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NICOTINAMIDE–ADENINE DINUCLEOTIDE PYROPHOSPHATASE OF *CAMBAROIDES JAPONICA*

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SUMMARY

1. The cleavage of NAD in crayfish hepatopancreas is catalyzed chiefly by a pyrophosphatase rather than by NAD glycohydrolase (EC 3.2.2.5). This fact was confirmed by the loss in the coenzyme function of NAD for yeast alcohol dehydrogenase without a significant concomitant loss in reactivity towards cyanide and the identification of the reaction products as NMN and AMP by means of paper chromatography and Dowex 1-X2 column chromatography.

2. NAD pyrophosphatase is localized chiefly in mitochondria and microsomes. The enzyme was partially purified by $(NH_4)_2SO_4$ fractionation. Using this preparation, the K_m value for NAD was determined as $1.1 \cdot 10^{-3}$ M. The activity with reduced NAD was about 3-fold higher than with NAD. ATP and NADP are not cleaved by the crayfish enzyme.

3. NAD pyrophosphatase is inactivated by heating at 60° for 1 min and no stimulation was observed by heating at 40° for 1 min. The pH optimum is 8.4.

4. Enzyme activity is inhibited by mononucleotides such as AMP, GMP and UMP, and AMP inhibition is competitive with substrate. Nicotinamide did not inhibit the enzyme activity at $1 \cdot 10^{-3}$ M.

5. The possible metabolic significance of AMP inhibition of NAD hydrolyzing enzymes in mammalian tissues is also discussed.

INTRODUCTION

NAD pyrophosphatase catalyzes the cleavage of the pyrophosphate linkage of NAD to produce nicotinamide mononucleotide and adenosine 5′-phosphate. This enzyme activity has been found in a number of organisms[1–6]. In pigeon liver, two different NAD pyrophosphatase enzymes have been found[7]. One enzyme splits only reduced NAD and NADP and is located in the soluble cytoplasm and another enzyme cleaves both the oxidized and reduced coenzymes. The mammalian enzyme has been found only in microsomes and cleaves the reduced coenzyme faster than the oxidized form. SHLISELFELD, VAN EYS AND TOUSTER[8] reported that a nucleotide pyrophosphatase in rat-liver nuclei catalyzed the cleavage of NAD, NADP and UDP-glucuronic acid as well as UDP-glucose. They suggested that NAD metabolism might be regulated

by the nuclear enzyme and that the formation of glucuronic acid from UDP-glucuronic acid is also catalyzed by the same enzyme. Recently, a specific pyrophosphatase for reduced NAD and NADP has been found by MATSUDA AND KATSUNUMA[9] in rat-liver mitochondria or microsomes which was activated by Mg^{2+} or NH_4^+. VILLELA[10] has reported that NAD cleavage in *Tetrahymena pyriformis* is catalyzed only by a NAD pyrophosphatase which is not inhibited by nicotinamide.

Recently, OLIVERA AND LEHMAN[24] and ZIMMERMAN *et al.*[25] presented most interesting evidence that the formation of the 3',5'-phosphodiester linkage of DNA which is catalyzed by the DNA-joining enzyme from *E. coli* is accompanied by splitting of the pyrophosphate linkage of NAD to produce NMN and AMP.

We have previously reported that the main enzymatic cleavage reaction of NAD in the crayfish hepatopancreas is probably catalyzed by the enzyme NAD pyrophosphatase, which is located in the particulate fraction and is strongly inhibited by purine mononucleotides such as AMP and GMP[11]. This enzyme has now been partially purified and its properties are discussed in this paper.

MATERIALS AND METHODS

Material

The crayfish used were raised on a stock rat diet (MF, Oriental Yeast Kogyo Co.) at room temperature for at least 7 days before each experiment. The hepatopancreas was used as the enzyme source.

NAD, reduced NAD, NADP, AMP, ADP, ATP and alcohol dehydrogenase were purchased from Sigma Chem. Co. The reagents for NADP, ATP, ADP and AMP measurement were purchased from Böhringer Chem. Co. Deamido-NAD, NMN, deamido-NMN and [*carbonyl*-^{14}C]NAD were kindly donated by Dr. O. HAYAISHI, Kyoto University.

