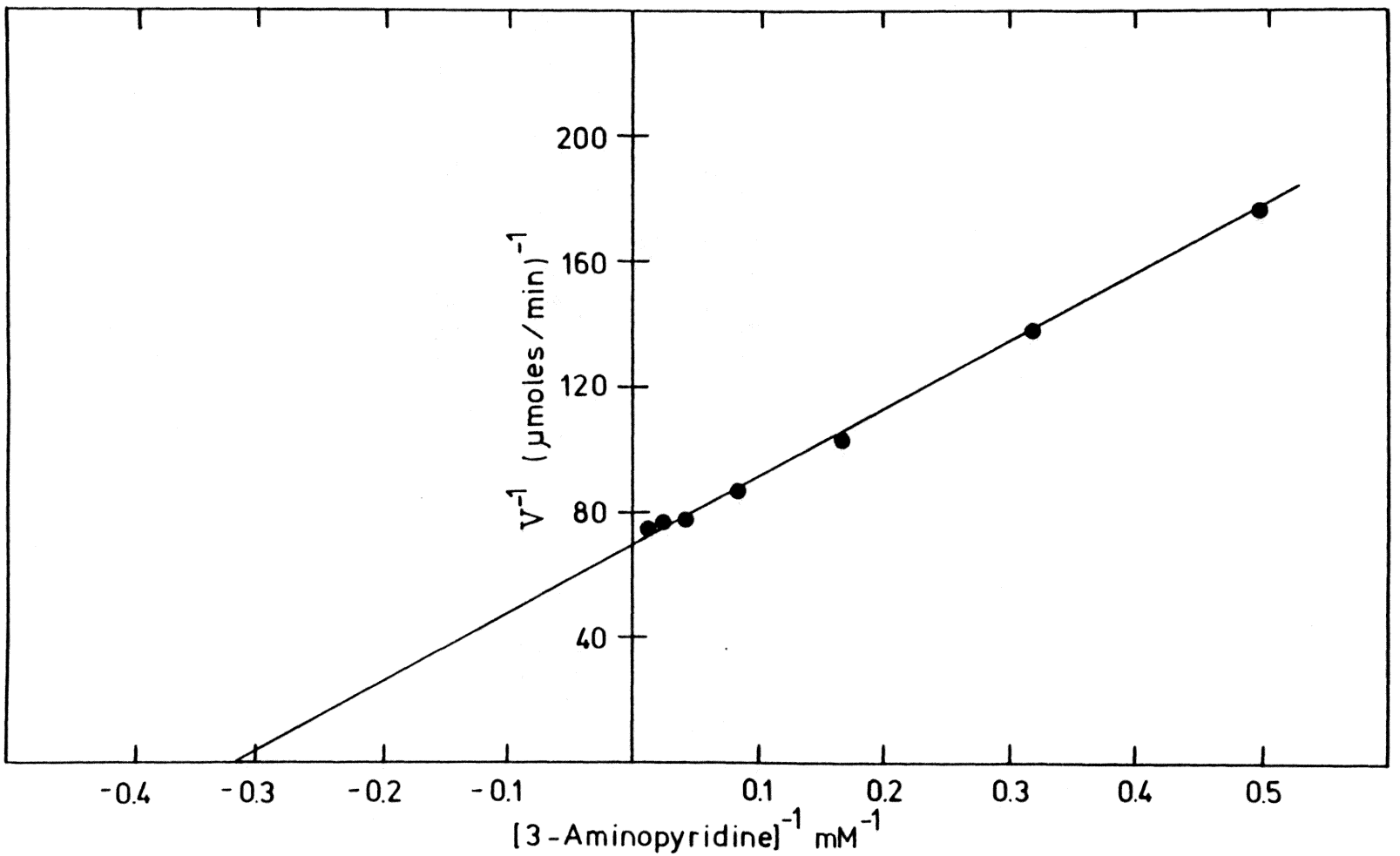

Relative Absorbance
A
B
C
D
1
2
3
0 5 10
Minutes

measured by transferring 0.1 ml aliquots at timed intervals during the first 15 minutes of the reaction to 0.5 ml of 50 mM potassium phosphate buffer, pH 3.3 and immediately freezing at -60°C. Kinetic parameters were determined from double reciprocal plots of initial rates of pyridine base exchange and free pyridine base concentration. The effect of different 3-aminopyridine concentrations on the rate of pyridine base exchange is shown in Figure 34. At saturating NAD concentrations the observed V_{max} for AAD formation was 18.2 μmoles formed/minute/mg protein. Under these experimental conditions a K_m equal to 3.2 mM for 3-aminopyridine was found. A summary of the kinetic parameters for other pyridine bases is shown in Table XVI.

The rate of 3-acetylpyridine adenine dinucleotide formation was the highest tested, with a V_{max} of 93.2 micromoles of analog formed/minute/mg. It is interesting to note that the apparent K_m for a given pyridine base (Table XVI) could not be equated to the inhibitor constant for that base determined in the hydrolytic reaction (Table XII). Only 3-acetylpyridine has values for the two parameters which are nearly equal.

Pyridine Base Exchange With Substrates Other Than NAD - The formation of 3-aminopyridine dinucleotide derivatives from NAD, NADP, nicotinamide 1, N^6-ethenoadenine dinucleotide, thionicotinamide adenine dinucleotide, and 3-acetylpyridine adenine dinucleotide was monitored by the HPLC assay. A routine reaction mixture consisted of 1.5 mM of NAD analog, 50 mM potassium phosphate buffer, pH 7.8, 1 unit of NADase (740 ng), and varying concentrations of 3-aminopyridine, in

FIGURE 34 - The effect of 3-aminopyridine concentration on the rate of the pyridine base exchange reaction. Reaction mixtures were prepared as described in the text. Formation of AAD was determined by the previously described HPLC assay.


200
160
120
80
40
v^{-1} (µmoles/min)$^{-1}$
-0.4
-0.3
-0.2
-0.1
0.1
0.2
0.3
0.4
0.5
[3-Aminopyridine]$^{-1}$ mM^{-1}

a final volume of 1 ml, at 37°C. The ratio of base exchange to hydrolysis for each of the ribosyl donors studied at 50 mM 3-aminopyridine is shown in Table XVII. The partitioning of the ribosyl intermediate was fairly constant regardless of the structure of the ribosyl donor.

Kinetic parameters for the formation of AAD from the various NAD analogs were determined from double reciprocal plots of initial velocities of analog formation as described in the legend of Figure 34. A summary of the kinetic parameters using alternate NAD analogs as ribosyl donors are listed in Table XVIII. The apparent K_m for 3-aminopyridine was not noticeably different when pyridine base exchange was carried out with alternate NAD analogs as ribosyl donors. The maximal velocities for base exchange varied directly to how well the donor substrate was hydrolyzed in the NADase-catalyzed reaction (Table XI). The faster a NAD analog was hydrolyzed, the faster it was converted by the base exchange reaction to the 3-aminopyridine derivative. NAD was utilized in the pyridine base exchange reaction faster than ε-NAD, NADP, or s-NAD. The formation of AAD from 3-acetylpyridine adenine dinucleotide was the slowest.

Nucleophilic Partitioning

The ability of the B. fasciatus venom NADase to catalyze the pyridine base exchange reaction supports the concept of the enzyme-catalyzed reaction proceeding through an active enzyme-bound intermediate. It was of interest to study reactions of nucleophiles other

TABLE XVI

KINETIC PARAMETERS OF THE PYRIDINE BASE EXCHANGE REACTION

Pyridine Base	pK_a	K_m	V_{max}
		(mM)	(μmoles/min/mg)
3-Acetylpyridine	3.26	20.0	93.2
3-Pyridylcarbinol	4.91	7.9	87.8
3-Methylpyridine	5.62	6.5	35.1
3-Pyridylacetonitrile	3.80	6.1	24.3
3-Aminopyridine	5.98	3.2	18.2
Pyridine	5.23	1.4	9.2

TABLE XVII

THE RATIO OF PYRIDINE BASE EXCHANGE:HYDROLYSIS AS A FUNCTION OF RIBOSYL DONOR

Ribosyl Donor	Base Exchange/Hydrolysis*
NAD	7.2
NADP	7.4
ε-NAD	7.1
s-NAD	7.5
AcPAD	7.3

* The concentration of 3-aminopyridine analog formed divided by the concentration of ADP-ribose formed.

TABLE XVIII

KINETIC PARAMETERS OF AAD(P) SYNTHESIS UTILIZING VARIOUS NAD ANALOGS

NAD Analog	Pyridine Base	K_m	V_{max}
		(mM)	(μmoles/min/mg)
NAD	3-Aminopyridine	3.2	18.2
s-NAD	3-Aminopyridine	3.3	17.8
AcPAD	3-Aminopyridine	3.1	11.5
NADP	3-Aminopyridine	3.3	16.2

than pyridine bases that could compete with water in a reaction with the proposed intermediate. Kinetic analyses of the venom NADase catalyzed hydrolysis of NAD in the presence of methanol as the nucleophilic competitor of water were investigated. Reaction rates were studied titrimetrically. If methanol acts as an alternate acceptor of the enzyme bound ADP-ribose, then methyl-ADP-riboside should be formed. This methanolysis reaction should also produce a proton as in the hydrolytic reaction. Derivation of the rate equations from steady-state analysis are shown in Appendix II. Reaction conditions were as described in the legend of Figure 35, except various concentrations of methanol were added. Results are shown in Figure 35. Increasing concentrations of methanol up to 5.0 M only decreased the initial rate of proton release by 20 percent. Since high concentrations of methanol did not affect the initial rate of NAD breakdown, another study to determine the effect of methanol on V_{max} and K_m was performed. Reaction mixtures were as described in the legend of Figure 11, except that varying concentrations of methanol were used. From double reciprocal plots of the initial rates of NAD breakdown versus NAD concentration at each methanol concentration, a family of parallel lines was generated (Figure 36). The K_m/V_{max} ratio was constant (Table XIX). Increasing concentrations of methanol up to 3.1 M resulted in a decrease in the observed V_{max} and K_m for NAD. The methanol effects on the kinetic parameters of the NADase-catalyzed hydrolysis of NAD are shown in Table XIX. There was less then a 2 percent variance in the K_m/V_{max} ratio.

FIGURE 35 - The effect of methanol on NADase activity. Reaction mixtures contained: 87 mM potassium chloride, 56 ng purified snake venom NADase, and methanol concentration was varied as indicated (0 to 5 M). Enzyme activity was measured by the titrimetric assay.

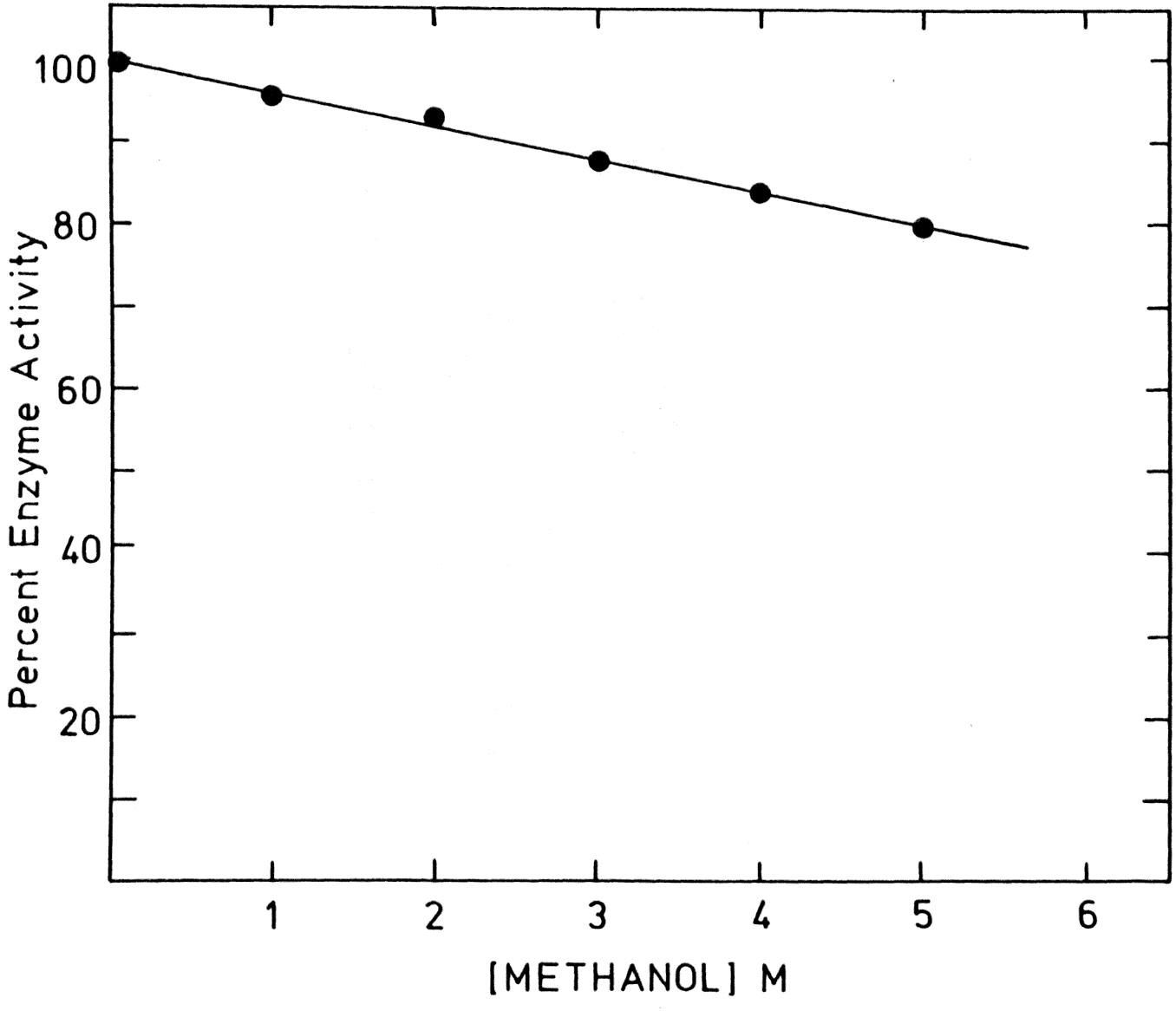

Percent Enzyme Activity
100
80
60
40
20
1
2
3
4
5
6
[METHANOL] M

FIGURE 36 - Kinetic parameters for NADase-catalyzed hydrolysis of NAD in the presence of methanol. Reaction mixtures contained 87 mM potassium chloride, 55 ng purified B. fasciatus venom NADase, methanol as indicated and NAD concentrations were varied from 12 to 90 μM. Initial velocities were measured by the consumption of 1 mM NaOH at 37°, pH 7.5. The concentrations of methanol used were as follows: Line 1, 0; Line 2, 1.5 mM; Line 3, 3.1 mM.

