STUDIES ON NICOTINIC ACID MONO-NUCLEOTIDE PYROPHOSPHORYLASE

By

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STUDIES ON NICOTINIC ACID MONO-

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CHAPTER I

LITERATURE REVIEW

Discovery and Identification of the Pyridine Nucleotides

Early in the twentieth century, Harden and Young (1, 2) noticed an increase in the fermentation of sugar to alcohol and carbon dioxide after the addition of boiled yeast juice to fermenting yeast extracts. Upon dialysing the boiled juice, this effect was lost and it was concluded that whatever was effecting the fermentation increase passed through the dialysis bag into the dialysate. These investigators proceeded to study the factor or factors involved by ultracentrifugation, separating the yeast juice into a residue and a filtrate. Each component was unable to ferment glucose alone whereas when combined, a fermentation rate comparable with that of the original juice was obtained.

These experiments led to the conclusion that not only was a proteinaceous substance required for fermentation but also an additional factor which von Euler and Myrbäck (3) called "cozymase." This name was given because Büchner (4) held the theory that fermentation of yeast was caused by a single proteinaceous substance called "zymase." As noted in the preceding paragraph, "cozymase" is dialysable and thermostable.

When Meyerhof (5, 6) observed that "cozymase" of alcoholic fermentation was also present in milk and mammalian tissue, a greater

interest was stimulated in the physiological role of what was to be identified later as pyridine nucleotides. Furthermore, he observed that yeast "cozymase" activated lactic acid formation in muscle while at the same time "cozymase" of muscle activated "zymase" of yeast.

After many efforts to isolate and purify "cozymase," von Euler and Myrbäck (7) succeeded in obtaining a purified preparation. The purified preparation was found to contain adenine, phosphate, and an unidentified carbohydrate upon acid hydrolysis (8).

During this time, Warburg and Christian (9), studying the oxidation of glucose-6-phosphate in red blood cells, found that they were able to separate the components of this system into "ferment" which was a protein factor and a "coferment" a non-proteinaceous substance. "Coferment" yielded two bases upon acid hydrolysis, one of which was adenine (10). Carbohydrate and phosphate were also found in this hydrolysate. The second base was found to take up three moles of hydrogen when a picrolinate salt of the base was stirred in with finely divided palladium (11). Later they showed this base to be nicotinamide (12). Von Euler et al. (13) then found that "cozymase" also contained nicotinamide.

Although not the same, "cozymase" and "coferment" were very similar. "Coferment" could not substitute for "cozymase" in the apozymase system as shown by Warburg and Christian (10). Furthermore, "coferment" contained a higher phosphate content than "cozymase" and von Euler and Adler (14) noticed that partially pure "cozymase" was active in the alcohol dehydrogenase system but not in the glucose-6-phosphate dehydrogenase system. In addition von Euler and Adler (15) and Warburg and Christian (16) working independently, showed that

"cozymase" and "coferment" were both present in horse erythrocytes and that "coferment" could be selectively adsorbed on aluminum oxide.

Enough evidence had accumulated by this time to show that both "cozymase" and "coferment" contained to one mole of adenine (8), one mole of nicotinamide (12, 13), and two moles of carbohydrate at least one of which was ribose (17, 18). In addition, "cozymase" contained two moles of phosphate while "coferment" contained three.

As a result of the investigations of von Euler, Schlenk, and Warburg, the structure of "cozymase," later known as diphosphopyridine nucleotide (DPN) and now called nicotinamide adenine dinucleotide (NAD^+)[1], was established (Figure 1). Hydrolysis in dilute base at low temperature yielded nicotinamide, adenylic acid, and adenosine diphosphate (19), while at high temperatures adenosine diphosphate was the most abundant product. Upon acid hydrolysis, D-ribose-5-phosphate (17, 20) was isolated. When incubated with a "phosphatase" from almond press cake, nicotinamide ribose was obtained (21-23). The ribose was identified as D-ribose by formation of a derivative with p-bromophenylhydrazone (24).

"Coferment" or triphosphopyridine nucleotide (TPN) now called nicotinamide adenine dinucleotide phosphate ($NADP^+$) had already been shown to contain an additional phosphate over "cozymase" (25). It

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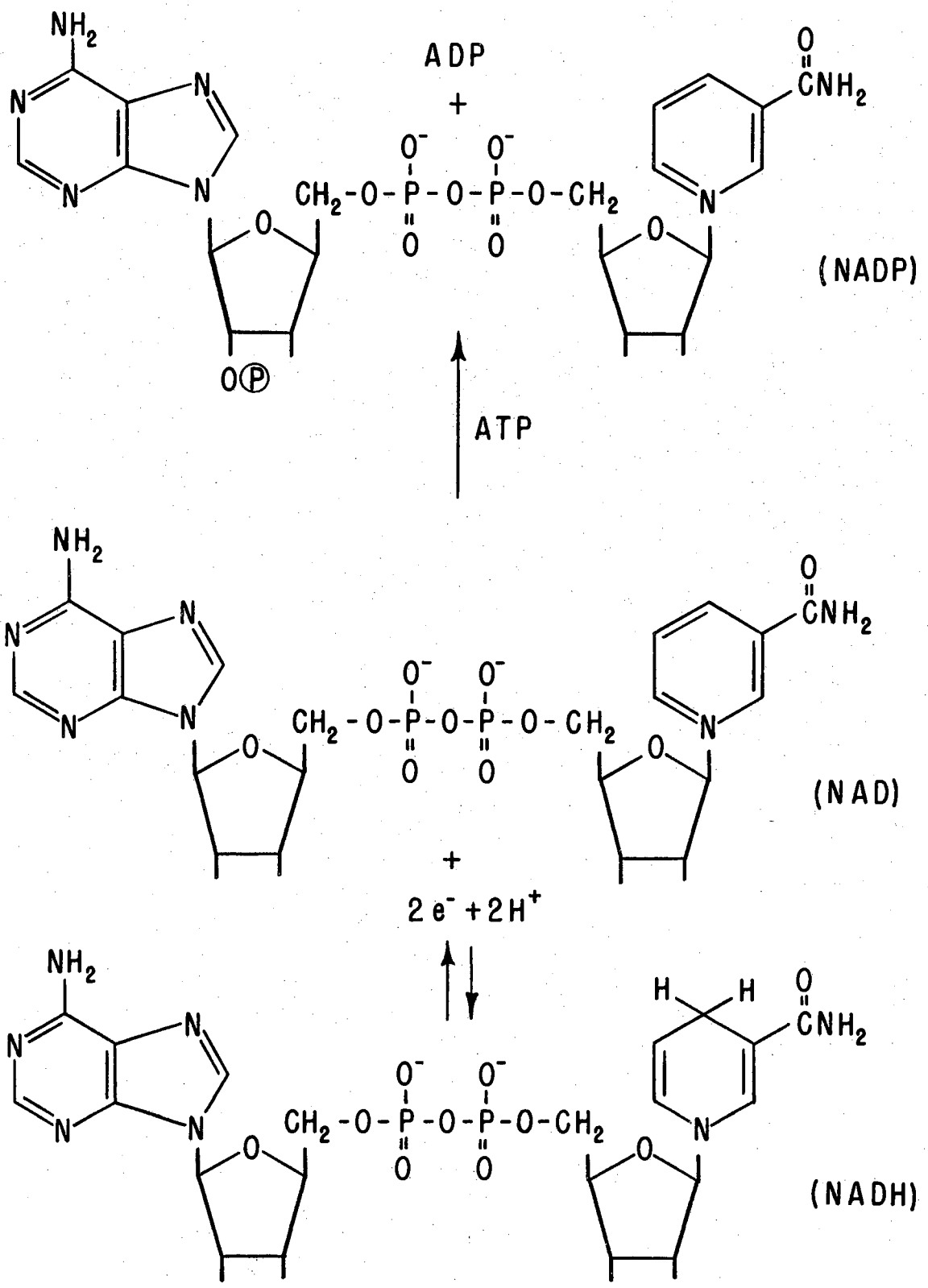
[1] All abbreviations used are those specified in the Journal of Biological Chemistry. For those not appearing in this journal, the following are used: NaMN, nicotinic acid mononucleotide; NAR, nicotinic acid ribose; des-NAD^+, desamido NAD^+; ARPPR, adenosine diphosphate ribose; $APNAD^+$, 3-acetylpyridine NAD^+; PRPP, 5-phosphoribosyl-1-pyrophosphate; NA, nicotinic acid; QA, quinolinic acid; ACTH, adrenocorticotropic hormone; POPOP, 1,4-bis-[2-(5-phenyloxazolyl)]-benzene; PPO, 2,5-diphenyloxazole; DEAE cellulose, diethylaminoethyl cellulose; ADP-methylene-P, adenyl diphosphonate (β,γ-methylene)-phosphate.

Figure 1.

Relationship Between NADH, NAD^+, and NADPH


ADP
+
(NADP)
ATP
(NAD)
+
2 e⁻ +2H⁺
(NADH)

could also be shown that "cozymase" was converted to $NADP^+$ in the presence of yeast extract and ATP (26) and although in a smaller yield, in the presence of phosphorus oxychloride (27), while $NADP^+$ could be converted to NAD^+ with a phosphatase preparation (28).

At first it was thought that the three phosphate groups were linked together in pyrophosphate linkages, but it was later found that one of the phosphates was not easily hydrolysed relative to the other two. Also ADP could be formed from NAD^+ whereas it could not be from $NADP^+$ (29). Therefore Schlenk suggested that the third phosphate might be attached to the second or third carbon atom of the ribose moiety of adenosine. In 1949, Cohn and Carter (30) showed that yeast adenylic acid could be separated into two factions, adenylic acid "a" and adenylic acid "b." Although it could not be determined for sure which compound was adenosine-3'-phosphate and which was adenosine-2'-phosphate, enzymatic degradation of $NADP^+$ produced an adenylic acid "a," and was different from adenosine-5'-phosphate, adenosine-3'-phosphate, or adenylic acid "b" (31, 32). The third phosphate was tentatively presumed to be attached to the second carbon atom of ribose and the validity of this assumption was proven unequivocally by Khym et al. (33). Dowex-50 hydrolyzed adenlyic acid "a" to the free base and the 2'-phosphate ribose ester which was immediately released from the resin, thus isomerization or exchange of the phosphate group to another carbon was improbable. This ribose phosphate consumed one mole of periodate when converted to methyl ribose pyranoside and had marked optical activity when converted to a ribitol. These findings would indicate that the phosphate was on carbon 2. The ribose phosphate from adenylic acid "b" had none of these properties, thus the phosphate was

on carbon-3. The structural formula of $NADP^+$ is also shown in Figure 1.

In addition to finding that nicotinamide was a component in the pyridine nucleotides, it was also observed that this moiety was actually involved in hydrogen transfer (18, 25, 34). Warburg et al. (25) demonstrated a stoichiometric relationship between the amount of glucose-6-phosphate and $NADP^+$ consumed and the amount of 6-phosphogluconic acid and NADPH produced in the presence of glucose-6-phosphate dehydrogenase which was free of the old yellow enzyme. Furthermore, in the presence of palladium, oxidized $NADP^+$ took up six moles of hydrogen while reduced $NADP^+$ took up only four. It was thus concluded that $NADP^+$ took up hydrogen during the oxidation of glucose-6-phosphate.

Warburg et al. (25) observed that $NADP^+$ exhibited an absorption band with a maximum at 340 mμ when reduced to NADPH, even when reduced chemically with $Na_2S_2O_4$. Similar results could be obtained with NAD^+ (35, 36). Other pyridinium compounds such as N'-methyl nicotinamide iodide (36), trigonelline (25), and tetraacetyl glucosidonicotinamide bromide (37) also could be observed to show changes in their absorption spectrum with a maximum at about 340 mμ. The latter two compounds released one mole of acid per mole of compound when reduced with dithionite (38). Thus, enzymatic and chemical reduction was concluded to proceed as shown in Figure 1.

Previous observations (39-41) gave indication that reduction of N'-methyl nicotinamide by $Na_2S_2O_4$ occurred at the ortho position. Colowick and coworkers (42, 43) later reduced NAD^+ chemically in D_2O and then enzymatically oxidized it by substrate. They found that when this NAD^+ was converted to 1-methyl-2-pyridone-3-carboxamide and 1-methyl-6-pyridone-3-carboxamide, the two derivatives contained equal

amounts of deuterium. They explained these observations by assuming that reduction occurred at the four or para position of the nicotinamide. Loewus et al. (44) confirmed Colowick's work and showed unequivocally that hydrogen transfer occurred at the para position by preparing 2, 4, and 6 deuterium labeled NAD^+. Only deuterium labeled in the 4 position transferred deuterium to pyruvate in the presence of lactic acid dehydrogenase. In addition to this evidence, two model compounds, 1-benzyl-dihydro-nicotinamide (45) and N'-methyl-nicotinamide (46) were shown to transfer hydrogen chemically from the para position.

In 1958, two pyridine nucleotides containing nicotinic acid were observed by Preiss and Handler (47, 48), to occur in human erythrocytes. These were identified as nicotinic acid mononucleotide and desamido NAD^+ by paper chromatography and chromatography over Dowex-1-formate. Des-NAD^+ was identified by comparing its chemical properties with the compound which Kaplan's group had chemically prepared earlier (49). NaMN was identified by observing its molar ratio of nicotinic acid, ribose, and phosphate in a ratio of 1:1.15:1.0, respectively. Yeast (50), mold (51), bacteria (52, 53), and plants (54) also contain NaMN.

Properties of the Pyridine Nucleotides

Several properties which are peculiar to the pyridine nucleotides were first observed during their isolation and characterization (10, 25, 55, 56). The oxidized coenzymes are labile in base but relatively stable in acid, while just the opposite is true for the reduced forms. Degradation at neutrality is primarily due to hydrolysis of the gly-

cosyl linkage between the pyridine and ribose moieties. This is shown by the fact that dilute alkali (0.1 to 1 N NaOH) at room temperature will hydrolyze NAD^+ to yield free nicotinamide much more readily than adenylic acid. In contrast, in strong base (5 N KOH) NAD^+ is degraded to yield a highly fluorescent substance or group of substances with an absorption maximum at 360 mμ (57), while only a small amount of free nicotinamide is given off under these circumstances.

The reduced forms of pyridine nucleotides are even more sensitive to acid degradation than are the oxidized to alkali. In 0.1 N acids at room temperature, both NADH and NADPH along with the reduced forms of N' substituted nicotinamide compounds instantly degrade to lose their fluorescence and simultaneously a hypochromic shift occurs in the 340 mμ band (10, 34, 52, 58, 59). The probable site of action of the hydronium ion is the nicotinamide ring where the primary product formed is slowly converted to another compound with a maximum absorption at about 290 mμ (60). This second reaction can be inhibited with bisulfite.

It was noted by Meyerhof and his coworkers (61) that bisulfite and cyanide form addition compounds with pyridine nucleotides which have absorption maximums at 320 and 325 mμ, respectively. It has since been found that this is a general reaction for all N' substituted pyridine compounds (62). The pyridine nucleotide can be easily regenerated from the bisulfite addition compounds with the addition of dilute alkali or merely by dilution with water. In spite of the fact that the NAD^+ addition compound closely resembles NADH in its absorption spectrum, it cannot replace NADH in enzymatic reactions.

Cyanide apparently attacks the para position of the pyridine ring

since it can be shown that a deuterium atom is acquired when the complex is formed in the presence of D_2O and this atom can be transferred in enzymatic reactions where NAD^+ is regenerated from the complex (63). Furthermore, decomposition of the complex in the presence of D_2O by reacting with dithionite to produce NADH, introduces a deuterium atom into NADH which also can be transferred in enzymatic reactions that reoxidize NADH to NAD^+ (64). Thus the pyridinium ring takes on an aldehydic nature and this can be further illustrated by reacting NAD^+ with carbonyl compounds (65) and hydroxylamine (66). Pyridine nucleotides containing nicotinic acid instead of nicotinamide also behave in this manner, each giving an absorption maximum of 315 mμ and 315 to 360 mμ, respectively.

Although much work has been done on the mechanism by which coenzymes function in biological oxidation reactions the detailed mechanism has yet to be explained. Vennesland and Westheimer (67) and Westheimer (68) and others have shown that a hydrogen is directly transferred (69) and that these transfers are stereospecific (70-72) by the use of deuterium. Even more interesting is the fact that some enzymes only transfer hydrogen from one side of the ring while other enzymes transfer hydrogen only from the other (73-76).

Using NADH and N'-benzyldihydronicotinamide to reduce thiobenzophenone as a model system for studying hydrogen ion transfer (77), a mechanism has been postulated from studies of reaction kinetics and solvent and isotope effects. This postulate involves a direct transfer of a hydride ion from the dihydro compound to the thioketone through an activated state. Another hypothesis includes a free radical but no evidence in so far as inhibiting the reaction with free radical traps

(45) have been shown, while in contrast, much data have been shown in the case of the hydride ion theory.

A destruction of NADH also occurs with triose phosphate dehydrogenase (78), giving a reaction product "NADH-X" with an absorption peak at 290 mμ and an altered optical rotation. "NADH-X" differs from the acid degradation product in its altered optical rotation. It has enzymatic activity in that it can be slowly converted to NADH by a yeast kinase (79). Data such as isotope and spectrophotometric studies give evidence that "NADH-X" is a hydrated product having one water molecule inserted into the pyridine ring (80, 81). The metabolic significance of these results cannot be assessed at this time.