Methods

Enzyme assay. The standard assay mixture consisted of 10 μmoles of NAD, 250 μmoles of phosphate buffer (pH 8.0) and enzyme in a final volume of 2.5 ml. The reaction was initiated with enzyme and then incubated for 10 min at 37°. The incubation was terminated by the addition of 2.5 ml of 7% $HClO_4$ and an aliquot of the supernatant was adjusted to pH 6.0–6.5 by the addition of an equal volume of neutralizing reagent (3 M KOH–0.5 M phosphate buffer (pH 6.0)–water, 1.1:1.9:2.0, by vol.). The supernatant was used for the measurement of the NAD cleaved after standing for 10 min in ice water to precipitate the insoluble $KClO_4$ formed.

The NAD content in reaction mixtures was measured by a minor modification of the method of BONNICHSEN[12] using alcohol dehydrogenase with semicarbazide. Full experimental details are given below. A 0.5-ml aliquot of the deproteinized reaction mixture was added to 5 ml of ethanol–semicarbazide solution in 0.05 M phosphate buffer (pH 8.0) followed by the addition of 0.5 ml of the alcohol dehydrogenase solution, which was prepared as a 1 mg per ml aqueous solution immediately before use. The resulting absorbance of the reduced coenzyme was read at 340 mμ after incubation for 10 min at 37°. The controls were carried out in same manner except for the addition of 0.5 ml of water instead of the alcohol dehydrogenase solution.

In some cases the cyanide addition method was used to determine the amount of NAD cleaved by the NAD glycohydrolase activity. This measurement was carried out by the addition of 5 ml of 1 M KCN to 0.5 ml of the samples and the resulting absorbance of the quaternary pyridinium cyanide complex was read at 325 mμ (ref. 13).

The unit of enzyme activity was defined as the amount of enzyme cleaving 1 μmole of the coenzyme in 1 h. The specific activity was represented as units/mg of protein.

Preparation of NAD pyrophosphatase. Fresh tissues were homogenized with Teflon pestle homogenizer in 3 vol. of 0.14 M KCl solution and centrifuged at 700 × *g* for 15 min. The supernatant was then treated by $(NH_4)_2SO_4$ fractionation. The fraction precipitating between 0 and 40% saturation of $(NH_4)_2SO_4$ was dissolved in 0.01 M phosphate buffer (pH 7.0) followed by dialysis against 4 l of the same buffer solution of 0.001 M for 16–18 h. Subcellular components were prepared by a standard procedure[14]. The supernatant after centrifugation at 700 × *g* for 15 min from the homogenate prepared as above was further centrifuged at 15 000 × *g* for 15 min to separate mitochondria and the resulting supernatant containing microsomes was subsequently centrifuged at 105 000 × *g* for 120 min to separate the microsomal fraction. All procedures were performed at 4°.

Other procedures. The amounts of NADP (ref. 15), ATP (ref. 16), ADP and AMP (ref. 17), P_i and PP_i (ref. 18) and pentose[19] were measured as described in the literature with minor modification.

Toyo filter paper No. 50 was used for paper chromatography. Development was carried out at room temperature by the ascending technique until the solvent front had moved 25–30 cm from the origin. The routine solvent was 1 M ammonium acetate–ethanol (7:3, by vol.), adjusted to pH 5.0 with 30% HCl. Nicotinamide, niacin and related compounds were detected as quenching spots under ultraviolet light or by exposure to cyanogenbromide vapor in a closed cabinet for 10 min, followed by spraying with 1% benzidine or *N*-(1-naphthyl)-ethylenediamine in alcoholic solution.

The radio activities of ^{14}C-labeled compounds on the developed paper were measured by the direct counting of paper strips using a Packard Tri-Carb liquid scintillation spectrometer. Pyridinium compounds were detected spectrophotometrically by the absorption at 260 mμ following elution from paper strips with 5 ml of water.

The enzymatic cleavage products were examined by column chromatography on a 2 cm × 12 cm column of Dowex 1 (formate) (200–400 mesh). Elution was conducted as described in RESULTS.

Protein was measured by the method of LOWRY *et al.*[20].

RESULTS

The partial purification of the NAD cleaving enzyme is summarized in Table I. The enzymic activity was completely recovered in the 0 to 40% $(NH_4)_2SO_4$ precipitate.

