PYRIDINE NUCLEOTIDE BIOSYNTHESIS IN RUMINANT LIVER MITOCHONDRIA

By

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RUMINANT LIVER MITOCHONDRIA

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PREFACE

This study was undertaken as an investigation of the biosynthesis of the pyridine nucleotides from nicotinic acid and/or nicotinamide by the liver mitochondria from beef and sheep. The origin of these compounds is of interest due to their importance in the electron transport chain. I would like to acknowledge the National Science Foundation for supporting this research under grant number NSF PCM77-18814. I would like to thank the Meat Science Department for providing the liver used in this study. I also extend my thanks to Miss Angelika Behr for her help with the laboratory techniques. Dr. R. K. Gholson, my major professor, has been of tremendous help throughout the laboratory work and the writing of this thesis. In addition, many others in the Biochemistry Department have been of considerable help throughout this study. To all, I extend my appreciation. A very special thank you goes to my husband, Alan, for his cooperation and understanding during the lab work and for the typing of this manuscript.

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LIST OF ABBREVIATIONS

ml	-	milliliter(s)
hr	-	hour
g	-	gram
M	-	Molar
mM	-	millimolar
NA	-	nicotinic acid
NAm	-	nicotinamide
µCi	-	micro-curie
µmol	-	micro-moles
NMN	-	nicotinamide mononucleotide
NAD^+	-	nicotinamide adenine dinucleotide
µl	-	microliter
NaMN	-	nicotinic acid mononucleotide
$NaAD^+$	-	nicotinic acid adenine dinucleotide
PRPP	-	5-phosphoribosyl-1-pyrophosphate
ATP	-	Adenosine Triphosphate
mmol	-	millimoles
pm	-	picomoles
g	-	gravity

CHAPTER I

INTRODUCTION

Most of the intracellular NAD^+ occurs within the mitochondria where it is an essential electron carrier for the dehydrogenases linked to the electron transport chain. The origin of the intramitochondrial pool of NAD^+ is not clear. The inner membrane is impermeable to pyridine nucleotides and the enzymes which synthesize NAD^+ from nicotinamide and nicotinic acid are localized in the cytoplasm and the nucleus. A recent study using intact rat liver mitochondria concluded that NAD^+ was formed from nicotinamide via NMN. However, no NAD^+ synthetic activity could be found in sonicated mitochondria. Another study, using yeast mitochondria, led to the conclusion that pyridine nucleotides were synthesized from nicotinamide via nicotinic acid and nicotinic acid mononucleotide.

In view of these conflicting results, we have undertaken this study designed to investigate some of the individual enzyme reactions in the synthesis of NAD^+ from nicotinic acid and/or nicotinamide in beef and sheep mitochondria.

CHAPTER II

LITERATURE REVIEW

The pyridine nucleotides are an important class of compounds. Although they are found throughout the cell, a large proportion of these compounds occurs in the mitochondria. There, they serve the essential function of electron carriers for the dehydrogenases linked to the electron transport chain.

The biosynthesis of NAD^+ from tryptophan, via quinolinic acid [1], and from nicotinic acid and nicotinamide has been widely studied. (See review by Chaykin [2]). The nicotinamide resulting from the degradation of NAD^+ is recycled [3,4]. Two salvage pathways have been elucidated. The first, known as the Preiss-Handler pathway [5,6] involves the series of reactions:

$$NAm + H_2O \rightleftharpoons NA + NH_3$$

$$NaMN + ATP \rightleftharpoons NaAD^+ + PP_i$$

$$NaAD^+ + \text{glutamine} + ATP \rightleftharpoons NAD^+ + \text{glutamate} + AMP$$

A second pathway involves the series of reactions:

$$NAm + PRPP \rightleftharpoons NMN + PP_i$$

$$NMN + ATP \rightleftharpoons NAD^+ + PP_i$$

All the pathways have been consolidated into the proposal by Gholson [4] of a pyridine nucleotide cycle. This cycle is shown in Figure 1. Different organisms use different portions of the cycle, and some may use more than one pathway, depending on the conditions. Streffer and Benes [7] showed that mouse liver utilizes the Preiss-Handler pathway when high levels of nicotinamide are given, but that at low levels the synthesis proceeds via NMN. Rat erythrocytes also use both pathways *in vitro*, depending on whether the starting material present is nicotinamide or nicotinic acid [8]. In contrast, Ehrlich ascites tumor cells [9], mammary gland tissue [10], and mouse embryos [11] use only the NMN pathway. Keller, Liersch, and Grunicke [12], working with perfused rat liver, found the incorporation of nicotinamide into NAD^+ with no evidence for any deamidation. This agrees with the earlier work of Dietrich *et al* [13]. In contrast, Ismande and Handler [14], and later Smith and Gholson [15] found an active nicotinic acid phosphoribosyl transferase in beef liver. Yeast [16,17], *Astasia longa*, [18], and *Escherichia coli* [19] also exhibit synthesis of NAD^+ through the Preiss-Handler pathway.

In order for cells to grow and divide, the pyridine

nucleotide content must be continuously increasing. The evidence indicates that new mitochondria are formed from pre-existing mitochondria, rather than de novo [20]. There is also evidence of a reaction in which the ADP-ribose portion of the NAD^+ molecule is transferred to a mitochondrial acceptor protein [21] resulting in cleavage of the NAD^+ molecule. It also appears that the mitochondrial membrane is impermeable to the pyridine nucleotides [22-25]. However, the enzymes discussed so far are found in the nucleus or cytoplasm. Consequently, the origin and pathway for the synthesis of NAD^+ within the mitochondria is of interest.

Evidence for the intramitochondrial synthesis of NAD^+ has been found by Grunicke, et al [26]. These workers, using intact rat liver mitochondria, showed synthesis of NAD^+ from nicotinamide but not nicotinic acid or quinolinic acid. Neither could they find evidence of synthesis with any of these substrates following sonnification of the mitochondria. These workers propose that the pathway of synthesis for rat liver mitochondria is NAm->NMN->NAD^+. They rule out involvement of the desamido forms because they found labelled NMN but not labelled NaMN in mitochondria incubated with ^{14}C-NAm, and because the addition of azaserine, an inhibitor of $NaAD^+$->NAD^+ [6] did not interfere with formation of NAD^+ in their system.

In contrast, the work of Lange and Jacobson [27] with yeast mitochondria indicates that the Preiss-Handler pathway is used. Both nicotinic acid and nicotinamide were

incorporated into the desamido nucleotides, but no NAD^+ was formed. The synthesis was enhanced by sonnification of the mitochondria. This evidence suggests that in the yeast mitochondria there is an active nicotinic acid pathway, and a nicotinamide deamidase. Other workers have reported active nicotinamide deamidases [28,29] in the cytoplasm and microsomes, but not in the mitochondria.