Determination of the Pyridine Nucleotides

Following is a list of the principles by which pyridine nucleotides are assayed in biological systems:

1) Measurements of the rate of CO_2 production, dye decoloration, or oxygen uptake which is proportional with the amount of pyridine nucleotide present.

2) Microbiological assays.

3) Spectrophotometry.

4) Fluorimetry.

5) Chromatography and electrophoresis.

6) Radioactive isotopes.

7) Employment of a recycling technique in which the nucleotide undergoes a cyclic oxidation and reduction as a component member of a series of coupled reactions, and the extent of the reaction is measured by the accumulation of an end product.

The most common method used at first for determining pyridine nucleotides was that used by Myrbäck (8), who found that dried washed brewers yeast would glycolyze at a rate dependent upon "cozymase" present when catalytic amounts of hexose diphosphate and magnesium ions were added. The reaction was followed by evolution of CO_2 and activity was expressed as volume of CO_2 per unit time per weight of "cozymase" tested, since the molecular weight was not known at that time. Since then, the development of the Warburg apparatus and NAD^+ standards (82-84) and later further modifications have proven this technique to be quite sensitive (84). This reaction may also be followed by assessing dye decoloration using such dyes as methylene blue (85), 2,6-dichlorophenol indophenol, or cytochrome c (86, 87). More recently, this method has been used with specific dehydrogenases [alcohol dehydrogenase (alcohol:NAD oxidoreductase EC 1.1.1.1) for NAD^+ and glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase EC 1.1.1.49) for $NADP^+$] and a non specific dehydrogenase (diaphorase) and followed by measuring the rate of decoloration or coloration of 2,6-dichlorophenol-indophenol (88).

Microbiological assay procedures were used extensively in the early 1940's. These involved determining the growth rate of Hemophilus influenza or H. parainfluenza on limiting quantities of pyridine nucleotides (82-92). The main objection to this method is that they are very slow and non specific as to the pyridine nucleotides (93, 94).

Spectrophotometry is currently one of the most common methods for determining pyridine nucleotide concentration. This method, which measures absorption at 340 mμ upon reduction of the coenzymes (25), will show an optical density increment of 0.06 absorbancy units with

as little change in concentration as 0.01 mM. NAD^+ may be assayed with either yeast or beef liver alcohol dehydrogenase (95, 96), reduction being complete at pH 10 in the presence of excess substrate. NADH is determined with these enzymes at neutral pH (97). $NADP^+$ can be assayed using $NADP^+$ specific isocitrate dehydrogenase (L_s-isocitrate:NADP oxidoreductase EC 1.1.1.42) (98-100), yeast glucose-6-phosphate dehydrogenase, or pigeon liver malic enzyme (101), while NADPH is assayed with glutathione or cytochrome c reductase (NADP:glutathione reductase EC 1.6.4.2 and NADP:cytochrome c reductase EC 1.6.2.3 respectively) (87, 102). Des-NAD^+ and NaMN can be assayed by conversion to NAD^+ with their respective enzymes and then assaying for NAD^+ (48, 103). Chemically induced spectral changes can be used in place of biologically introduced changes using cyanide formation to detect nicotinamide ribose compounds (62). These compounds exhibit an absorption maximum at 325 mμ while the nicotinic acid analogs have a band at 315 mμ (47, 49).

Another method used for determination of pyridine nucleotides is the method of fluoremetry (54), which is sensitive enough to determine a concentration as low as 10^{-8} M (104). Thus since volumes as small as 0.01 ml may be used, as little as 10^{-13} moles of oxidized or reduced pyridine nucleotide may be detected. Two major objections to this method are that various foreign substances are known to influence fluorescence (104, 105) and the difficulty encountered in rate studies. However this latter difficulty has been eased somewhat by more sophisticated instrumentation (106). Fluorescence of the oxidized form is developed by strong base (6 N NaOH). The undesired form of the nucleotide is destroyed by changing the pH and then determination of the remaining form is performed. This method is not as sensitive for the

nicotinic acid analogs of the pyridine nucleotides (101).

Methods for separation of pyridine nucleotides by either ion exchange or paper chromatography are well known. Preiss and Handler (47, 48) were among the first to use such methods and found that seven parts of 95 percent ethanol and three parts ammonium acetate, pH 5.0, was particularly useful as a solvent system for separation on paper. As will be described later, n-butanol:glacial acetic acid:water in a ratio of 4:1:2 also proves to be a successful method for separation of the pyridine nucleotides.

Radioisotopes can also be used with conjunction of evolution of $^{14}CO_2$. Pastan and his coworkers (107) have described a method whereby 6-phosphoglucose dehydrogenase is used with ^{14}C-6-phosphogluconate and the amount of $^{14}CO_2$ produced is proportional to the $NADP^+$ concentration. If glutathione reductase and oxidized glutathione are also included, NADPH is oxidized to $NADP^+$, which is then measured by the same assay system. Sensitivity is in the range of 10^{-12} moles $NADP^+$ or NADPH.

The last method involves using two enzymes coupled together with all substrates of the respective reactions in excess except the nucleotide in question. Lowry *et al.* (108) were the first to use this technique. NADPH or NADP could be measured with the glutamic acid dehydrogenase (L-glutamate:NADP oxidoreductase EC 1.4.1.3) reaction coupled with the oxidation of glucose-6-phosphate to 6-phosphogluconate dehydrogenase. As mentioned previously all substrates and enzymes were present in excess except of course the nucleotides. Again the undesired nucleotide could be previously destroyed by adjusting the pH. After the reactions had been incubated several minutes in order to get an accumulation of 6-phosphogluconate proportional to the original

nucleotide present, the concentration of this product was determined using 6-phosphogluconate dehydrogenase in an excess of $NADP^+$ and measuring the fluorescence of NADPH. NAD^+ and NADH can be assessed the same way using NAD^+ glutamate dehydrogenase (L-glutamate:NAD oxidoreductase EC 1.4.1.2) forming glutamate from α-ketoglutarate coupled with lactate dehydrogenase to form pyruvate. After incubation to allow accumulation of pyruvate, the product is measured with lactate dehydrogenase in the presence of excess NADH and the production of NAD^+ is followed fluorometrically. Sensitivity as low as 10^{-9} M can be obtained if careful technique is used. Other coupled enzymes can be used in place of those described and seem to work just as well (109).

Biological Degradation of Pyridine Nucleotides

Pyridine nucleotide destruction in mammalian liver tissue was observed early according to the literature (2, 6, 105, 106), and also in other organisms including microorganisms (86, 107), castor beans (113), and sweet almond extracts (21, 114). However, the responsible enzymes were characterized only recently.

One of the first enzymes discovered was NAD^+ pyrophosphatase which splits the pyrophosphate bond between the two ribose moieties producing nicotinamide mononucleotide and adenylic acid from NAD^+ while NMN and 2',5'-diphosphoadenosine are the products of $NADP^+$ hydrolysis. This enzyme has been observed in potatoes (115), kidney (116), and sweet almonds (117), while Proteus vulgaris contains a "heat activated" enzyme which carries out the same reaction with NAD^+ and NADH (118).

Kidney and liver of rabbit and swine (119, 120) converts $NADP^+$ to NAD^+. This activity has also been observed with preparations of milk

alkaline phosphatase (121) and prostate phosphatase (122). Snake venom phosphatase along with the phosphatase of human or bull semen (123) and the 3' nucleotidase of barley (124) can attack the 3' and 5' nucleotides but not the third phosphate of $NADP^+$ attached to the ribose moiety of adenosine (32).

Takadiesterase contains a deaminase which converts the adenosine moiety to the inosinic acid analog of NAD^+ called deamino NAD^+ (122). While neither $NADP^+$ or 2'-adenylic acid is attacked by this enzyme, 3'-adenylic acid is, thus providing more evidence for a phosphate attached to the 2' carbon of ribose.

NAD^+ pyrophosphorylase catalyzes the reversible decomposition of NAD^+ to NMN and ATP (125). Preiss and Handler reported that this enzyme also catalyzes the reverse reaction yielding NAD^+ (48); however, they found that NaMN is a better substrate for the reverse reaction than is NMN.

The degradative enzyme which has been studied in greater detail is diphosphopyridine nucleotidase (NADase) which forms free nicotinamide from both $NADP^+$ and NAD^+ but not from nicotinamide riboside (126, 127). Kornberg reported that NMN is also split by this enzyme prepared from rat brain (116) and this has been confirmed by Leder and Handler (93). Spleen NADase, however, will not attack either NMN or nicotinamide riboside (128).

Although NADase is inhibited by a high concentration of nicotinamide (129), NAD^+ is not resynthesized from the split products even with a high concentration of nicotinamide or ARPPR, the other product of NAD^+ degradation. Therefore a simple mass law effect will not explain the inhibition of NADase by nicotinamide.

Neurospora NADase, although possessing the same specificity as spleen NADase, is not inhibited by nicotinamide (130). Furthermore, the enzyme is particularly abundant when the organism is grown on a zinc deficient medium (131).

It was found by Zatman and his coworkers (128) that nicotinamide could act as a noncompetitive inhibitor of NADase and furthermore, they observed nicotinamide-^{14}C could exchange with the nicotinamide moiety of NAD^+. This led to the theory that nicotinamide may be competing with H_2O for the ARPPR-enzyme complex and this theory was given additional support when it was found that analogs of nicotinamide could replace nicotinamide, forming analogs of NAD^+ (132). This phenomena does not occur with Neurospora NADase.

Since this time, many analogs have been prepared by the NADase exchange reaction (133-138) and many of these analogs have replaced NAD^+ in in vitro systems (134, 135, 139, 140). This does not mean that the nicotinamide moiety is without significance since most of these analogs are not as efficient as NAD^+ itself in these reactions. Des-NAD^+ and the 3-acetylpyridine analog are the two most widely known of these analogs. Des-NAD^+ has been shown to be an intermediate in NAD^+ biosynthesis (47, 48, 141) while $APNAD^+$ has been used in transhydrogenase assays (139, 142) and in the quantitative determination of glutamate (139).

A specific NADPase has also been observed in erythrocytes (143-145). The partial purification of both NADase and NADPase has been reported and it was observed that while nicotinic acid inhibits both enzymes from this tissue (129), nicotinic acid does not effectively inhibit NADase from other tissues (145).

The function of NADase in the organism is still unclear (146); however, because it releases free nicotinamide, it may be that this is part of a salvage mechanism for regulating the level of NAD^+. Nicotinamide deamidase has been found in a number of organisms, and recently this enzyme has been found in rat liver by Petrack et al. (147) who later showed that it may exist in vivo in an inhibited state (148). They concluded from this that deamination of nicotinamide is the first step in the biosynthesis of NAD^+ via the Preiss, Handler pathway (48). The significance of this will be discussed later.

Enzymatic cleavage of the riboside linkage has been reported in human and bovine erythrocytes (149, 150). While the enzyme is sensitive to nicotinamide in the crude state, partial purification of the enzyme relieves the sensitivity. The responsible factor for its sensitivity appears to be ergothionine (2-thiolhistidine) but the mechanism by which it functions has not been elucidated as yet. Ergothionine also produces sensitivity of Neurospora NADase promoting an exchange reaction of nicotinamide-^{14}C into NAD^+ (150).

Biosynthesis of Pyridine Nucleotides

The earliest indication of NAD^+ biosynthesis was noted by Harden (151), who observed that the rate of degradation of NAD^+ was much faster in the absence of active fermentation than in the presence of fermentation. Later Lennerstrand (152, 153) reported that "apozymase" destroyed NAD^+ when not supplemented with the requirements necessary for fermentation. In contrast, when nicotinamide, adenylic acid, inorganic phosphate, glucose, and hexose diphosphate were present with the "apozymase" preparation, partial resynthesis of NAD^+ could be ob-

served. As would be expected, synthesis was greatest when all were added simultaneously and when NAD^+ was added in addition to the components, the rate of destruction was reduced even more. Fluoride would completely inhibit synthesis of NAD^+ from either nicotinamide and adenylic acid or from nicotinamide and adenosine.

In 1937, nicotinic acid and nicotinamide were found to cure pellegra in man (154, 155) and black tongue in dog (156). Subsequently, many attempts were made to correlate the concentration of pyridine nucleotides and the physiological status in man and animals. With these studies, it was found that "cozymase," assayed as "V" factor, had a concentration level in liver and muscle, about 30 percent lower in those subjects which had the disease than in the normal subjects (157-159). On the contrary, Kohn and his coworkers (91) found that the nucleotide concentration in erythrocytes did not vary with the physiological state of the animal.

Although there were no significant differences in NAD^+ concentration in erythrocytes of animals possessing pellegra, it was found that when experimental animals ingested large amounts of nicotinic acid, an elevation of about two fold in the concentration of NAD^+ in this tissue was observed (160-164). This was confirmed with *in vitro* studies (162, 164) but little or no synthesis in erythrocytes was observed with nicotinamide. Examples of these data were that of Leifer *et al.* (165) who noted that when nicotinic acid-^{14}C was incubated with defibrinated human blood, radioactivity was incorporated into the cells which could not be washed out, while on the other hand, when nicotinamide-^{14}C was incubated with the same tissue, the radioactivity which was incorporated was easily washed out. In mouse erythrocytes, however, both compounds

gave rise to non-extractable radioactivity.

The first indication of formation of NAD^+ on the enzymatic level came from the laboratory of Kornberg (125, 166) in 1948 when he and his colleagues could show the production of NAD^+ and PPi with a yeast enzyme supplemented with ATP and NMN. The reaction is freely reversible and when NAD^+ and radioactive PPi are incubated with the enzyme, ATP takes up the radioactivity with the two terminal phosphate coming from the PPi (167). The NAD^+ molecule on the other hand, failed to take up any of the phosphate.

Leder and Handler (93) reported the accumulation of NMN when erythrocytes were incubated with large amounts of glucose and nicotinamide. When these data were compared to the above mentioned observation of the presence of a large amount of NAD^+ pyrophosphorylase and the reversibility of this enzyme was considered a possible pathway for NAD^+ biosynthesis was provided. Preiss and Handler (103) investigated this possibility and did indeed find a NMN phosphorylase present in human erythrocytes which formed NMN from nicotinamide and 5-phosphoribosyl-1-pyrophosphate. However, the K_m for nicotinamide was not physiological, being in the order of about 0.1 M. This high K_m taken along with the fact that erythrocytes obtain a low NAD^+ pyrophosphorylase activity (103, 168) and that erythrocytes are capable of synthesizing NAD^+ at low levels of nicotinic acid but not nicotinamide (164, 169) discouraged this idea and led to the possibility of an alternate pathway.

In a preliminary report (170) and later in a detailed publication (47, 48), Preiss and Handler elucidated the biosynthetic pathway of NAD^+ from nicotinic acid, establishing nicotinic acid mononucleotide

and desamido-NAD^+ as intermediates (Figure 2).

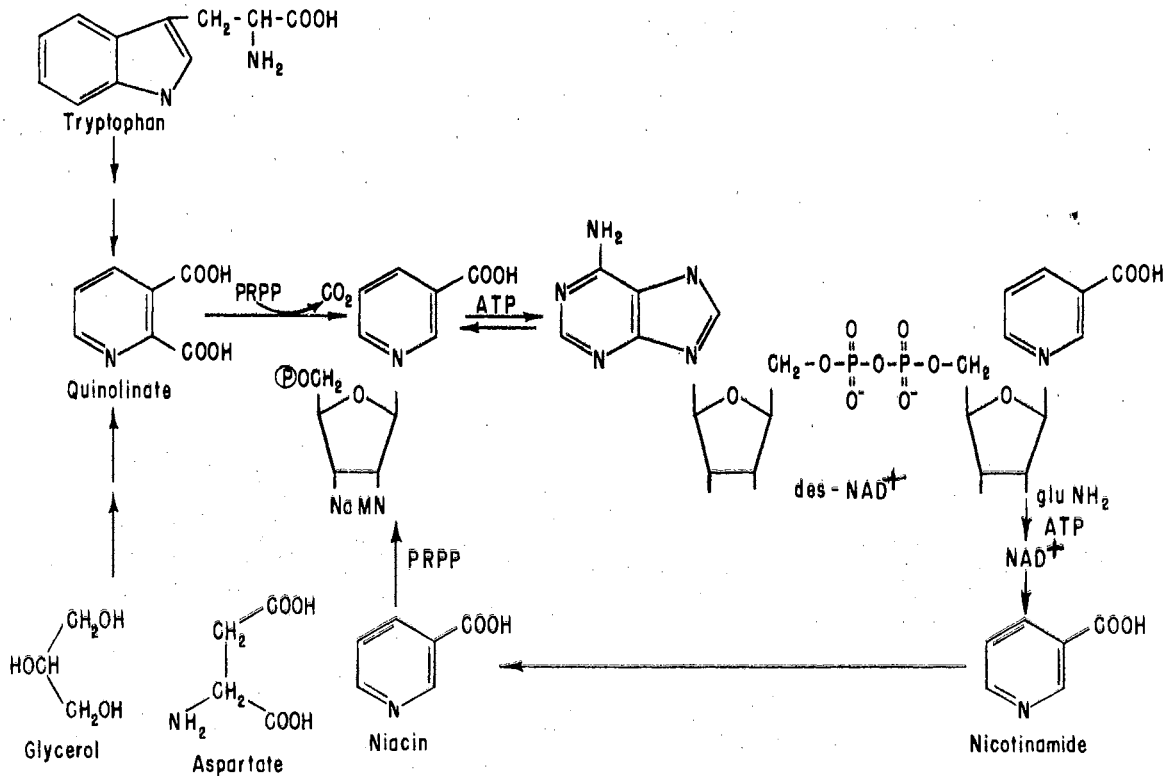
The enzyme NaMN pyrophorylase which catalyzes the formation of NaMN from nicotinic acid was partially purified from erythrocytes and later purified to a greater extent from beef liver acetone powder (171). It was found that the enzyme required nicotinic acid, PRPP, Mg^{++}, ATP and orthophosphate. This system of enzymes had every appearance of being the primary pathway for NAD^+ biosynthesis in that all of the responsible enzymes showed physiological K_ms and velocity. In addition this pathway seemed to be present in a number of organisms besides the mammalian system including E. coli (52), Mycobacterium (53) and yeast (47, 172). Thus for a matter of two or three years, the main pathway of biosynthesis of NAD^+ was thought to have been elucidated.