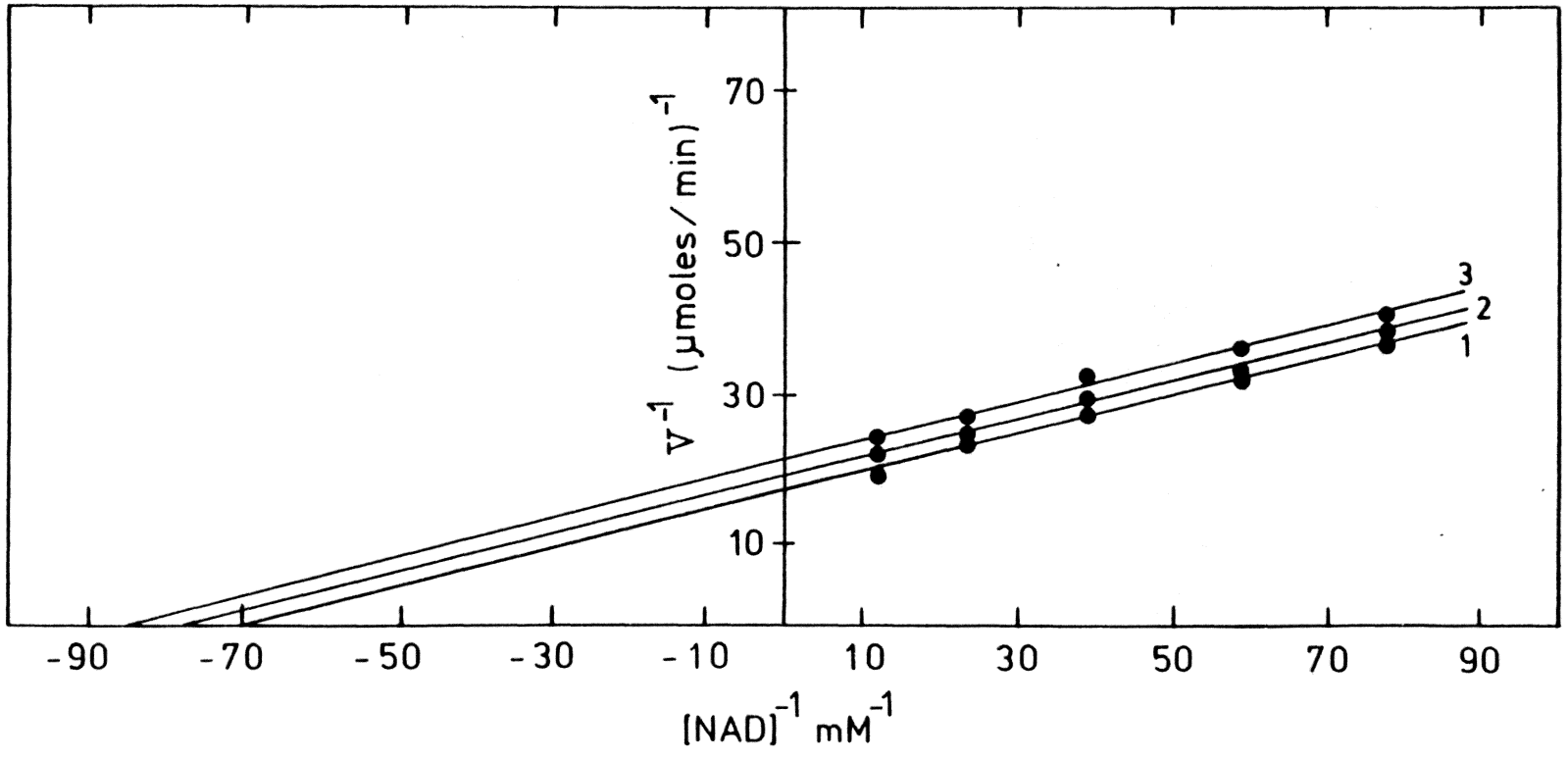

70
50
30
10
v^{-1} (µmoles / min)$^{-1}$
3
2
1
-90
-70
-50
-30
-10
10
30
50
70
90
[NAD]$^{-1}$ mM^{-1}

TABLE XIX

EFFECT OF METHANOL ON THE KINETIC PARAMETERS OF NAD HYDROLYSIS

[Methanol]	K_m	V_{max}	K_m/V_{max}
	(μM)	(μmol/min/mg)	
0	14	1350	0.01
1.5	13	1230	0.01
3.1	12	1120	0.01

The previous proposal of methanol acting as an ADP-ribose acceptor relies on kinetic results. The demonstration that methyl-ADP-riboside was formed was confirmed by HPLC. As shown in Figure 37, increasing amounts of methanol resulted in an increase in the ratios of product concentrations [methyl-ADP-riboside]/[ADP-ribose]. The absence of saturation kinetics indicates that methanol is acting as a true nucleophile and does not bind to the enzyme. From the slope of the line in Figure 37, methanol was calculated to react 38-fold faster than water with the enzyme-ADP-ribose intermediate, assuming $[H_2O]$ is 55 M at the active site.

The observation that snake venom NADase catalyzed a methanolysis reaction in competition with the normal hydrolytic reaction prompted the investigation of other alcohols as ADP-ribose acceptors. The experimental protocol for alcoholysis was similar to the methanolysis experiments described in the legend of Figure 37. The extent of alcoholysis to hydrolysis was calculated by the ratio of the concentrations of products formed. The only notable difference in the assays used was that when analyzing the longer chain alcohols the O-alkyl-ADP-ribosyl products could only be eluted from the HPLC anion exchange column when 5% methanol was present in the mobile phase. Results of alcoholysis experiments are summarized in Table XX. $K_{partition}$ values were determined as for methanol (Figure 37). The effect of chainlength on the free energy change of the reactivity of the various alcohols with the enzyme-ADP-ribosyl intermediate was calculated from the linear relationship obtained in a plot of the logarithms of $K_{partition}$ values versus

FIGURE 37 - The effect of methanol concentration on the distrubution of products in the NADase-catalyzed reaction. The reaction mixtures were as described in the text. Methanol concentration was varied as indicated. Product analysis was determined by the HPLC assay described in the experimental procedures.

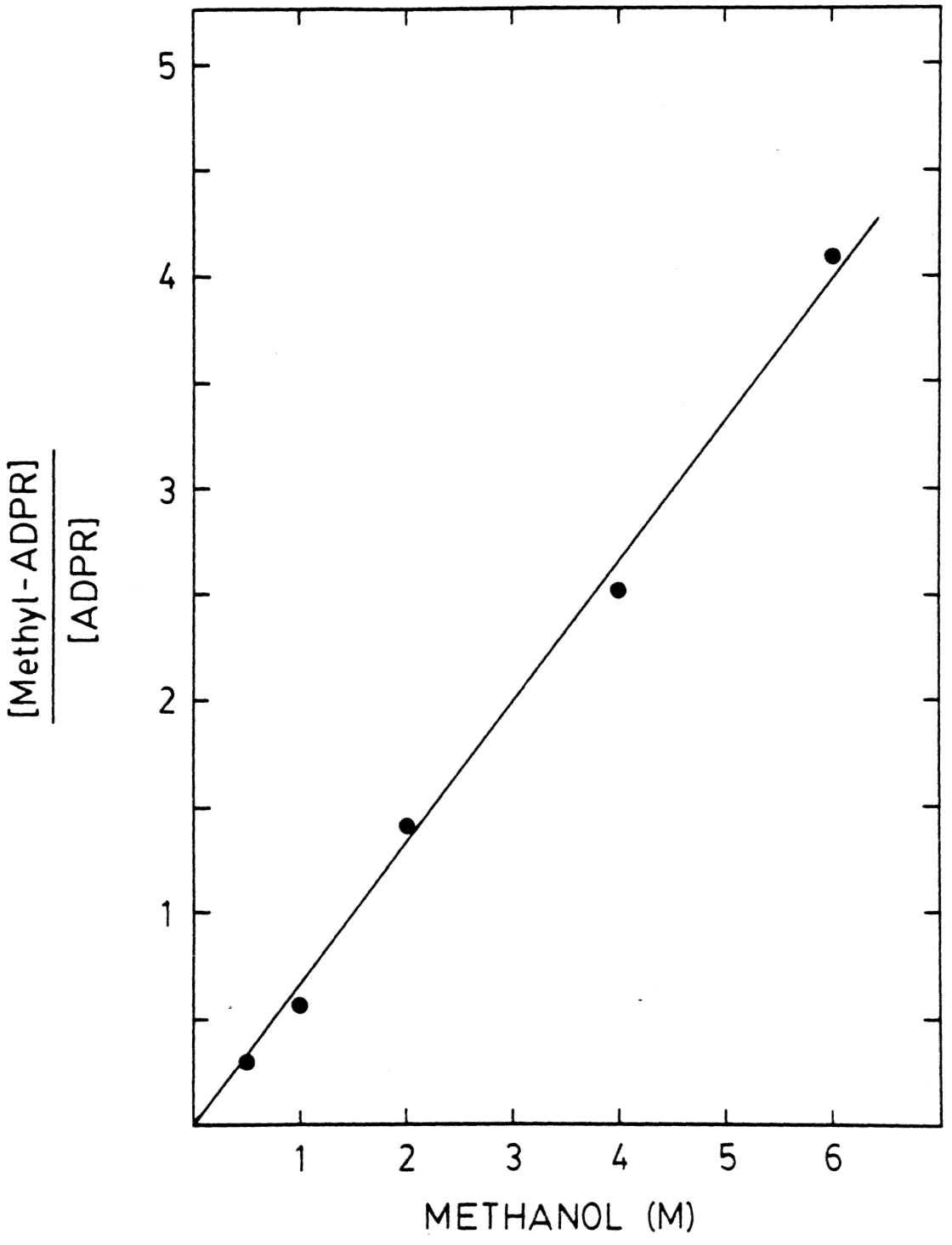

[Methyl-ADPR] / [ADPR]
5
4
3
2
1
1
2
3
4
5
6
METHANOL (M)

TABLE XX

EFFECT OF ALCOHOL CHAINLENGTH ON THE PARTITION INDEX

Alcohol	# carbons	$K_{partition}$
Methanol	1	38
Ethanol	2	87
n-Propanol	3	193
n-Butanol	4	315
n-Pentanol	5	560
n-Hexanol	6	508
2-Propanol	3	15

the number of alkyl chain carbons, Figure 38. Deviations from linearity occurs at a chainlength of four carbons with a maximum effect noted at five and six carbons. From the initial slope of the line a $\Delta\Delta G$ of 0.51 kcal/mole can be calculated using the formula, $\Delta\Delta G = 2.3\ RTS$, where S = the change in the logarithm of $K_{partition}$ per methylene group. Low solubility interfered with the use of alcohols larger than hexanol in these studies. Using a secondary alcohol such as isopropanol resulted in a large decrease in the $K_{partition}$ value.

Methanolysis When Analogs Other Then NAD Were Used - Methanolysis was studied with ε-NAD, NADP, s-NAD, AcPAD used as substrates in place of NAD. Experimental conditions were performed as described in the legend of Figure 37. The ratio of [methyl-ADP-riboside] to [ADP-ribose]* was determined by the HPLC assay. The $K_{partition}$ values for each of the dinucleotides investigated were calculated from graphs as described in Figure 37. A summary of these results is shown in Table XXI. Varying the structure of the ADP-ribosyl donor had little effect on hydrolysis/methanolysis ratio.

Physiologically Related ADP-ribose Acceptors - The observation that various primary alcohols could effectively compete with water for the postulated enzyme-ADP-ribose intermediate prompted an investigation of other nucleophiles as ADP-ribose acceptors. The experimental protocol was as described in the legend of Figure 37 except various nucleophiles other then methanol were used. Resulting products were analyzed by the HPLC assay. A summary of these results are shown in Table XXII. The

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*ADP-ribose or etheno-ADP-ribose or phospho-ADP-ribose

FIGURE 38 - The effect of increasing number of carbons in the aliphatic alcohol on the partition coefficient. Reactions were assayed as described in Figure 37 legend.