Figure 1. The Pyridine Nucleotide Cycle

a. Tryptophan to quinolinate pathway (many steps)

b. Quinolinate phosphoribosyl transferase

c. Desamido NAD^+ pyrophosphorylase

d. NAD^+ synthetase

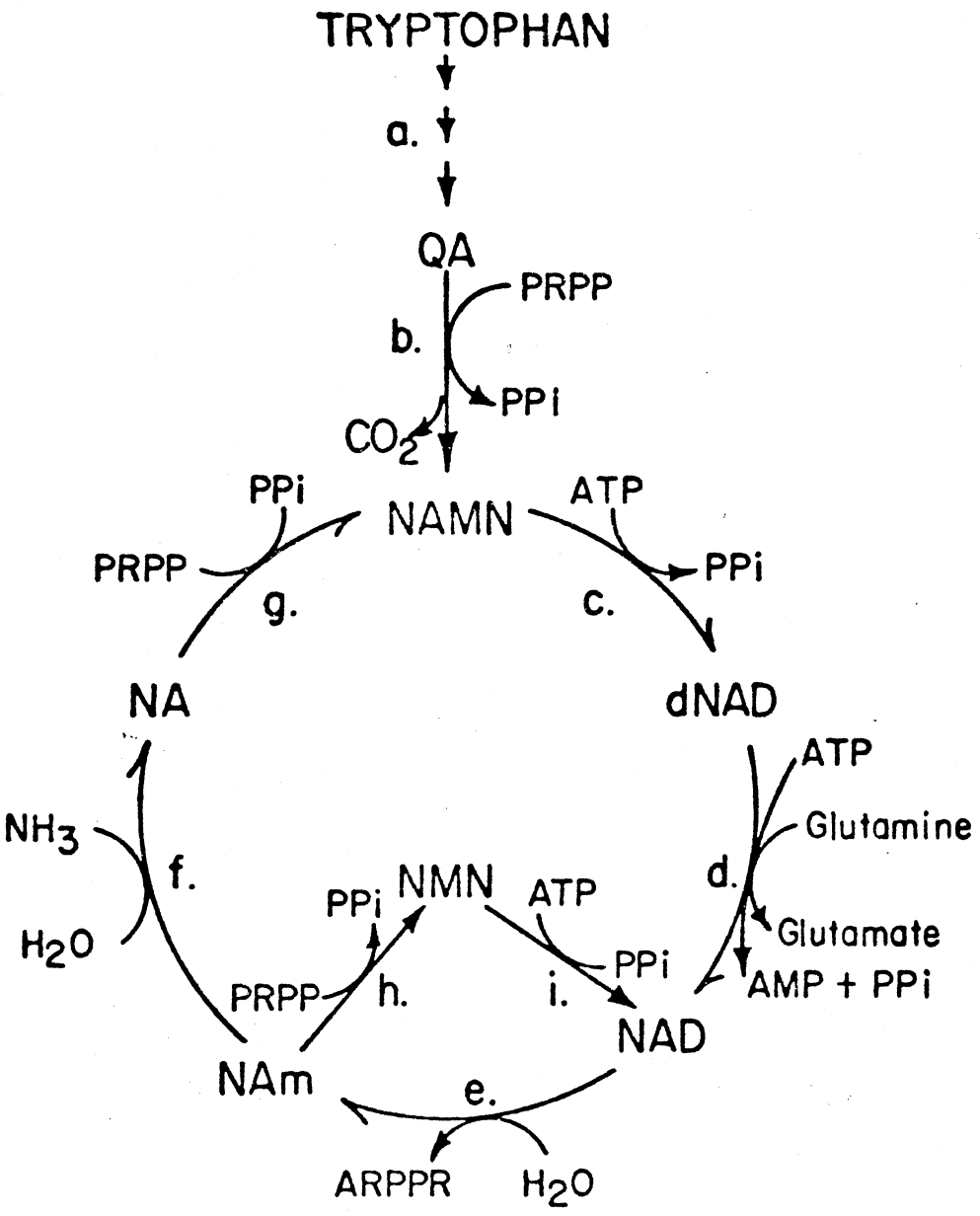
e. NAD^+ glycohydrolase

f. Nicotinamide deamidase

g. Nicotinic acid phosphoribosyl transferase

h. Nicotinamide phosphoribosyl transferase

i. NAD^+ pyrophosphorylase (NMN adenyl transferase)


TRYPTOPHAN
a.
QA
PRPP
b.
PPi
CO_2
PPi
NAMN
ATP
PRPP
g.
c.
PPi
NA
dNAD
ATP
Glutamine
NH_3
f.
d.
PPi
NMN
ATP
Glutamate
H_2O
PRPP
h.
i.
PPi
AMP + PPi
NAD
NAm
e.
ARPPR
H_2O

CHAPTER III

MATERIALS AND METHODS

A. Methods

1. Preparation of Mitochondria

The mitochondria used in these experiments were isolated from the liver of freshly (< 2 hours) killed beef or sheep. All procedures were carried out at 4°C. The procedure is a modification of that of Schaitman and Greenawalt for the isolation of mitochondria [30]. After removing connective tissue and veins approximately 15 g. (accurately weighed) of liver was diced and rinsed in cold isolation medium (0.22 M mannitol, 0.07 M sucrose, 0.02 M HEPES, pH 7.4). This material was then homogenized in a Potter Elvehjem glass homogenizer with a teflon pestle, brought to a total volume of 150 ml., and centrifuged at 120 g for 15 minutes in a Sorvall RC 2-B centrifuge. The supernate was decanted and recentrifuged at 6000 g for 15 minutes. The pellet was resuspended in 75 ml. chilled isolation medium, and centrifuged as before at 6000 g for 15 minutes. The resulting pellet was suspended in 30 ml. cold isolation medium, and centrifuged at 120 g for 15 minutes. The super-

natant material was removed and centrifuged at 6000 g for 15 minutes. The resulting pellet was then brought to a final volume of 4 ml. using isolation medium. The mitochondria may be used for whole mitochondria testing at this stage. This material may be frozen for use as disrupted mitochondria, if desired, or used immediately.

Two methods of disruption were used, sonification and solubilization with Triton X-100 (0.75% final concentration). The latter was more satisfactory, primarily because it was easier to control and reproduce. Using 2 ml. of either fresh or frozen mitochondria, 0.150 ml. of 10% Triton X-100 was added and the mixture was incubated at 0°C for 30 minutes, with occasional swirling. For some experiments, this solution was centrifuged at 20,000 g in a Sorvall RC 2-B centrifuge for 15 minutes at 4° C. The supernate was then carefully pipetted off for use as an enzyme source; the pellet was brought to a final volume of 2 ml. with isolation medium, centrifuged at 20,000 g for 15 minutes, and again resuspended in a 1 ml. final volume of isolation medium. This material was also tested as an enzyme source.