For several years tryptophan was known to substitute for niacin in the diet (173). Additional studies revealed that addition of tryptophan to the diet resulted in an increase in the excretion of N^1-methylnicotinamide in the urine (174, 175). A number of nutritional studies have shown that tryptophan can replace niacin in the rat, dog, rabbit, pig, monkey, chick, duck, and several other mammals and birds (176, 177). The studies with mutant strains of N. crassa and with rat have led to recognition of the fact that kynurenine (178), 3-hydroxykynurenine (179), and 3-hydroxyanthranilic acid (179-181) are intermediates in the conversion of tryptophan to niacin. Direct evidence for the metabolic relationship between tryptophan and niacin has come from isotope experiments in which labeled tryptophan or 3-hydroxyanthranilic acid was given to the rat and the niacin from the tissues and urine analyzed. The results indicated that position 3 of the indole ring becomes the carboxyl carbon of niacin (182-185) and that the

Figure 2.

Pathways of NAD^+ Biosynthesis From Tryptophan,

Nicotinic Acid, and Glycerol + Aspartate


Tryptophan
Quinolinate
PRPP
CO2
ATP
NaMN
PRPP
des - NAD+
glu NH2
ATP
NAD+
Glycerol
Aspartate
Niacin
Nicotinamide

nitrogen of the indole ring is converted to the pyridine ring nitrogen of niacin (185, 186).

In 1951, Preist et al. (187) reported that a soluble enzyme system from liver and kidney catalyzes the conversion of 3-hydroxyanthranilic acid to quinolinic acid. Several investigators subsequently showed that the primary product of oxygenation reaction was 2-amino-3-acroleyl-fumaric acid and the cyclization of the latter resulted in the formation of quinolinic acid (QA) (188-191). The latter reaction appeared to be a spontaneous reaction not requiring an enzyme. However, quinolinic acid had up until this time been considered a by-product of tryptophan metabolism rather than an intermediate in niacin biosynthesis since the compound is a very poor growth factor in the rat and Neurospora (192). In the several mammalian liver preparations studied by Mehler and May (193), picolinic carboxylase was the only enzyme found to react with 2-amino-3-acroleylfumaric acid. The reaction produced picolinic acid and did not result in the formation of niacin.

In 1963, Nishizuka and Hayashi (192) obtained a system from rat liver which converted 3-hydroxyanthranilic acid to niacin ribonucleotide (nicotinic acid mononucleotide) in the presence of PRPP. Evidence was presented that free nicotinic acid is not an intermediate in this pathway which is now known as the NAD^+ pathway of tryptophan metabolism (Figure 2). The niacin ribonucleotide produced is converted to des-NAD^+ in the presence of ATP by a pyrophosphorylase and the latter is then amidated to NAD^+ in the presence of glutamine and ATP by NAD^+ synthetase via the Preiss-Handler pathway (47, 48).

Subsequently, the same reaction was shown to take place in E. coli (194), a number of mycobacteria (195) and plants and fungi (194, 196).

Gholson et al. (197) were able to isolate the enzyme converting QA to NaMN proving that it was a separate enzyme from NaMN pyrophosphorylase. They also showed that the pathway had kinetic properties which allowed sufficient NAD^+ biosynthesis to account for the pyridine nucleotides needed by the body. In addition, it was found that this enzyme is about 20 times as active as the NaMN pyrophosphorylase and thus they concluded that this was the main pathway for NAD^+ biosynthesis.

While the above discussion reveals two pathways for NAD^+ biosynthesis, it is known that still another must exist. For example, Andreoli et al. (194) have shown the PRPP dependent formation of NaMN from quinolinate in E. coli and this reaction has also been shown to occur in plants (54). Since neither E. coli (198) nor plants (199) are able to convert tryptophan to niacin it must be concluded that in these organisms quinolinate arises by a mechanism other than the degradation of tryptophan.

The details of the third pathway for the synthesis of NAD via NaMN have not been elucidated as yet. Ortega and Brown (200) have shown that nicotinic acid could be synthesized in vivo in E. coli, from a 3-carbon compound (glycerol or a closely related compound) plus a 4-carbon dicarboxylic acid. Other studies with Bacillus subtilis (198), and M. tuberculosis (201) indicated that both aspartate and glycerol could serve as precursors of nicotinic acid in these organisms. Ahmad and Moat (202) presented evidence that under anaerobic conditions, yeasts incorporate radioactivity from uniformly labeled aspartate-^{14}C and glutamate-^{14}C suggesting that an alternative pathway other than the tryptophan pathway also operates in yeast under these conditions. Furthermore, evidence was shown which established quinolinic acid as a

precursor of des-NAD^+ under either aerobic or anaerobic conditions in this organism. Working with Clostridium butylicum, Isquith and Moat (203) found that crude extracts were able to incorporate ^{14}C from uniformly labeled aspartate and glycerol in the same manner as did growing cultures. However, upon fractionation of the extract, only aspartate, acetyl Co A, and formate gave rise to an increase in nicotinic acid production. Furthermore, quinolinate was shown to be intermediate in this pathway.

Chandler et al. (204) reported that cell free preparations of E. coli have been shown to convert aspartate-^{14}C and succinate-^{14}C to quinolinate-^{14}C. Degradation of the product formed from uniformly labeled aspartate-^{14}C showed a labeling pattern consistent with a direct incorporation of aspartate into carbons 2, 3, 7 and 8 of quinolinate. Variation of the concentration of a niacin requiring mutant of E. coli produced changes in the extent of incorporation of succinate-^{14}C into quinolinate-^{14}C by cell free preparations of this organism suggesting that the pathway of quinolinate biosynthesis is under repression control.

Thus, the pathway of niacin mononucleotide biosynthesis may proceed from at least three different routes. Under various circumstances the same organism may use one particular pathway although the potential for one or both of the other pathways may exist. It is seen, therefore, that the control and the level of each individual pathway is very complicated and the details to date remain obscure.

Regulation of the Biosynthesis of Pyridine Nucleotides

Regulation via ATP

Since there exist at least three routes for pyridine nucleotide synthesis, there should be some means of control whereby the different pathways are regulated. Imsande and Pardee (205) made one study of this regulation using E. coli. They found that a repression-derepression mechanism existed with the NA $\longrightarrow$ NaMN $\longrightarrow$ NAD^+ pathway. This conclusion was based on the finding that if the concentration of nicotinic acid is below 10^{-6} M, the activity of nicotinic acid mononucleotide pyrophosphorylase is increased over 5-fold. It was also concluded that this enzyme is rate limiting in the biosynthesis of NAD^+ as would be expected. When a culture of the organism which had been grown in the derepressed state was incubated with a high concentration of niacin the pyrophosphorylase activity reverted to that of the repressed state. Salmonella typherium and possibly Serratia marcesens, two organisms very similar genetically to E. coli, also exhibited this phenomenon. Contrastingly, this repression is apparently absent in most other organisms including yeast, tetrahymena, and the rat (206).

Adenosine triphosphate apparently plays some role in this regulation. In another study using Bacillus subtilis (207), it was found that ATP is the limiting substrate for nicotinic acid mononucleotide pyrophosphorylase. This was evidenced by the fact that while the apparent K_m for ATP was 4×10^{-4} M the intracellular concentration was in the neighborhood of $5 - 10 \times 10^{-5}$ M. Conditions that increased the intracellular concentration of ATP, while inhibiting all growth, resulted in no decrease in the rate of pyridine nucleotide formation. Although this constituted no proof of regulation of NAD^+ biosynthesis by ATP, it certainly suggested that ATP may be involved in this regulation.

Upon partial purification of the enzyme from B. subtilis (208) it was found that ATP could be replaced by other triphosphate nucleotides with GTP replacing ATP the best. Even tripolyphosphate substituted to some extent, being approximately 30 percent as efficient as ATP when added at high concentrations. When the enzyme was treated with urea, binding of ATP was inhibited, thus providing evidence that there is a separate binding site for ATP on the enzyme. It was also found that ATP is required for activity and that it is converted to ADP during the process of the reaction. A two step reaction involving an ADP or Pi and either of the two atoms which comprise the ribosyl-niacin bond must be ruled out since the pyrophosphate moiety of PRPP is of the α-configuration, whereas the ribosyl-nicotinamide bond of NAD^+ is of the β-configuration.

While considering the mechanism of control in the B. subtilis organism it should be noted that although ATP may play a significant role in regulation of NAD^+ biosynthesis from niacin, NAD^+ also arises via the QA to NAD^+ pathway. In this connection, Gholson and Kori (209) found that the synthesis of NaMN from QA is under a repression-derepression control in this organism.

Imsande and Handler (171) reported that ATP stimulates the activity of beef liver NaMN pyrophosphorylase. More recently Nakamura et al. (210) have suggested that ATP may play the role of an allosteric modifier of NaMN pyrophosphorylase. These workers reported that in the absence of ATP, NaMN pyrophosphorylase dissociates into subunits which have nicotinate-ribonucleosidase activity. These subunits reassociate in the presence of ATP to form NaMN pyrophosphorylase which has no ribonucleotidase activity. They concluded that this was a potent

mechanism for the regulation of NAD^+ biosynthesis. In the presence of ATP, NAD^+ would be synthesized from niacin and in the absence of ATP, the system would degrade NaMN to nicotinic acid by the ribonucleotidase activity. The study of this phenomenon is an important part of this thesis.

Effect of Hormones

In 1956 Kaplan et al. (211) found that when nicotinamide (250-500 mg/kg body weight) was injected into rats, a large increase in the concentration of hepatic pyridine nucleotides resulted. The increase is largely in the form of oxidized NAD^+ and is associated with a parallel rise of liver acid-soluble adenine, nicotinamide, ribose, and phosphate (212). No changes of concentrations of nucleic acids, or of 5'-adenylic acid, ADP and ATP have been detected. These results show that formation of the newly synthesized pyridine coenzyme does not occur at the expense of the various components already present in the liver. Greengard et al. (214) stated that in hypophysectomized rats this effect is more pronounced and lasts for a longer period of time. For example, 30 hours following nicotinamide administration, the level of NAD^+ in normal animals had returned to the endogenous level of about 450 µg NAD^+ per gm wet weight of tissue, whereas that in the hypophysectomized animals remained as high as 3000 µg/gm. Apart from the liver, only the kidney showed more than a doubling of NAD^+ content upon the administration of nicotinamide, but even this was not as pronounced. When the experiment was repeated with nicotinic acid or tryptophan, some increase in the level of NAD^+ occurred, but not nearly to the extent of the increase following nicotinamide administration.

Experiments were carried out to determine which of the known anterior pituitary hormones might be involved in this effect by testing their ability to reverse the effect of hypophysectomy on the change in the level of liver NAD^+ which occurs following nicotinamide administration. ACTH and cortisone acetate, reversed this effect to some extent. The response of the NAD^+ level to nicotinamide administration was also affected by adrenalectomy, but not as greatly as by hypophysectomy (215). These results suggested to the authors that this effect may be mediated through the pituitary-adrenal system. Additional evidence supporting the view that this is a fairly specific hormonal effect is that parathyroid extracts cause bone resorption but do not increase the nicotinamide coenzyme level following injection of nicotinamide (216).

The increase in the concentration of liver NAD^+ due to nicotinamide injections can be prolonged by the administration of chlorpromazine or reserpine as shown by Burton et al. (217). Both chlorpromazine and reserpine, as many authors have shown (218), have marked effects on the pituitary-adrenal axis. Greengard and Quinn (219) investigated this effect further. They confirmed earlier work that ACTH could reverse the effect of nicotinamide in hypophysectomized rats. Taking all of the evidence together, it seemed quite possible that the effect of chlorpromazine and reserpine on liver NAD^+ may be the result of these drugs causing a decreased pituitary activity. They found a paralleled correlation existed between the sedative action of these drugs and increased NAD^+ levels upon challenging with both nicotinamide and tranquilizer, thus further confirming that decreased pituitary activity is associated with NAD^+ biosynthesis.

Several types of tumors have been found to contain much lower levels of NAD^+ and other pyridine nucleotides than corresponding normal tissues (220-222). Shimoyama et al. (223) found that 3-hydroxyanthranilic acid oxidase activity is greatly reduced in tumor tissue. QA phosphoribosyl-transferase and NaMN pyrophosphorylase were also greatly reduced or absent in tumor cells. These results are in agreement with the work of Clark et al. (224) who found that treating with various carcinogens such as 4-methylamino-3-methylazobenzene and 4-dimethylamine-4'-fluoroazobenzene, even in precancerous conditions, causes a definite over all decrease in pyridine nucleotides. To date the mechanism of this phenomenon has not been worked out.

Pyridine Nucleotide Cycle

According to the Preiss Handler pathway (47, 48) nicotinic acid is an intermediate in NAD^+ biosynthesis. However, nicotinamide appears to be a much more efficient precursor of hepatic NAD^+ than nicotinic acid, when these two compounds are injected intraperitoneally (211, 212). Several workers (145, 225, 228) have investigated this apparent paradox. When nicotinamide was injected, the formation of des-NAD^+ was increased in conjunction with NAD^+ and then, as would be expected of an intermediate, the concentration of des-NAD^+ falls back to normal while that of NAD^+ remains high (141). Also, 45 minutes following intraperitoneal injection of radioactive nicotinamide, the specific activity of hepatic des-NAD^+ was approximately twice as high as NAD^+ itself.

Experimentation with low doses of nicotinic acid revealed that when nicotinic acid is injected at a dose level of 50 mg/kg body weight,

the amount of NAD^+ formed approaches that obtained from 500 mg/kg of nicotinamide and is substantially higher than the amount produced by 50 mg/kg of nicotinamide (226). Thus at doses lower than 50 mg/kg, nicotinic acid produces a greater synthesis of NAD^+ than nicotinamide, while at doses of 100 to 500 mg/kg the relative effectiveness of these two compounds is reversed. The accumulation of the des-NAD^+ in the nicotinic acid injected animals occurs only at a dose of 50 mg/kg, the maximal dose for NAD^+ biosynthesis, while in the animals given injections of nicotinamide, accumulation occurs only at doses of 200 mg/kg or above (141).

A study by Minard and Hahn (227) on the relative efficiency of precursors of NAD^+ as related to the rate of biosynthesis showed that with injections of the order of 50 mg/kg of nicotinic acid, nicotinamide, NaMN, and NAD^+ that NaMN gave the fastest rate of biosynthesis. Therefore, these authors suggested that the rate limiting step in this biosynthetic pathway is the formation of NaMN. Thus the evidence indicates that even though nicotinamide in high concentrations leads to a greatly increased biosynthesis of NAD^+, it appears that this synthesis occurs through the Preiss-Handler pathway (48).

Nicotinamide has a significantly longer half-life in hepatic tissue than nicotinic acid (227). When both compounds were injected at a concentration of 500 mg/kg, the nicotinamide disappeared from the liver with a half-life of 4.4 hours whereas the half-life of nicotinic acid was only 1.0 hour. This difference in rate of disappearance was also observed in whole blood. Excretion of nicotinamide-^{14}C was mainly in the unchanged form when these compounds were given at this high dosage level. However, when these compounds were injected at a low level,

five mg/kg, the radioactivity was excreted primarily in the form of metabolites. Moreover, the rate of excretion of radioactivity was approximately the same for both forms of the vitamin. Although the percentage of administered nicotinic acid and nicotinamide excreted as metabolites was lower with a 500 mg/kg dose than with a five mg/kg dose, the absolute amount of metabolites derived from the five mg/kg dose was lower.

It should be brought out in this connection that nicotinic acid at high concentration (225, 227), that is 500 mg/kg, either with nicotinamide or without, shows an inhibitory action on the biosynthesis of NAD^+. The mechanism of this inhibition is not understood. The short half-life of nicotinic acid in liver and the inhibition of NAD^+ biosynthesis by high concentrations of nicotinic acid may explain the relatively low efficiency of this compound as a precursor of NAD^+. Nicotinamide could slowly be deaminated by the liver nicotinamidase (147) keeping the concentration of the nicotinic acid low enough to prevent inhibition and allow NAD^+ biosynthesis to be carried on.

