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NAD GLYCOHYDROLASE FROM THE SMALL INTESTINE OF THE RAT*

PURIFICATION, PROPERTIES AND POSSIBLE ROLE OF THE ENZYME

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SUMMARY

1. In the investigation of NAD glycohydrolase (EC 3.2.2.5) in the small intestine of the rat, the properties of this enzyme solubilized by lipase (glycerol-ester hydrolase, EC 3.1.1.3) and purified 250-fold were found to be similar in essentials to its properties found in other tissues. The K_m values for NAD and NADP were $2.7 \cdot 10^{-5}$ and $3.8 \cdot 10^{-6}$ M, respectively.

2. An over 70% inhibition of NAD hydrolysis was observed in the presence of less than $1 \cdot 10^{-6}$ M NADP when NAD was used at a concentration of $6.25 \cdot 10^{-6}$ M as substrate. In contrast to the NADP effect on the NAD cleavage, a slight effect of NAD on the hydrolysis of the NADP was observed.

3. The permeability of pyridine nucleotide in the small intestine was studied. No penetration of [^{14}C]NAD prior to degradation to [^{14}C]nicotinamide was observed. This result would indicate that NAD glycohydrolase may contribute to the absorption of the pyridine moiety of NAD, which would be found significant in the diet.

INTRODUCTION

NAD glycohydrolase (EC 3.2.2.5) catalyzes the reaction NAD + H_2O → nicotinamide + ADP-ribose and is perhaps the most important enzyme in animal tissues which destroys NAD, although the existence of this enzyme reaction in cellular metabolism is yet to be proven. This enzyme (originally named NAD nucleosidase) appears to be bound to microsomal particles from several of the mammalian tissues, and it is also capable of transferring nicotinamide or some other pyridine base to the ADP-ribose moiety of NAD[1-8]. Furthermore, it has been reported that the specificity for

Abbreviation: PCMB, *p*-chloromercuribenzoate.

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NAD and NADP is not altered by purification[9,10]. Thus it appears that the hydrolytic activity of glycohydrolase toward NAD and NADP is found in a single enzyme; however, Hofmann[11], and Hofmann and Rapoport[12] have solubilized, separated and purified specific NAD and NADP nucleosidase from rabbit erythrocytes. Leone and Bonaduce[13] obtained similar data to those of Hofmann in their investigation of soluble pyridine nucleotide nucleosidase found in seminal vesicles. Although the properties of microsomal NAD glycohydrolase has been investigated in several parenchymal organs[8,10,14–17], no report of this enzyme from the digestive canal has yet been published.

From their experiments on the *in vivo* efficacy of the precursors of NAD, Minard and Hann[18] concluded that intraperitoneally injected NAD is cleaved extra-hepatically and that the pyridine moiety is resynthesized to NAD in the liver because of the prolonged rise of NAD and also because of the maximal level common to those produced with the other precursors. On the other hand, Everse *et al.*[19] reported that pyridine nucleotides may enter the liver without cleavage following an intraperitoneal injection of NAD. Deguchi *et al.*[20] could find no permeability of pyridine nucleotide into cell membrane prior to degradation to at least the ribonucleoside level in brain. Since pyridine nucleotide would be found in significant amounts in the diet, the behavior of NAD glycohydrolase in the intestinal tract might be of interest.

We purified the enzyme from rat small intestine 250-fold and studied its properties. Furthermore, it will be seen to be probable that NAD glycohydrolase in the small intestine may contribute to the absorption of the pyridine moiety of NAD.

MATERIALS AND METHODS

Materials

White male rats of the Wistar strain, weighing between 180–200 g, were used in all experiments except where indicated. They were maintained on an Oriental Yeast solid diet fed *ad libitum*, and were allowed free access to drinking water. Lipase (glycerol-ester hydrolase, EC 3.1.1.3) (from hog pancreas, type II), NAD, NADP and alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) were obtained from Sigma Chemicals. Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49) was purchased from the Boehringer Co. The [^{14}C]NAD (nicotinamide-7-labeled) was obtained from the Radioactive Centre, Amersham. The other chemicals were purchased from Nakarai Chemicals, Kyoto. Germ-free rats were obtained from Drs Y. Kotake and T. Hama, Kobegakuin University, Kobe.