2. Acetone Powder

For some experiments, an acetone powder was used. The mitochondria were isolated and the acetone powder prepared following the procedure of Chang and Lane [31] with the addition of an extra sucrose solution wash of the mitochondria. For use in the transferase assays, 0.5 g. of the

acetone powder was extracted with 5 ml. of 0.05 M Tris buffer, pH 7.5, at 0°C for 30 minutes with continuous stirring. The mixture was then centrifuged in a clinical centrifuge at maximum speed in the cold for 5 minutes, and the supernatant used in the assays described below.

3. Protein Determination

Protein was determined by the Bio-Rad assay system, using the Bio-Rad protein standard as a reference [32]. This procedure was selected for use because it is easier to use than the Lowrey assay system. Only one reagent is required, and a shorter time span is involved. The only interference with this method was from the Triton-X 100 at 0.75%. This can be overcome by either determining the protein concentration of the mitochondria before solubilization with Triton, or diluting the sample to 0.1% Triton and including the appropriate amount of Triton in the standards. This dilution factor was quite acceptable at the protein concentrations typically used. Whenever possible, however, an aliquot of the mitochondrial solution was removed for protein concentration determination before the Triton was added.

4. Dialysis

For some procedures, it was necessary to dialyse the enzyme material prior to testing. In this case, both enzyme solution and buffer were made 10% in glycerol. The buffer

used was the isolation medium described for use in the isolation of the mitochondria. A volume of 2.0 ml of crude enzyme solution was dialysed against a volume of 1.0 liter of buffer for 1 hour, then a fresh liter of buffer for a second hour. This system appears to be adequate for removal of endogenous cofactors but does not seem to lower the enzyme activity.

5. Assays for Enzyme Activity

The assay for nicotinic acid phosphoribosyl transferase activity was a modified version of that described in Methods of Enzymology [33]. The standard incubation mixture was 1.33 mM PRPP, 0.67 mM ATP, 26.0 mM phosphate buffer, pH 7.5, 3.3 mM $MgCl_2$, 0.56 µM ^{12}CNA, 0.60 µCi ^{14}CNA, and 0.4 µm NaF, added to inhibit ATPase activity. Total volume was 0.300 ml. The tubes were routinely incubated at 35° for 90 minutes. The reaction was stopped by placing the tubes in boiling water for 90 seconds, then spinning them in a clinical centrifuge for 5 minutes at maximum speed.

For the assay of nicotinamide phosphoribosyl transferase, the same reaction system as that above was used except that 0.27 mM ^{12}CNAm and 0.20 µCi ^{14}CNAm were used in place of the nicotinic acid. This system was incubated at 35° for 10 minutes.

In both these assays a dinucleotide (NAD^+ or $NaAD^+$) was usually formed in addition to the mononucleotide product (NMN or NaMN). Both mono- and dinucleotides were counted in

determining the phosphoribosyl transferase activities.

When these systems were used to test activity in intact mitochondria, the assay solution used was made 100 mM sucrose to prevent lysis of the mitochondria during incubation. The reaction was stopped by centrifugation at 20,000 g in a Sorvall RC 2-B centrifuge (4° C) for 15 minutes. An aliquot of the supernate was then removed for testing for pyridine nucleotide formation.

The reaction system for testing for pyridine nucleotide formation in intact mitochondria was that described by Grunicke and coworkers [26]. Final concentrations were 100 mM KCl, 100 mM sucrose, 33 mM Tris, pH 7.5, 10 mM $MgCl_2$, 10 mM KH_2PO_4, 10 mM succinate and 0.01 mM NAm or NA. ATP was added (2 mM) to insure that sufficient quantities were present. Total volume was 2.0 ml originally, but for some experiments only 0.500 ml total volume was used. The tubes were incubated at 35° for 10 minutes. The reaction was stopped by adding 0.2 ml (50 µl for 0.500 ml assays) of 3N $HClO_4$. The tubes were then centrifuged in a clinical centrifuge at maximum speed for 5 minutes, the supernate was neutralized with $KHCO_3$ (tested with pH paper) and recentrifuged to remove $KClO_4$. A 50 µl aliquot of the supernate was tested by paper chromatography as for other assays.

6. Chromatography

To test for the formation of pyridine nucleotides in

the above assays, 50 µl of the supernate material was spotted on Whatman #1 Chromatography Paper and developed (descending) for 8 hours in a solvent system of n-butanol : acetic acid : water (4:1:2). This solvent solution was made fresh every 3 to 7 days. The pyridine nucleotides stayed on or near the origin while the starting material (NA or NAm) was well separated. The NAD^+ had an R_f value of 0.06, the NMN had an R_f value of 0.15, and the NA had a value of about 0.75. The identity of the products was checked with authentic compounds in the same system and locating the corresponding absorption bands under UV light. The area corresponding to product was cut into 1 cm. strips and counted in a Beckman LS 3150 T Liquid Scintillation Counter. The scintillation liquid was a solution of 0.2 g. POPOP and 4.0 g. PPO per liter of sulfur-free toluene.

B. Materials

The 7-^{14}C Nicotinic acid (50 mCi / mm) used in these experiments was obtained from Amersham Corp., as was the 7-^{14}C nicotinamide (60 mCi / mm). These labelled compounds were stored at 0^{o} C. Each sample was split into several smaller diluted samples to avoid excessive numbers of thawing-refreezing cycles. The PRPP and ATP were from Sigma and were also stored frozen.

CHAPTER IV

RESULTS

A. Enzyme Characteristics

1. Time Course

The nicotinic acid phosphoribosyl transferase showed a standard time course reaction curve. The product formation was approximately linear for 90 minutes; during 45 minutes of subsequent incubation, there was no decrease in the amount of product present (see Figure 2). For this reason, all assays were routinely incubated for 90 minutes. The nicotinamide phosphoribosyl transferase, in contrast, showed maximum product accumulation at 8 to 10 minutes, with subsequent rapid loss of product (see Figure 3). Therefore, all nicotinamide assays for this enzyme were incubated routinely for 10 minutes.

The rapid decrease in level of product present in the nicotinamide assays indicates the presence of an active enzyme which degrades NMN. The failure of the product level to decrease in the nicotinic acid assays indicates that NaMN is being formed, and is not degraded by this enzyme.

Figure 2. Nicotinic Acid Phosphoribosyl Transferase Activity as a Function of Time

Assay conditions were the standard conditions described in Chapter III, Methods, with incubation times as shown. All 'o' and '△' data points are from the same isolation of the enzyme.