In contrast to the results discussed above, Ijichi et al. (228) found that following pulse-labeling with nicotinic acid-^{14}C, nicotinamide-^{14}C, or quinolinic acid-^{14}C, nicotinamide disappeared quite rapidly from the liver. For instance, when nicotinamide-^{14}C (78 μmoles) was injected into the portal vein of mice weighing 25 to 30 g, a large portion of the radioactivity disappeared rapidly from the liver during the first three minutes, and less than 10 percent of the total radioactivity in the liver appeared to increase slightly. In contrast, the radioactivity of injected nicotinic acid remained in the liver in much larger quantities. More than 20 percent of the total radioactivity

was recovered from the liver even after four hours. When quinolinic acid-^{14}C was injected by a similar procedure, less than 10 percent of the radioactivity was recovered in the liver even 20 seconds after the injection, showing that QA cannot penetrate the cell. When an analysis of the nicotinic acid-^{14}C metabolites was made, it was found that nicotinic acid-^{14}C disappeared quite rapidly in the liver. After three minutes less than five percent of the radioactivity was recovered as nicotinic acid-^{14}C, and by 10 minutes it had disappeared almost completely. The rapid disappearance of nicotinic acid-^{14}C was accompanied by the appearance of NaMN-^{14}C and des-NAD^+-^{14}C.

In contrast to nicotinic acid-^{14}C, there was a small but definite incorporation of nicotinamide-^{14}C immediately after the injection but there occurred no increase of NAD^+-^{14}C during the first 10 minute period. After several hours, NAD^+-^{14}C in the liver started to increase slightly. No other radioactive nicotinic acid derivatives accumulated to any appreciable extent.

As already noted, when small doses of nicotinic acid were injected, nicotinic acid is a better stimulator than nicotinamide in increasing the NAD^+ content of the liver while at large doses, the effect is reversed. These authors injected a large quantity of nicotinamide-^{14}C directly into the portal vein and then removed the livers and analyzed them at various time intervals. The radioactivity decreased from the liver rapidly during the first hour; however, an increase of radioactivity began after one or two hours which was associated with a net increase of NAD^+. This suggested that the nicotinamide was rapidly excreted from the liver except for the small amount that appeared to be transformed into NAD^+ <u>via</u> nicotinamide mononucleotide adenyl transferase,

and then returned to the liver after an interval of one or two hours presumably in the form in which it can be used in the synthesis of NAD^+.

Survey of the distribution of radioactivity after intraperitoneal injection of a large dose of nicotinamide-^{14}C showed that although there was a large proportion excreted in the urine, as much as 20 percent of the radioactivity was found in the gastrointestinal tract one hour after injection. The radioactivity in the tract then decreased to about one-third after four hours with a concomitant increase in liver NAD^+. Preliminary analysis showed that 90 percent of this radioactivity was located in the contents of the tract rather than in the stomach or intestinal wall. When nicotinamide was injected intraperitoneally, as much as 50 percent of the injected radioactivity accumulated in the tract. Furthermore, an increase of radioactive nicotinic acid was observed with a concomitant decrease of nicotinamide-^{14}C during the four hours after injection.

These authors suggest, in view of these experiments, that nicotinamide is rapidly excreted from the liver and is sent to the gastrointestinal tract where it is deaminated to nicotinic acid. The nicotinic acid formed is returned to the liver where it is used in the biosynthesis of NAD^+.

Which, if either, of the two theories mentioned above is correct remains to be seen. It should be pointed out that the liver nicotinamidase has a high K_m but this would still release enough nicotinic acid to allow maximum NAD^+ biosynthesis and at the same time keep the concentration low enough to prevent the inhibition that is associated with a high concentration of nicotinic acid.

Considering these facts and the following information Gholson (229)

proposed a pyridine nucleotide cycle (Figure 2). This theory is based on the following facts. Tryptophan serves as the de novo precursor of QA in mammals (193, 197) and a number of other organisms. In higher plants (230) and some bacterial species, QA is synthesized from glycerol and asparate (34, 194, 198-204) by an unknown series of reactions. Once at the level of NaMN, the biosynthesis pathway appears to be via the so called Preiss-Handler pathway (48). NAD^+ is split by a number of enzymes, the most common being the glycohydrolase type which splits the NAD^+ molecule into nicotinamide and ARPPR. Nicotinamide released by NADase activity may be reutilized by deamidation to nicotinic acid (147). Greengard et al. have reported that nicotinamide deamidase is in an inhibited state in vivo and upon hypophysectomy, the activity of this enzyme is greatly elevated (231). If the effect of hypophysectomy on NAD^+ metabolism is to increase nicotinamide deamidase activity, then an increase in the activity of this cycle could explain the fact that synthesis of NAD^+ from nicotinic acid and tryptophan is also increased by hypophysectomy.

Other evidence for the existence of this cycle is provided by the observation that when nicotinamide-^{14}C-^{15}N is injected into rats, the carboxyl group is almost undiluted while there is considerable dilution of the amide nitrogen in hepatic NAD^+ (232). Data consistent with the operation of this cycle in plants have been reported by Waller et al. (233).

Since the regulation of the various pathways of NAD^+ biosynthesis appears to be varied and complex, this thesis will deal with a small phase of this topic. The enzyme, nicotinic acid mononucleotide pyrophosphorylase, is believed to be one of the regulatory sites of NAD^+

biosynthesis from nicotinic acid (205-208, 210). Therefore, this thesis will describe work which shows that this enzyme displays unusual properties allowing it to be a controlling site in this pathway.

CHAPTER II

EXPERIMENTAL PROCEDURES

Methods

The assay for NaMN pyrophosphorylase activity was a modification of the system of Imsande and Handler (171). The standard incubation mixture included in a total volume of 0.15 ml the following: nicotinic acid, 0.067 mM; PRPP, 1.33 mM; ATP, 0.667 mM; $MgCl_2$, 3.33 mM; phosphate buffer, pH 7.5, 26.6 mM; and approximately 0.25 units of enzyme. When assays of crude extracts were carried out, 0.4 µmole of NaF was included in order to inhibit ATPase activity. The incubation mixtures were then allowed to react for two hours at 37° C and were deproteinized by heating in a boiling water bath for one minute. A 50 µl aliquot was then spotted on Whatman #1 chromatography paper and developed in a descending manner for three to four hours in n-butanol:glacial acetic acid:water (4:1:2 v/v/v). The product is well separated from nicotinic acid with this system and stays on or close to the origin; therefore, the area surrounding the origin was routinely cut out, put in a scintillation solution containing four g PPO and 0.2 g POPOP per 1 of sulfur-free toluene, and counted on a Packard Instrument Company tri-carb scintillation spectrophotometer for a minimum of five minutes. A unit of enzyme activity is that amount which will catalyze the formation of one mµmole of NaMN per hour. Protein was determined by the Lowry modification of the Folin-Ciocalteau procedure using bovine

serum albumin as a standard (234).

NaMN was identified by comparing its R_f with that of known NaMN in two different solvent systems: one M ammonium acetate:95 percent ethanol (3:7 v/v) adjusted to pH 5.0 with HCl, and the previously mentioned system of n-butanol:acetic acid:water. When the chromatograms were developed in the latter system for 14-16 hours, the NaMN migrated off the origin with an R_f of 0.17. This system quite easily separated NaMN and des-NAD^+ which had an R_f of 0.05.

In order to determine the amount of ATP converted to ADP, a slight modification of the normal assay was used. In a total volume of 0.75 ml, the following were added: nicotinic acid-^{14}C, 0.0067 mM; $MgCl_2$, 3.33 mM; phosphate buffer, pH 7.5, 26.6 mM; ATP-^{14}C, 0.21 mM; PRPP, 0.27 mM; and approximately three to four units of enzyme. The system was allowed to incubate two hours at 37° C after which time the reaction was stopped by immediately freezing in dry ice. The products were separated by placing the contents of the incubation mixture, after diluting to 50 ml, over a Dowex-1-formate column 0.5 x 7 cm^2. Non-radioactive carrier nicotinic acid and ADP (one and 0.5 umoles, respectively) were added in order to detect them as they were eluted off of the column by reading the absorbance at 260 mμ. The column was then eluted with six ml of 0.1 N HCOOH, eight ml of 0.25 N HCOOH, 12 ml of 1.0 N HCOOH, and finally with 30 ml of 4.0 N HCOOH. Using this procedure, it was possible to determine both the amount of NaMN formed and the amount of ATP converted to ADP simultaneously. After the products were separated the fractions (one ml each) were collected and aliquots were spotted on paper and counted on a Packard tri-carb scintillation counter as described above.

The purification of NaMN pyrophosphorylase was also a slight modification of Imsande and Handler's system (171). The purification procedure was as follows:

Step 1. One hundred and twenty g of beef liver acetone powder was extracted at room temperature with 1200 ml of phosphate buffer, 0.05 M, pH 7.5 for 30 minutes. The suspension was then centrifuged at 10,000 x g for 20 minutes and the precipitate discarded. All subsequent steps were performed at 0-4° C.

Step 2. Ammonium sulfate (238 g) was added to 1020 ml of the supernatant from the preceding step. This is approximately a 0-40 percent fraction. The precipitate was collected by centrifugation at 10,000 x g for 10 minutes and then taken up in 800 ml of 0.01 M phosphate buffer pH 7.5 containing 10^{-2} M mercaptoethanol. It was then dialyzed for four hours against 20 l of water. The supernatant from the ammonium sulphate fractionation was discarded.

Step 3. Sixteen ml of 0.4 N HCl was added to 830 ml of the dialyzed solution to bring the pH down to approximately 5.3. After stirring for one hour, the precipitate was centrifuged down and discarded. The supernatant was then adjusted to pH 7.5 with 8.5 ml of one N KOH.

Step 4. One hundred and eighty ml of calcium phosphate gel suspension (30 mg/ml) was then added to the 820 ml of step 3. After stirring for 15 minutes, the suspension was centrifuged and the precipitate discarded.

Beyond this step the enzyme became unstable; therefore, the preparation was routinely stored at -20° C at this step. When assays were performed, small aliquots were taken and further purified. A summary

of purification through this step is shown in Table I.

Step 5. A 25 ml aliquot of step 4 enzyme was dialyzed for seven hours against four l of water. It was then placed on a DEAE cellulose column 1.8 x 12 cm^2 which had been previously equilibrated in 0.015 M phosphate buffer pH 7.5. The enzyme was subsequently eluted from the column with a linear gradient consisting of two reservoirs containing 130 ml each of 0.015 M and 0.05 M phosphate buffer pH 7.5, respectively. After the gradient was completed, an additional 100 ml of the 0.05 M buffer was passed through in order to elute all of the enzyme off of the column. All buffers contained 10^{-2} M mercaptoethanol.

The fractions containing the enzyme were then combined and the enzyme was concentrated by adding ammonium sulfate (0-80 percent saturation), centrifuged and taken up in two ml of 0.001 M phosphate buffer, pH 7.5.

Step 6. To each mg of protein obtained in step 5, three mg of alumina C_γ was added and the mixture stirred for ten minutes. After centrifugation the supernatant was discarded. Two ml of phosphate buffer 0.05 M, pH 7.5 containing 10^{-2} M mercaptoethanol, were then added to the precipitate and after an additional ten minutes of stirring the suspension was again centrifuged. Finally to the precipitate from this centrifugation was added one ml of 0.1 M phosphate buffer pH 7.5 containing 10^{-2} M mercaptoethanol, and after ten minutes of extraction the suspension was centrifuged and the gel discarded. The final specific activity varied with each preparation but normally fell in the range of 50-80 units per mg protein.

TABLE I

SUMMARY OF PURIFICATION THROUGH THE CALCIUM PHOSPHATE STEP

	Volume (ml)	Protein (mg)	Units (total)	$\frac{U}{mg}$	Percent Yield
Crude extract	1,020	42,432	61,812	1.46	
Ammonium sulfate	830	15,272	62,650	4.10	100
pH 5.3	820	5,945	56,170	9.45	92
Ca P	930	5,440	70,680	13.00	100

Materials

Nicotinic acid-7-^{14}C obtained from Calbiochem was routinely purified before use by applying it on Whatman #1 chromatography paper and developing in n-butanol:acetic acid:water. The radioactive nicotinic acid was then eluted off the water and subsequently used in enzyme assays.

The DEAE cellulose was routinely washed before use. The cycle used was essentially that given by Peterson and Sober (235). After washing, the resin was equilibrated in 0.015 M phosphate buffer, pH 7.5 containing 10^{-2} M mercaptoethanol.

Dowex-1-formate was regenerated by washing with several volumes of one N HCl and then with four M ammonium formate until no more chloride ion could be detected coming off of the column. Finally the column was flushed thoroughly with deionized water before use.

NaMN-7-^{14}C was prepared in the following way. Eight ml of partially purified erythrocyte acetone powder extract was incubated seven hours at 37° C with the following in a total volume of 17 ml: nicotinic acid-^{14}C, 0.6 mM; $MgCl_2$, 8.1 mM; phosphate buffer pH 7.5, 3.2 mM; ribose-5-phosphate, 2.7 mM; ATP, 3.1 mM. The reaction was stopped and deproteinized by heating in boiling water for two minutes. After centrifugation, the supernatant was applied to Whatman #1 chromatography paper and developed in the ammonium acetate:ethanol solvent mentioned above. After determining the location of the radioactive NaMN by a Nuclear Chicago Strip Scanner, the area was cut out and the NaMN-7-^{14}C eluted with water. After diluting this solution to 500 ml with water, it was applied to a Dowex-1-formate column (1.7 x 30 cm^2) and eluted in a stepwise manner with 0.1 N HCOOH, 190 ml; 0.25 N HCOOH,

560 ml; 1.0 N HCOOH, 370 ml. The NaMN-7-^{14}C which was eluted in the 1.0 N acid was concentrated to dryness by lyophilization. After taking up in a minimum volume of water, the NaMN-7-^{14}C was again applied to Whatman #1 chromatography paper and developed this time in butanol: acetic acid:water (4:1:2 v/v/v). The NaMN-7-^{14}C was then eluted and was by this time essentially free of all other radioactive components. After purification, it was then incubated with phosphodiesterase and acid phosphatase. Phosphodiesterase would not react thus ruling out the possibility of it being des-NAD$^+$, while acid phosphatase reacted with it producing nicotinic acid ribose-7-^{14}C. This plus the fact that its R_f compared favorably with that reported in the literature (192) proved that it was NaMN-7-^{14}C.

ATP-8-^{14}C, obtained from Nuclear Chicago Co., was purified before use in the ATP splitting experiment No. 4, Table V by ion exchange chromatography on DEAE cellulose. The DEAE cellulose was washed in acid and base as described by Peterson and Sober (235) and then equilibrated in two to three volumes of one M acetic acid-triethylamine pH 5.0. After equilibration the resin was filtered and washed two to three times with deionized water and finally suspended in water. The ATP-8-^{14}C was then applied to a column 1.1 x 7 cm^2 and eluted with a linear gradient consisting of 100 ml each of 0.5 and 1.0 M acetic acid-triethylamine pH 5.0, respectively. This system quite easily separated ADP and AMP from ATP. The fractions containing ATP were then lypholyzed, taken up in methanol and dried down five to six times in order to free the ATP of any remaining triethylamine. The ATP-8-^{14}C was then taken up in one ml of water and used in the ATP splitting experiment.

The enzymes used for identification of NaMN were the following:

potato acid phosphatase which was dissolved in a solution of five mg/ml in water and then assayed in the presence of 0.1 N acetate buffer pH 4.8 for two hours, and phosphodiesterase type II from Crotalus adamanteus venom which was used at pH 7.5. Both enzymes were obtained from Sigma Chemical Co.

Chemicals

PRPP, AMP, ADP, and ATP were obtained from P-L Biochemical Inc. Nicotinic acid-7-^{14}C was obtained from Calbiochem Laboratories, and ATP-8-^{14}C from Nuclear Chicago Corporation. GTP, UTP, CTP, ITP, and TTP were from Sigma Chemical Co. ADP-methylene-P was obtained from Miles Laboratories Inc. DEAE cellulose and Dowex-1-chloride were purchased from Bio-Rad Laboratories, and Sephadex-G-100 from Pharmacia Fine Chemicals, Inc. All other chemicals used were of reagent grade.

CHAPTER III

RESULTS

Time Course of Reaction

The standard reaction mixture, as described in the experimental procedures section, was incubated with 0.01 ml of the enzyme, purified through the alumina C_γ step, at 37° C, and the reaction stopped at different time intervals by heating for two to three minutes in a boiling water bath in order to determine the time course of the reaction. As shown in Figure 3, formation of NaMN appears to be linear with time until two and one-half hours, at which time the increase in velocity approaches a plateau. Routine assays were subsequently run for two hours.

Effect of Protein Concentration on the Reaction

The standard reaction mixture was incubated with various levels of enzyme purified through the alumina C_γ step, for two hours at 37° C. The reaction is linear with protein concentration up to 15-20 µg of protein per 0.15 ml total volume (Figure 4). Normal assays therefore contained enough enzyme to give 0.1 to 0.5 mµmole NaMN per hour depending upon the preparation.

pH Optimum

The dialyzed calcium phosphate gel enzyme was used to determine

Figure 3.