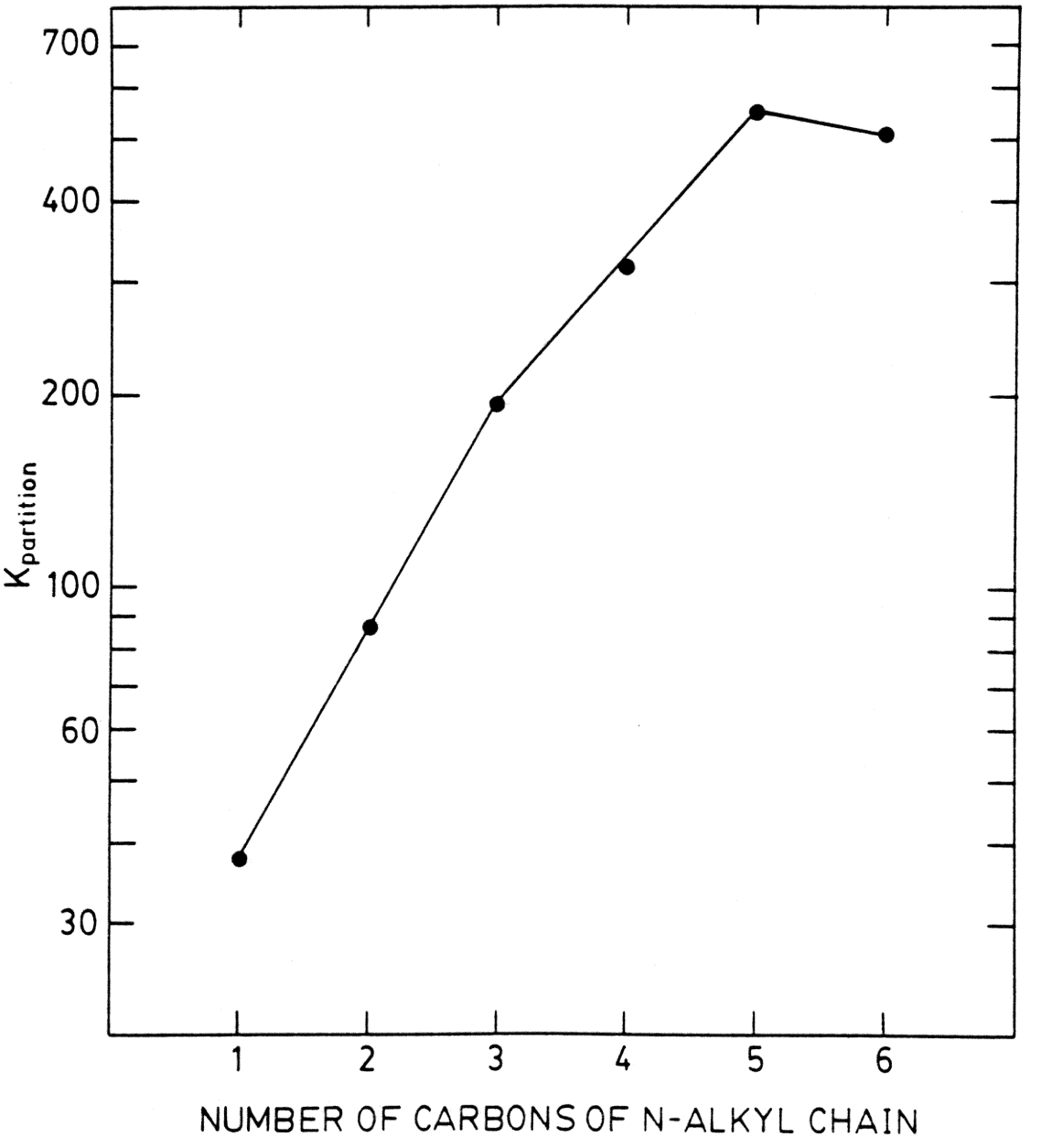

700
400
200
100
60
30
$K_{partition}$
1
2
3
4
5
6
NUMBER OF CARBONS OF N-ALKYL CHAIN

TABLE XXI

PARTITIONING INDEX OF METHANOL WITH VARIOUS NAD ANALOGS AS SUBSTRATES

NAD analog	$K_{partition}$
Nicotinamide adenine dinucleotide	38
Thionicotinamide adenine dinucleotide	34
3-Acetylpyridine adenine dinucleotide	36
Nicotinamide $1,N^6$-ethenoadenine dinucleotide	37
Nicotinamide adenine dinucleotide phosphate	32

TABLE XXII

PARTITIONING INDEX FOR VARIOUS ADP-RIBOSE ACCEPTORS

Acceptor	$K_{partition}$
Imidazole	540
Histamine	170
Histidine	120
Ergothioneine	8
Hydroxylamine	200
N-Acetyl-serine Amide	3
N-Acetyl-threonine Amide	0
Arginine Methyl Ester	0
N-Alkyl Amines	0

hydroxyl groups of serine and tyrosine were not very effective nucleophiles in comparison to water. At neutral pH the effective concentration of the attacking nucleophile of the N-alkylamines was too low and no ADP-riboside was observed. Arginine methyl ester which has been shown to be an ADP-ribose acceptor for the choleragen-catalyzed mono ADP-ribosylation (9) was not an acceptor for the snake venom NADase catalyzed transfer of ADP-ribose. The ring nitrogens of imidazole and imidazole derivatives were found to be very effective nucleophilic acceptors. Imidazole was the most effective non-pyridine compound tested in competing for the enzyme-ADP-ribosyl intermediate, with a $K_{partition}$ of 540. The reactivity of the imidazole derivatives followed the order imidazole > histamine > histidine > ergothioneine. In the concentration ranges used, saturation kinetics were not observed with these compounds. No evidence for selective binding of these nucleophiles to the snake venom NADase was observed.

DISCUSSION

Purification and Properties of the Snake Venom NADase

NAD glycohydrolases catalyze the hydrolysis of the N-ribosidic bond of nicotinamide in NAD. NADases have been demonstrated in various microorganisms and in a variety of animal tissues (41-87). Most mammalian NADases are membrane bound. Recent investigations by Schuber and others (103-105) indicate that these NADases may exist as ecto-enzymes on the plasma membrane, and not microsomal enzymes as originally thought. The only known non-membraneous mammalian NADase is found in bovine seminal plasma as a freely soluble extracellular glycoprotein, originating from the seminal vesicles (77). This enzyme does not catalyze the pyridine base exchange reaction like the majority of mammalian membrane bound NADases (121); however, it does catalyze a self-inactivation reaction (110) in which enzyme activity terminates prior to complete NAD hydrolysis. Addition of fresh enzyme to reaction mixtures results in a continued hydrolysis of NAD. Self-inactivation is a time dependent process which may involve an internal ADP-ribosylation of the enzyme resulting in the formation of an inactive enzyme molecule. Since the seminal fluid NADase did not catalyze a pyridine base exchange reaction, it could not serve as an experimental system for the investigation of ADP-ribose transfer reactions and studies were initiated to identify other soluble NADases that could be used for this purpose.

The presence of a NADase in snake venom was first demonstrated by Bhattacharya (111), who found that when NAD was incubated with crude Bungarus fasciatus venom, nicotinamide was released. Bhattacharya

however, did not purify or characterize this enzymatic activity. Several recent reports (63,83) have indicated the presence of NADase activity in other snake venoms and only in one case were attempts made to purify the enzyme (83). Tatsuki et al. (83) reported a 26-fold purification of a NADase from Agkistrodon halys blomhoffi venom to a final specific activity of 0.7 units/mg protein.

All snake venoms do not contain NADase activity (Table III p. 44); however, of these that do, venoms from the genus Bungarus were shown to contain the largest amounts. Bungarus fasciatus venom was chosen as a source of NADase since it was readily available and on a cost per unit of enzyme activity basis, this venom was the least expensive.

The NADase from B. fasciatus venom was purified to electrophoretic homogeneity through a 3-step chromatographic procedure involving phosphocellulose ion exchange, gel filtration, and affinity chromatography on Amicon Matrex gel Blue A. The snake venom enzyme was purified over 1000-fold with routine recoveries of over 90% of the original enzyme activity (Table IV). The purification procedure thus allowed a high recovery of a minor component of B. fasciatus venom.

The purified enzyme migrated as a single band during SDS gel electrophoresis and stained positively for both protein and carbohydrate. The glycoprotein nature of this NADase was further indicated by the difficulty encountered in eluting the enzyme from a Concanavalin-A agarose column. When purified enzyme was applied to such a column, no NADase activity could be eluted from the column until the eluting buffer contained 2 M potassium chloride plus 0.5 M α-methyl mannoside. (data

not shown). Several other NADases have been shown to exist as soluble glycoproteins. The purified bovine seminal plasma NADase was shown to contain 10.8% carbohydrate (126). Everse and Kaplan have recently shown (49) that the NADases from N. crassa and B. subtilis are highly glycosylated proteins. The only other glycosylated NADase obtained from an animal source, was purified from rat liver by Diaugustine and co-workers (64). This enzyme preparation was the only membraneous NADase shown to exist as a glycoprotein.

Under non-denaturing conditions, the purified B. fasciatus venom NADase was observed to have an apparent molecular weight of 125,000 determined by gel filtration on Sephacryl S-200. This was confirmed by results obtained from zone sedimentation sucrose density gradient centrifugation in which a native molecular weight of 130,000 was determined. Under the denaturing conditions of SDS polyacrylamide electrophoresis in the absence or presence of dithiothreitol, the enzyme exhibited an apparent molecular weight of 62,000. These results would be consistent with the enzyme existing as a dimer composed of two 62,000 molecular weight subunits.

The molecular weight of 125,000 for the B. fasciatus venom NADase is the largest reported for NADases (Table II). As a freely soluble glycosylated dimer the B. fasciatus venom NADase is unique when compared to other known NADases.

Purified B. fasciatus venom NADase contained no residual phosphodiesterase activity. All phosphodiesterase activity remaining after the Sephadex G-100 gel filtration step during purification was

removed during the frontal elution of the Cibacron Blue:affinity chromatography step (data not shown). The disappearance of NAD in the presence of the purified snake venom NADase was attributed solely to the hydrolysis of the nicotinamide-ribosidic bond, yielding only ADP-ribose and nicotinamide as products. The 1:1 stoichiometry of these products, along with the absence of any other ultraviolet absorbing products was verified by high pressure liquid chromatography. Consistent with these results was the observation that identical rates of hydrolysis were obtained when NADase activity was measured by either the cyanide addition assay or the titrimetric assay.

Through the use of the continuous titrimetric assay an accurate and direct measurement of both the initial rates and other kinetic parameters for NAD hydrolysis was accomplished. A K_m for NAD of 14 μM and a V_{max} of 1380 μmoles NAD cleaved/minute/mg protein was determined. In comparison, the membrane associated bovine erythrocyte NADase (72) was reported to have a K_m for NAD hydrolysis of 16 μM, while other membrane bound erythrocyte NADases exhibited K_m values from 10 to 80 μmM (73). The purified calf spleen NADase, also a membrane bound enzyme, exhibited a K_m for NAD of 56 μM (75). Yuan and Anderson determined a K_m of 100 μM for NAD hydrolysis catalyzed by the soluble bovine seminal plasma NADase (77). The only other snake venom NADase examined was the partially purified NADase from A. halys blomhoffi venom which exhibited a K_m of 830 μM for NAD (83).

The kinetic parameters for the B. fasciatus venom NADase exhibited little change over the pH range from 6.0 to 8.5. Maximum rates of

hydrolysis were obtained at pH values between 7.5 and 8.0. The V_{max}/K_m ratio varied less than 20% from pH 6.0 to 8.5. This would suggest that the interaction between the substrate and enzyme is relatively pH independent, an observation fairly consistent with other extensively studied NADases (64,72,75,77).

The purified snake venom NADase was labile at pHs below 6.0 and above 8.5 (Figure 10). At pH 5.0 and pH 9.0 the enzyme lost approximately 50 percent of its catalytic activity in 10 minutes. A similar observation was also described by Tatsuki and coworkers (83) with A. halys blomoffi venom NADase. This instability is not readily explained since one might expect these glycoproteins to be less sensitive to pH changes. In contrast, the calf spleen and bull semen NADases appear to be more stable to pH changes than the snake venom enzyme (75,77).

The temperature dependence of NAD hydrolysis catalyzed by the snake venom enzyme when plotted as an Arrhenius plot indicated an activation energy of 15.7 kcal/mol. As expected the Arrhenius plot was linear over the temperature range studied. This energy of activation is similar to that obtained with the purified calf spleen NADase, which exhibited an activation energy of 17.8 kcal/mol (117). Other NADases where the activation energy has been measured, indicated much lower values, between 5 and 10 kcal/mol for the enzyme catalyzed hydrolysis of NAD (65,72).

The purified snake venom NADase was thermolabile. When enzyme solutions were incubated at temperatures of 50°C and above, catalytic activity decreased. A rate constant for inactivation of the enzyme at

50° of 0.087 min^{-1} was observed. Higher incubation temperatures resulted in much faster rates enzyme inactivation. A similar observation was observed by Tatsuki et al. (83) with the partially purified A. halys blomhoffi venom NADase which also rapidly lost activity at temperatures above 50°C. Yuan and Anderson (126) observed that bull semen NADase was relatively stable to pH changes and was thermally denatured at 60°C with a rate constant for inactivation of 0.012 min^{-1} ($T_{1/2}$ = 60 minutes).

Substrate Specificity and Substrate Binding Properties

Studies of substrate specificity of the purified Bungarus fasciatus venom NADase using the titrimetric assay indicated that of 25 NAD analogs tested, only NAD, NADP, nicotinamide 1,N^6-ethenoadenine dinucleotide, thionicotinamide adenine dinucleotide, thionicotinamide adenine dinucleotide phosphate, 3-acetylpyridine adenine dinucleotide, 3-acetylpyridine hypoxanthine dinucleotide, nicotinamide hypoxanthine dinucleotide, and nicotinamide guanine dinucleotide served as substrates. The importance of a carbonyl function at the 3-position of the pyridine ring for recognition of the dinucleotide as a substrate was indicated by Schuber et al. (117) in a study of NAD analogs with solubilized calf spleen NADase also observed a specifity for analogs possessing a carbonyl function at the 3-position of the pyridine ring. A similar specificity has been observed in the functioning of analogs with dehydrogenases (1). It was speculated that a 3-position carbon double bonded to oxygen, sulfur or nitrogen was an absolute requirement for the functioning of the NAD analogs in dehydrogenase-catalyzed reactions (113).