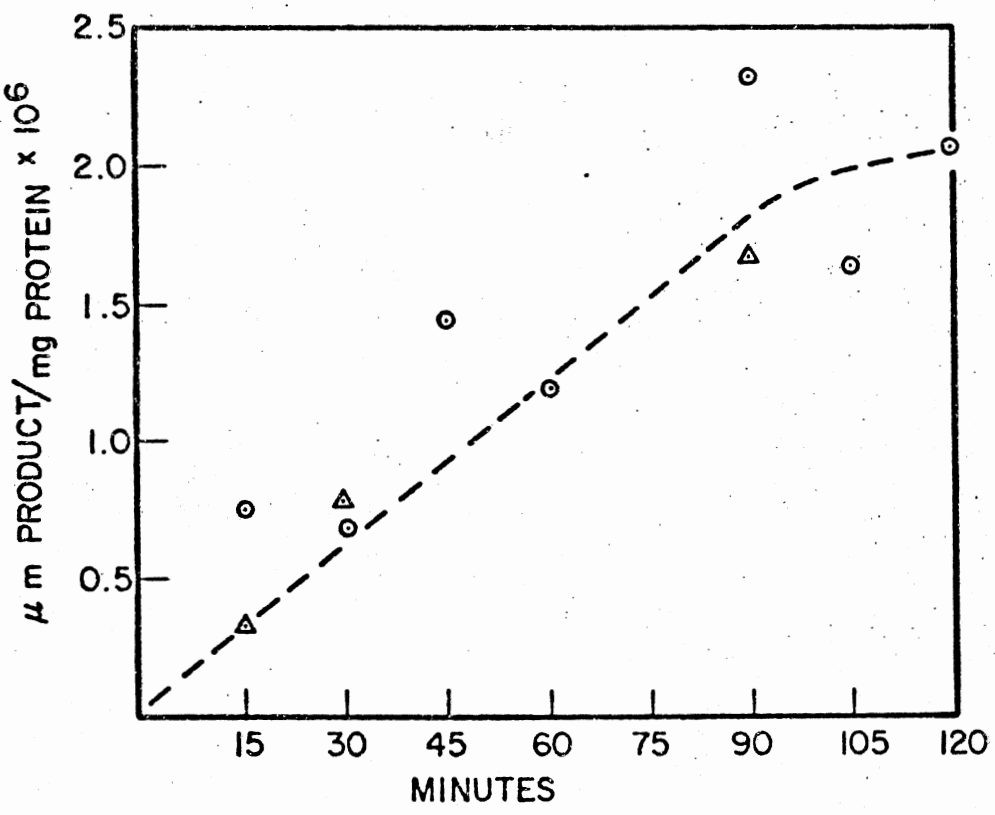
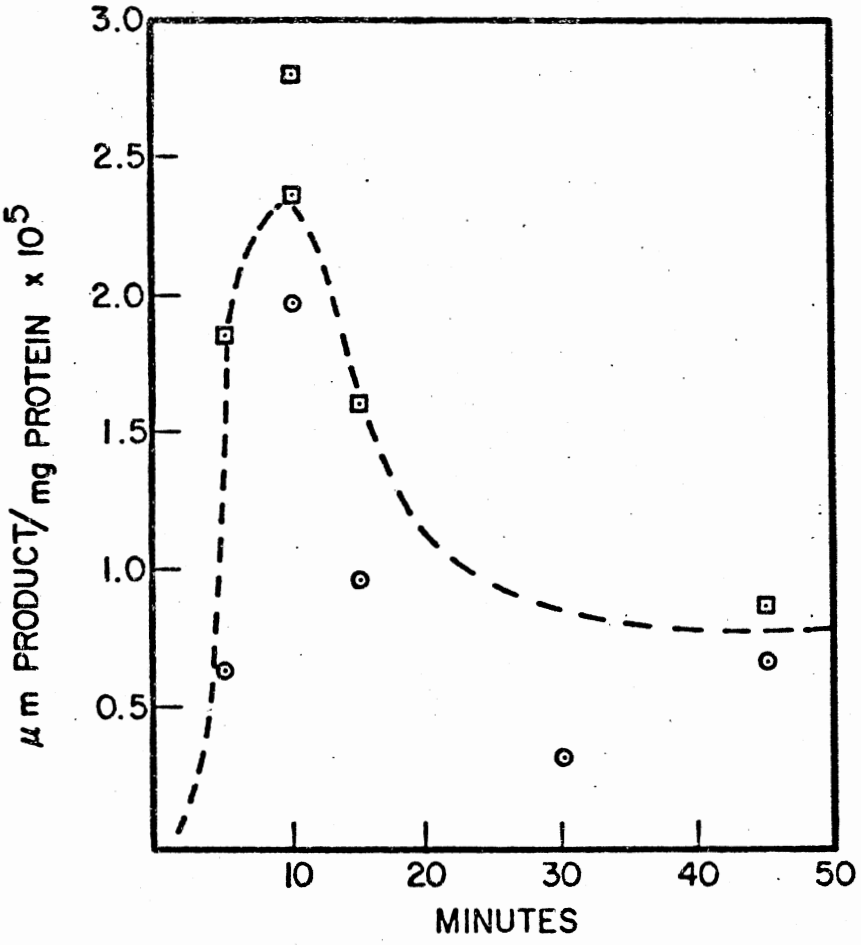

2.5
2.0
1.5
1.0
0.5
μ m PRODUCT/mg PROTEIN x 10⁶
15
30
45
60
75
90
105
120
MINUTES

Figure 3. Nicotinamide Phosphoribosyl Transferase Activity as a Function of Time

Assay conditions were the standard conditions described in Chapter III, Methods, with incubation times as indicated.


3.0
2.5
2.0
1.5
1.0
0.5
μm PRODUCT/mg PROTEIN x 10^5
10
20
30
40
50
MINUTES

2. Temperature

The activities of the transferases were compared at 25° and 35° C. Neither the nicotinic acid nor the nicotinamide enzyme showed a consistent increase in activity at either temperature. Therefore, most assays were run at 35° C. This temperature was chosen for purely pragmatic reasons: the ambient temperature was above 25° during part of the study, and a 35° water bath was readily available.

3. Protein Dependence

Both the nicotinamide and nicotinic acid showed product formation linear with protein concentration up to a protein concentration of about 30 mg./ml., under standard assay conditions. For more details, refer to Figures 4 and 5. Most of the work done in this study was done at a protein concentration of 25 to 30 mg./ml.

Figure 4. Nicotinic Acid Phosphoribosyl Transferase Activity as a Function of Protein Concentration

Standard assay conditions were used, varying only the amount of protein. The 'o' and '□' symbols indicate data from the same preparation.