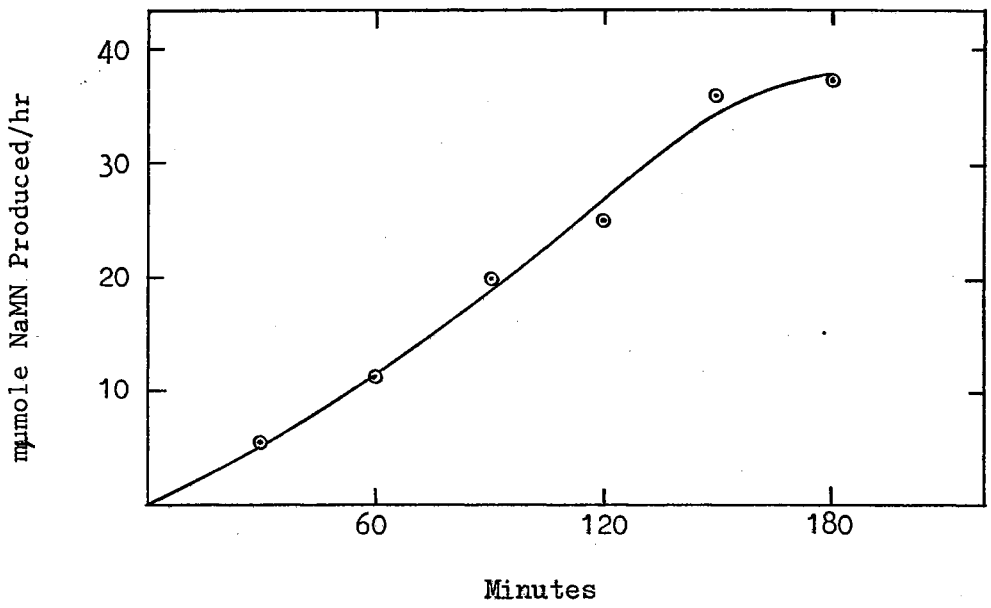
Time Course of Reaction

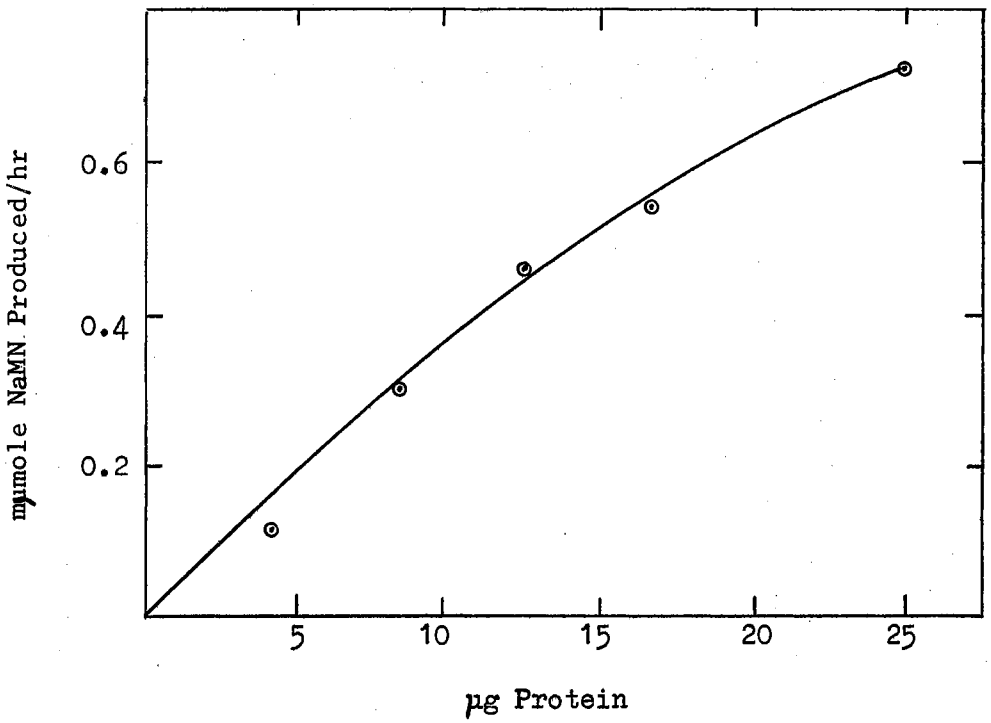
Incubation mixtures containing a final concentration of nicotinic acid-7-^{14}C, 0.067 mM; PRPP, 1.33 mM; ATP, 0.667 mM; $MgCl_2$, 3.33 mM; phosphate buffer, pH 7.5, 26.6 mM; and 0.25 units of enzyme in a final volume of 0.15 ml were allowed to react for the various time intervals indicated at 37° C and then assayed for the amount of NaMN formed as described under experimental procedures.

Figure 4.

Velocity Versus Protein Concentration

Incubation mixtures as described under Figure 3 were incubated for two hours at 37° C with various levels of enzyme purified through the alumina C_γ step. The incubations were then stopped and assayed for the amount of NaMN formed.


mμmole NaMN Produced/hr
40
30
20
10
60
120
180
Minutes


mμmole NaMN Produced/hr
0.6
0.4
0.2
5
10
15
20
25
μg Protein

the pH optimum. This was used since the alumina C_γ enzyme was in 0.1 M phosphate buffer pH 7.5. This buffer was too concentrated to allow a wide variable range of pH even when other buffers were present in four-fold excess. The dialyzed enzyme was therefore incubated for two hours at 37° C in the normal way with the exception that the pH of the phosphate buffer was varied. As can be seen a fairly normal pH curve is obtained with an optimum at pH 7.5 (Figure 5).

Determination of Niacin Ribonucleotidase Activity

It has been mentioned previously that Nakamura *et al.* (210) reported the conversion of NaMN pyrophosphorylase to niacin ribonucleotidase in the absence of ATP. When NaMN-7-^{14}C (eight mμmole) was incubated with the purified NaMN pyrophosphorylase in the presence of $MgCl_2$ and phosphate buffer, pH 7.5 for two hours at 37° C, there was no apparent conversion to nicotinic acid ribose-^{14}C or nicotinic acid-^{14}C (Table II). When the same compounds were incubated with the crude extract of acetone powder under similar conditions, there was considerable ribonucleotidase activity present. Thus the protein responsible for the conversion of niacin to NaMN appears to be separable from the protein which catalyzes the dephosphorylation of NaMN. Results reported here are in contrast with those reported by Nakamura *et al.* (210) and will be discussed in more detail later.

Velocity Versus Nicotinic Acid Concentration

Imsande and Handler (171) reported the stimulation of NaMN pyrophosphorylase activity by ATP. A study was made of this phenomenon and the results are shown in Figure 6. In this experiment, the purified

Figure 5.

Effect of pH on the Reaction

Reaction mixtures as described under Figure 3, using phosphate buffers with different pH values, were incubated under standard conditions for two hours with 0.27 units of enzyme purified through the calcium phosphate gel step. The amount of NaMN produced was then determined as described under experimental procedures.

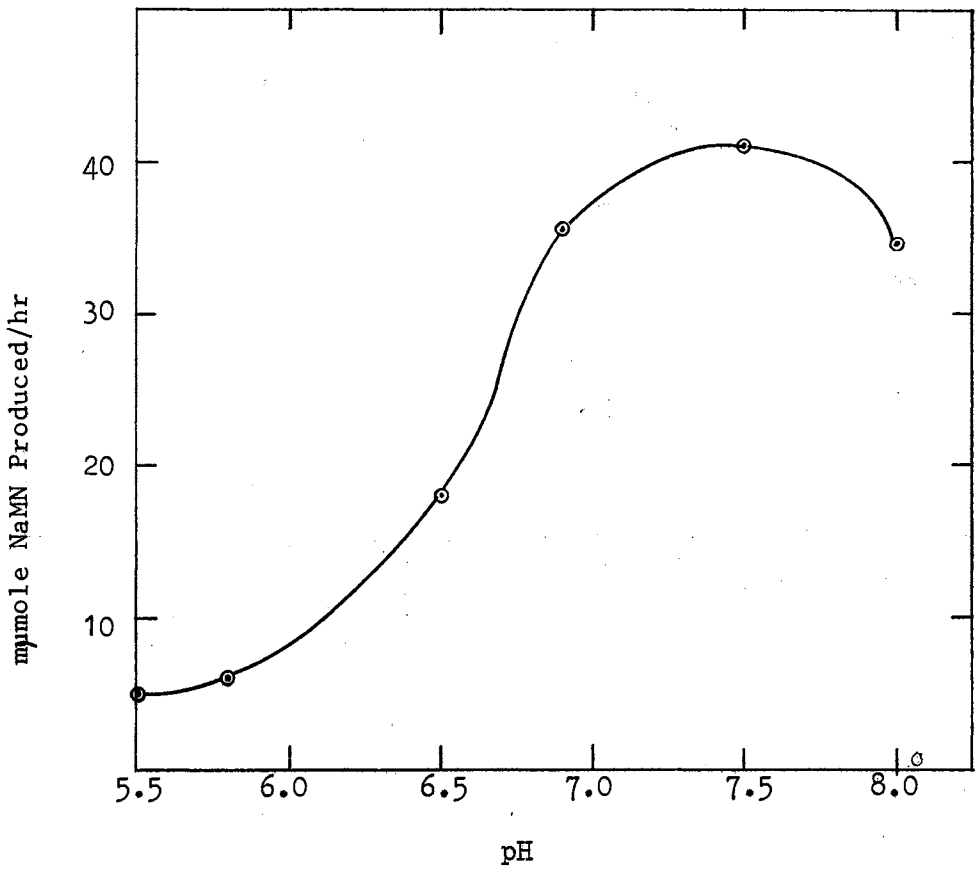

mμmole NaMN Produced/hr
40
30
20
10
5.5
6.0
6.5
7.0
7.5
8.0
pH

TABLE II

SEPARATION OF NaMN-PYROPHOSPHORYLASE AND -PHOSPHATIDASE

NaMN Pyrophosphorylase (mμmole/hr)				
	NaMN	des-NAD^+	Total	Crude / Pur.
Crude	19.0	63	82.0	
Purified	35.5	0	35.5	2.3

NaMN Phosphatidase (mμmoles/hr)				
	NA	NAR	Total	Crude / Pur.
Crude	210.0	107	317.0	
Purified	2.2	0	2.2	143

enzyme was incubated for two hours at 37° C with increasing concentrations of nicotinic acid-^{14}C both in the presence and absence of 6.67 x 10^{-4} M ATP. (The concentration of PRPP and Mg^{++} were as described under Figure 3.) As can be seen, at low concentrations of nicotinic acid, ATP shows a marked stimulatory effect, i.e., at 6.7 x 10^{-6} M a five-fold stimulation occurs. However, at high concentrations of substrate, ATP apparently has no effect upon the initial velocity of the reaction. When a Lineweaver-Burk plot was made, it was found that ATP lowers the apparent K_m for nicotinic acid from 2.1 x 10^{-5} M to 2.5 x 10^{-6} M. Thus ATP definitely appears to stimulate pyrophosphorylase activity at low concentrations of nicotinic acid. (See Figure 7.)

In order to be sure that there was no binding of ATP to the enzyme, the purified enzyme was further treated with charcoal (one mg/ml). After treatment, the enzyme was again incubated as before with increasing concentrations of nicotinic acid-^{14}C both plus and minus ATP. Table III shows essentially the same results as described above. PRPP was essentially free of ATP and nicotinic acid-^{14}C is routinely purified before use so that no ATP is present in this material. Therefore, unless the ATP is so tightly bound to the enzyme that treatment with charcoal cannot remove it, the reaction can proceed without ATP as long as the concentrations of the substrates are high enough.

Velocity Versus PRPP Concentration

Since ATP stimulated pyrophosphorylase activity at low concentrations of nicotinic acid, it was of interest to investigate the effect of ATP at low concentrations of PRPP. As shown in Figure 8, ATP, 6.67 x 10^{-4} M, causes an even more striking stimulation under these conditions.

Figure 6.

Velocity of Reaction Versus Nicotinic Acid Concentration

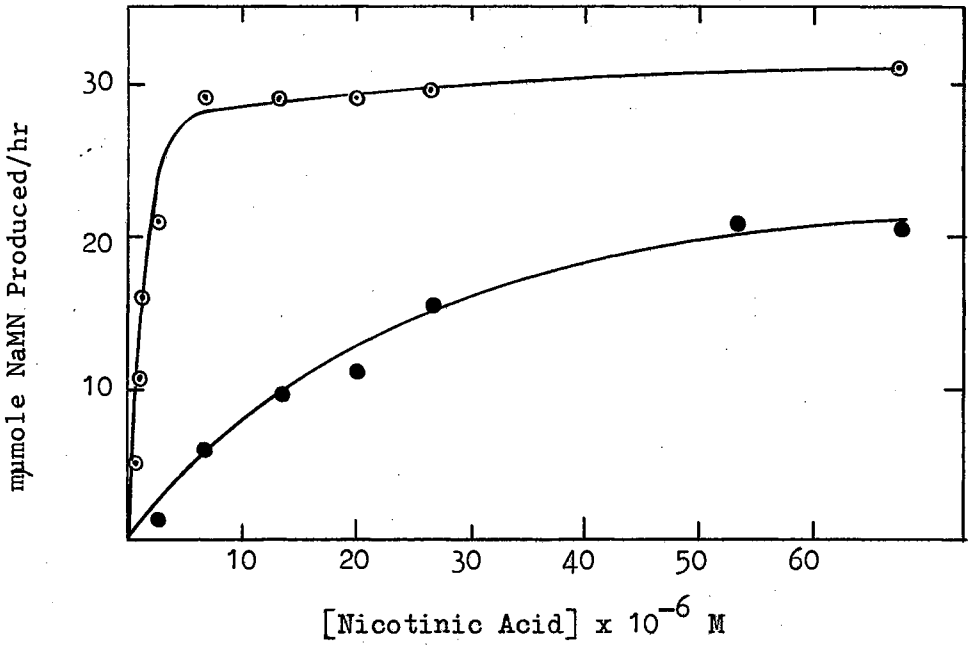
Standard reaction mixtures as described under Figure 3 with various levels of nicotinic acid-^{14}C concentrations were incubated with 0.27 units of enzyme for two hours at 37° C and then assayed for the amount of NaMN formed in the usual manner.

o = + 6.67 x 10^{-4} M ATP; ● = - ATP.

Figure 7.

Lineweaver-Burk Plot of Reaction Velocity Versus Nicotinic Acid Concentration

o = + 6.67 x 10^{-4} M ATP; ● = - ATP.


mμmole NaMN Produced/hr
30
20
10
10
20
30
40
50
60
[Nicotinic Acid] x 10⁻⁶ M

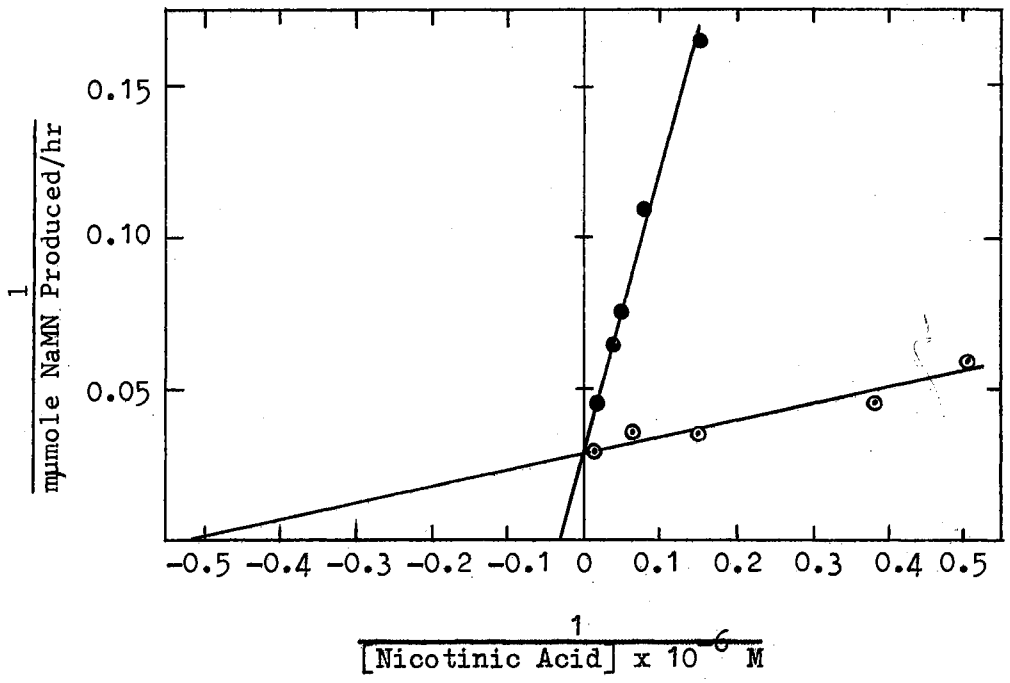

1 / mμmole NaMN Produced/hr
0.15
0.10
0.05
-0.5 -0.4 -0.3 -0.2 -0.1 0 0.1 0.2 0.3 0.4 0.5
1 / [Nicotinic Acid] x 10⁻⁶ M

TABLE III

VELOCITY OF REACTION VERSUS NICOTINIC ACID CONCENTRATION WITH CHARCOAL TREATED ENZYME

Nicotinic Acid	mμmole/hr of NaMN Produced	
μM	+ATP	-ATP
6.7	25±4.5[1]	12±3
19.8	31±0	19±2
66.0	27±5[2]	37±1
193.0	40±1	46±1

[1](±) refers to range of experiment

[2]This figure unusually low and when repeated experiment, did not observe this low figure.

