Nicotinamide-Adenine Dinucleotide Pyrophosphatase in the Growing and Aging Mosquito

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1. The disappearance of pyridine nucleotides during incubation with mosquito homogenates proceeds through the hydrolysis of the pyrophosphate linkage of these compounds as demonstrated by the formation of NMN and AMP from NAD⁺. This reaction was also demonstrated by the loss in the coenzyme functioning property of NAD⁺ (yeast alcohol dehydrogenase reaction) without a concomitant loss in reactivity towards cyanide. Transglycosidase activity was not observed in the mosquito homogenates, and low concentrations of nicotinamide did not inhibit the NAD⁺ splitting activity of these homogenates. These observations are all in accord with the presence in these homogenates of a NAD⁺ pyrophosphatase rather than a NADase. 2. The NAD⁺ pyrophosphatase is destroyed by boiling, is not heat-activated, and has a pH optimum at pH 8.75. In addition to NAD⁺, other dinucleotides such as NADP⁺, the 3-acetylpyridine and thionicotinamide analogues of NAD⁺ and the thionicotinamide analogue of NADP⁺, function as substrates in the hydrolysis catalysed by the pyrophosphatase. 3. A decrease in the specific activity of NAD⁺ pyrophosphatase was observed during larval development, and a barely detectable activity was found in the pupa and adult. 4. Enzyme activity per organism increased in the larva but decreased to a very low value in the pupa and adult. These results indicate that the decrease in specific activity was due to a decrease in enzyme concentration rather than an increase in amounts of protein.

Previous papers in this series have described experiments on the activities of pyridine nucleotide-linked enzyme systems in the growing and aging mosquito (Lang, 1959, 1961). It was of further interest to investigate the activity of a related enzyme which degrades pyridine nucleotides. Such an enzyme could serve as a control mechanism during development or aging by regulating the concentration of available pyridine nucleotides essential for other reactions.

Enzymes catalysing the splitting of NAD⁺ and NADP⁺ have been demonstrated in tissue preparations from both animal and plant sources. The nicotinamide-adenine dinucleotidases (NADases) catalyse the hydrolysis of the nicotinamide ribosidic linkage and in many cases also possess trans-*N*-glycosidic activity (Zatman, Kaplan & Colowick, 1953; Zatman, Kaplan, Colowick & Ciotti, 1954; Kaplan & Ciotti, 1956). The disappearance of NAD⁺ in reactions catalysed by these enzymes can be followed by the yeast alcohol dehydrogenase method (Kaplan, 1957*a*). This method can also be used to follow the cleavage of NAD⁺ at the pyrophosphate linkage catalysed by pyrophosphatases

obtained from various sources (Kornberg, 1950; Kornberg & Lindberg, 1948; Jacobsen & Kaplan, 1957; Swartz, Kaplan & Lamborg, 1958; Swartz & Merselis, 1962). Although a convenient method of analysis, the yeast alcohol dehydrogenase method will not differentiate between NADase and pyrophosphatase activities. The cyanide addition reaction was suggested as a means of distinguishing between these two activities (Colowick, Kaplan & Ciotti, 1951). This reaction is dependent on the quaternary nitrogen of the pyridine moiety and thus on the integrity of the nicotinamide-ribose linkage. NADase cleaves this linkage whereas a pyrophosphatase will not.

In the present investigation the enzyme activity of mosquito homogenates responsible for the disappearance of NAD⁺ in reaction mixtures has been studied to identify the type of enzyme present. Studies were also carried out to determine the changes in activity occurring during various phases of growth and aging.

EXPERIMENTAL

Materials. NAD⁺, NADP⁺, AMP and ATP were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. The

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3-acetylpyridine analogue of NAD⁺ (AcPyAD⁺) and the thionicotinamide analogue of NAD⁺ (TNAD⁺) were prepared from NAD⁺ by using the transglycosidation reaction catalysed by pig-brain NADase (Kaplan & Stolzenbach, 1957b). The thionicotinamide analogue of NADP⁺ (TNADP⁺) was prepared in the same manner from NADP⁺. NMN was prepared from NAD⁺ by using phosphodiesterase (Kaplan & Stolzenbach, 1957a) partially purified from *Crotalus adamanteus* venom obtained from Ross Allen's Reptile Institute, Silver Springs, Fla., U.S.A. Adenosine diphosphoribose (ADPR) was prepared from NAD⁺ by using *Neurospora* NADase (Kaplan, 1957b). AcPyAD⁺, TNAD⁺, TNADP⁺, NMN and ADPR were purified on Dowex 1 (Cl⁻ form) columns.

Neurospora NADase and yeast alcohol dehydrogenase (twice-crystallized) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Glucose 6-phosphate, disodium salt, and glucose 6-phosphate dehydrogenase, type II, were obtained from Sigma Chemical Co.