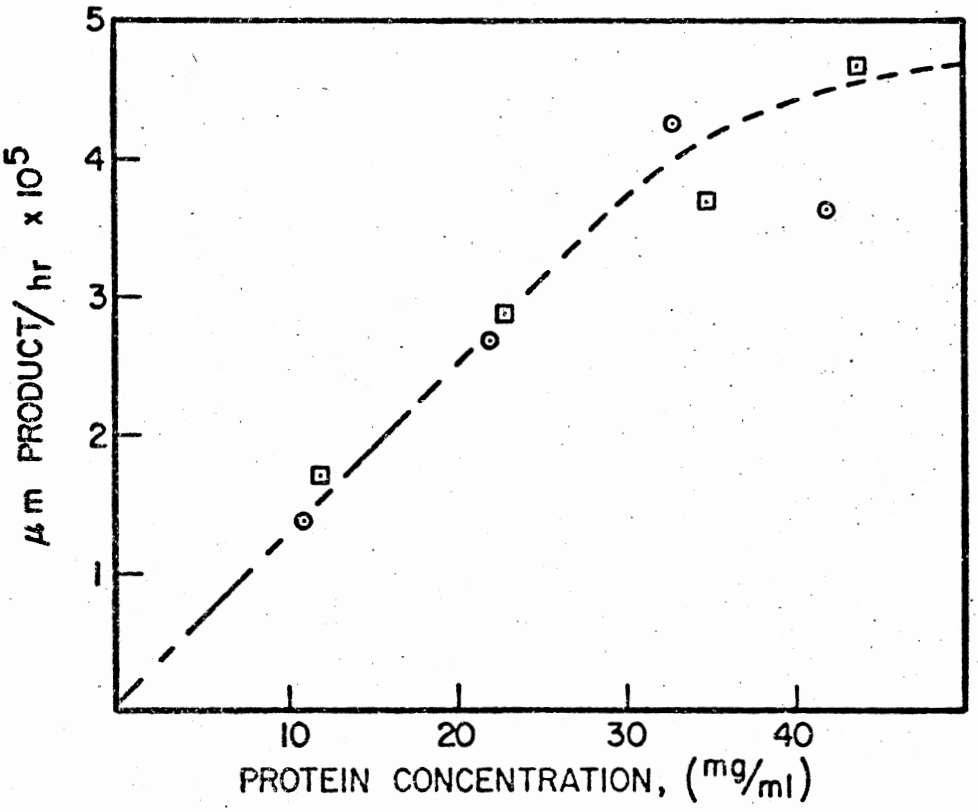
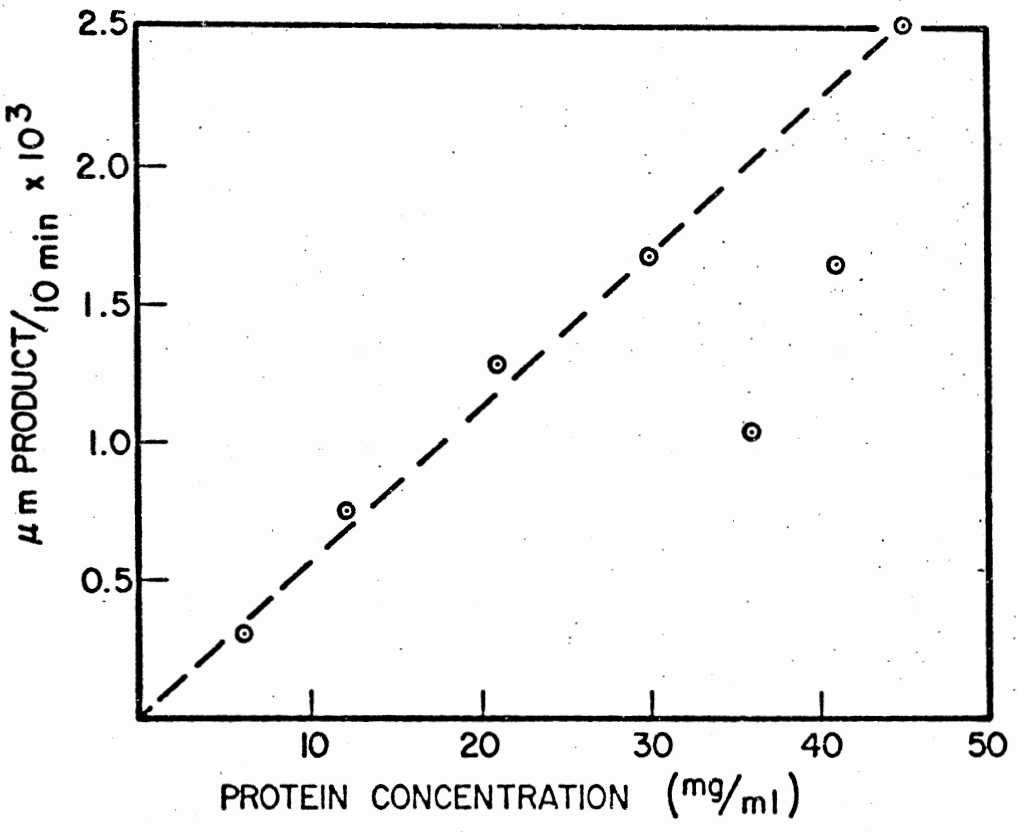

5
4
3
2
1
μm PRODUCT/hr x 10^5
10
20
30
40
PROTEIN CONCENTRATION, (mg/ml)

Figure 5. Nicotinamide Phosphoribosyl Transferase Activity as a Function of Protein Concentration

Standard assay conditions were used, varying only the amount of protein.


2.5
2.0
1.5
1.0
0.5
μm PRODUCT/10 min x 10³
10
20
30
40
50
PROTEIN CONCENTRATION (mg/ml)

4. Substrate and Cofactor Requirements

Routinely, the NA phosphoribosyl transferase and NMN phosphoribosyl transferase activity were determined in the presence of added ATP and PRPP. Because the enzyme source was a crude mixture of disrupted mitochondria, these compounds and other cofactors were presumably present in the enzyme mixture, possibly in large enough quantities to obscure requirements for exogenous substrates. Consequently, to test for substrate and cofactor requirements, the disrupted mitochondria were dialysed as described in Chapter III. This dialysis method seemed sufficient to remove endogenous compounds required for the reactions, while leaving the enzymes active. After dialysis, the enzymes were tested, with the results shown in Tables I and II. Both enzymes require added PRPP. There was essentially no incorporation of either nicotinic acid or nicotinamide in the absence of added PRPP. Magnesium was also absolutely required for the activity of both enzymes. No reaction would be expected in the absence of PRPP since it is a substrate for both enzymes. Demonstration of a PRPP requirement however is important since it rules out mononucleotide formation by exchange reactions. However, only the nicotinamide phosphoribosyl transferase required added ATP. There is essentially no nicotinamide utilization in the absence of added ATP. The nicotinic acid phosphoribosyl transferase was fully activity in the absence of any added

ATP. This is in contrast to previous reports on enzymes from whole liver [15] and yeast [16], but similar to the lack of ATP requirement reported for the enzyme from Astasia longa [18].