The subcellular distrubution of the enzymic activity is shown in Table II. The activity is chiefly localized in particulate fractions with the activities in mitochondrial and microsomal fractions being almost equal.

Attempts to solubilize the particulate enzyme have so far not been successful.

TABLE I

PURIFICATION OF NAD CLEAVING ENZYME

Preparation	*Vol. (ml)*	*Total protein (mg)*	*Total units**	*Specific activity**
Whole homogenate	20.0	206.7	957.9	4.65
Supernatant at 700 × *g*, 15 min	17.9	138.0	890.1	6.45
$(NH_4)_2SO_4$ fraction 0 to 40% precipitate	19.1	37.3	373.3	10.00

* The units are μmoles of NAD cleaved per h and specific activities are units per mg of protein.

The pH–activity curve is shown in Fig. 1. The enzymic activity exhibited a peak between pH 8.0 and 8.6 with a maximum around pH 8.4. A sharp decline of enzymic activity was observed at pH values above 8.6.

Identification of enzymic reaction product

The main reaction products were postulated to be NMN and AMP from the results obtained from the alcohol dehydrogenase and KCN assays as shown in Table III, since the NAD cleavage estimated by the cyanide method was far less than that obtained by the alcohol dehydrogenase method even when whole homogenate was used. No significant activity was detected using the cyanide method in partially purified enzyme preparations (Table III). These results suggest that the NAD cleavage by this enzymic reaction may be due to the hydrolysis of the pyrophosphate linkage of the coenzyme molecule to produce NMN and AMP rather than hydrolysis of the glycosidic linkage.

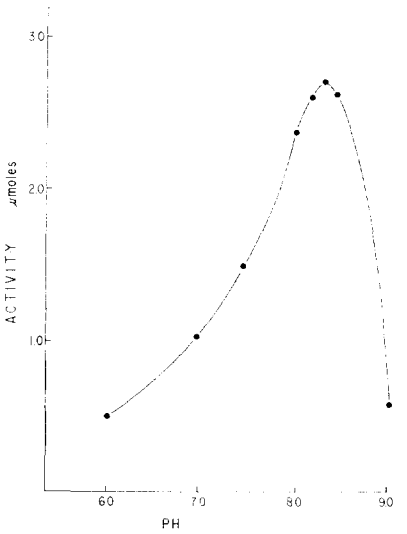

Fig. 1. pH optimum of crayfish NAD cleaving enzyme. The assay was carried out under standard conditions except for pH. 0.1 M phosphate buffers were used after adjusting to the designated pH. Activities are expressed as μmoles of NAD cleaved at 37° for 30 min. $(NH_4)_2SO_4$ fractionated enzyme was used.

TABLE II

SUBCELLULAR DISTRIBUTION OF NAD CLEAVING ENZYME

Experimental details are given in the text.

Fraction	*Distribution (%)*	*Specific activity*
Whole homogenate	100	4.65
Mitochondria and nuclei. Precipitate at 15 000 × *g*, 15 min	43.4	4.84
Microsomes. Precipitate at 105 000 × *g*, 120 min	47.8	4.87
Supernatant at 105 000 × *g*, 120 min	2.1	0.42

Further investigations have been carried out to identify the reaction products. Two main products having an absorption maximum at 260 mμ were detected by means of column chromatography using a Dowex 1-X2 (formate) column. A large scale reaction was carried out in order to identify these products. The reaction mixture consisting of 5 ml of NAD solution (10 μmoles/ml), 10 ml of 0.5 M phosphate buffer (pH 8.0) and enzyme in a total volume of 20 ml was incubated for 2 h at 37°. The initiation, termination and deproteinization were performed as described above. The deproteinized reaction mixture was placed on the Dowex 1-X2 (formate) column (200–400 mesh, 2 cm × 12 cm), and was eluted stepwise with 200 ml of 0.01 M formic acid and 300 ml of 0.25 M formic acid. The first peak ($A_{260\ m\mu}$ maximum) eluted with 0.01 M formic acid reacted with cyanide to produce a cyanide complex which had an absorption maximum at 327 mμ, but was not reduced by alcohol dehydrogenase. Mild alkaline hydrolysis of this compound with NH_4OH at pH 10 overnight at 25° resulted in the release of a product which behaved identically with nicotinamide on paper chromatography. The second peak having an absorption maximum at 260 mμ was eluted with 0.25 M formic acid following the small peak of remaining NAD. The content of P_i and ribose in this peak was measured and equimolar amounts of these components were observed but the compound reacted with neither cyanide nor alcohol dehydrogenase. The paper chromatographic identification of ^{14}C-labeled products formed from [*carbonyl*-^{14}C]NAD by the enzymic reaction is shown in Table IV. The reaction mixture containing [^{14}C]NAD 0.5 μmole (60 000 counts/min), 0.5 M phosphate buffer (pH 8.0) and 0.05 ml of enzyme solution in a total volume 0.5 ml was