However, Biellmann et al. (145) have shown that the 3-cyanopyridine adenine dinucleotide can be enzymatically reduced. The cyano-derivative is highly electron withdrawing, the pK_a of the parent pyridine base is 1.39. Biellmann and coworkers (146,147) have more recently shown that 3-halopyridine analogs of NAD can also be enzymatically reduced. Apparently, sufficient electron withdrawing power by the substituent in the 3-position will promote coenzymatic function. The 3-halopyridine and the 3-cyanopyridine analogs were not substrates for calf spleen NADase (117) suggesting that other factors are involved in the NADase catalyzed hydrolysis of NAD analogs.

In the present study a slow hydrolysis of 3-pyridylacetonitrile adenine dinucleotide was catalyzed by B. fasciatus venom NADase. ADP-ribose and 3-pyridylacetonitrile were formed with the quantitative loss of 3-pyridylacetonitrile adenine dinucleotide. Control experiments without enzyme confirmed the absence of a nonenzymatic hydrolysis of 3-pyridylacetonitrile adenine dinucleotide. The enzyme could be recognizing a conjugated ¶-orbital electron cloud of the 3-pyridylacetonitrile analog. This constitutes the first report of a NAD analog without a true carbonyl type function at C-3 of the pyridine ring serving as a substrate for a NADase.

The nicotinic acid analog would be expected to serve as a substrate for the snake venom NADase. The fact that it did not may indicate negative interactions between the enzyme and the charged carboxyl group. The importance of the adenyl moiety for nucleotide binding and hydrolysis is suggested, since nicotinamide mononucleotide did not serve as a substrate for the snake venom enzyme and was poorly bound.

However, other substituted purines in place of adenine in the dinucleotide were all functional as substrates. The inability of α-NAD to function as a substrate indicated the B. fasciatus venom NADase to be stereospecific for the β-configuration.

These observations suggest that the snake venom NADase is more sensitive to alterations in the pyridinium moiety than in the purine moiety of NAD. Specifically, the venom NADase exhibited only a slight tolerance to alterations in the carboxamide group at the C-3 position of the pyridine moiety. Carbonyl like functional groups were recognized the best, although the 3-pyridylacetonitrile derivative of NAD was slowly hydrolyzed in the presence of the snake venom enzyme. Similar results were obtained for substrate specificity for the calf spleen and bull semen NADases (117,121). The erythrocyte ghost NADase was similar, but was even more sensitive to alterations in the nicotinamide moiety (72).

Inhibitor Studies

The substrate binding site of Bungarus fasciatus venom NADase was investigated by studying the inhibition patterns of a number of specific structural analogs of NAD. These analogs were divided into three structurally different classes. The first class of inhibitors studied were compounds structurally related to nicotinamide. Nicotinamide was shown to be a linear noncompetitive inhibitor with respect to NAD in the hydrolytic reaction with an inhibitor constant of 1.4 mM. In addition to nicotinamide, 21 other nicotinamide analogs were studied as possible

inhibitors of the NADase-catalyzed reaction. All of the pyridine bases tested were noncompetitive inhibitors of the NADase-catalyzed hydrolysis of NAD (Table XII). Other heterocylic bases tested were not inhibitors of the NADase catalyzed reaction suggesting the nicotinamide portion of the NAD binding site to be very specific for the pyridine ring. From Table XII it can be seen that there was a wide range of inhibitor constants determined for the different pyridine bases. The ineffectiveness of nicotinic acid, as in the case of the corresponding analog again suggests interferring ionic interactions. Steric hindrance is the most likely factor in the poor inhibition by 2-acetylpyridine and 6-aminonicotinamide. The high K_i observed for 3-acetylpyridine in the hydrolytic reaction is interesting in that the acetyl group is isosteric with the carboxamido group of nicotinamide. Both 3-acetylpyridine and nicotinamide demonstrate very similar pK_as suggesting similar electron withdrawing power. Therefore, it is not readily obvious why there is a 13-fold difference in the K_is of these two pyridine bases.

The moderately effective inhibitors, nicotinamide, thionicotinamide, 3-pyridinecarboxyaldehyde, 3-pyridylcarbinol, and nicotinic acid hydrazide all have substituent groups which could interact with the enzyme to form a hydrogen bond, i.e. all have a carbonyl like functional group at the 3-position.

The most effective inhibitors are those containing a substituent in the 4-position of the pyridine ring. 4-Acetyl pyridine is bound approximately 392-fold tighter than is 3-acetylpyridine. The carboxylate derivatives also exemplify this effect. Isonictonic acid had a K_i of

250 μM as compared to 7.9 mM for nicotinic acid. This represents a 32-fold increase in binding. The smaller the substituent group, the least pronounced the position effect. This is exemplified by comparing the inhibitor constants for 3-aminopyridine and 4-aminopyridine, 66 and 40 μM respectively. The low inhibitor constant observed for unsubstituted pyridine is not readily explained. The snake venom NADase is more sensitive to alternations at the 3-position than at the 4-position of the pyridine ring. NAD analogs hydrolyzed by the NADase all have pyridine bases which were moderately bound to the enzyme.

The second class of inhibitors tested were those which were structurally related to the adenosine moiety of NAD. ADP-ribose was a linear competitive inhibitor with a K_i of 0.36 mM. Other adenosine derivatives, AMP, ADP, ATP, and phospho-ADP-ribose, were also competitive inhibitors of the NADase (Table XIII). The snake venom enzyme was not inhibited by adenosine at concentrations as high as 25 mM. The similar inhibitor constants for ADP and ADP-ribose imply that the additional ribose on ADP-ribose does not enhance the binding of the nucleotide. Schuber *et al.* (141) have shown that the binding of ADP and ADP-ribose to calf spleen NADase was likewise similar. Yuan and Anderson (121) on the other hand, found that ADP-ribose was bound 2-fold better than ADP to bull semen NADase. Although the terminal ribose in ADP-ribose does not appear to play a major role in the binding of the dinucleotide to the enzyme, the substitution of this ribose with a phosphate (ATP) increases the inhibitor constant by 2-fold. The 2'-phosphate of phospho-ADP-ribose decreases the inhibitor constant 2-fold

which is consistent with the K_ms of NAD and NADP (Table XI). The additional phosphate facilitates the binding of substrate or inhibitor, presumably through electrostatic interactions with a positively-charged amino acid residue of the enzyme.

Kinetic features obtained from product inhibition studies of B. fasciatus venom NADase suggest a ping pong bi-bi type mechanism. As with other enzyme-catalyzed hydrolytic reactions where water is the second substrate, the mechanism reduces to an apparent ordered uni-bi. NAD binds first to the free form of the enzyme, followed by the catalytic steps with nicotinamide being the first product released. The second and last product released is ADP-ribose. Derivation of rate equation for ordered uni-bi mechanism is described in Appendix II. This basic mechanism will be discussed later.

Product inhibition studies with nicotinamide and ADP-ribose were consistent with those observed with the bovine seminal fluid and calf spleen NADases (116,121) and represent a major difference with those found for bovine and other mammalian erythrocyte ghost NADases (72,73). Erythrocyte ghost NADases have an opposite pattern of product inhibition.

A third class of inhibitors studied were NAD analogs which were not utilized as substrates in the enzyme catalyzed hydrolytic reaction. A representative sample of NAD analogs tested as inhibitors of the enzyme is shown in Table XII. Nicotinamide mononucleotide was shown to have a relatively high K_i when compared to the other NAD analogs tested. This would imply that the adenyl portion is important in the binding of the nucleotide derivatives to the enzyme. NADH and ADP-ribose exhibited

similar inhibitor constants, suggesting the positively charged nicotinamide ring contributes significantly in the binding process. It is of interest to note that both the bovine erythrocyte ghost NADase and the purified bovine seminal plasma NADase have much lower K_i values for NADH then does the snake venom enzyme (148). Previous results indicated that the snake venom enzyme was stereospecific for the β-isomer of NAD. Inhibition studies with the α-isomer indicated that it was bound approximately 5-fold less effectively than β-NAD, if we assume the K_m to be nearly equal to the K_s for the enzyme-substrate complex (see Appendix II). This result would be expected if one considers that in relation to β-isomer, the α-linkage is inverted. Another weakly bound NAD analog was nicotinic acid adenine dinucleotide, with a K_i 76 μM. As in the case of free nicotninic acid a negative charge at the 3-position of the pyridine ring decreases the effectiveness of binding to the enzyme.

NAD analogs which do not have a carbonyl substituent at the 3-position of the pyridine ring all appeared to have low inhibitor constants. Of these compounds 3-aminopyridine-, pyridine-, and isonicotinic acid hydrazide adenine dinucleotides were the tightest bound with K_is of 300, 220 and 180 nanomolar, respectively. The free bases of these analogs were similarly much better bound than other pyridine bases (Table XII). The importance of the pyridine moiety in the binding of these dinucleotides to the snake venom NADase is indicated by comparing the K_is for a particular pyridine base and dinucleotide of that pyridine base. A direct relationship between the

binding of a pyridine base and the binding of the corresponding dinucleotide was observed. This is best exemplified by isonicotinic acid hydrazide which had the lowest K_i for any pyridine base studied and isonicotinic acid hydrazide adenine dinucleotide which was the best bound of the dinucleotides studied.

Procion Blue HB (Cibacron Blue F3GA) as indicated in Table XIV was shown to be an extremely effective linear competitive inhibitor of the purified B. fasciatus venom NADase, having the lowest K_i of any of the inhibitors studied. The inhibitor constant of 0.033 μM for Procion Blue HB represents a 10 to 100-fold tighter binding of this triazine dye to the snake venom enzyme than that observed for any other NAD-requiring enzyme so far investigated. Schuber and Pascal (149) demonstrated the dye to be a competitive inhibitor of calf spleen NADase with a K_i of 0.4 μM, a value similar to those observed with several other NAD requiring enzyme (150).

Many investigators (150-152) have suggested that the polysulfonated aromatic chromophores of the triazine dyes mimic naturally occuring biological molecules such as NAD, NADP, and certain flavins. However, Biellmann et al. (153) have recently demonstrated by X-ray diffraction studies, that the triazine dyes are not completely specific for nucleotide binding sites. Their results indicated that only part of the chromophore accurately mimics the coenzyme binding to the enzyme. Beissner and Rudolph (150) suggested that these dyes interact through their hydrophobic properties. The hydrophobic binding domain in several dehydrogenases has been extensively investigated by Anderson and

coworkers (154-156). Therefore, Procion Blue HB may be binding to the snake venom enzyme through a number of interactions both ionic and nonpolar in nature.

The importance of the high affinity of the B. fasciatus NADase for Procion Blue HB has allowed the use of this immobilized triazine dye for an affinity matrix during enzyme purification. The extremely tight binding of the triazine dye to the B. fasciatus was one of the major reasons the enzyme was purified to homogeneity after a 3-step purification procedure.

Inhibitor studies with the purified B. fasciatus venom NADase indicated that there are several important interactions between the substrate molecule and the active site of the enzyme. There appear to be at least three distinct regions or sites of interactions of substrates or inhibitor molecules with the NADase. The first, the nicotinamide binding site is fairly specific for the orbital characteristics of the pyridine ring. Other aromatic rings such as pyrimidine, aniline, or benzene were not bound by the enzyme. The importance of the positively charged nicotinamide ring was evident by the relative poor binding of NADH, ADP-ribose, and free pyridine base. The adenyl portion of the dinucleotides was also important since nicotinamide mononucleotide was only poorly bound; however, only a purine base need be present, adenine is not essential. The distal ribose of ADP-ribose does not appear to be important in the binding since both ADP and ADP-ribose have similar inhibitor constants. The importance of the pyrophosphate group is suggested by the fact that AMP is bound only about 19 percent as

tightly as ADP-ribose or ADP. Therefore, it is suggested that the pyrophosphate group is important in substrate or inhibitor recognition.

Substrate activity required all of the above mentioned interactions plus specific interactions of the enzyme with substituents in the 3-position of the pyridine ring. The hydrolysis of the N-pyridinium-ribosidic bond involves specific interactions between the substituent at the 3-position of the pyridine ring and the active site of the enzyme. Such interactions only occur with carbonyl and carbonyl-like functional groups. One might expect the carboxamido group of NAD to provide additional interactions with the enzyme that would result in a lower energy of activation in the enzyme-catalyzed reaction. However, 3-amino-pyridine adenine dinucleotide, which is not a substrate for the enzyme is bound more effectively than NAD. Jencks (157) has suggested that this is typical for induced substrate destabilization, in which part of the intrinsic energy of the binding of the specific substrate to the enzyme may be utilized as necessary energy required for the catalytic process. Because some of the binding energy is used for catalysis, a lowering of the apparent affinity of the substrate to the enzyme occurs. It is interesting to note (Table XII) that all of the NAD analog substrates which were hydrolyzed contained pyridine bases which were equally effective as inhibitors, K_i value approximately 1 mM, whereas, the majority of other pyridine bases studied had much lower K_i values.

Amino Acid Modification

The effects on B. fasciatus venom NADase activity of various reagents known to modify enzyme amino acid side chains were investigated.

Incubation with N-ethylmaleimide, iodoacetamide, or p-chloromercuribenzoate resulted in no inactivation of the enzyme. Using the affinity label, diazotized AAD described by Fisher, et al. (124) to probe the possibility of a hidden sulfhydryl buried in the active site also proved negative. Therefore, no evidence was found to indicate an essential sulfhydyl group in the enzyme.