The mosquito strain, *Aedes aegypti* L., used in the study, has been maintained in this Laboratory for several years. A standardized rearing procedure with a diet of tap water and desiccated hog liver was used (Lang, 1956; Lang, Lau & Jefferson, 1965). Samples of larvae and pupae of known age were washed in distilled water and homogenized (6–12%, w/v) in 0.25 M-sucrose solution at 0–4°. Adult samples were homogenized without washing but in a similar manner. A portion was removed for protein analysis by the method of Lowry, Rosebrough, Farr & Randall (1951). Since preliminary experiments indicated that enzymic activity was unchanged by freezing, some of the characterization studies were also carried out on samples of frozen homogenates.

Methods. The disappearance of NAD⁺, AcPyAD⁺ and TNAD⁺ in reaction mixtures incubated with mosquito homogenates was followed by the yeast alcohol dehydrogenase method (Kaplan, 1957a) applied to portions removed from the reaction mixtures and deproteinized with trichloroacetic acid. Unless otherwise stated, the incubation reactions were carried out in 0.1 M-tris buffer, pH 8.75, at 37°. All reactions were run in a constant-temperature water bath. Cyanide addition reactions (Colowick *et al.* 1951) were carried out on neutralized samples of the trichloroacetic acid supernatant solutions. Enzymic activity was defined as the μ moles of NAD⁺ split in 30 min. Specific activity was expressed as the enzymic activity per milligram of protein.

Spectrophotometric measurements were carried out on a Zeiss PMQ II spectrophotometer equipped with a thermostatically controlled cell compartment. Measurements of pH were made with a Radiometer pH-meter, type PHM 4c, with G-200-B glass electrode.

RESULTS AND DISCUSSION

The stability of NAD⁺ in the presence of mosquito homogenates was studied at 37° in 0.1 M-tris buffer, pH 8.75, by using both the yeast alcohol dehydrogenase method and the cyanide addition reaction to detect changes in this dinucleotide with time. Fig. 1 illustrates the changes in NAD⁺ observed by incubating the dinucleotide with a

Fig. 1. Disappearance of NAD⁺ with time. Reactions were carried out in 0.5 ml. of reaction mixture containing 0.1 M-tris buffer, pH 8.75, 4.5 μ moles of NAD⁺ and 0.25 ml. of 10% mosquito larval homogenate. The reactions were followed by the yeast alcohol dehydrogenase method (●) and the cyanide addition method (○).

10% mosquito larval homogenate. The ability of NAD⁺ to function as a coenzyme with yeast alcohol dehydrogenase was much decreased, and during the same time-interval no loss in cyanide-reactive material was observed. Although the inability of these two analytical methods to parallel one another would indicate the absence of NADase activity, studies were carried out to investigate the possibility of a transglycosidation reaction being present. When the incubation of NAD⁺ with the 10% mosquito larval homogenate was repeated in the presence of 0.1 M-3-acetylpyridine, the rate of splitting of NAD⁺ was inhibited by 20%, and no formation of the 3-acetylpyridine analogue of NAD⁺ was observed. Nicotinamide at 0.1 M also caused a slight inhibition (17%) of the splitting of NAD⁺ in these reaction mixtures; however, at lower concentrations (5–50 mM) nicotinamide had no effect.

The products of the NAD⁺ degradation reactions were investigated. A reaction mixture containing 20 mg. of NAD⁺ in 1.00 ml. of 0.1 M-tris buffer, pH 8.75, plus 7.0 ml. of a 20% (w/v) homogenate of fourth-instar larvae was incubated at 37° until more than 90% of the NAD⁺ had disappeared as measured by yeast alcohol dehydrogenase. The reaction mixture was deproteinized with trichloroacetic acid, neutralized and applied to a Dowex 1 (Cl⁻ form) column. On the basis of extinction at 260 μ two major components were eluted from the column, which accounted for 95% of the 260 μ -absorbing material initially applied. The first fraction to be removed contained a compound which reacted with cyanide to yield a product absorbing maximally at 325 μ . This material was not reduced in the yeast alcohol dehydrogenase

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reaction. The second fraction was found to be inactive in both the cyanide and yeast alcohol dehydrogenase reactions. The two eluted fractions were freeze-dried, redissolved in a small amount of distilled water and applied to Whatman 3MM paper for chromatography in 0.1N-acetic acid-95% ethanol (1:1, v/v). The fractions were chromatographed for 16 hr. in this solvent system along with known samples of NAD⁺, NMN, AMP, nicotinamide and adenosine diphosphoribose. The first fraction gave a u.v.-absorbing spot corresponding in R_f to that of NMN. A trace of nicotinamide was also observed in this sample, which may indicate a slight degradation during the preparative procedures. The second fraction yielded a u.v.-absorbing spot corresponding in R_f to that of AMP. On the basis of the differences observed in the changes in NAD⁺ as measured by the cyanide and yeast alcohol dehydrogenase methods and the products formed during the incubation of NAD⁺ with mosquito homogenates the catalytic reaction observed was attributed to NAD⁺ pyrophosphatase activity.