TABLE III

COMPARISON OF % NAD REMAINING AS MEASURED BY ALCOHOL DEHYDROGENASE ASSAYS AND KCN ASSAY

The reaction mixture contained 10 μmoles NAD, 0.1 M phosphate buffer (pH 8.0) and $(NH_4)_2SO_4$ fractionated enzyme in a final volume of 10 ml and was incubated at 37°. The system in which enzyme was omitted showed no significant cleavage of NAD. Aliquots were used for NAD determination at the times designated above.

Assay	*% NAD remaining after*				
	0 min	*10 min*	*20 min*	*40 min*	*60 min*
KCN	100	100	99	92	95
Alcohol dehydrogenase	100	69	38	30	20

TABLE IV

IDENTIFICATION OF PRODUCTS FROM [^{14}C]NAD BY PAPER CHROMATOGRAPHY

All flasks contained 0.5 μmole (60 000 counts/min) [^{14}C]NAD, 0.05 ml enzyme and 50 μmoles phosphate buffer (pH 8.0) in 0.5 ml final volume. Aliquots of deproteinized reaction mixture were used for alcohol dehydrogenase assay to estimate NAD cleavage. 50 μl of the mixture containing non-labeled NAD, NMN and nicotinamide as markers were used for paper chromatography using the solvent system; 1 M ammonium acetate–ethanol (7:3, by vol.) adjusted to pH 5.0.

Flasks	*[^{14}C]Compounds detected (counts/min)*			*NAD cleaved by alcohol dehydrogenase assay (μmole)*
	NAD (R_F 0.09)	*NMN (R_F 0.23)*	*Nicotinamide (R_F 0.80)*	
Boiled enzyme	59 520 (0.5 μmole)	---	---	---
Active enzyme	13 270	49 550 (0.39 μmole)*	---	0.40

* Calculated from radioactivities.

incubated for 30 min at 37°. The control was prepared in the same manner except that boiled enzyme was used instead of active enzyme. The reaction was terminated by heating in a boiling water bath for 3 min.

An aliquot (0.05 ml) of the deproteinized reaction mixture after centrifugation at 10 000 rev./min for 10 min was analyzed by paper chromatography and another portion (0.1 ml) was used for NAD assay by the alcohol dehydrogenase method. Only one radioactive product from [^{14}C]NAD was obtained which behaved in an identical manner to NMN on paper chromatography. A stoichiometric relationship was observed between the amount of NMN produced as calculated from radioactivity and the amount of NAD decomposed as measured by the alcohol dehydrogenase method (Table IV). In another experiment, equimolar production of AMP from NAD was observed but no ADP was produced. These results strongly suggested that the enzyme obtained from crayfish hepatopancreas catalyzes cleavage at the pyrophosphate linkage of the NAD molecule.

Kinetic studies on crayfish NAD pyrophosphatase

The NAD cleavage was linear with time up to 60 min using 0.25 ml of enzyme solution and up to an amount of enzyme of 0.5 ml for 10 min. A decline of apparent velocity was observed at prolonged reaction times or with excess enzyme.

The apparent affinity for substrate is shown in Fig. 3 in which a double reciprocal plot of velocity against substrate concentration is shown. The K_m value for substrate is $1.1 \cdot 10^{-3}$ M.