Methods

Enzyme assay

Glycohydrolase activity toward NAD and NADP was measured by a modification of Kaplan's method[21]. In this procedure, the activity was assayed by measuring the decrease in absorbance at 327 nm as follows except where indicated: a reaction mixture containing 200 μmoles citrate–phosphate buffer (pH 6.5), 0.2 μmole pyridine nucleotide and 0.2 ml of enzyme preparation in a volume of 0.5 ml was prepared. After incubation of the mixture at 37 °C for 10 min, 5 ml of 1.0 M KCN were added and the mixture was read at 327 nm with a Shimazu multipurpose spectrometer, MPS 50. A control tube was added with cyanide at zero time. When the crude preparation was

used as enzyme, the reaction was stopped by the addition of 10% $HClO_4$ and the neutralized supernatant solution, after centrifugation at 5 000 × *g* for 10 min, was employed for assay as reported previously[22]. In some cases, alcohol dehydrogenase and glucose-6-phosphate dehydrogenase methods[21,23] were also carried out for the enzyme assay. The amount of nucleotide was calculated by using the extinction coefficients $5.90 \cdot 10^6$ cm²/mole in the KCN addition method and $6.22 \cdot 10^6$ cm²/mole in both dehydrogenase methods. 1 unit of enzyme activity was defined as the amount cleaving 1 μmole of coenzyme per 10 min. Enzyme concentration was so adjusted that nucleotide hydrolysis would be less than 15% at any substrate concentration. The 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) and glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) activities were measured by methods previously described[24]. Protein was determined by the method of Lowry *et al.*[25]. ADP-ribose and *P*-ADP-ribose were prepared as previously described[26].

Solubilization and purification of enzyme

A crude preparation of the whole homogenate, removed cell debris and nuclear fraction was made by centrifugation at 600 × *g* for 10 min and this was employed as the first step towards enzyme purification. This step was adopted because the mitochondrial fraction frequently exhibited much cross-contamination of microsomes involving NAD glycohydrolase. The purification of the enzyme was carried out as follows:

Step 1. The rats were sacrificed by exsanguination. The small intestine was removed, cut longitudinally, washed thoroughly with ice-cold 0.85% NaCl solution and cut into small pieces with scissors. The intestinal sections were then homogenized with 9 vol. of 0.25 M sucrose solution in a Teflon homogenizer. The homogenate was centrifuged at 600 × *g* for 10 min and the supernatant solution was referred to as crude preparation.

Steps 2 and 3. The enzyme solubilization was carried out as reported by Swislocki and Kaplan[9]. The crude preparation was recentrifuged at 105 000 × *g* for 60 min and the precipitate was suspended in a 0.01 M phosphate buffer (pH 6.5) at 0.2 vol. of original homogenate. The suspension was incubated at 37 °C for 15 min with 2.5 mg of lipase per ml. After incubation, the suspension was immediately chilled at 0 °C in an ice water bath and then centrifuged at 105 000 × *g* for 60 min. Under these conditions, incubation for about 15 min was required for a 60% solubilization of the enzyme. If the incubation time was increased, however, the activity of the enzyme solubilized by lipase was reduced as reported by Swislocki and Kaplan[9].

Step 4. The solubilized enzyme was applied to a DEAE-cellulose column, 2.5 cm × 60 cm, equilibrated with 0.01 M potassium phosphate at pH 7.5. Elution of the enzyme was carried out with the same buffer. Enzyme activity was associated with the first peak which was not absorbed under these conditions.