When the amounts of PRPP and/or ATP were doubled in the standard assay conditions, there was no increase in the maximum activities of either enzyme.

TABLE I

Substrate and Cofactor Requirements for Nicotinamide Phosphoribosyl Transferase

Deletions	Enzyme Activity (pm/mg/10m)	
	undialysed	dialysed
none	37.9	30.0
ATP	15.8	0
PRPP	8.6	0
$MgCl_2$	-	0

Tubes were incubated at 35° C for 30 minutes. The tubes contained 1.33 mM PRPP, 3.3 mM $MgCl_2$, 26.0 mM phosphate buffer (pH 7.5), and where indicated, 0.67 mM ATP. The NA concentration was as indicated. The enzyme was dialysed against buffer for 2 hours before use. The results shown are the average of two or more determinations.

TABLE II

Substrate and Cofactor Requirements for Nicotinic Acid Phosphoribosyl Transferase

Deletions	Enzyme Activity (pm/mg/10m)	
	undialysed	dialysed
none	2.74 ± 0.35	2.77 ± 0.16
ATP	2.55	2.76 ± 0.55
PRPP	0.79 ± 0.23	0.06 ± 0.03
$MgCl_2$	--	0

For experimental conditions, see Table I.

5. Exchange Reaction

Undialysed nicotinamide phosphoribosyl transferase showed good activity as determined by incorporation of the ^{14}C- label into pyridine derivatives. The dialysed enzyme showed activity in the presence of added ATP and PRPP, but the level was generally lower than for the undialysed enzyme. In the absence of ATP or PRPP, there was no incorporation seen at all. To test for the possibility of an exchange reaction taking place, NMN or NAD^+ were added to assay tubes utilizing dialysed enzymes, without added ATP and PRPP. As shown in Table IV, ^{14}C-NAm was incorporated into NMN or NAD^+ under these conditions. Since ATP and PRPP can be replaced with NMN of NAD^+, it seems likely that an exchange reaction is occurring (see Figure 6).

When these same experiments were tried with nicotinic acid, no exchange could be demonstrated. The addition of NAD^+ or NMN did not result in any incorporation of ^{14}C-NA into pyridine nucleotides in the absence of PRPP and ATP. Since the addition of PRPP to dialysed enzyme restores the activity for the incorporation of ^{14}C-NA into pyridine nucleotides to pre-dialysis levels, it may be assumed that this is a true synthetase activity. See Table III.

TABLE III

Nicotinic Acid Exchange Activities

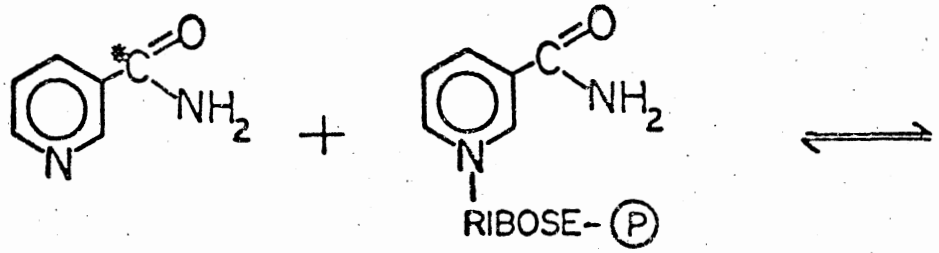
Deletions	Additions	Activity (pm / mg / hr)
none	none	8.09
PRPP	none	0.16
PRPP	NMN	0.23
PRPP	NAD	0.09

TABLE IV

Nicotinamide Exchange Activities

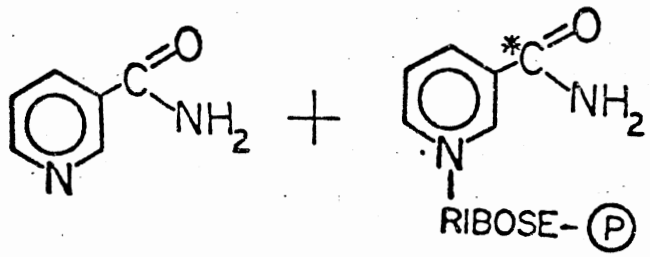
Deletions	Additions	Activity (pm / mg / 10 min.)
none	none	20.8
PRPP,ATP	none	0
PRPP,ATP	NMN	72.7
PRPP,ATP	NAD	34.6

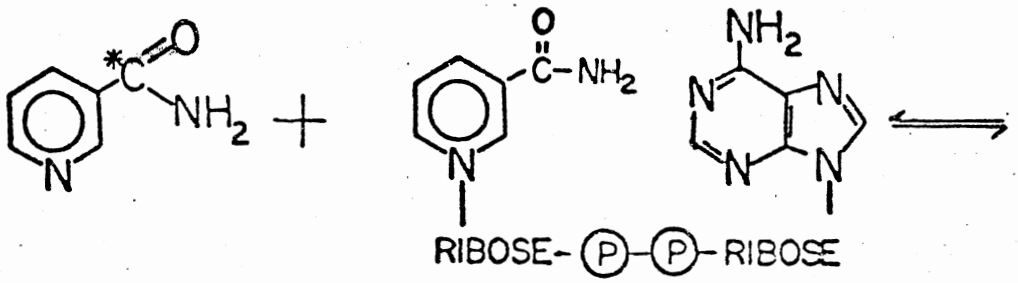
Figure 6. Pathways for the Exchange Reactions



Nicotinamide

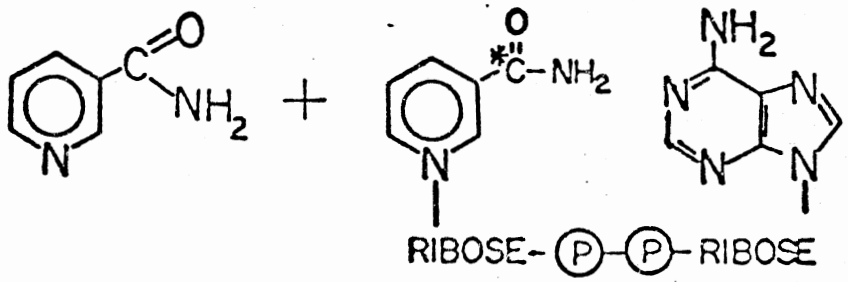
Nicotinamide mononucleotide





Nicotinamide

Nicotinamide adenine dinucleotide



6. Acetone Powder

The results of the nicotinic acid phosphoribosyl transferase assays with the acetone powder extracts are summarized in Table V. The nicotinic acid phosphoribosyl transferase showed good activity, with a requirement for PRPP and $MgCl_2$, but no dependence upon ATP. No activity with nicotinamide could be demonstrated. A sample of isolated mitochondria was prepared and boiled and the supernatent material tested with the acetone powder extract to see if an unidentified cofactor was necessary for the nicotinamide phosphoribosyl transferase activity. Even with the addition of this supernate, no activity could be observed.