The conversion of NAD⁺ into NMN and AMP was not observed when NAD⁺ was incubated with boiled samples of mosquito larval homogenate. Two minutes at 100° was sufficient time to destroy completely the NAD⁺ pyrophosphatase activity of the homogenates. Samples of the mosquito larval homogenate were preincubated in 0.1M-tris buffer, pH 8.75, at 37° in the absence of NAD⁺ to determine the stability of the NAD⁺ pyrophosphatase at this temperature. These incubations were carried out for as long as 150 min. without apparent loss in activity. No indication of heat activation of the pyrophosphatase enzyme was observed during these studies on the effect of temperature on activity. The activity of these homogenates was also stable to freezing and thawing, and homogenates could be kept in the frozen state for several weeks without loss of activity.

Mosquito larval homogenates were incubated for 30 min. with NAD⁺ at temperatures in the range 20–65°. Reactions were studied at 5° intervals in this temperature range, and a temperature optimum was observed at 50°. The effect of pH on these reactions was studied in three different buffers. Mosquito larval homogenates were incubated with NAD⁺ in the presence of 0.1M-ethanolamine, glycylglycine and tris buffers in the range pH 7.5–9.5 (Fig. 2), and a pH optimum 8.75 was observed at 9 mM-NAD⁺. The pH optima of other NAD⁺ pyrophosphatases studied vary from pH 6.5 to 9.5 (Kornberg, 1950; Swartz *et al.* 1958; Swartz & Merselis, 1962).

NADP⁺, the 3-acetylpyridine analogue of NAD⁺ (AcPyAD⁺) and the thionicotinamide analogues of NAD⁺ (TNAD⁺) and NADP⁺ (TNADP⁺) were

Fig. 2. Effect of pH on enzymic activity. Reactions were carried out in 0.5 ml. of reaction mixture containing 4.5 μmoles of NAD⁺, 0.25 ml. of 10% mosquito larval homogenate, and 0.1M-buffer at the indicated pH. □, Glycylglycine; ●, tris; ○, ethanolamine.

Table 1. NAD⁺ pyrophosphatase activity with various dinucleotide substrates

Reaction mixtures containing 9 μmoles of dinucleotide, 0.5 ml. of mosquito larval homogenate (10%) and 0.1M-tris buffer, pH 8.75, in a total volume of 1.0 ml. were incubated at 37° in a constant-temperature bath. Portions (0.1 ml.) were removed at timed intervals, deproteinized with trichloroacetic acid and analysed for remaining dinucleotide by the appropriate dehydrogenase method.

Dinucleotide	Splitting (μmoles in 30 min.)	Relative rate of splitting
NAD ⁺	2.53	1.00
TNAD ⁺	2.67	1.05
AcPyAD ⁺	3.61	1.43
NADP ⁺	2.10	0.83
TNADP ⁺	2.56	1.01

studied as possible substrates in the dinucleotide-splitting reaction catalysed by the mosquito larval homogenate. The rates of splitting of these dinucleotides relative to the rate observed with NAD⁺ are given in Table 1. The splitting of NADP⁺ and TNADP⁺ was followed by the glucose 6-phosphate dehydrogenase reaction. The group in the 3-position of the pyridine rings of these dinucleotides, as well as the additional phosphate group of NADP⁺ and TNADP⁺, have little effect on the rate of splitting of these compounds. This can be compared with the specificity of other enzymes such as dehydrogenases where these portions of the dinucleotide molecule have a much greater effect on the rate of the reaction being catalysed (Kaplan & Ciotti, 1956; Anderson & Kaplan, 1959).

The reverse reaction between AMP and NMN to form NAD⁺ was studied at 37°. The reaction mixtures contained 18 μ moles of AMP, 30 μ moles of NMN, 0.1 M-tris buffer, pH 8.5, and a sample of mosquito larval homogenate capable of splitting 2 μ moles of NAD⁺ in 30 min. Portions were removed from these reaction mixtures, deproteinized with trichloroacetic acid and assayed for NAD⁺ with the yeast alcohol dehydrogenase method. After 2 hr. incubation no formation of NAD⁺ was observed. These reactions were repeated with 20 μ moles of ATP in place of the AMP, and again no formation of NAD⁺ was observed in the 2 hr. incubation period.