Figure 8.

Velocity of Reaction Versus PRPP Concentrations

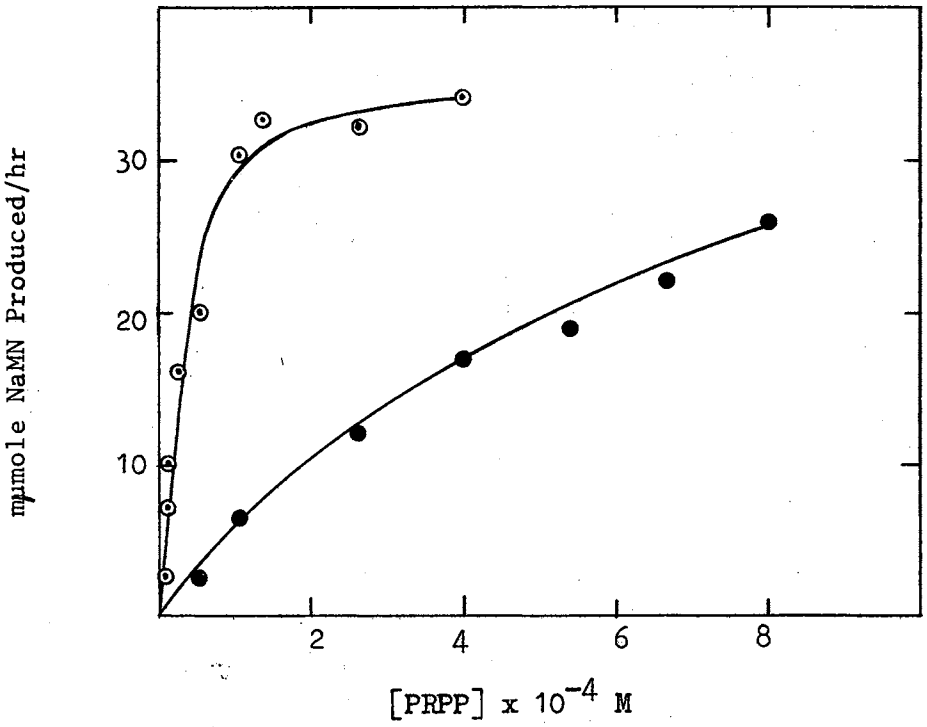
Standard reaction mixtures as described under Figure 3 with various levels of PRPP concentrations were incubated with 0.26 units of enzyme for two hours at 37° C and then assayed for the amount of product formed in the usual manner.

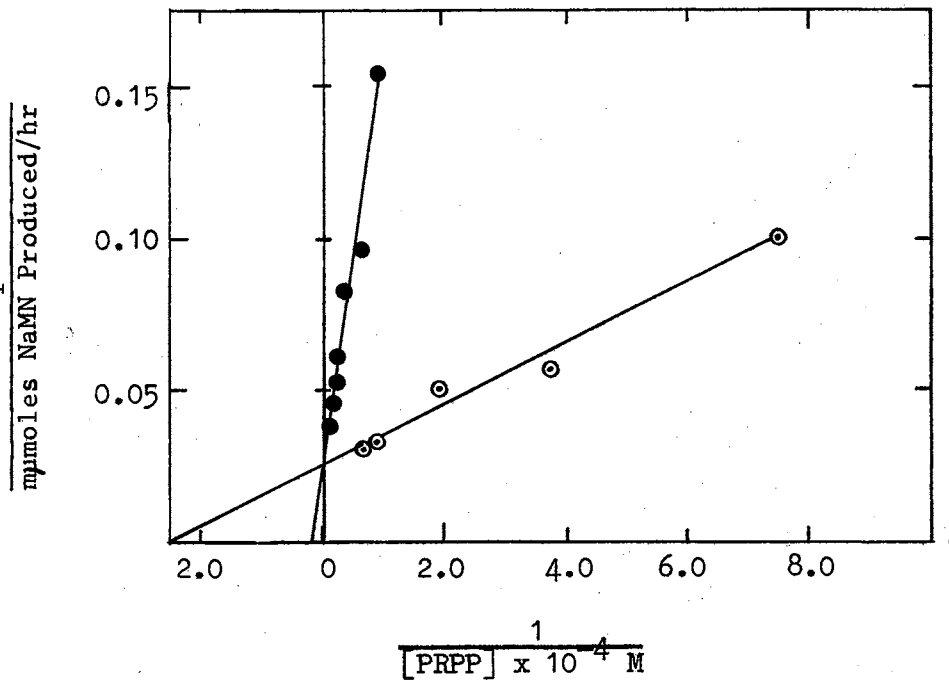
o = + 6.67 x 10^{-4} M ATP; • = - ATP.

Figure 9.

Lineweaver-Burk Plot of the Reaction Velocity Versus PRPP Concentration

o = + 6.67 x 10^{-4} M ATP; • = - ATP.


mμmole NaMN Produced/hr
30
20
10
2
4
6
8
[PRPP] x 10^{-4} M


$\frac{1}{\text{mμmoles NaMN Produced/hr}}$
0.15
0.10
0.05
2.0
0
2.0
4.0
6.0
8.0
$\frac{1}{[\text{PRPP}] \times 10^{-4}\ \text{M}}$

At low concentrations of PRPP, ATP causes an eight to ten-fold increase in activity while at higher concentrations the stimulation is not as pronounced. The apparent K_m for PRPP is decreased from 5×10^{-4} M in the absence of ATP to 4×10^{-5} M in the presence of ATP (Figure 9).

In order to determine if PRPP may be an activater of the enzyme as well as a substrate, a Hill plot was made (Figure 10). However, the slope did not indicate this, being very close to one both in the presence and absence of ATP. Therefore, PRPP apparently functions only as a substrate for the enzyme.

Molecular Weight

Due to the fact that ATP stimulates the reaction at low substrate concentration, a study to determine if ATP caused a change in the molecular weight of the enzyme was carried out. A Sephadex G-100 column (1.7×100 cm^2) was packed in 0.05 M phosphate buffer pH 7.5 containing 0.1 M KCl. After the column had equilibrated for two or three days, the column was calibrated using bovine serum albumin, ovalbumin, and cytochrome c as standards. When the column was calibrated, 95 mg of the enzyme, purified past the calcium phosphate step, six mg cytochrome c and two mg blue dextran were suspended in two ml of the phosphate buffer mentioned above and eluted.

Three ml fractions were collected and every other tube was assayed for enzyme activity. Figure 11 shows a typical profile as the protein is eluted off of the column. It can be seen that enzyme activity was localized between fractions 34-48 with a peak at 41. When the experiment was repeated after the column had been equilibrated with 10^{-4} M ATP, it was found that the same elution pattern existed with enzyme

Figure 10.

Hill Plots of NaMN Pyrophosphorylase Activity as a Function of PRPP Concentrations

v = amount of NaMN produced per ml enzyme solution per hour at 37° C under normal reaction conditions. PRPP concentration expressed as moles/liter. (A) - ATP, (B) + ATP.

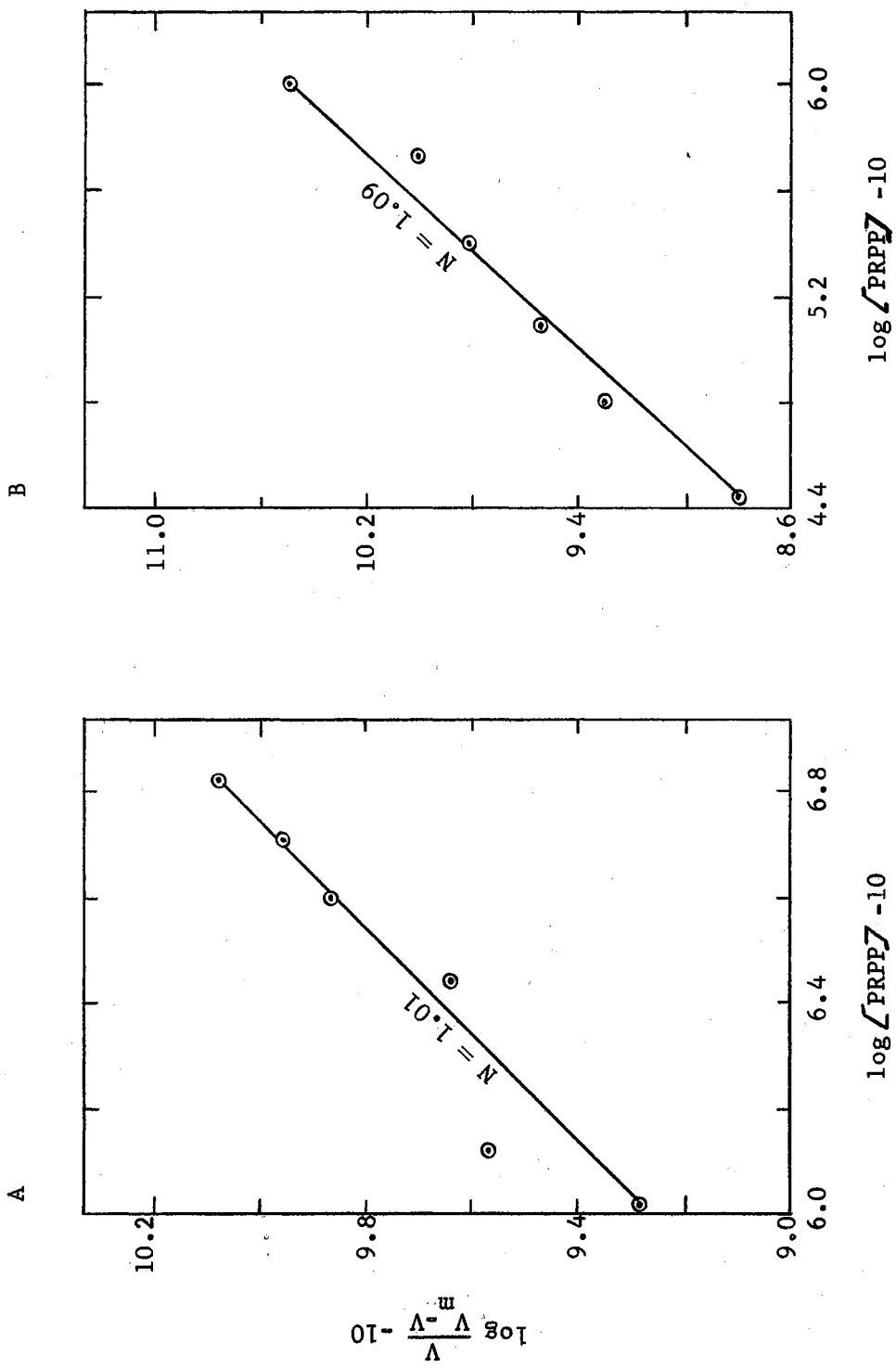
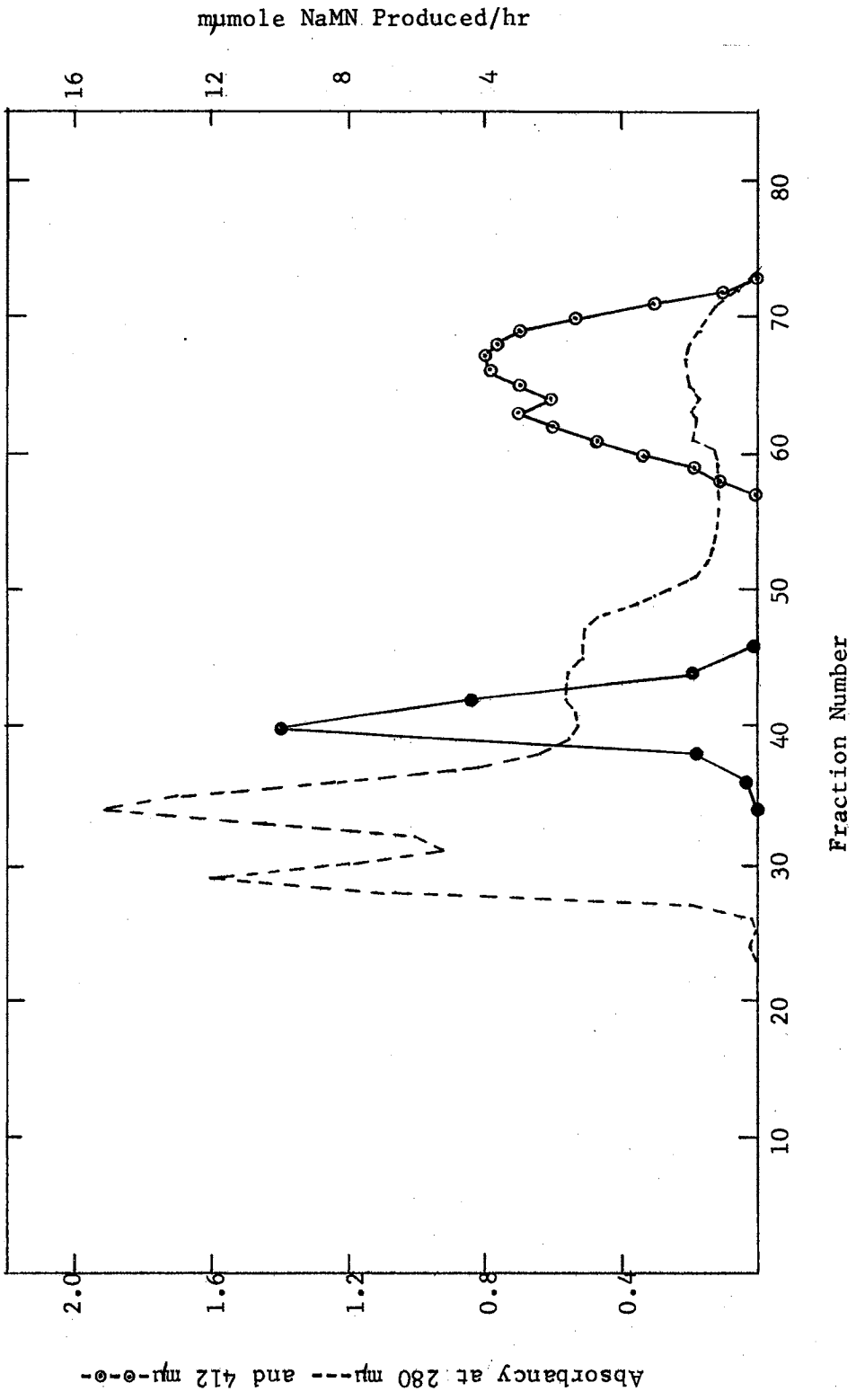

A
B
N = 1.01
N = 1.09
log [PRPP] -10
log [PRPP] -10
log V/(V_m - V) -10
10.2
9.8
9.4
9.0
6.0
6.4
6.8
11.0
10.2
9.4
8.6
4.4
5.2
6.0

Figure 11.

Elution of NaMN Pyrophosphorylase From

a Sephadex G-100 Column

After reading the absorbancy at 260 and 412 mμ, 0.05 ml of every other fraction were incubated at the conditions under Figure 3 and assayed for the amount of product formed. ---- = absorbance at 280 mμ, -o-o- = absorbance at 412 mμ, -●- = enzyme activity.


mμmole NaMN Produced/hr
16
12
8
4
Absorbancy at 280 mμ--- and 412 mμ-o-o-
2.0
1.6
1.2
0.8
0.4
10
20
30
40
50
60
70
80
Fraction Number

activity coming off in the same fractions. Thus it was concluded that the enzyme does not dissociate in the absence of ATP. The concentration of the enzyme was one half or less than the concentration used in the standard assays thereby eliminating any dilution factor as the prevention of dissociation. Figure 12 shows the calibration curve for the G-100 column and the molecular weight for NaMN pyrophosphorylase which is approximately 77,000.

The calcium phosphate step was used for the determination of the molecular weight because any purification beyond this step caused the enzyme to become extremely labile.

Nucleotide Specificity

Several different di- and trinucleotides were substituted for ATP to test their effect upon the velocity of the reaction at low substrate concentration. As can be seen in Table IV, when the enzyme, purified through the alumina C_γ step, was incubated with 6.67×10^{-6} M nicotinic acid and 13.3×10^{-4} M PRPP, all other conditions being standard, none of the nucleotides were as effective as ATP. The final concentration of all nucleotides was 6.7×10^{-4} M. Guanosine triphosphate (GTP) partially stimulated activity while on the other hand thymidine triphosphate and possibly adenosine monophosphate caused a slight inhibition. Adenyl diphosphate (β,γ-methylene) phosphate (ADP-methylene-P) was also tested for stimulatory activity. As can be seen, instead of stimulation of activity, it appeared to inhibit the enzyme. Thus the enzyme seems to be fairly specific in its requirement of nucleotides for activation.

Figure 12.