Inhibition studies

It has been reported by several investigators that NAD pyrophosphatases from microorganisms are associated with heat labile inhibitors[3,4]. Excess enzyme seems to inhibit apparent enzymic activity as mentioned above. This suggests the existence of an endogenous inhibitor as in the bacterial enzymes. In order to examine this possibility, whole homogenates were preincubated for 1 min at 20°, 40°, 60° and 80°.

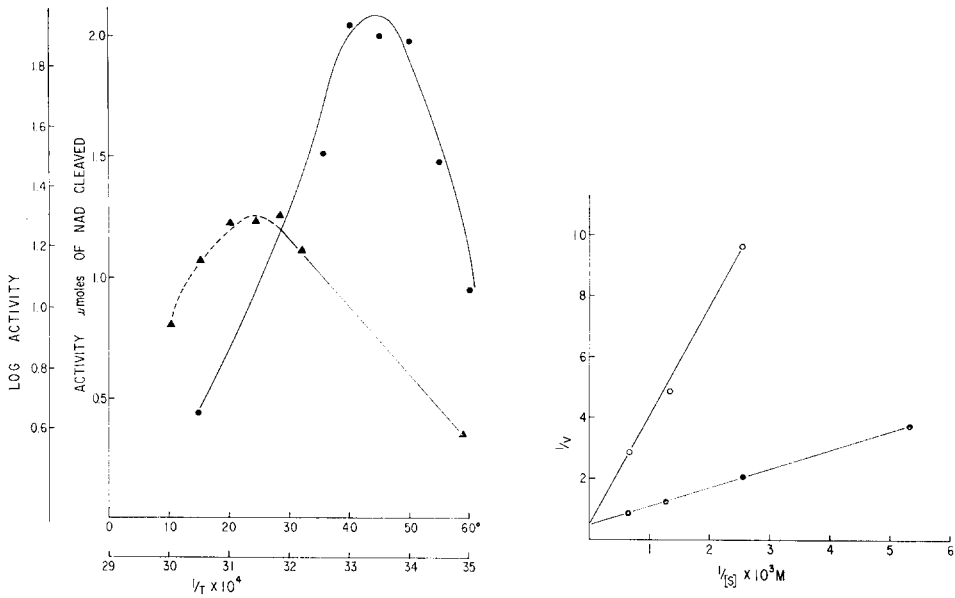

Fig. 2. Temperature optimum of NAD cleaving enzyme. $(NH_4)_2SO_4$ fractionated enzyme was used. Reaction mixtures contain 2.5 μmoles of NAD, 250 μmoles of phosphate buffer and 0.25 ml of enzyme solution. Incubations were carried out at various temperatures for 20 min. The activities are expressed as μmoles of NAD cleaved during incubation. ▲—▲, Arrhenius plot; ●—●, enzyme activities plotted against temperature.

Fig. 3. Competitive inhibition by AMP. $(NH_4)_2SO_4$ fractionated enzyme were used. Incubations were carried out under standard assay conditions. ●—●; no addition. ○—○; $1 \cdot 10^{-3}$ M AMP added. Activities are expressed as μmoles of NAD cleaved per h per ml of enzyme. The final concentration of AMP added is $1 \cdot 10^{-3}$ M.

Neither stimulation nor decrease in enzymic activity was observed up to 40°, whereas very rapid inactivation was observed at temperatures higher than 60° and the optimum temperature was around 40° as shown in Fig. 2. An Arrhenius plot is shown in Fig. 2. The activation energy was calculated as 12 300 cal/mole. These results appear to rule out the possibility of the presence of a heat-labile endogenous inhibitor as in the bacterial enzymes and the decline of apparent velocity observed at prolonged reaction time or with excess enzyme may be partially due to inhibition by AMP produced as a product of the reaction.

Studies comparing the effects of various compounds on the activity of NAD pyrophosphatase indicated that mononucleotides such as AMP, GMP, CMP and UMP strongly inhibited enzyme activity while nicotinamide, nicotinic acid, hydroxylamine and sodium pyrophosphate showed no effect at the same concentration ($1 \cdot 10^{-3}$ M). ADP and adenine ($1 \cdot 10^{-3}$ M) inhibited slightly either at the concentration of $1 \cdot 10^{-3}$ M or $4 \cdot 10^{-3}$ M NAD by almost the same extent (Table V).