The NAD⁺ pyrophosphatase activity of homo-

genes of larvae, pupae and adult mosquitoes of different ages are presented in Fig. 3. The highest specific activity of this enzyme was found in the 1-day-old larval samples. This activity then dropped upon larval development and reached a plateau by the fourth day to 25% of the initial value. During the pupal period a further decrease in activity to a barely detectable value was observed. This low specific activity remained without change throughout the adult stage and senescence.

The decrease in specific activity of NAD⁺ pyrophosphatase could have been attributed to an increase in protein concentration rather than to a decrease in enzyme activity. In this case the enzyme activity per organism would be constant. A graph of the enzymic activity per organism (Fig. 4), however, indicates otherwise for the activity increases during larval development. Comparison of these data with that of specific activity in Fig. 3 leads to the conclusion that the increase of this enzyme per organism during larval development is at a lower rate than that of total protein increase. The low activity per organism and the low specific activity values in both pupae and adults are the result of a low enzyme concentration since the mg. of protein per mosquito in these two developmental stages is constant.

GENERAL DISCUSSION

NAD⁺ pyrophosphatase was demonstrated in the mosquito, instead of NADase, which is found in many other organisms. The presence of NADase in the mosquito has been reported by Gonda, Traub & Avi-Dor (1957). In their studies the disappearance of NAD⁺ was followed by the alcohol dehydrogenase method. The results of our experiments indicate that their activity was probably due to NAD⁺ pyrophosphatase rather than a NADase since the alcohol dehydrogenase assay alone would not distinguish between the two activities. To support this view our analysis of reaction products indicates that under these conditions NAD⁺ is being hydrolysed only at the pyrophosphate linkage. Additional evidence is that NAD⁺ pyrophosphatase, unlike NADase, is not inhibited by low concentrations of nicotinamide.

The constancy of the extinction values at 325 $m\mu$ upon incubation is inconsistent with the presence of an amidase. If a deamidation reaction had been present, one would have expected a lower extinction with time for two reasons. First, the molar extinction coefficient of the cyanide addition product of nicotinic acid pyridine nucleotide is 3.7×10^3 , compared with 6.2×10^3 for NAD⁺ (Lamborg, Stolzenbach & Kaplan, 1958). Secondly, the extinction of the nicotinic acid form at 325 $m\mu$,

Fig. 3. Specific activity of NAD⁺ pyrophosphatase in mosquitoes of different ages. Each point is the average of three to six samples, with the exception of the adult 35-day point, which is a single sample. The vertical broken lines delineate the pupal stage.

Fig. 4. NAD⁺ pyrophosphatase activity per organism. Each point is the average of three to six samples with the exception of the adult 35-day point, which is a single sample. Each sample contained 50–100 mosquitoes/0.6 ml. of homogenate volume with the exception of the earliest larval sample, which consisted of 2000 larvae in the same volume. The vertical broken lines delineate the pupal stage.

the wavelength at which our measurements were made, is lower than at its absorption maximum at approx. 315m μ . In addition, the disappearance of AcPyAD⁺ observed in this study would not be explained by an amidase.

The metabolic changes that occur during different periods of the life span could be dependent upon the concentration of pyridine nucleotide co-enzymes. In turn these concentrations could be regulated by a NAD⁺ pyrophosphatase. The changes observed in our experiments indicate that the activity of NAD⁺ pyrophosphatase decreases during larval development and remains at a low activity until death. This enzyme profile is in contrast with that of another NAD⁺-splitting enzyme, NADase, reported by Nemeth & Dickerman (1960). Their experiments indicate that NAD⁺ activity in different organs of the guinea pig increase to a maximum during the latter part of gestation and remain at this value in the adult. No data were presented regarding the activity in the aged animal. A direct comparison may not be valid because of the differences in enzymes and in experimental animals. The different patterns observed with these two enzymes with age may indicate different physiological roles for these enzymes and may be related to the transglycosidase activity, which is associated with NADases but is absent from NAD⁺ pyrophosphatase.

Our earlier studies of the enzymic profiles of NADH-cytochrome *c* reductase and NADPH-cytochrome *c* reductase demonstrate that the highest specific activity for the NADH enzyme was attained in the adult. On the other hand, the NADPH enzyme activity was high in the larval stage and decreased to a low value during pupal and adult stages. The present results on the enzymic hydrolysis of NADP⁺ are difficult to relate with the reductase information. One interpretation is that the NAD⁺ pyrophosphatase enzyme is at a high activity during early larval development to produce the pyridine and adenine mononucleotides for other metabolic pathways. It appears unlikely that this enzyme could regulate preferentially the concentration of NADP⁺ since the relative rates of splitting of this coenzyme and NAD⁺ were the same.

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