Steps 5 and 6. The contents in the tubes corresponding to the enzyme activity peak were pooled. The pooled solution was concentrated to 10 ml by ultrafiltration through a G 10 T membrane (Nihonshinku, MC-2 type) at 4 °C under a nitrogen atmosphere at a pressure of 4 kg/cm². The concentrated enzyme was applied to a Sephadex G-200 column, 2.5 cm × 40 cm, equilibrated with a 0.01 M potassium phosphate buffer at pH 7.5. The enzyme activity was eluted between tubes No. 22–29, when 5 ml of eluate was collected per each tube. The enzyme was pooled and concen-

trated to 5 ml by ultrafiltration as described above. Further purification was achieved by a second gel filtration on a Sephadex G-200 column, 2.5 cm × 40 cm, under the same conditions as those of the first column chromatography. Tubes presenting the highest specific activity (about 40–50 units per mg) were pooled and were referred to as the purified enzyme.

Studies on absorption of NAD in small intestine

Rat intestine, rinsed out *in situ*, was removed from the animals and everted. One end of a 8-cm segment of ileum was closed with a ligature while the opposite end was tied around the lower end of the canula according to the method described by Crane and Wilson[28]. The samples were incubated at 37 °C with 0.8 ml saline solution on the serosal side and the same solution containing [^{14}C]NAD (0.25 μCi, 0.25 μmole) on the mucosal side. At the times indicated, aliquots of both solutions were pipetted and the radioactivity was measured by a Horiba scintillation spectrometer. For the identification of ^{14}C-labelled compound, the samples were chromatographed on Toyo No. 51 filter paper with authentic samples. Nicotinamide and NAD were identified by chromatography of the samples with Solvent I (1-butanol–water–conc. NH_4OH, 680:114:6, by vol.), Solvent II (1 M ammonium acetate (pH 5.1)–ethanol, 3:7, v/v) and Solvent III (pyridine–water, 2:1, v/v). The radioactive spots on the paper corresponding to the ultraviolet absorption of the authentic samples were counted by scintillation spectrometer.

RESULTS

Tissue and subcellular distribution of NAD glycohydrolase

Comparative NAD glycohydrolase activity in various rat tissues is shown in Table I. Next to the activity in the spleen, that in the small intestine proved to be highest when compared with that found in other tissues tested, and it appears to be rather evenly localized, although the proximal and middle portions of the small intestine demonstrate a somewhat higher activity than do the distal portion and the large intestine. Significant activity could not be detected in the stomach.

TABLE I

COMPARISON OF NAD GLYCOHYDROLASE ACTIVITY IN VARIOUS RAT TISSUES

A portion (1 g) of each organ was removed and homogenized with 9 vol. of 0.25 M sucrose solution. The homogenate was employed as enzyme. The assays of NAD glycohydrolase were performed as described in the text. Each value is an average determination for three rats. The small intestine was divided into three portions. A indicates the proximal, B the middle and C the distal portion.

Tissue	*Activity (units/g wet tissue)*
Spleen	71.3
Liver	21.1
Brain	12.2
Stomach	3.5
Small intestine A	29.7
Small intestine B	26.8
Small intestine C	16.3
Ascending colon	17.1

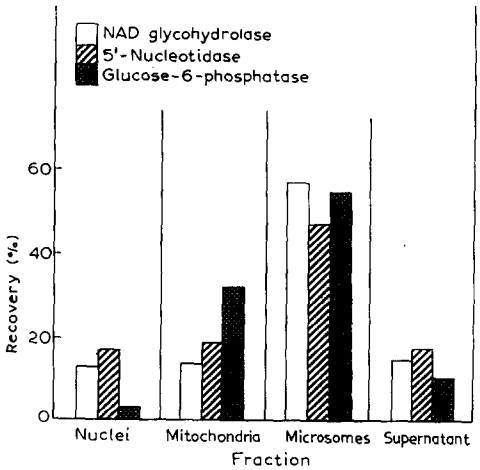

Fig. 1. Subcellular distribution of NAD glycohydrolase, 5'-nucleotidase and glucose-6-phosphatase in the small intestine. 5 g of the middle part of the small intestine were homogenized with 9 vol. of cold 0.25 M sucrose solution. The homogenate was separated into the principal subcellular fractions by the differential centrifugation described by Hogeboom and Schneider[27]. 5'-AMP and glucose 6-phosphate were used as substrates for the assays of 5'-nucleotidase and glucose-6-phosphatase, respectively. The other conditions are described in the text.