Because the acetone powder extract showed very good activity with the nicotinic acid, but no demonstrable activity with nicotinamide, the two activities would appear to be separate. The tentative conclusion therefore is that these are two separate enzymes rather than one enzyme using two substrates.

The effect of ATP on the nicotinic acid phosphoribosyl transferase was further studied by testing the activity at a variety of substrate levels in the presence and absence of ATP. Smith and Gholson [15] have reported activation of the enzyme at low nicotinic acid concentrations with the activation effect disappearing at higher substrate levels. The acetone powder of sheep liver mitochondria was used to test for this activation effect, with the results shown in

Figures 6 and 7. The levels of nicotinic acid were taken as low as practical.

These results are in marked contrast to those obtained by Smith and Gholson using nicotinic acid phosphoribosyl transferase prepared from acetone powders of whole beef liver. Lineweaver-Burk plots of their data showed that ATP appeared to be lowering the K_m of the beef liver nicotinic acid phosphoribosyl transferase for nicotinic acid about 10 fold. On the other hand, in the results with sheep liver mitochondria acetone powder, ATP appears to be more stimulatory at higher nicotinic acid concentrations. This may be due to the fact that in this crude system, a considerable quantity of $NaAD^+$ is formed when ATP is present. This may relieve some product inhibition of the nicotinic acid phosphoribosyl transferase and allow more total NaMN plus $NaAD^+$ to be formed than when ATP is absent and only NaMN accumulates.

TABLE V

Substrate and Cofactor Requirements for Nicotinic Acid Phosphoribosyl Transferase in the Acetone Powder

Deletions	Activity (pm / mg / hr)
none	47.5
ATP	41.8
PRPP	0.5
$MgCl_2$	5.7

Conditions for assays were as described under Section III.A.5, Assays, with the exception that the concentration of nicotinic acid used was 37 µM. The enzyme was dialysed against buffer for 1.5 hours before testing.

Figure 7. Nicotinic Acid Phosphoribosyl Transferase Activity as a Function of Nicotinic Acid Concentration, Showing Effect of ATP on Activity

'●' = +ATP

'■' = -ATP

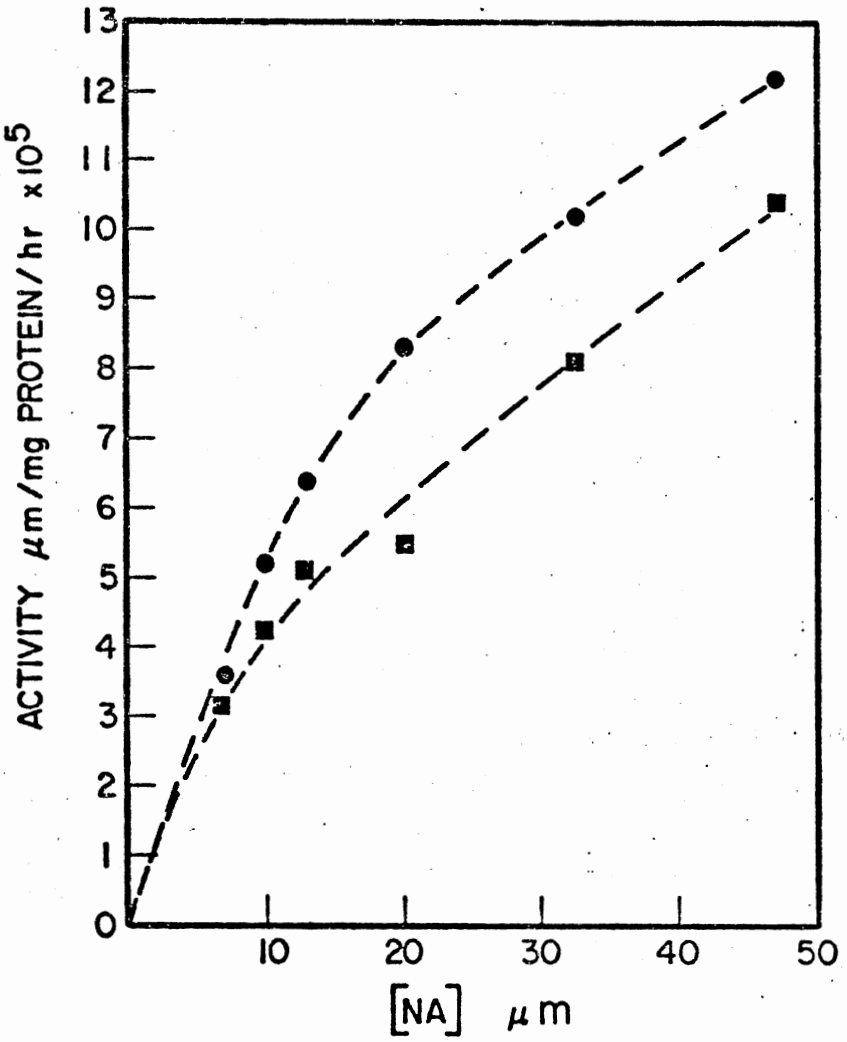
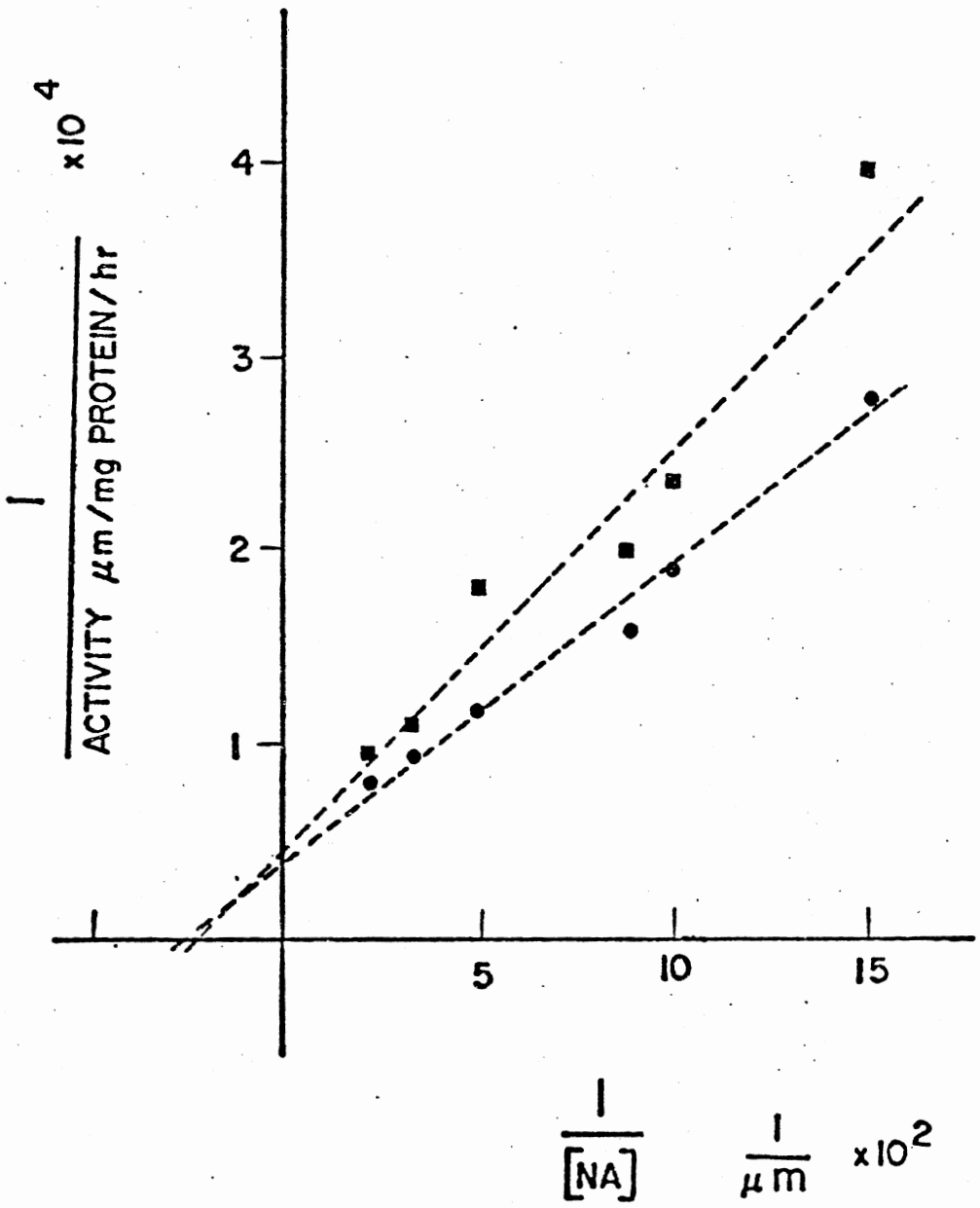