Molecular Weight of NaMN Pyrophosphorylase

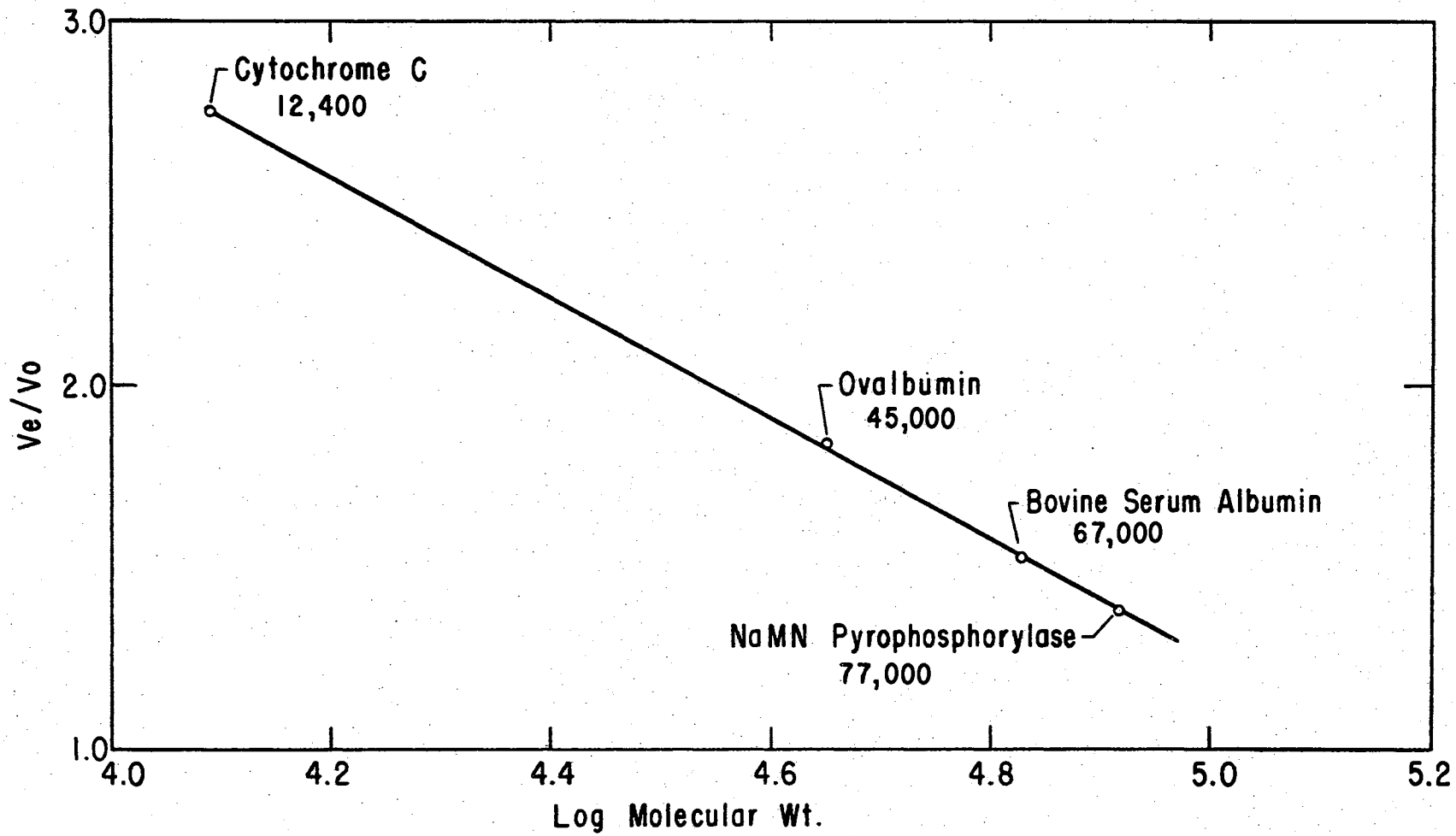

3.0
2.0
1.0
Ve/Vo
Cytochrome C
12,400
Ovalbumin
45,000
Bovine Serum Albumin
67,000
NaMN Pyrophosphorylase
77,000
4.0
4.2
4.4
4.6
4.8
5.0
5.2
Log Molecular Wt.

TABLE IV

NUCLEOTIDE SPECIFICITY FOR ACTIVATION OF NaMN PYROPHOSPHORYLASE ACTIVITY

Nucleotide 6.7×10^{-4} M	mμmole/hr
ATP	23.0
ADP	8.7
AMP	6.0
GTP	15.0
ITP	9.0
TTP	5.0
UTP	8.0
ADP-methyl-P	3.0
None	8.0

Effect of Increasing Concentrations of ATP

NaMN pyrophosphorylase was incubated with nicotinic acid-^{14}C (6.67×10^{-6} M), PRPP (13.3×10^{-4} M) and increasing concentrations of ATP (Figure 13). There appeared to be little or no stimulation of the enzyme at concentrations below 5×10^{-5} M ATP. Above this concentration an increase in initial velocity is seen with increasing concentrations until a maximum is approached at 6.67×10^{-4} M. When a Lineweaver-Burk plot was made, linearity could not be obtained as seen in Figure 14A. At high concentrations, the reaction velocity is greater than that expected from extrapolation of data obtained at lower concentrations. When the amount of NaMN formed in the absence of ATP was subtracted from the amount formed when ATP was present and a Lineweaver-Burk plot of this data was made, a straight line could be observed however (Figure 14B). The apparent K_m obtained from this plot was 2×10^{-3} M. This unusually high K_m may be explained since the V_{max} is automatically lowered upon subtraction of the constant amount of NaMN produced in the absence of ATP, therefore increasing the K_m. The significance of this "K_m" is presently not understood. The fact that a straight line relationship can be observed under the latter conditions indicates that a site on the enzyme is being saturated. Whether or not this site is independent of the sites responsible for binding PRPP and nicotinic acid is impossible to say from this experiment. Thus ATP does not behave as a normal substrate for this reaction and the kinetic behavior of this compound would indicate that it activates the pyrophosphorylase in some manner analogous to an allosteric modifier.

Figure 13.

Velocity of Reaction Versus ATP Concentration

Incubation mixtures contained the standard reaction mixture with the exception that the concentration of nicotinic acid-^{14}C was 6.67×10^{-6} M and various concentrations of ATP were used.

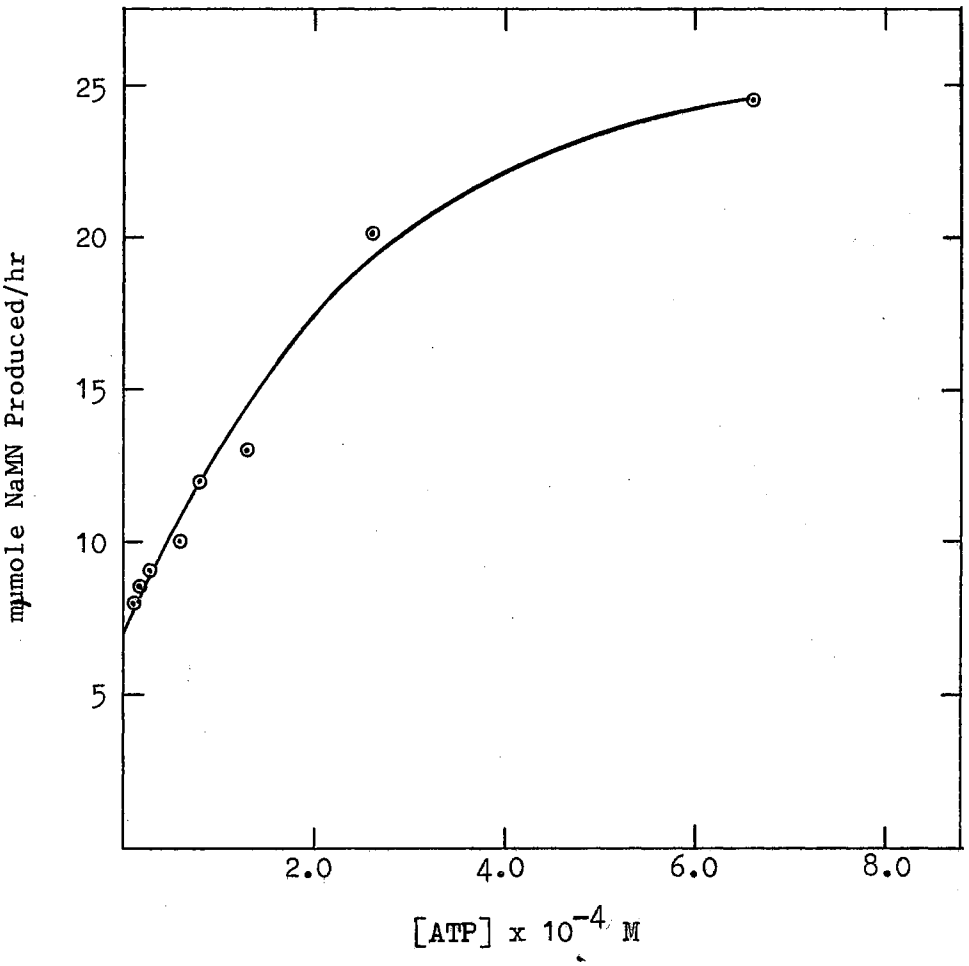

25
20
15
10
5
2.0
4.0
6.0
8.0
mμmole NaMN Produced/hr
[ATP] x 10^{-4} M

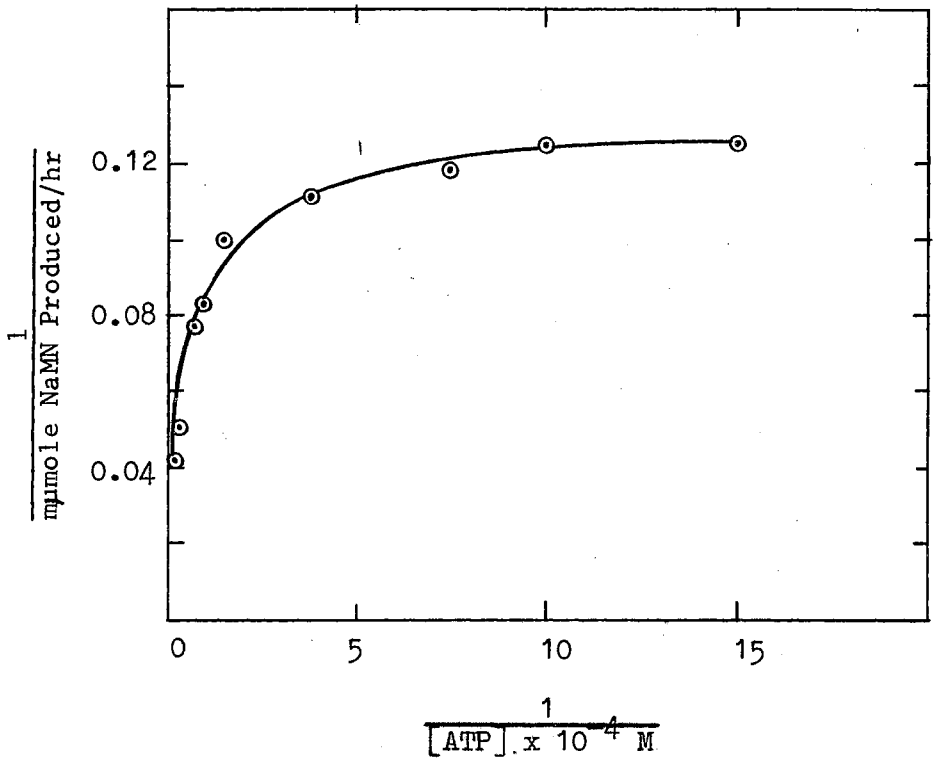
Figure 14.

Lineweaver-Burk Plot of the Reaction Velocity Versus the ATP Concentration

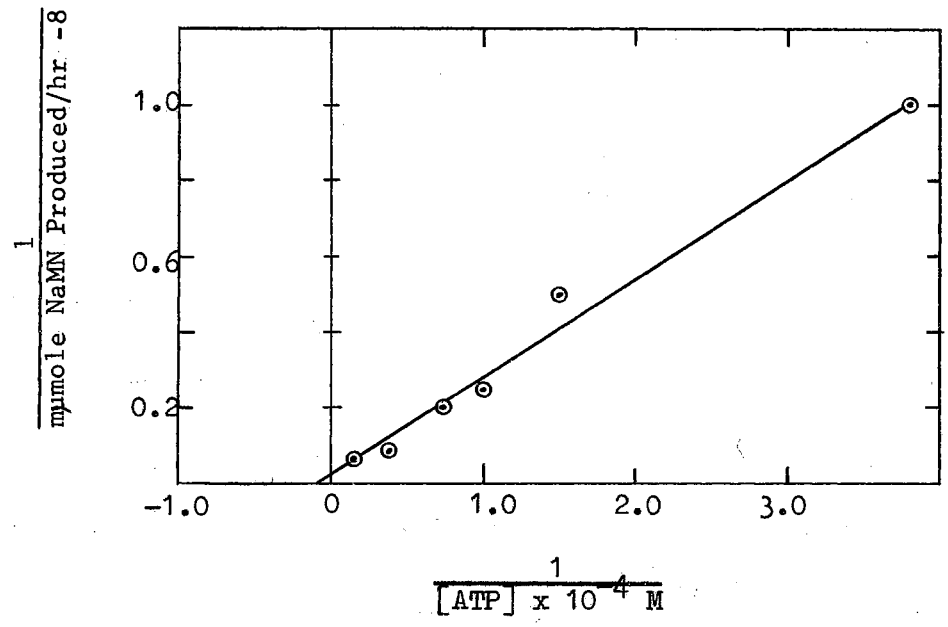
A. Before subtracting the amount of NaMN produced in the absence of ATP.

B. After subtracting the amount of NaMN produced in the absence of ATP.

A


1 / mμmole NaMN Produced/hr
0.12
0.08
0.04
0
5
10
15
1 / [ATP] x 10⁻⁴ M

B


1 / mμmole NaMN Produced/hr -8
1.0
0.6
0.2
-1.0
0
1.0
2.0
3.0
1 / [ATP] x 10⁻⁴ M

Substrate Cooperation Effects

When low levels of PRPP were used i.e., 6.7×10^{-5} M, in conjunction with varying the concentration of nicotinic acid-^{14}C, it was found that in the absence of ATP, an effect known as substrate cooperation was observed (Figure 15). Thus at low concentrations of PRPP, nicotinic acid activates the enzyme in the absence of ATP. At high levels of PRPP (13×10^{-4} M) this effect is lost. When ATP was present, this effect is also lost. When Hill plots of the data of Figure 6 were made for nicotinic acid it was found that a slope greater than one existed both in the presence and absence of ATP (Figure 16). This indicates that nicotinic acid is involved both as a substrate and an activator in contrast to PRPP which functions only as a substrate for the reaction (Figure 10).

Conversion of ATP to ADP During the Reaction

The kinetics of the reaction catalyzed by NaMN pyrophosphorylase in the presence and absence of ATP seemed to indicate that ATP acts as an allosteric modifier. Although no change in molecular weight could be observed, it seemed possible that ATP may change the conformational structure of the enzyme without affecting the molecular weight.

Experiments using nicotinic acid-7-^{14}C and ATP-8-^{14}C were performed to determine whether or not ATP actually took part in the reaction when it was present. To do this, 2.5 units of enzyme purified through the alumina C_γ step were incubated with 6.67×10^{-4} M nicotinic acid-7-^{14}C, 2.4×10^{-4} M ATP-8-^{14}C, and 2.67×10^{-4} M PRPP as described under experimental procedures. After two hours incubation time, assays for the amount of NaMN-^{14}C and ADP-^{14}C formed were made by eluting the

Figure 15.

Substrate Cooperative Effect With Nicotinic Acid

Standard reaction conditions were used with 6.67×10^{-5} M PRPP and various levels of nicotinic acid-^{14}C in the absence of ATP. The incubation mixtures were then assayed for the amount of NaMN-^{14}C formed in the usual manner.