The subcellular distribution of small intestinal NAD glycohydrolase was studied. As shown in Fig. 1, the subcellular distribution of this enzyme in the small intestine was found to be mostly localized in microsomes similar to that appearing in other tissues previously reported upon[29]. Glucose-6-phosphatase and 5'-nucleotidase activities were also recovered in the same fractions found with NAD glycohydrolase under the conditions described here.

Purification of the enzyme

A typical result of the purification according to the method described above is given in Table II. The last purification step on the 2nd Sephadex G-200 column chromatography yielded a 250-fold purification of the original material with an 8.5% recovery rate. Furthermore, the cleavage ratio for NADP and NAD was found to be

TABLE II

PURIFICATION OF ENZYME

The enzyme activity of each fraction was assayed as described in the text. One unit of enzyme activity hydrolyzes 1 μmole of NAD per 10 min.

Fractions	*Volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Yield (%)*	*Specific activity (units/mg)*
Crude preparation	500.0	3173.9	634.7	100	0.19
105 000 × *g* precipitate	114.2	1225.7	444.3	70.0	0.36
Preparation in solution	90.0	570.5	270.0	42.0	0.47
DEAE-cellulose	82.0	157.9	166.1	26.0	1.05
1st Sephadex G-200	40.4	4.9	76.7	12.0	15.65
2nd Sephadex G-200	35.1	1.3	54.6	8.6	42.00

about 0.35 during purification, when the substrate had been adjusted to a concentration of $4 \cdot 10^{-4}$ M (data not shown).

Time course of reaction, and effects of enzyme concentration and pH on activity

As to the effect of incubation time at 37 °C on NAD hydrolysis, the reaction was barely linear for at least 15 min and the hydrolysis of NAD was found to be quite proportional to the amount of enzyme present under the given conditions (data not shown). As had been seen in other tissues, small intestinal glycohydrolase also exhibited a relatively broad pH optimum in the range 5.5 to 8.0 when NAD was used as substrate, with the highest activity obtained at 6.5 (data not shown).

Inhibition studies

It has been reported by many investigators that NAD glycohydrolase in microsomes are inhibited by nicotinamide, 3-acetylpyridine and several metals. We also studied this point with the small intestinal enzyme. As shown in Table III, nicotin-

TABLE III

INHIBITORS OF NAD GLYCOHYDROLASE

Inhibitors were added to the standard assay system which contained 0.3 unit of purified enzyme. All additions to the solutions were adjusted to pH 6.5 immediately before use.

Compounds	*Concentration (M)*	*Inhibition (%)*
None		0
Nicotinamide	$1 \cdot 10^{-2}$	81.2
	$1 \cdot 10^{-3}$	57.5
	$1 \cdot 10^{-4}$	14.8
$HgCl_2$	$1 \cdot 10^{-2}$	96.2
	$1 \cdot 10^{-3}$	72.5
	$1 \cdot 10^{-4}$	8.8
PCMB*	$1 \cdot 10^{-4}$	0
3-Acetylpyridine	$1 \cdot 10^{-2}$	17.7
	$1 \cdot 10^{-3}$	5.0
N-Diethyl nicotinamide	$1 \cdot 10^{-3}$	0
N-Ethyl nicotinamide	$1 \cdot 10^{-3}$	0
N-Methyl nicotinamide	$1 \cdot 10^{-3}$	0
ATP	$1 \cdot 10^{-3}$	0
ADP	$1 \cdot 10^{-3}$	0
AMP	$1 \cdot 10^{-3}$	0
Cyclic AMP	$1 \cdot 10^{-3}$	0
Dithiothreitol	$1 \cdot 10^{-3}$	0