Modification of possible essential arginine residues which might be involved in the binding of the pyrophosphate group of NAD was also investigated. Phenylgloxal and 2,3-butanedione have been shown to be very effective reagents for the modification of arginine residues in proteins (137). Using either of these reagents resulted in no observable loss in enzyme activity. However the presence of 2,4-pentanedione, a reagent specific for ε-amino groups of lysine residues (138) resulted in a complete loss of B. fasciatus venom NADase enzymatic activity. The indicated essentiality of a lysine residue may well result from a required electrostatic stabilization of the negatively-charged pyrophosphate groups of substrates in the selective binding processes. Further confirmation that the modified residue was a lysine and not an arginine was provided by the reversal of inactivated enzyme by treatment with hydroxylamine. The enamine of 2,4-pentanedione and the ε-amino group can go through a transimination reaction with hydroxylamine, whereas, the arginyl derivative is stable to such treatment. Protection experiments with AAD suggested the lysinyl residue(s) was at the active site. The addition of 2 μM AAD to incubation mixtures provided the enzyme with 87 percent protection from inactivation by 2,4-pentanedione. Dunlap

(158) has shown that an essential lysinyl residue is required for dihydrofolate reductase activity which appears to be located at the pyridine dinucleotide binding site. In contrast, an essential arginyl residue has been postulated for the functioning of calf spleen NADase (141).

Previous investigators (141) have suggested that an essential carboxyl group exists at the active site of certain NADases. Two possibilities for carboxylate interactions have been presented. The carboxyl group could serve as the ionic site of interaction with the positive charged pyridinium ring of the substrate, or the site of stabilization of a postulated oxycarbonium intermediate in the catalytic sequence. Woodward's Reagent K, N-ethyl-5-phenyl-isoxazolium-3'-sulfonate has been shown recently to be a specific reagent for carboxyl group modification (139). Results obtained in the present study indicated that Woodward's Reagent K rapidly inactivated the snake venom NADase. Addition of AAD to inactivation mixtures protected the snake venom enzyme from inactivation by this reagent. These results provide evidence that a carboxyl group is involved in the B. fasciatus venom NADase catalyzed hydrolysis of NAD. Schuber and coworkers (141) have observed similar results with the purified calf spleen NADase, which also was rapidly inactivated with Woodward's Reagent K. Schuber (141) has suggested that the carboxyl group is essential for catalytic activity due to the stabilization of a proposed oxocarbonium ion, ADP-ribosyl intermediate. However, at present it is not known whether this carboxyl group is involved in binding of the positively charged pyridium

ring of NAD or oxocarbonium ion stabilization in the reaction catalyzed by B. fasciatus venom NADase. It is conceivable that the same carboxyl group could be involved in both functions, since the positive charge being stabilized in both cases is in essentially the same place on the substrate.

Pyridine Base Exchange Reaction

In addition to the hydrolytic reaction, several mammalian NADases have been shown to catalyze a transglycosidation reaction, also referred to as the pyridine base exchange reaction (schematic representation, Figure 4). In this reaction, the nicotinamide moiety of NAD is replaced by another substituted pyridine base. To date over 70 NAD analogs have been successfully synthesized by this pyridine base exchange reaction (112).

Kaplan et al. (56) suggested that the noncompetitive inhibition of NADases by nicotinamide was due to the binding of the free pyridine base to an enzyme-ADP-ribose intermediate. This binding of nicotinamide resulted in the resynthesis of NAD. Therefore, according to Kaplan, the pyridine base exchange reaction is simply a nucleophilic competition between water and a pyridine base for an enzyme-ADP-ribosyl intermediate. The existence of this intermediate can explain the hydrolysis of NAD, the noncompetitive inhibition by nicotinamide or other pyridine bases, and the pyridine base exchange reaction (Figure 26). This model has been generally accepted by most researchers studying NADase catalyzed reactions. However, until now the ability to study kinetically the pyridine base exchange reaction with a soluble, stable, and purified

preparation of NADase has been impossible. Analysis of the pyridine base exchange reaction with Bungarus fasciatus venom NADase indicated that the accepted model proposed by Kaplan and coworkers could not fully explain the results of the present study.

In the presence of 3-acetylpyridine and NAD, the snake venom enzyme catalyzed the pyridine base exchange reaction. The formation of 3-acetylpyridine adenine dinucleotide was monitored spectrophotometrically and the kinetic parameters for the reaction were investigated. The K_m for 3-acetylpyridine as a substrate for transglycosidation was found to be 21 mM. As a noncompetitive inhibitor of the hydrolytic reaction, 3-acetylpyridine had K_I = 19.5 mM (Table XII). In this one case, a relationship between the K_I for the noncompetitive inhibition by a particular pyridine base and the observed K_m for that base in the pyridine base exchange reaction existed. This was consistent with Kaplan's model for the pyridine base exchange reaction. However, when pyridine bases other than 3-acetylpyridine were used in the base exchange reaction, results inconsistent with the Kaplan model were obtained. The formation of NAD analogs not reducible in dehydrogenase reactions was studied through HPLC analysis. If such pyridine bases compete effectively with water for a reactive enzyme-ADP-ribose intermediate, then the analysis of products will provide information about the partitioning of the intermediate. By measuring the ratio of the concentrations of the products formed, NAD analog: free ADP-ribose, the relative reactivity of a particular pyridine base compared to water can be determined. This value, called $K_{partition}$, was determined for a

number of pyridine bases. The results of these experiments indicated that there was no relationship of the $K_{partition}$ to the K_I of that particular pyridine base as a noncompetitive inhibitor. For example, the K_I values for 3-pyridylcarbinol and pyridine (Table XII) were 457 and 32 μM, respectively whereas $K_{partition}$ values of 7,400 to 3,600 respectively were determined (Table XII). On an intuitive basis and according to Kaplan's model one would expect that pyridine would react faster than 3-pyridylcarbinol in the exchange reaction. However, the exact opposite was found. 3-Pyridylcarbinol was essentially two times more reactive in the base exchange reaction than pyridine. One would have predicted from the K_I data, that pyridine being better bound should have been more effective in competing with water for the enzyme-ADP-ribose intermediate. These apparent contradictory results indicate that a more complex process is involved rather than that indicated by the Kaplan model.

A quantitative assessment of the sensitivity of a particular reaction to electron donation or withdrawal in the attacking nucleophile may be made by measuring the second-order rate constants for the attack of a series of nucleophiles in a particular reaction (157). With the pyridine bases, the pK_a of the ring nitrogen should be directly correlated with the nucleophilicity of that pyridine base. A logarithmic plot of the $K_{partition}$ values versus the pK_a of a series of pyridine bases indicated a linear relationship between these parameters. The ability of a particular pyridine base to compete with water for the enzyme-bound intermediate can be related to the nucleophilicity of the

attacking base. The slope from this relationship is $\beta = 0.43$. A β value of 0.43 means that the $K_{partition}$ for the pyridine base exchange reaction is 0.43 as sensitive to electron donating groups as is the equilibrium constant for deprotonation of those pyridine bases investigated. The correlation of the pyridine base pK_a and the ability of that base to compete effectively with water for the enzyme-ADP-ribosyl intermediate is an interesting observation, since earlier results (117) indicated that the pK_a of the leaving pyridine was not related to the rate at which that particular NAD analog was hydrolyzed. The results in Table XII also demonstrated no correlation with the inhibitor constants of the different pyridine bases and their pK_as.

Other evidence has been obtained to indicate that the pyridine base exchange reaction is more complicated than the model presented by Kaplan. The previously explained $K_{partition}$ results do not deal with the rate of formation of a particular NAD analog, but only with the relative rate at which the free pyridine base competes with water for the enzyme bound ADP-ribose. Determination of the kinetic parameters, K_m and V_{max} for various pyridine bases in the transglycosidation reaction indicated that 3-acetylpyridine adenine dinucleotide was synthesized faster than any of the other analogs. The free base 3-acetylpyridine also had the highest K_m value of any of the bases tested in the transglycosidation reaction. There was no relationship between the maximum velocity of the transglycosidation reaction and the pK_a of the attacking pyridine base. Comparing the results in Table XII with those on Table XVI, one observes that the K_m of a particular pyridine base for the transglycosidation

reaction greatly differs from the K_I of that pyridine base as an inhibitor of the hydrolytic reaction. Comparing 3-aminopyridine in both the transglycosidation and inhibition of the hydrolytic reaction, nearly 50 times more 3-aminopyridine is needed to obtain half maximal velocity of the base exchange reaction than to cause 50 percent inhibition of NAD hydrolysis. These results can be accomodated by proposing more than one affinity binding site for a particular pyridine base. (A complete model will be presented below.)

A number of NAD analogs were also substrates for the pyridine base exchange reaction. The formation of 3-aminopyridine adenine dinucleotide (phosphate) was observed when s-NAD, 3-acetylpyridine adenine dinucleotide, or NADP were used as the ADP-ribosyl (phosphate) donor with 3-aminopyridine. All enzyme intermediates appeared to react the same way with 3-aminopyridine. The $K_{partition}$ measured with any of the donors was the same as that observed with NAD. The measured kinetic parameters for 3-aminopyridine varied little when these NAD analogs were used as substrates in the pyridine base exchange reaction. A K_m for 3-aminopyridine of approximately 3.2 mM was observed regardless of the structure of the ADP-ribose (phosphate) donor. In these cases, the maximum velocity of transglycosidation varied directly with the maximum velocity of NAD analog hydrolysis. 3-Acetylpyridine adenine dinucleotide was hydrolyzed about 50% as fast as NAD and the maximum rate of transglycosidation with this NAD analog was approximately 63% of that for NAD.

The snake venom NADase catalyzed the pyridine base exchange reaction regardless of the ADP-ribose doner. These results support the existence of an enzyme-ADP-ribose intermediate. According to the proposed kinetic scheme the ability of the snake venom enzyme to catalyze the pyridine base exchange is indicative that $k_{-1} >> k_2$. Product inhibition studies indicated that all of the pyridine bases examined were pure noncompetitive inhibitors. Derivation of the rate law (Appendix II) provided further information about individual rate constants. If $k_{-1} >> k_2$ and the pyridine base are pure noncompetitive inhibitors (intersecting on X axis) then $k_2 << k_3$ and the formation of the enzyme intermediate is slower than its breakdown. The absence of a relationship between the rate of pyridine base exchange and the pK_a of the pyridine base also indicated that the bond forming step is not rate limiting.

Nucleophilic Partitioning

The observation that B. fasciatus venom NADase catalyzes a transglycosidation reaction with NAD and a number of pyridine bases is consistent with the existence of a reactive enzyme-ADP-ribose intermediate. A number of nucleophiles other than pyridine bases have been found to function as acceptor molecules in the ADP-ribosyl transfer reaction. For example, methanol as an alternate nucleophile competed with water to form the methyl riboside of ADP-ribose. The following scheme illustrates the uni-bi kinetic mechanism including nucleophilic partitioning of the enzyme intermediate.

$$E + NAD \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot NAD \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-ADP-ribose} + Nam \begin{cases} \xrightarrow{k_3[H_2O]} ADP\text{-ribose} + E \\ \xrightarrow{k_4[Nuc]} Nuc\text{-ADP-ribose} + E \end{cases}$$

Derivation of the rate equations is presented in Appendix II. Expressions of the general rate equations were derived in accord with the release of a proton during either hydrolysis or methanolysis. That is, the measured rate will be the sum total of the number of protons released during hydrolysis and methanolysis. The derived expressions for V_{max} and K_m are shown below.

$$V_{max} = \frac{k_2(k_3 + k_4[N])\ [E_o]}{(k_2 + k_3 + k_4[N] + k_{-2}[P])}$$

$$K_m = \frac{(k_3 + k_4[N] + k_{-2}[P])}{(k_2 + k_3 + k_4[N] + k_{-2}[P])} \cdot K_{ia}$$

where: [N] = methanol concentration

$$K_{ia} = \frac{k_{-1}}{k_1}$$

[P] = nicotinamide concentration

However, when measuring the initial rates the concentration of nicotinamide is essentially zero, therefore, the rate equation reduces to:

$$V_{max} = \frac{k_2(k_3 + k_4[N])\ [E_o]}{(k_2 + k_3 + k_4[N])}$$

$$K_m = \frac{(k_3 + k_4[N])}{(k_2 + k_3 + k_4[N])}$$

Therefore, in the presence of added nucleophile, when represented in double reciprocal form, the rate equation converts to the following:

$$1/v = \frac{K_{ia}}{k_2[E_o]}\frac{1}{[A]} + \frac{(k_2 + k_3 + k_4[N])}{k_2(k_3 + k_4[N])\ [E_o]}$$

which is of the general form; $1/v = \frac{K_m}{V}\frac{1}{[A]} + \frac{1}{V}$

One observes that the addition of nucleophile should not alter the slopes of double reciprocal plots. Depending on the individual rate constants, an effect on the intercept term may or may not be observed. Results obtained with the B. fasciatus venom NADase in the presence of 5.0 M methanol indicated that the initial velocities decreased less than 15 percent. Experiments performed to determine the kinetic parameters of the NADase catalyzed reaction in the presence of methanol described a family of parallel lines (Figure 36). These results could be predicted from the rate equation derived for the addition of methanol in the absence of nicotinamide.