AMP inhibition seems to be competitive with substrate as shown in Fig. 3. GMP also inhibited in a manner identical to AMP inhibition. ATP did not reverse AMP inhibition. The comparison of phosphate buffer with Tris–HCl buffer is presented in Table VI. Higher activity was observed in phosphate buffer than in Tris–HCl buffer. No difference in enzymic activity was observed between potassium phosphate

TABLE V

INHIBITORS OF NAD CLEAVING ENZYME

Inhibitors were added to the standard assay system which contained $(NH_4)_2SO_4$ fractionated enzyme. All addition solutions were adjusted to pH 8.0 immediately before use.

Addition ($1 \cdot 10^{-3}$ *M*)	*Inhibition at* $1 \cdot 10^{-3}$ *M NAD* (%)	*Inhibition at* $4 \cdot 10^{-3}$ *M NAD* (%)
None	0	0
AMP	74.0	48.3
GMP	72.7	47.8
CMP	50.0	36.4
UMP	50.0	6.9
Adenine	12.0	12.0
ADP	24.6	21.4
ATP	3.4	—
Nicotinamide	0	—
Nicotinic acid	0	—
Hydroxylamine	0	—
Sodium pyrophosphate	0	—
4-Quinolylalanine	37.5	—
2-Quinolylalanine	18.7	—

buffer and sodium phosphate buffer. The addition of PP_i ion to either Tris buffer or phosphate buffer had no effect on the activity of NAD pyrophosphatase as shown in Table VI. Nicotinamide did not inhibit enzymic activity in contrast to NAD glycohydrolase which is strongly inhibited by nicotinamide.

Substrate specificity and distribution of enzymic activity

NADP and ATP were not decomposed by the enzyme from crayfish tissue. The specific activity for reduced NAD is about 3-fold higher than that for NAD.

NAD cleavage activities of muscle and eggs were tested but significant cleavage reaction was not detected in these tissues.

Correlation between AMP inhibition and the portion of NAD cleavage due to pyrophosphatase in various rabbit tissues

If AMP is a specific inhibitor for NAD pyrophosphatase, AMP inhibition may

TABLE VI

EFFECTS OF PHOSPHATE ION AND PYROPHOSPHATE ION

Final concentration of buffer was 0.1 M. Activities are expressed as μmoles of NAD cleaved in 30 min at 37°. Final volume was 2.5 ml containing 0.1 ml enzyme and 10 μmoles of NAD.

Buffer	*Addition*	*NAD cleaved* (μmoles)	*Relative activity*
Phosphate	None	3.14	100
Phosphate	$1 \cdot 10^{-3}$ M sodium pyrophosphate	3.22	101
Tris	None	1.35	43
Tris	$1 \cdot 10^{-3}$ M sodium pyrophosphate	1.36	43

TABLE VII

COMPARISON OF AMP AND NICOTINAMIDE INHIBITION OF THE NAD CLEAVAGE ACTIVITIES OF VARIOUS RABBIT TISSUES

The determination of NAD cleavage by the alcohol dehydrogenase and KCN assays is given in the text. Activities in columns headed alcohol dehydrogenase and KCN are values estimated by the alcohol dehydrogenase assay and the KCN assay, respectively.

*Tissue**	*NAD cleavage activity***		*Ratio of (1) − (2)/ (1) × 100 (%) (3)*	*Relative activity of inhibited assay system to control****			
	Alcohol dehydrogenase (1)	*KCN (2)*		*Alcohol dehydrogenase*		*KCN*	
				AMP (4)	*Nicotinamide (5)*	*AMP (6)*	*Nicotinamide (7)*
Cecum	3.89	3.69	5.1	107	55	102	57
Liver	1.46	1.30	11.0	95	42	81	38
Kidney	1.82	0.06	96.7	14 (42)	78	nil	nil
Spleen	4.74	4.57	3.6	113	56	111	58

* Tissues were obtained from one animal and immediately homogenized in 5 vol. of 0.14 M KCl. The whole homogenates were used for enzyme assay.

** Enzyme activity is expressed as μmoles of NAD cleaved in 10 min per 0.5 ml of homogenate at 37°. Reaction mixtures consisted of 10 μmoles of NAD, 250 μmoles of phosphate buffer (pH 8.0) and enzyme in a final volume of 2.5 ml. The additions were $1 \cdot 10^{-3}$ M in the standard assay system.