* Preincubated at 37 °C for 10 min.

amide and $HgCl_2$ were found to be potent inhibitors, but 3-acetylpyridine was found weak and *p*-chloromercuribenzoate (PCMB) impotent. Furthermore 50% of the hydrolytic activity could be inhibited with $0.9 \cdot 10^{-3}$ M nicotinamide (data not shown). The inhibition was seen to be noncompetitive with a K_i for nicotinamide of $8.8 \cdot 10^{-4}$ M (Fig. 2). The other chemicals tested here did not inhibit the hydrolysis of NAD when these compounds were added to the reaction mixture at a concentration of $1 \cdot 10^{-3}$ M, although Apitz *et al.*[31] reported that dithiothreitol produced a marked inhibition in the pig brain enzyme.

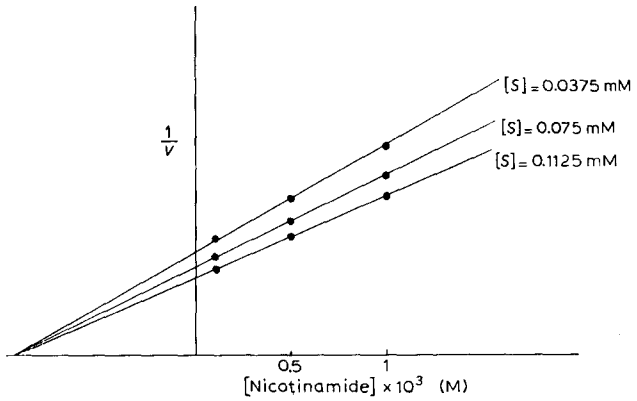

Fig. 2. Dixon plot[30] to determine the K_i for nicotinamide of NAD glycohydrolase activity. [S] is the concentration of NAD.

Effect of substrate concentration

Fig. 3 shows a plot of enzyme activity *versus* substrate concentration. A Lineweaver–Burk plot of this enzyme shows the apparent K_m for NAD and NADP to be $2.7 \cdot 10^{-5}$ and $3.8 \cdot 10^{-6}$ M, respectively. The values are almost identical with those obtained from the purified spleen enzyme[10], but not with those obtained from the other tissues[32–34].

Effect of NADP and NAD on the hydrolysis of NAD and NADP, respectively

From the data described above, it seemed possible that NADP could inhibit NAD cleavage but that NAD would have less influence on NADP hydrolysis. As anticipated, the NADP added to the reaction mixture progressively inhibited the glycoside cleavage of NAD. As shown in Fig. 4, over 70% inhibition of NAD hydrolysis was observed in the presence of less than $1 \cdot 10^{-6}$ M NADP when the NAD concentra-