If $k_{-1} >> k_{-2}$ which is quite probable owing to the existence of the pyridine base exchange reaction, one can question which is faster the formation or breakdown of the enzyme-ADP-ribose intermediate. In terms of rate constants, the same question exists, how does k_2 compare to k_3? Expressions for kinetic parameters:

$$K_m = \frac{k_{-1}}{k_1} \cdot \frac{(k_3 + k_4[N])}{(k_2 + k_3 + k_4[N])}$$

$$\text{if } k_3 >> k_2 \text{ then } K_m = \frac{k_{-1}}{k_1} = K_{ia}$$

$$\text{if } k_2 >> k_3 \text{ then } K_m = \frac{k_4[N]}{(k_2 + k_4[N])} \cdot K_{ia}$$

V_{max} can be equated to k_{cat} by simply dropping $[E_o]$ since $V_{max} = k_{cat}[E_o]$.

$$k_{cat} = \frac{k_2(k_3 + k_4[N])}{(k_2 + k_3 + k_4[N])}$$

again if $k_3 >> k_2$ and $[N] \neq 0$ then k_{cat} reduces to $k_{cat} = k_2$

$$\text{if } k_2 >> k_3 \text{ then } k_{cat} = \frac{k_2(k_3 + k_4[N])}{k_2 + k_4[N]}$$

If $k_2 >> k_3$, the presence of an added nucleophile will affect both the K_m and the V_{max} of the enzyme catalyzed reaction. However, if $k_3 >> k_2$ then the K_m should equal the disociation constant for the enzyme-NAD complex (K_{ia}) and the V_{max} should equal k_2. In other words if $k_2 << k_3$ then added nucleophiles (methanol) should not alter either the K_m or the V_{max} for the observed enzyme catalyzed reaction.

The results in Table XX indicate that this is the case. These kinetic data utilizing an alternate nucleophile to compete with water in the breakdown of NAD suggest the existence of an enzyme-ADP-ribose intermediate.

Kinetic data are supportive but not conclusive evidence. Further verification of the existence of an enzyme-bound intermediate relies on the isolation of the products of the suggested reactions. With methanol, as the alternate nucleophile, one should observe the formation of methyl-ADP-riboside along with the hydrolytic product ADP-ribose. This hypothesis was confirmed by the isolation of the postulated products by high pressure liquid chromatography. Methanol functioned as a true nucleophile in that saturation kinetics were not observed at increasing concentrations of methanol. Assuming that the concentration of water at the active site is 55 M, methanol can be shown to react about 37 times faster than water with the postulated E-ADP-ribose. Obviously this assumption is not completely valid if water has a distinct binding site which becomes saturated under the experimental conditions used.

Schuber and coworkers (122) concluded that with respect to nucleophilic attack, methanol would be expected to be more reactive than water with an activated carbonyl derivative such as an oxocarbonium ion. Inward and Jencks (159) studying the deacylation of acyl-chymotrypsin suggested that other factors may be involved than just the basicity of the attacking nucleophile. These investigators determined that the reactivity of a series of alcohols with acyl-chymotrypsin varied little with the bascity of the attacking nucleophile. Inward and Jencks (159) further indicated that the alcohols studied were binding

to a hydrophobic domain near or at the active site of chymotrypsin. Hydrophopic domains have been experimentally shown to exist in a number of dehydogenases and reductases (154-156,160). The purified seminal plasma NADase (148) exhibited a slight chainlength effect when a number of N^1-alkylnicotinamide chlorides were studied as inhibitors of the catalyzed hydrolysis of NAD. These observations of possible hydrophobic domains existing in the active site of pyridine nucleotide-requiring enzymes could explain the increased reactivity observed for methanol over water in the B. fasciatus venom NADase catalyzed reactions. When a number of primary aliphatic alcohols were tested for the relative effectiveness as alternate nucleophiles a positive chainlength effect was observed. Increasing the number of carbon atoms in the aliphatic alcohols resulted in an increase in the ratio of alcoholysis/hydrolysis. N-pentanol reacted with the E-ADP-ribose intermediate about 550-fold faster than water. This increase in the reactivity observed with n-pentanol can not be explained solely by the bascity of the hydroxyl group. An alternate explanation is the existence of a hydrophobic domain in the active site of B. fasciatus.

The change in free energy per methylene group was determined to be 0.51 kcal/mole. This value falls well within the range 0.39 to 0.95 kcal /mole suggested as the criteria for a positive chainlength effect due to chain-chain interactions (161). It is therefore possible that these aliphatic alcohols interact with a hydrophobic pyridine binding site on the enzyme. Isopropanol, a secondary alcohol was only 1/13 as reactive when compared to n-propanol as a nucleophilic reactant with the postulated enzyme-ADP-ribose intermediate. These data indicate that steric factors

must be considered in addition to hydrophobic interactions.

If the formation of an enzyme-ADP-ribose intermediate is rate limiting, $k_2 << k_3$, the given intermediate will not accumulate but, must react in the same way regardless of the precursors from which it was formed. In other words, if $k_2 << k_3$ no matter what the V_{max} is for a particular substrate the ratio of methanolysis to hydrolysis should be the same if the enzyme catalyzed reactions proceeds through the same intermediate. When various NAD analogs were used as substrates for the snake venom NADase-catalyzed reaction in the presence of methanol, similar ratios for methanolysis to hydrolysis were observed. These results are further evidence that the formation of an enzyme-ADP-ribose intermediate is slower than its breakdown.

A variety of other nucleophiles were found to compete with water for the enzyme-ADP-ribose intermediate. N-Acetyl-serine amide was shown by Anderson et al. (162) to exhibit unusually high reactivity with acetyl-chymotrypsin. This was however, not found to be the case when alcoholysis with N-acetyl-serine amide was compared to hydrolysis in the snake venom NADase-catalyzed reaction. Alcoholysis proceeded about 3 times faster than hydrolysis. Compared to the aliphatic alcohols, N-acetyl-serine amide is a fairly poor ADP-ribosyl acceptor. Under similar conditions the ADP-ribose moiety of NAD was not transferred to N-acetyl-tyrosine amide.

The absence of reactivity of primary amines probably reflects the low concentration of the unprotonated attacking nucleophile. Due to enzyme lability at higher pHs, further investigation could not be done.

However, hydroxaminolysis did occur at about 200 times the rate of hydrolysis. This increased rate is most likely due to the alpha effect. That is compounds containing a free pair of electrons on an electronegative atom adjacent to the attacking atom often have usually high nucleophilic reactivity (157).

Recent evidence by Moss et al.(9) has suggested that choleragen, an ADP-ribose transferase, will ADP-ribosylate arginine methyl ester. Under conditions with up to 150 mM arginine methyl ester, the B. fasciatus venom NADase did not transfer ADP-ribose to arginine methyl ester.

Van Ness et al.(8) have demonsrated that diphtheria toxin catalyzes the ADP-ribosylation of a unique amino acid, 2-[3-carboxyamido-3-(trimethylammonia)propyl] histidine. In light of these results, several imidazole derivatives were tested as possible ADP-ribosyl acceptors in the NADase-catalyzed reactions. Of the imidazole derivatives investigated, unsubstituted imidazole proved to be the most effective compound. The substituted imidazole derivatives probably were less reactive with the enzyme-ADP-ribose intermediate because they were all charged. Presumably the imidazole ring is as reactive but interaction with the enzyme is hindered by these charges. Ergothioneine which has some similarities to the compound found ribosylated in elongation factor 2 by diphtheria toxin was not a very good acceptor of the ADP-ribose moiety of NAD catalyzed by the snake venom NADase. Solubilized beef spleen NADase was previously shown to catalyze the histaminolysis of NAD (58). However, other investigators failed to see similar results of histamine and NAD in the presence of bull semen NADase (163). The ability of the purified B. fasciatus venom NADase to catalyze the imidazolysis and

histaminolysis of NAD suggests that this enzyme may be more mechanistically similar to beef spleen NADase than seminal plasma NADase. Both the snake venom and beef spleen NADases catalyze the pyridine base exchange reaction, whereas, the bull semen NADase does not.

None of the nucleophiles described above exhibited saturation kinetics; suggesting that these compounds were not specifically bound to the snake venom enzyme or that saturation of this binding site requires abnormally high concentrations of these nucleophiles. It is more likely that these nucleophiles function like water in attacking the enzyme intermediate. The pyridine bases on the other hand, are specifically bound and demonstrate a much higher reactivity with the NADase.

Model of NADase-Catalyzed Reactions

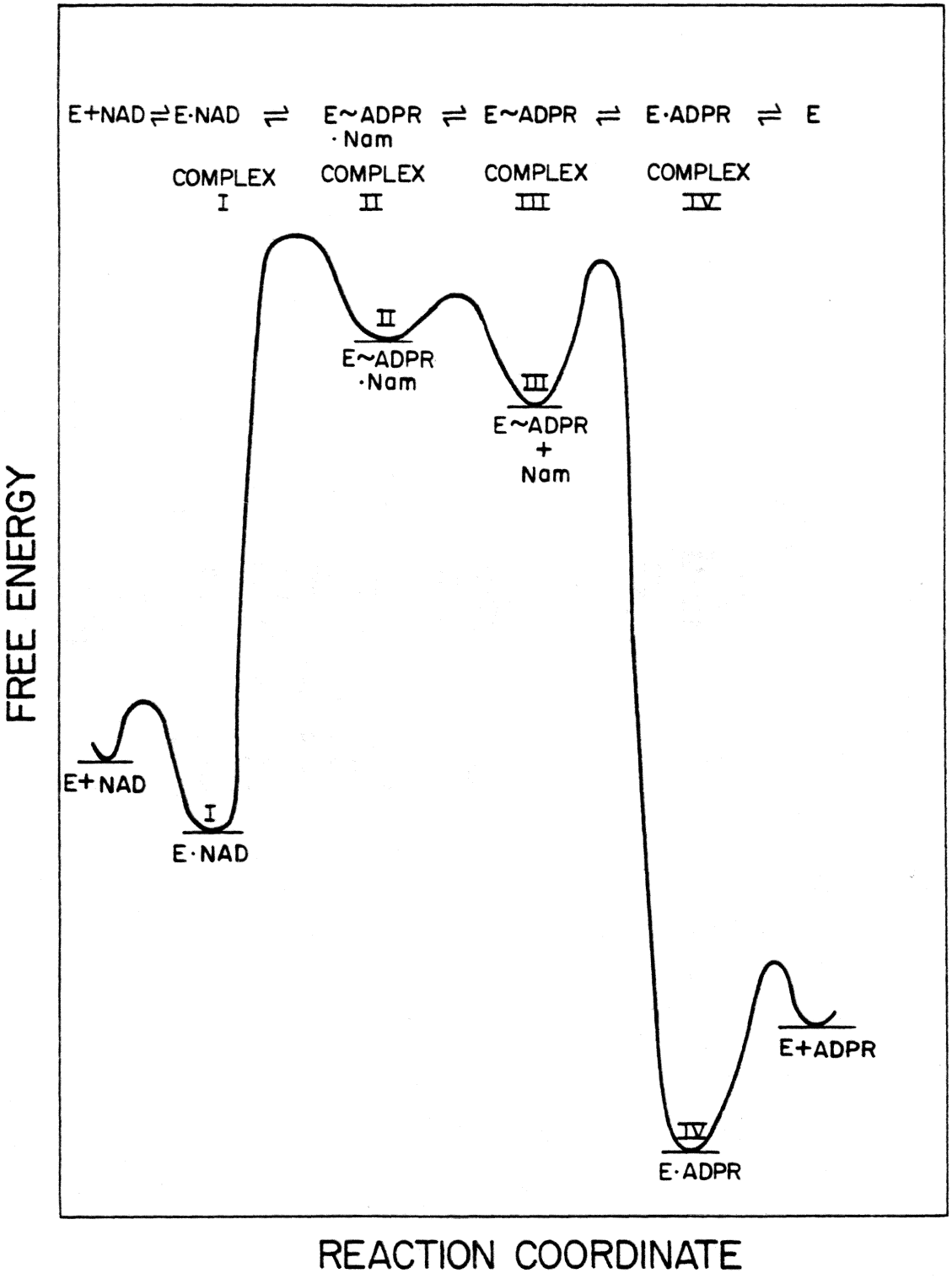
A model for the reactions catalyzed by the B. fasciatus venom NADase must explain the following:

1) the rate limiting step must occur at or prior to the bond breaking step.
2) the presence of two pyridine binding sites of differing affinity
3) noncompetitive inhibition by pyridine bases
4) incubation of free pyridine base with ADP-ribose does not yield NAD analog
5) competition between various added nucleophiles and water

The proposed model is shown in Figure 39. The upper portion of the figure describes the overall scheme and designates important complexes during the enzyme-catalyzed reaction. The lower portion of the figure is an energy diagram proposed for the B. fasciatus venom NADase catalyzed hydrolytic and base exchange reactions.

According to the scheme presented in Figure 39 NAD binds to the free form of the enzyme to form the Michaelis complex, designated complex I. Activation of the Michaelis complex by the enzyme results in the bond breaking of NAD with the concominant formation of complex II, in which nicotinamide and ADP-ribose are still bound to the enzyme. Complex II can be considered a ternary complex with ADP-ribose bound to the enzyme in such a way as to conserve the high energy inherent in the original N-ribosidic bond of NAD. The free energy of hydrolysis of the nicotinamide-ribosidic bond has a $\Delta G = -8.2$ kcal/mol (56). Dissociation

FIGURE 39 - Schematic representation and possible energy diagram of proposed model for the NADase-catalyzed reactions.


E+NAD ⇌ E·NAD ⇌ E~ADPR ·Nam ⇌ E~ADPR ⇌ E·ADPR ⇌ E
COMPLEX I
COMPLEX II
COMPLEX III
COMPLEX IV
II
E~ADPR ·Nam
III
E~ADPR + Nam
E+NAD
I
E·NAD
E+ADPR
IV
E·ADPR
FREE ENERGY
REACTION COORDINATE

of nicotinamide results in the formation of complex III, the enzyme-ADP-ribose intermediate. The nature of the intermediate will be discussed later. Hydrolysis of the enzyme-ADP-ribose intermediate converts complex III to complex IV. This latter complex is the binary complex of enzyme and reversibly bound ADP-ribose. Dissociation of ADP-ribose from complex IV yields free enzyme.

Not shown in the scheme presented in Figure 39, pyridine bases such as nicotinamide can be bound only to forms of the enzyme containing ADP-ribose, i.e. complex III or complex IV. Pyridine bases were shown to be linear noncompetitive inhibitors of the NADase-catalyzed hydrolysis of NAD and this inhibition occurs by the binding of the bases to the binary complex IV.