ACTIVITY μm/mg PROTEIN/hr x10⁵
0
1
2
3
4
5
6
7
8
9
10
11
12
13
10
20
30
40
50
[NA] μm

Figure 8. Lineweaver-Burk Plot of Nicotinic Acid Phosphoribosyl Transferase Activity as a Function of Nicotinic Acid Concentration

'●' = +ATP

'■' = -ATP


1 / ACTIVITY µm/mg PROTEIN/hr
x10^4
4
3
2
1
5
10
15
1 / [NA]
1 / µm
x10^2

B. Discussion

Both nicotinic acid and nicotinamide can be incorporated into pyridine nucleotides by disrupted mitochondria from beef and sheep liver. Apparently, two different enzymes are involved, since the time course profile and ATP requirements are different, and selective destruction occurs with the acetone powder.

The nicotinamide phosphoribosyl transferase shows maximum activity within a short time, with a subsequent decrease in total product present. In contrast, the nicotinic acid enzyme shows continued accumulation of product, with a maximum reached at about 90 minutes. One possible explanation for this difference is the presence of a degradative enzyme which attacks NMN but not NaMN. Thus the NaMN could accumulate and continue on to $NaAD^+$, whereas the NMN would be constantly being destroyed.

The two transferases also differ in their requirements for ATP. The nicotinamide phosphoribosyl transferase shows no activity after dialysis unless Mg^{2+}, PRPP, and ATP are added to the reaction mixture. However, the nicotinic acid phosphoribosyl transferase incorporates nicotinic acid into NaMN after dialysis with only Mg^{2+} and PRPP being added. No ATP is required.

With the acetone powder of the mitochondria, only the nicotinic acid enzyme was demonstrable. The specific activity of the acetone powder enzyme was about 10 times the

activity seen in the Triton-disrupted mitochondrial preparation, consistent with removal of an inhibitor. The acetone powder activity shows the identical time course and ATP independence as the fresh enzyme. However, no activity could be observed with nicotinamide in the acetone powder preparation. Because of the presence of only one of the activities in this preparation, it would appear that two separate enzymes are involved in the formation of the pyridine nucleotides from nicotinic acid and nicotinamide.

Also demonstrated was a rapid exchange reaction between nicotinamide, NMN and NAD^+. In the absence of added ATP and PRPP, the addition of either NMN or NAD^+ results in a high amount of labelling of these compounds, apparently due to exchange of the nicotinamide moiety. It would seem that both the synthesis and exchange reactions are operating, since adding ATP and PRPP results in product formation after dialysis in the absence of any NMN or NAD^+.

To insure that the activities investigated were truly intramitochondrial rather than cytoplasmic, several controls were run. The last buffer wash of the mitochondrial preparation procedure was examined, and showed no activity, indicating the enzymes are not in free solution at that time (i.e., cytoplasmic enzymes have been removed). Also, the whole-mitochondria preparation was centrifuged at a very high speed in order to remove all the mitochondria. Then, the remaining supernatant was tested. Again, no activity was found.

CHAPTER V

CONCLUSIONS

Beef and sheep mitochondria are capable of utilizing both nicotinic acid and nicotinamide for the synthesis of NAD^+. Two separate transferases are responsible, rather than one transferase and a deamidase. The first enzyme, nicotinic acid phosphoribosyl transferase, uses NA and PRPP to form NaMN. No ATP is required for this synthesis. Maximum product accumulation is found after about 90 minutes of incubation. An acetone powder of the mitochondria retains this activity. The second enzyme, nicotinamide phosphoribosyl transferase, catalyses the reaction NAm -> NMN. Both PRPP and ATP are absolute requirements for activity. The maximum amount of product is found in 8 to 10 minutes. This enzyme could not be demonstrated in the acetone powder.

In addition to the transferase activities, a rapid exchange of ^{14}C-NAm with NMN as NAD^+ was found. This activity obscures the synthesis reactions in undialysed preparations. No corresponding nicotinic acid exchange activity could be demonstrated.

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