*** Relative activity is expressed as % of control activity remaining in the assay system after addition of inhibitor. The final concentration of additions was $1 \cdot 10^{-3}$ M. The number in parentheses expressed the relative activity when GMP was added instead of AMP. The addition to Column 4 and 6 was AMP and that to Column 5 and 7 was nicotinamide.

be utilized to distinguish NAD cleavage by pyrophosphatase from that catalyzed by NAD glycohydrolase. In order to test this possibility, the NAD cleavage activities in various rabbit tissues were determined by alcohol dehydrogenase and KCN assay and the differences of NAD cleavage behavior in response to the inhibitory effects by AMP and nicotinamide were compared. As shown in Table VII, AMP inhibition is closely correlated with the portion of NAD cleavage due to the pyrophosphatase reaction as detected by comparison of the alcohol dehydrogenase and KCN assays in various rabbit tissues. A clear correlation was observed between the portions of NAD pyrophosphatase in various tissues presenting in Column 3 of Table VII and the relative activities of the AMP-inhibited system presenting in Column 4 of Table VII.

DISCUSSION

The cleavage of nicotinamide–adenine dinucleotide in crayfish hepatopancreas is catalyzed by the enzyme NAD pyrophosphatase but not NAD glycohydrolase.

This conclusion is based on the following facts. (1) The enzymic cleavage of NAD could be detected by the alcohol dehydrogenase method but not by the cyanide addition reaction, (2) the enzymic activity was inhibited by AMP in contrast to NAD glycohydrolase, which is inhibited by nicotinamide and (3) analysis of the reaction products indicated that NAD was hydrolyzed at the pyrophosphate linkage to produce NMN and AMP.

The question might arise whether deamidation could have occurred at the nucleotide level. This possibility may be ruled out by the constancy of the absorbance

values of cyanide products at 325 mμ upon incubation (Table III). If deamidation had been occurring, one would have expected a lower absorbance with time because of the differences of molar absorbance values at 325 mμ between cyanide deamido-NAD and NAD which are $3.7 \cdot 10^3$ and $6.2 \cdot 10^3$, respectively[21,23].

The existence of heat labile endogenous inhibitors for NAD glycohydrolase[22] or NAD pyrophosphatase[3,4] have been reported. When the enzyme preparation was preincubated at various temperatures for 1 min neither stimulation nor decrease in enzymic activity was observed up to 40°. This suggests that this enzyme is not associated with an endogenous inhibitor.

Since the cleavage activity for reduced NAD was higher than that for NAD, the concentration of reduced NAD in crayfish hepatopancreas may also be regulated by the enzyme pyrophosphatase.

ANDERSON AND LANG[23] reported that the cleavage of NAD in mosquito was catalyzed only by the enzyme NAD pyrophosphatase, which underwent metabolic changes during different periods in the life span, and in turn the concentration of the coenzyme could be regulated by this NAD pyrophosphatase. In crayfish no data has been presented concerning changes in NAD pyrophosphatase activity and in the coenzyme concentration during different periods of the life span. Attempts to clarify these metabolic changes are now proceeding in our laboratory.

The cleavage of NAD by pyrophosphatase from crayfish was strongly inhibited by purine mononucleotides such as AMP and GMP. It appears likely that the relative concentration of purine mononucleotides and ATP, which stimulates NAD synthesis at several steps, might regulate the pyridine nucleotide level in crayfish and also in mammals (Table VII). Recently, OLIVERA AND LEHMAN[24] and ZIMMERMAN *et al.*[25] presented most interesting evidence that the DNA-joining enzyme activity from *E. coli* was accompanied by cleavage of the pyrophosphate linkage of NAD to produce NMN and AMP. OLIVERA AND LEHMAN[24] observed that in the absence of polynucleotide, the enzyme catalyzed an exchange reaction between NAD and NMN suggesting that an adenylate–enzyme intermediate is formed. The AMP inhibition of crayfish NAD pyrophosphatase is interesting in this regard.

Attempts to further purify this enzyme and to clarify the significant role of this enzyme in pyridine nucleotide metabolism are now proceeding in our laboratory.

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