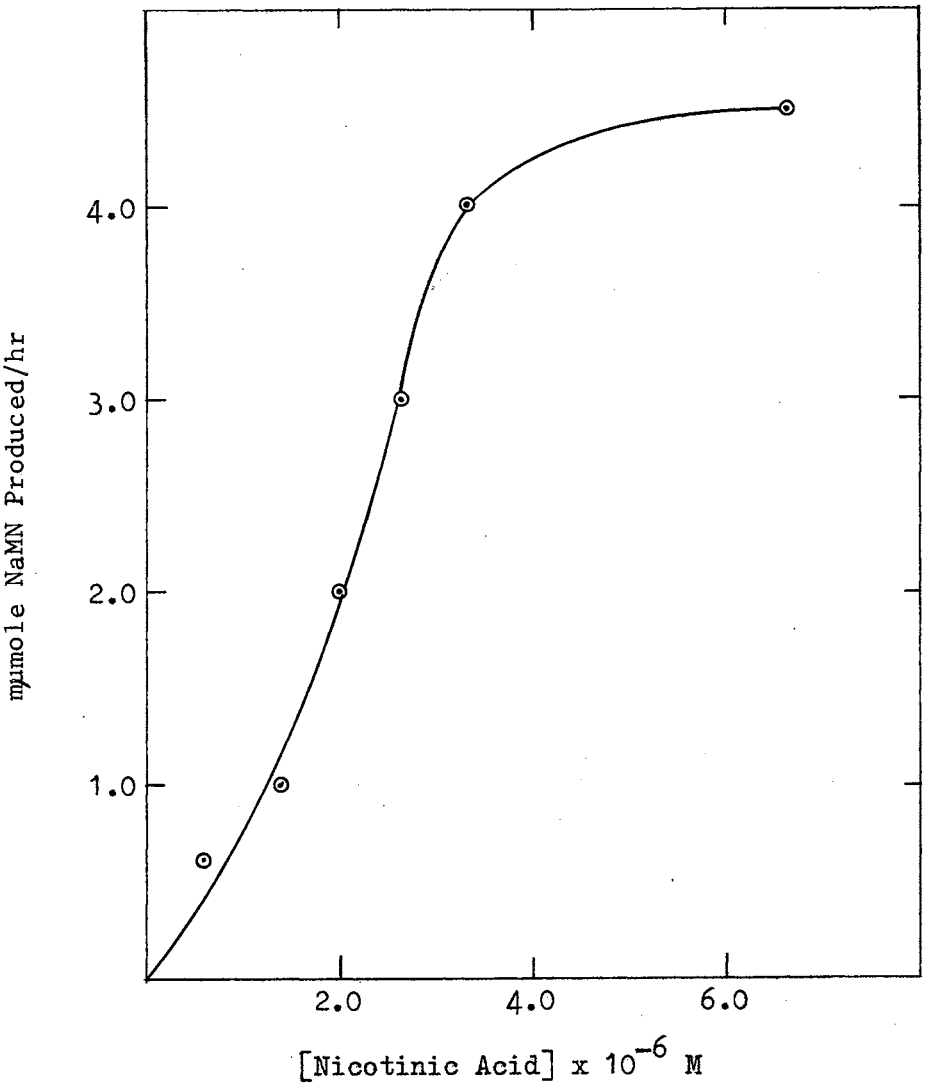
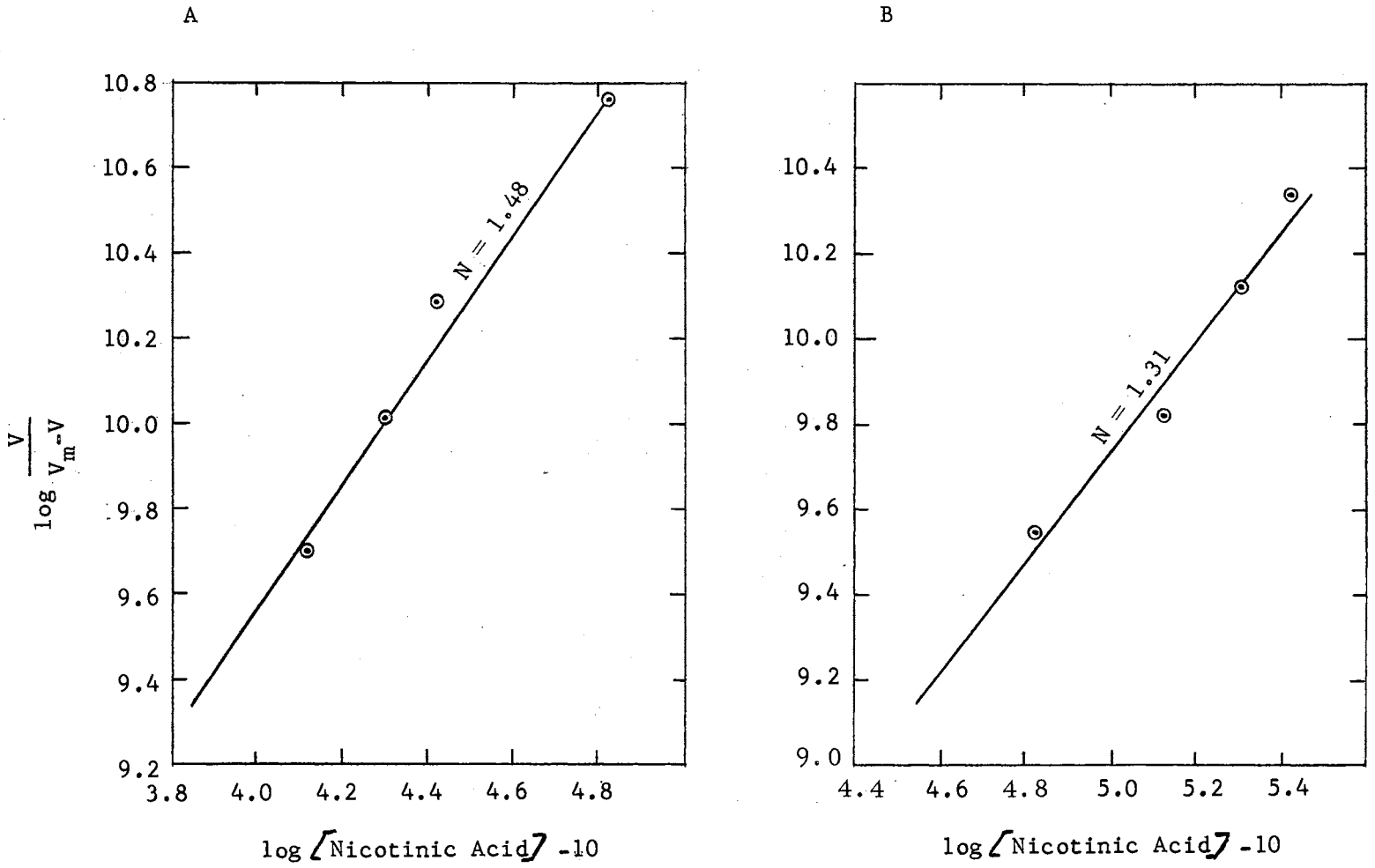

mμmole NaMN Produced/hr
4.0
3.0
2.0
1.0
2.0
4.0
6.0
[Nicotinic Acid] x 10⁻⁶ M

Figure 16.

Hill Plots of NaMN Pyrophosphorylase Activity as a Function of Nicotinic Acid Concentrations

v = amount of NaMN produced per ml of enzyme solution per hour at 37° C under standard reaction conditions. Nicotinic acid concentration is expressed as moles/liter. (A) + ATP, (B) - ATP.


A
B
$\log \frac{V}{V_m - V}$
10.8
10.6
10.4
10.2
10.0
9.8
9.6
9.4
9.2
3.8
4.0
4.2
4.4
4.6
4.8
N = 1.48
log [Nicotinic Acid] -10
10.4
10.2
10.0
9.8
9.6
9.4
9.2
9.0
4.4
4.6
4.8
5.0
5.2
5.4
N = 1.31
log [Nicotinic Acid] -10

reaction mixture from a Dowex-1-formate column. As can be seen in Table V, there appeared to be some ATP converted to ADP. Much difficulty was encountered with this experiment since the ATP-^{14}C obtained from Nuclear Chicago Corporation contained about five percent contaminating ADP-^{14}C or some substance that co-chromatographed with this compound. All efforts to purify the ATP (including paper chromatography, column chromatography over Dowex-1-formate, and high voltage electrophoresis) were in vain (with the exception of purification over DEAE cellulose-acetate). Either such efforts failed to purify the ATP to any significant extent or else the recovery was so low as to make the method impractical. Although ATP could be purified with the use of DEAE cellulose-acetate as described under experimental procedures and as used in experiment 4 of Table V, the triethylamine salt of the ATP obtained in this manner inhibited the enzyme 30 to 50 percent. Efforts to convert this to the sodium salt resulted in such low recovery as to again make the method impractical. When the concentration of nicotinic acid was increased ten fold there followed an increase in the amount of NaMN formed, experiment 3, Table V, but no increase in the amount of ATP converted to ADP was observed. Thus at the present time it is impossible to say if there is a stoichiometric relationship between the amount of NaMN produced and the amount of ATP converted to ADP, but it is evident that some ADP is formed during the reaction when ATP is present. Therefore, ATP takes part in the reaction when it is present; however, as shown earlier, it appears not to be an absolute requirement for the reaction when nicotinic acid and PRPP are present in high enough concentrations.

TABLE V

CONCOMITANT FORMATION OF ADP WITH NaMN PRODUCTION

Omission	mμmole Produced			
	NaMN	Δ NaMN	ADP	Δ ADP
Experiment 1				
Nicotinic Acid	0.49[1]	3.09	8.24	2.61
None	3.58	——	10.85	——
Experiment 2				
PRPP	0.88	3.07	11.84	3.56
None	3.95	——	15.4	——
Experiment 3				
Nicotinic Acid	1.16	3.41 (4.42)[2]	13.6[3]	0.83 (0.79)
PRPP	1.11	3.36 (4.47)	12.0	2.43 (2.39)
Enzyme	1.07	3.40 (4.51)	10.8	3.63 (3.59)
None; NA = 6.7×10^{-6} M	4.47	——	14.43	——
None; NA = 6.7×10^{-5} M	5.58	——	14.39	——
Experiment 4[4]				
Nicotinic Acid	0.6	1.65	5.82	1.57
PRPP	0.93	1.32	5.99	1.40
Enzyme	0.48	1.77	5.39	2.00
None	2.25	——	7.39	——

[1]Background for NaMN and ADP inherent in experiment since detected products by isotopic technique and thus observed some background in system.

[2]Numbers in parentheses refer to amount of NaMN and ADP formed when nicotinic acid concentration increased to 6.7×10^{-5} M.

[3]Unusually high blank not usually observed.

[4]Experiment 4 performed after purification of ATP over DEAE cellulose as described in methods and procedures.

CHAPTER IV

DISCUSSION

It has been observed previously in this dissertation that the pathways and control of NAD^+ biosynthesis are varied and complex. This seems to be true especially in the case of mammalian systems. In this system, both tryptophan and nicotinic acid may serve as starting materials for NAD^+ biosynthesis; thus it must be very important for the cell to have a means of control whereby it can regulate one or both of these pathways.

NaMN pyrophosphorylase appears to be one of the control points of this biosynthesis when nicotinic acid is used as the starting material. As mentioned above, this enzyme is rate controlling in many organisms including E. coli (205), other related coliforms (206), B. subtilis (207-209), and last but not least with mammalian systems (170, 210). Imsande (208) mentioned that ATP stimulated the activity of NaMN pyrophosphorylase of beef liver. Curiously enough, this and the erythrocyte system appear to be the only two systems which do not show an absolute requirement for ATP (208).

When it was reported that NaMN pyrophosphorylase could be dissociated in the absence of ATP to form NaMN ribonucleotidase (210), it became of interest to us to study this system. However, when we tried to separate these two activities it was obvious that they were quite easily separable and apparently have no direct relationship to one

another. This has been confirmed with the yeast enzyme where Ogasawara and Gholson (236) found that the two enzyme activities were again quite easy to separate. Since this time, Honjo et al. (237) have retracted their previous statement concerning this point and thus it appears to be well established that the two enzymes are non-related.

When a study was made to determine the effect of ATP on the initial velocity it was found that as had been previously reported (170) ATP stimulated the activity five to ten fold at low nicotinic acid concentrations. On the other hand, it had not been reported that the reaction velocity was independent of ATP at higher concentrations of nicotinic acid (Figure 6). Therefore the ATP in some unknown manner changes either the reaction mechanism or the conformation of the enzyme causing a pronounced change of the K_m for both the nicotinic acid and the PRPP. Since the maximum velocity is the same whether in the presence or absence of ATP it is reasoned that without ATP the enzyme requires more substrate in order to be saturated. Thus at higher concentrations of nicotinic acid and PRPP maximum velocity is attained in the absence of ATP.

The substrate cooperative effect of nicotinic acid may also be of some significance in the regulation of the enzyme activity (Figure 15). The Hill plots (Figure 16) also show that nicotinic acid is involved with activation of the enzyme. This effect is not uncommon with systems requiring two or more substrates. Sanwal et al. (238) reported that isocitrate has an activating effect on isocitrate dehydrogenase of Neurospora. Another example of this is the activation of deoxythymidine kinase by ATP (239). Probably the most famous system of this type is the substrate cooperative effect of hemaglobin with oxygen uptake (230). Therefore nicotinic acid serves two functions with this enzyme:

that of a substrate and as an activator.

ATP also stimulates the velocity of NaMN pyrophosphorylase at low concentrations of PRPP (Figure 8). This effect is again striking in that an eight to ten fold stimulation in velocity is observed at low concentrations of PRPP and the K_m is decreased about ten fold (Figure 9). It is also interesting to note that PRPP is apparently not an activator of the enzyme as is nicotinic acid as noted by the Hill plots made for this substrate in the presence and absence of ATP (Figure 10). Therefore the two substrates are not equivalent in their functions.

These effects may play a significant role in the control of NaMN pyrophosphorylase. In the absence of ATP, the enzyme activity may be controlled to some extent by the concentration of nicotinic acid and PRPP. Since the K_ms of these substrates are much higher in the absence of ATP, the physiological concentrations of substrates may not be sufficient to saturate the enzyme and the rate of reaction would be proportional to substrate concentration. The effect of ATP stimulation is another means of controlling NaMN pyrophosphorylase activity at low concentrations of substrate. In all probability, this is the more significant of the two methods of regulating this activity. By some means yet unknown, ATP appears to act by drastically lowering the K_m for both substrates suggesting that ATP is acting as an allosteric modifier. However, this hypothesis does not explain the observed cleavage of ATP to ADP which may or may not be stoichiometric with NaMN formation. The fact that ADP-methylene-P inhibits the enzyme activity may also suggest that ATP is split when present. Thus when an analog such as this one is present, the reaction is inhibited because the analog cannot be cleaved. Although GTP can substitute for ATP to some degree it is not

as efficient as ATP. In this respect, the enzyme of B. subtilis (207, 208) is similar to the beef liver system. Although ATP is an absolute requirement in B. subtilis for NaMN formation, it apparently acts as a regulating mechanism as evidenced by the fact that ATP is the rate limiting substrate for the reaction (207). Furthermore, ATP appears to have a binding site distinct from that of the other two substrates (208). This could also be speculated of the beef liver enzyme since the reaction can certainly proceed in the absence of ATP.

It has been suggested that ATP may in some manner activate the pyridine ring (208). This is reasoned since the free energy of hydrolysis of the ribosyl-nicotinamide bond of NAD^+, which is formed in the presence of NaMN pyrophosphorylase, has been estimated to be approximately 9000 calories/mole (240). On the other hand, the free energy of hydrolysis of the ribosyl-pyrophosphate bond of PRPP should not be too different from that of ribose-1-phosphate, which is approximately 4800 calories/mole. Thus it appears that a bond is broken releasing 4800 calories/mole which drives the formation of another bond requiring 9000 calories/mole. This cannot be explained in terms of the established law of thermodynamics and it may be that ATP activates the pyridine ring perhaps in a concerted reaction which results in the formation of ADP and P_i. On the other hand this could not explain why the reaction can proceed in the absence of ATP.

The possibility also exists that ATP may change the conformational structure of the enzyme and in so doing require the conversion of ATP to ADP. This may alter the conformational structure such that as the enzyme catalyzes the reaction enough energy is released from the enzyme to enhance the formation of the pyridine-ribose bond. Again this does

not explain the large quantity of ATP converted to ADP since the amount of enzyme present compared to the substrate should be quite small. Thus neither of these hypotheses can completely explain all of the data.

In summary, NaMN pyrophosphorylase appears to be regulated by two mechanisms. The first and least significant is that of activation by nicotinic acid. The second, and most pronounced is by ATP, where it causes a marked stimulation of activity at low substrate concentrations, but is not essential for activity at high substrate concentrations. ATP may not be an allosteric modifier as described by Monod et al. (241) because it is cleaved to ADP. Therefore this enzyme appears to be very unique in its mechanism of action. It is regulated in some manner by ATP but this mechanism is as yet not understood. Further investigation will be necessary in order to elucidate the details of this mechanism.

SUMMARY

Nicotinic acid mononucleotide pyrophosphorylase was partially purified from beef liver acetone powder. NaMN pyrophosphorylase was shown to be distinct from NaMN phosphatidase. When the enzyme was incubated with high levels of PRPP and various levels of nicotinic acid, both in the presence and absence of ATP, it was found that ATP stimulated the activity of the enzyme five to ten fold and decreased the K_m approximately ten fold. Conversely, when the concentration of nicotinic acid was kept constant and the concentration of PRPP was varied, again an eight to ten fold stimulation by ATP was observed at low concentrations of PRPP and the K_m was lowered about ten fold. The molecular weight of the enzyme did not appear to change in the presence and absence of ATP being approximately 77,000 in both cases. The enzyme is rather specific for ATP with GTP replacing ATP to a slight degree and other nucleotides tested showing no effect. Nicotinic acid also appeared to have a slight stimulatory effect as shown by substrate cooperation in addition to serving as a substrate while PRPP appears to only serve as a substrate for this enzyme. When ATP-^{14}C and nicotinic acid-^{14}C were incubated simultaneously and assayed for the amount of NaMN-^{14}C and ADP-^{14}C formed, it was found that ATP was converted to ADP. The data obtained were not sufficiently precise to determine whether ADP formation is stoichiometric with NaMN formation. The kinetics of the reaction indicate that NaMN pyrophosphorylase is an allosteric protein with ATP serving as the allosteric modifier. However,

the fact that ATP is converted to ADP during the reaction appears to be inconsistent with this view.

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