For the purposes of this discussion, complex IV is considered the high affinity nicotinamide binding form, while complex III will be considered the low affinity nicotinamide binding form. All of the pyridine bases which have been examined bind to these forms of the enzyme in a similar fashion as nicotinamide.

Complex III is the form of the enzyme which will ultimately proceed to the formation of NAD or NAD analogs when high enough concentrations of pyridine base are present and if all conditions of the transglycosidation reaction are met. Much higher concentrations of free pyridine base are required to saturate complex III than needed for complex IV. The implied change in geometry of the active site regions in complexes III and IV may require the postulation of an enzyme conformational change in the interconversion of these forms of the enzyme. Other

models reported for the pyridine base exchange reaction propose the binding of pyridine bases to complex III as an explanation of both noncompetitive inhibition and transglycosidation.

One can consider how well the proposed model explains the reactions catalyzed by B. fasciatus venom NADase. The hydrolysis of NAD or NAD analogs by B. fasciatus venom NADase is first discussed. The first step in the hydrolytic reaction is the binding of substrate to the free enzyme to form the Michaelis complex (complex I). The enzyme catalysis involves the conversion of complex I to complex II with the concominant cleavage of the N-ribosidic bond of the substrate. Complex II, the high energy ternary complex is favorable for the release of nicotinamide (or alternate pyridine base). The expulsion of nicotinamide converts the ternary, high energy complex II to the binary high energy complex III. The addition of water to the activated complex III results in hydrolysis of the enzyme-ADP-ribose intermediate to form the reversibly bound ADP-ribose, complex IV. The dissociation of ADP-ribose from the binary complex IV results in the formation of free enzyme which can reenter the catalytic cycle.

Inhibition by ADP-ribose results from the binding of ADP-ribose to the free form of the enzyme. Since ADP-ribose binds to the same form of the enzyme as NAD it behaves as a competitive inhibitor. Inhibition exerted by nicotinamide was previously explained as the binding to the binary complex IV of the enzyme. Nicotinamide binding to complex IV results in the further lowering of the concentration of free enzyme, thus decreasing the observed maximal velocity.

Enzyme catalyzed hydrolysis of thionicotinamide-, or 3-acetylpyridine adenine dinucleotide proceeds in a similar manner as described above, although the individual free energy changes may vary. With either of these substrates, the conversion of complex II to complex III results in the formation of identical forms of the enzyme (enzyme-ADP-ribose).

The explanation for the pyridine base exchange reaction is not quite as simple. For pyridine base exchange to occur, NAD (analog) plus a high concentration of pyridine base to be exchanged are required. As in the hydrolytic reaction NAD binds first to the free enzyme and proceeds to complex III, the enzyme-ADP-ribose intermediate. Under conditions where high concentrations of free pyridine bases exist, complex IV the high affinity binding form is rapidly saturated. Additional pyridine base will result in binding to the low affinity form, complex III. The binding of pyridine base to complex III provides for a competition between the pyridine base and water in reactions with the high energy enzyme-ADP-ribose intermediate. At concentrations of pyridine base that promote the reverse reaction to form analog, complex IV should be at saturation, therefore blocking the formation of free enzyme in the forward reaction. After the formation of the ternary complex III, conditions may be more energetically favorable for complex III to revert to complex II, especially in light of the high concentration of pyridine base present, which saturates the enzyme form, complex IV. From complex II a bond forming step occurs between the new pyridine base at the active site and the high energy enzyme-ADP-ribose resulting

in the formation of an enzyme•NAD analog complex, followed by release of this NAD analog from the enzyme.

The reaction of nucleophiles other than pyridine bases is explained similarly. NAD or analog binds to the free enzyme to form complex I followed by catalysis resulting in the formation of complex III. Added nucleophiles react with this high energy complex III through a bimolecular process akin to that observed with water. The major difference in the functioning of non-pyridine nucleophiles is the absence of any selective binding of these compounds to any form of the enzyme.

Implicit in a mechanistic discussion of NADase catalyzed reactions is the existence of an enzyme-ADP-ribose intermediate. The nature of this intermediate will dictate the most probable chemical steps involved during NADase-catalyzed reactions. Alivisatos (113) described the enzyme catalysis to involve a double displacement reaction, where an imidazole ring of a histidine residue displaces the pyridine ring of NAD followed by a second displacement of this ribosyl-imidazole intermediate with a molecule of water. A double displacement mechanism would involve a covalent enzyme-bound intermediate.

Crodes (119) and Schuber (117) investigating the mechanism of calf spleen NADase have suggested an alternate mechanism involving the unimolecular decomposition of NAD, resulting in the formation of an oxocarbonium ion. This ion is visualized as forming a stabilized ion-pair at the active site of the enzyme. Bull et al. (119) measured a kinetic alpha secondary deuterium isotope effect of 1.0. The absence of a secondary deuterium isotope effect requires: 1) irreversible substrate

binding; or 2) carbon-nitrogen bond cleavage is not rate determining; or 3) participation of a nucleophilic reagent in carbon-nitrogen bond cleavage; or 4) an enzyme conformational change. Conclusions that the rate determining step of the reaction is an enzyme conformational change was described as the reason for the apparent lack of an observable secondary isotope effect (119). Schuber et al. (122) indicated that the following observations are consistent with the formation of an oxocarbonium ion intermediate: 1) methanol reacts 100-fold more rapidly with the enzyme intermediate than water, 2) the NADase-catalyzed methanolysis of NAD occurs with retention of configuration, and 3) pyridine base exchange of NAD occurs with retention of configuration. None of these observations excludes the possibility of a double displacement mechanism.

Other hydrolytic enzymes such as lysozyme, β-galactosidase, and more recently β-glucosidase have all been described as proceeding through a carbonium ion intermediate (120). Isolation of such enzyme-stabilized intermediates is unlikely due to the lability of carbonium ions and the possibility of decomposition of these ions to stable tetrahedral intermediates.

The existence of an enzyme-ADP-ribose intermediate during the snake venom NADase catalyzed hydrolysis of NAD is indicated by the ability of the enzyme to catalyze the pyridine base exchange reaction. This was further confirmed by nucleophilic competition between water and a series of aliphatic primary alcohols. Analysis of kinetic data in the absence or presence of added nucleophiles indicated that the rate determining step of the enzyme-catalyzed reaction occurs at or prior to the formation of the enzyme-ADP-ribose intermediate. Derivation of the

kinetic rate equation for the snake venom NADase- catalyzed reaction is shown in Appendix II.

Alcoholysis experiments indicated that an increase in the number of methylene groups within an aliphatic carbon chain increased the ratio of enzyme catalyzed alcoholysis to hydrolysis. A rate increase of approximately 15-fold was found when comparing methanol to pentanol. This increase in chain length does not affect the chemical reactivity of the hydroxyl group of these alcohols to any great degree. An explanation involving hydrophobic interactions of these alcohols with the B. fasciatus venom NADase appears warranted. These results imply that the ability of methanol to react faster than water is not necessarily a result of the nucleophilic reactivity of methanol and suggests other factors may be involved in nucleophilic competition for the enzyme-ADP-ribose intermediate. Inactivation of snake venom NADase by Woodward's Reagent K indicated the importance of a carboxyl group for enzyme activity. The observed protection of the enzyme by AAD would be consistent with the carboxyl group(s) in question to be located at the enzyme active site. One can envision the essential carboxyl group functioning through the stabilization of an oxocarbonium ion intermediate, however, functioning through the stabilization of the positively-charged pyridinium moiety in substrate binding is an alternative that can not be ruled out. However, the absence of inactivation of enzyme activity with diethyl pyrocarbonate, strongly suggests that a double-displacement reaction involving an enzyme histidine seems unlikely.

The proposed model must explain the results obtained with B. fasciatus venom NADase. In the present study the K_m values for different pyridine bases determined for the pyridine base exchange reaction did not equate to the inhibitor constants determined for these pyridine bases in inhibition of the hydrolytic reaction (Table XII and Table XVI). The K_i determined for 3-aminopyridine was 66 μM for the inhibition of the enzyme-catalyzed hydrolysis of NAD. The apparent K_m for 3-aminopyridine as a substrate for the pyridine base exchange reaction was 3.2 mM. The 50-fold difference between these values represents the difference in binding to complex III or complex IV. The V_{max} for analog formation was related to how well the newly formed analog was released from the enzyme and not related to the pK_a of the incoming pyridine base. The presence of two pyridine binding sites of differing affinity was observed with other pyridine bases as well. 3-Acetylpyridine is the exception to this generalization. It was the only pyridine base for which the apparent K_m in the pyridine base exchange reaction was essentially equal to the observed K_i in the hydrolytic reaction. The V_{max} for 3-acetylpyridine adenine dinucleotide formation was greater than that for the formation of other analogs. The rate of formation of 3-acetylpyridine compared to 3-aminopyridine adenine dinucleotide was 10-fold greater. Comparison of 3-acetylpyridine adenine dinucleotide to other NAD analogs studied is not entirely valid since it was the only analog formed which also served as a substrate for the enzyme-catalyzed hydrolysis.

The rate and extent of pyridine base exchange is determined by many factors in addition to simply the binding of free pyridine base to an

enzyme-ADP-ribose intermediate. It is proposed that binding of the pyridine base to the enzyme is required but not the determining factor to whether or not the transglycosidation reaction will be catalyzed. For example, both 6-aminonicotinamide and 2-acetylpyridine exhibit K_i values in the hydrolytic reaction similar to that observed for 3-acetylpyridine. However, neither of these bases are utilized to form NAD analogs in the snake venom NADase-catalyzed pyridine base exchange reaction. Presumably, the steric hinderance resulting from the presence of a substituent ortho- to the pyridine ring nitrogen has little effect on binding but a pronounced effect on reactivity in the pyridine base exchange reaction. According to the presented model, these pyridine bases can bind to complex IV but not complex III. Or, if binding to complex III is possible, these bases can not compete with water for the enzyme-ADP-ribose intermediate. The relative reactivity of a particular pyridine base for the enzyme-ADP-ribose intermediate as compared with water, $K_{partition}$, was shown to be a function of the pK_a of the ring nitrogen, (Figure 33). Therefore with 3-aminopyridine which is 11,000-fold more reactive than water with the enzyme-ADP-ribose intermediate, one observes almost 100% conversion to analog at saturating concentrations of 3-aminopyridine. A K_i of 66 μM was observed for 3-aminopyridine in the hydrolytic reaction. Isonicotinic acid hydrazide which was bound almost 7-fold better than 3-aminopyridine could never be converted 100% to analog. According to Table XV isonicotinic acid hydrazide was 6-fold less reactive than 3-aminopyridine competing with water for the enzyme-ADP-ribose intermediate. This is another example indicating additional properties are involved for pyridine base exchange

to occur than just tight binding between enzyme and pyridine base. These results indicate that although the overall rate of the pyridine base exchange is independent of the nucleophilicity of the incoming pyridine base, the ability for a pyridine base to compete with water is not. Mechanistically this implies that once the pyridine base binds to complex III, if the pyridine base is not sufficiently nucleophilic to compete with water then hydrolysis proceeds. However, if the pyridine base is a better nucleophile, then complex III rapidly is converted to complex I, the enzyme·NAD analog complex. Similar results were observed with all pyridine bases studied.

The reactions of nucleophiles other than pyridine bases with the enzyme-ADP-ribose intermediate proceed through a bimolecular process similar to that observed with water. Although most of the compounds studied were more nucleophilic than the pyridine bases, lower rates of transglycosidation were observed since facilitation due to selective binding at the enzyme site was lacking. Since these nucleophiles were not selectively bound by the enzyme, they also did not function as inhibitors in the hydrolytic reaction.

In summary, the proposed model can account for all of the reactions catalyzed by the snake venom enzyme. Although the presently discussed data can not distinguish between a covalent intermediate and a stabilized oxocarbonium ion, the existence of an active intermediate has been verified. Kinetic evaluation of the pyridine base exchange reaction and nucleophilic partitioning data indicate that the rate limiting step occurs at or before formation of complex III. Carbon-nitrogen bond

breaking can not be ruled out as the rate limiting step, since a direct relationship to a pyridine base nucleophilicity and reactivity compared to water was observed.

Schuber and coworkers (116) investigating the mechanism of calf spleen NADase catalysis proposed an isomerization of a central complex. Kinetic data obtained with the purified snake venom NADase supported the existence of multiple forms of enzyme-bound ADP-ribose (central complex). The proposed model for the mechanism of B. fasciatus venom NADase fully explains the results obtained. NADase-catalyzed hydrolysis of NAD proceeds through a bimolecular addition of water to an enzyme-ADP-ribose intermediate. The reaction of alcohols and other nucleophiles also involve a bimolecular addition of the nucleophile to the enzyme intermediate. The present model indicates that the pyridine base exchange reaction is more complex than originally proposed (56,117). Pyridine bases are more effective at competing with water for enzyme-ADP-ribose than other nucleophiles through their ability to bind to the NADase. Although efficient binding of a pyridine base is important for catalysis of transglycosidation to occur, it is not essential. Other factors important for snake venom NADase-catalyzed pyridine base exchange include nucleophilicity of the attacking pyridine base and absence of steric hindrance.

Accumulating evidence suggests an importance of ADP-ribose transfer reactions in metabolic regulation. In the present study the purified Bungarus fasciatus venom NADase has provided an ideal system to study some of these reactions in detail.

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APPENDIX I

The kinetic constants were determined by the following calculations:

$$K_m^o = \frac{\beta\gamma - \alpha\delta}{\Delta}$$

$$V_{max}^o = \frac{\beta\varepsilon - \delta^2}{\Delta}$$

where $\alpha = \Sigma\, v^3$

$\beta = \Sigma\, v^4$

$\gamma = \Sigma\, \frac{v^3}{s}$

$\delta = \Sigma\, \frac{v^4}{s}$

$\varepsilon = \Sigma\, \frac{v^4}{s^2}$

$\Delta = \alpha\varepsilon - \delta\gamma$

where s = substrate conc.

v = initial velocity

Fine adjustments to these parameters;

$$f = \frac{V_{max}^o\, s}{s + K_m^o}$$

$$f' = \frac{V_{max}^o\, s}{(s + K_m^o)^2}$$

Now $V_{max} = b_1 V_{max}^o$

$K_m = K_m^o + b_2/b_1$

where $\alpha = \Sigma\, f^2$ $\qquad \varepsilon = \Sigma\, vf'$

$\beta = \Sigma\, f'^2$ $\qquad \Delta = \alpha\beta - \gamma^2$

$\gamma = \Sigma\, ff'$ $\qquad b_1 = \frac{\beta\delta - \gamma\varepsilon}{\Delta}$

$\delta = \Sigma\, vf$

$$b_2 = \frac{\alpha\varepsilon - \gamma\delta}{\Delta}$$

$$\text{Standard error of } K_m = \frac{Z}{b_1\sqrt{\alpha/\Delta}}$$

$$\text{where } Z = \sqrt{(\Sigma v^2 - b_1 - b_2)/(n - 2)}$$

$$\text{Standard error of } V_{max} = V^{o}_{max}\, Z\sqrt{\beta/\Delta}$$

APPENDIX II

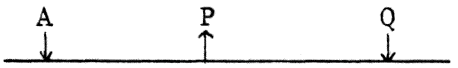
If the NADase-catalyzed reaction procceds via the following scheme:

$$E + NAD \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot NAD \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}ADPR + Nam \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + ADPR$$

which is of the form:

$$E + A \rightleftharpoons E \cdot A \rightleftharpoons E\text{-}Q + P \rightleftharpoons E + Q$$

or in Cleland notation:



where A = NAD, P = Nam, and Q = ADPR

Assuming that this is the reaction that is catalyzed by the snake venom NADase, then, the following rate expressions can be derived using King-Altman Methods.

$$v = \frac{k_2(k_1k_{-2}[A][P] + k_{-3}k_{-2}[Q][P] + k_1k_3[A])\ [E_o]}{k_3(k_{-1} + k_2) + k_1[A](k_2 + k_3) + k_{-1}k_{-2}[P] + k_{-3}[Q](k_{-1} + k_2) + k_1k_{-2}[A][P] + k_{-3}k_{-2}[P][Q]}$$

$$V_{max\ f} = \frac{num_1}{coef_A} = \frac{k_1k_2k_3[A][E_o]}{k_1(k_2 + k_3)\ [A]}$$

$$= \frac{k_2k_3[E_o]}{k_2 + k_3}$$

If $V_{max} = k_{cat}[E_o]$ and $k_3 << k_2$

THEN: $k_{cat} = k_3$

HOWEVER IF: $k_3 >> k_2$ THEN $k_{cat} = k_2$

$$K_m = \frac{const.}{coef_A} = \frac{k_3(k_{-1} + k_2)}{k_1(k_2 + k_3)}$$

IF $k_3 << k_2$ THEN $K_m = \frac{k_3(k_{-1} + k_2)}{k_1k_2}$

IF $k_3 >> k_2$ THEN $K_m = \frac{k_{-1} + k_2}{k_1}$

the rate of the reaction is of the form:

$$v = \frac{V_1[A] - (V_1/K_e)\ [P][Q]}{(K_A + [A] + ([A][P]/K_{ip}) + (K_{ia}/K_{ip}K_q)[P][Q] + (K_{ia}/K_{ip})[P] + (K_A/K_{iq})[Q]}$$

if [P] and [Q] = 0, initial conditions then,

$$v = \frac{V_1[A]}{K_A + [A]}$$

If vary [Q] while keeping [P] constant,

$$v = \frac{V_1[A]}{K_A + [A] + (K_A/K_{iq})[Q]}$$

$$v = \frac{V_1[A]}{K_A(1 + ([Q]/K_{iq})) + [A]}$$

or in reciprocal form;

$$1/v = \frac{K_A(1 + ([Q]/K_{iq}))}{V_1} \quad \frac{1}{[A]} + \frac{1}{V}$$

varying concentrations of [Q] only effects the slope term and not the ordinal intercept ($1/V_1$) this implys competitive inhibition by the second product released from the enzyme.

If vary [P] but keep [Q] constant. then the rate equals:

$$v = \frac{V_1[A]}{K_A(1 + (K_{ia}[P]/K_AK_{ip})) + [A](L + ([P]/K_{ip}))}$$

in reciprocal form;

$$1/v = \frac{K_A(1 + (K_{ia}[P]/K_AK_{ip}))}{V_1} + \frac{1}{[A]} + \frac{(1 + ([P]/K_{ip}))}{V_1}$$

this implys mixed inhibition since both the slope and intercept terms are effected by [P].

From double reciprocal plot the x intercept equals the inverse of K_m. Therefore, if P is a pure non-competitive inhibitor then all lines should intersect on the x-axis.

Solving for the x-intercept term:

$$\frac{\dfrac{1 + ([P]/K_{ip})}{V_1}}{\dfrac{K_A\{1 + (K_{ia}[P]/K_A K_{ip})\}}{V_1}}$$

which reduces to:

$$= \frac{K_{ip} + [P]}{K_A K_{ip} + K_{ia}[P]}$$

expanding with the individual rate constants;

$$= \frac{\dfrac{k_1(k_2 + k_3)}{k_1 k_{-2}} + [P]}{\dfrac{k_3(k_{-1} + k_2)}{k_1(k_2 + k_3)} \cdot \dfrac{k_1(k_2 + k_3)}{k_1 k_{-2}} + \dfrac{k_{-1}k_2}{k_1 k_2}[P]}$$

which reduces to:

$$\frac{k_1(k_2 + k_3 + k_{-2}[P]}{k_3(k_{-1} + k_2) + k_{-1}k_{-2}[P]} = \frac{1}{K_m}$$

since enzyme catalyzes the pyridine base exchange $k_2 << k_{-1}$

therefore;

$$\frac{1}{K_m} = \frac{k_1}{k_{-1}} \cdot \frac{(k_2 + k_3 + k_{-2}[P]}{k_3 + k_{-2}[P]}$$

However, if $k_2 << k_3$ then $\frac{1}{K_m} = \frac{k_1}{k_{-1}}$ since $K_{ia} = \frac{k_{-1}}{k_1}$

then $K_{ia} = K_m$

This indicates that the determined K_m is indepentent of the [P].

if $k_2 >> k_3$ then $\frac{1}{K_m} = \frac{k_1}{k_{-1}} \cdot \frac{(k_2 + k_{-2}[P])}{(k_3 + k_{-2}[P])}$

Under these conditions the K_m is dependent on the [P]. This implies from a double reciprocal plot in the presence of varying concentrations of P there is no effect on the measured K_m when $k_2 << k_3$

The rate equations in the presence of added nucleophile are derived below. If the reaction procceds through the following scheme:

Where [N] = the concentration of added nucleophile

$$E + NAD \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot NAD \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}ADPR \begin{array}{l} \xrightarrow{k_3[H_2O]} ADPR \\ \xrightarrow{k_4[Nuc]} Nuc\text{-}ADPR \end{array}$$

$$V_{max} = \frac{k_1k_2[A](k_3 + k_4[N])}{k_1k_2[A] + k_1k_3 + k_1k_4[N][A] + k_1k_{-2}[A][P]}$$

which reduces to:

$$V_{max} = \frac{k_2(k_3 + k_4[N])\ [E_o]}{(k_2 + k_3 + k_4[N] + k_{-2}[P]}$$

$$K_m = \frac{k_3(k_{-1} + k_2) + k_4[N](k_{-1} + k_{-2}) + K_{-1}k_{-2}[P]}{k_1(k_2 + k_3 + k_4[N] + k_{-2}[P])}$$

$$= \frac{(k_{-1} + k_2)(k_3 + k_4[N]) + k_{-1}k_{-2}[P]}{k_1(k_2 + k_3 + k_4[N] + K_{-2}[P])}$$

owing to the existence of the pyridine base exchange $k_{-1} >> k_2$ then;

$$K_m = \frac{k_{-1}k_3 + k_2k_3 + k_4k_{-1}[N] + k_{-2}k_4[N] + k_{-1}k_{-2}[P]}{k_1(k_2 + k_3 + k_4\ [N] + k_{-2}[P]}$$

which reduces to:

$$= \frac{k_{-1}}{k_1} \cdot \frac{(k_3 + k_4[N] + k_{-2}[P]}{(k_2 + k_3 + k_4[N] + k_{-2}[P]}$$

$$K_m = K_{ia} \cdot \frac{k_3 + k_4[N] + k_{-2}[P]}{k_2 + k_3 + k_4[N] + k_{-2}[P]}$$

if $k_3 >> k_2$ then $K_m = K_{ia}$

if $k_2 >> k_3$ then $K_m = K_{ia} \cdot \frac{(k_4[N] + k_{-2}[P]}{(k_2 + k_4[N] k_{-2}[P]}$

the effects on V_{max}

if $k_3 >> k_2$ then

$$V_{max} = \frac{k_2k_4[N] + k_2k_3}{k_{-2}[P] + k_4[N] + k_3}$$

if [N] and [P] = 0 then $V_{max} = k_2$

if [N]>>0 then $V_{max} = \frac{k_2k_4[N] + k_2k_3}{k_4[N] + k_3}$

$= k_2$

if $k_2 >> k_3$ then $V_{max} = \frac{k_2k_3 + k_2k_4[N]}{k_2 + k_4[N] + k_{-2}[P]}$

if [N] and [P]= 0 then $V_{max} = k_3$

if [N]>>0 then $V_{max} = \frac{k_2k_3 + k_2k_4[N]}{k_2 + k_4[N]}$

$$= \frac{k_2(k_3 + k_4[N])}{k_2 + k_4[N]}$$

In the absence of nicotinamide [P] = 0

$$V_{max} = \frac{k_2(k_3 + k_4[N])[E_o]}{k_2 + k_3 + k_4[N]}$$

$$K_m = K_{ia} \cdot \frac{k_3\, k_4[N]}{k_2 + k_3 + k_4[N]}$$

Knowing $1/v = \frac{K_m}{V} \frac{1}{[A]} + \frac{1}{V}$

then :

$$1/v = \frac{K_{ia} \cdot \frac{(k_3 + k_4[N])}{(k_2 + k_3 + k_4[N])}}{\frac{k_2(k_3 + k_4[N])[E_o]}{(k_2 + k_3 + k_4[N])}} \frac{1}{[A]} + \frac{(k_2 + k_3 + k_4[N])}{k_2(k_3 + k_4[N])[E_o]}$$

$$K_{ia} \cdot \frac{(k_3\, k_4[N])}{(k_2 + k_3 + k_4[N])} \cdot \frac{(k_2 + k_3 + k_4[N])}{k_2(k_3 + k_4[N])[E_o]} \frac{1}{[A]} + \frac{(k_2 + k_3 + k_4[N])}{k_2(k_3 + k_4[N])[E_o]}$$

$$= \frac{K_{ia}}{k_2[E_o]} \frac{1}{[A]} + \frac{k_2 + k_3 + k_4[N]}{k_2(k_3 + k_4[N])[E_o]}$$

This implys that added nucleophile should not alter the slope term in a double reciprocal plot, but may vary the intercept term depending on the individual rate constants, These derivations indicate that if $k_3 >> k_2$ in the presence of added nucleophile the $K_m = K_{ia}$ or the dissociation constant of enzyme·NAD complex equals the K_m, while the V_{max} in the presence of added nucleophile remains unchanged.

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PURIFICATION AND PROPERTIES OF *BUNGARUS FASCIATUS* VENOM NAD GLYCOHYDROLASE

by

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(ABSTRACT)

The NAD glycohydrolase (NADase) from *Bungarus fasciatus* venom was purified over 1000-fold to electrophoretic homogeneity through a 3-step procedure which included affinity chromatography on Cibacron Blue agarose. The enzyme exhibited a broad pH profile with the optimum range between 7-8. Studies on the substrate specificity of *B. fasciatus* venom NADase demonstrated that alterations in the purine ring were less pronounced then alterations in the pyridinium moiety of NAD. Product inhibition studies indicated nicotinamide to be a noncompetitive inhibitor with a K_i = 1.4 mM and ADP-ribose to be a competitive inhibitor with a K_i = 0.4 mM. The purified enzyme was inactivated by both 2,4-pentanedione and Woodward's Reagent K suggesting the involvement of a lysine and carboxyl group in the catalytic process. In contrast to other known NADases, the snake venom enzyme did not self-inactivate.

The purified *B. fasciatus* venom NADase catalyzed a transglycosidation reaction (ADP-ribose transfer) with a number of acceptor molecules. The functioning of a variety of substituted pyridine bases as acceptor molecules was demonstrated through the formation of the corresponding NAD analogs. The enzyme also catalyzed the transfer of ADP-ribose to aliphatic alcohols (methanol to hexanol, inclusive) and

a positive chainlength effect was observed in the functioning of these acceptors. Kinetic studies of transglycosidation reactions were consistent with the partitioning of an enzyme-ADP-ribose intermediate between water and nucleophilic acceptors as has been proposed in earlier studies of mammalian NADases. The partitioning of this intermediate between water and pyridine bases can be correlated with the basicity of the ring nitrogen of the pyridine derivative. The K_i of pyridine bases in the hydrolytic reaction did not equate to the K_m of these bases in the pyridine base exchange reaction suggesting two forms of the NADase with varying affinity for the pyridine bases. This implys the pyridine base exchange reaction to be more complicated than originally proposed.