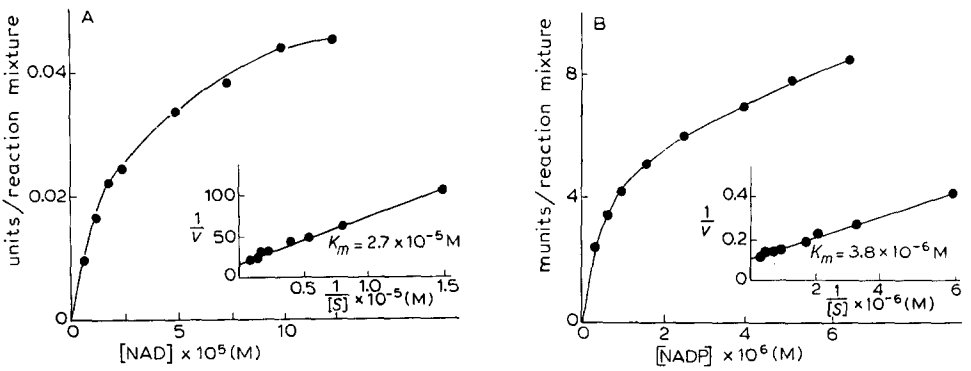

Fig. 3. Rate of hydrolysis as a function of NAD or NADP concentration. Reaction mixture containing 200 μmoles citrate–phosphate buffer (pH 6.5), 0.05 units of purified enzyme and several concentrations of substrate in a total volume 2.5 ml. After incubation at 37 °C for 10 min, 2.5 ml of a 2 M KCN solution were added and the mixture was read at 327 nm in the expanding spectrophotometric system. The other conditions are described in the text. (A) Rate of hydrolysis as a function of NAD concentration. (B) Rate of hydrolysis as a function of NADP concentration.

tion as substrate was $6.25 \cdot 10^{-5}$ M. These results are not due to the effect of an interaction between NADP or *P*-ADP-ribose and alcohol dehydrogenase on the determination of NAD glycohydrolase activity. Furthermore, *P*-ADP-ribose, at any concentration, had no inhibitory effect on enzyme activity (data not shown). In contrast to the NADP effect on NAD cleavage, the effect of NAD on the NADP hydrolysis under the same conditions was slight (Fig. 4). These phenomena would be in accord with the difference in K_m values for the two nucleotides. Therefore, when NAD was employed as substrate at a concentration of $2 \cdot 10^{-3}$ M, the inhibitory effect due to NADP was found to decrease clearly, but no significant influence of NAD could be observed on

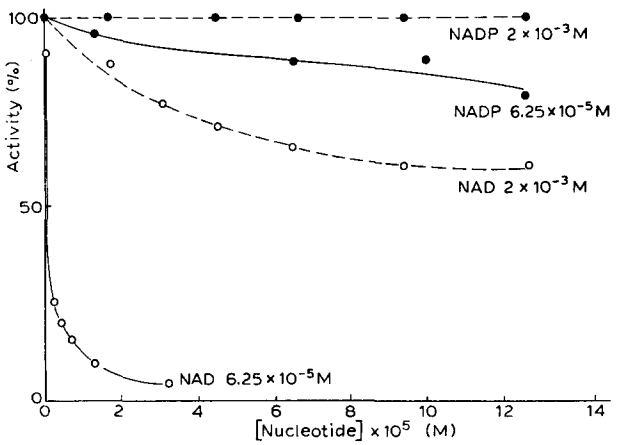

Fig. 4. Effects of NADP and NAD on the hydrolysis of NAD and NADP, respectively. The assay reaction mixture contained 200 μmoles of citrate–phosphate buffer (pH 6.5), 0.1 unit of purified enzyme, $6.25 \cdot 10^{-5}$ or $2 \cdot 10^{-3}$ M of the substrate and various concentrations of the other coenzymes in a total volume of 2.5 ml. After incubation for 10 min at 37 °C 2.5 ml of a 0.01 M Tris–ethanol solution containing sufficient yeast alcohol dehydrogenase for assay of NAD cleavage or 2.5 ml of a glucose-6-phosphate solution containing sufficient glucose-6-phosphate dehydrogenase for assay of NADP cleavage were added, respectively. The absorbance of the solution was read at 360 nm. The rate of coenzyme cleavage with no other nucleotide additions was set at 100%.

the hydrolysis of NADP as substrate at a concentration of $2 \cdot 10^{-3}$ M. Although *P*-ADP-ribose was found to be a very specific inhibitor for NADP dehydrogenase[35], the small amount of *P*-ADP-ribose produced by NADP cleavage had no influence on the assay of NADP glycohydrolase activity by the glucose-6-phosphate dehydrogenase method under the conditions described in the legend to Fig. 4. These results, together with the data on substrate specificity during enzyme purification, indicate that the catalytic site for NAD appears to be identical with that of NADP in the small intestine.

Molecular weight

The estimation of molecular weight was carried out by gel filtration on a calibrated Sephadex G-200 column giving an elution proportional to the logarithm of the molecular weight as described by Andrews[36]. In the linear range, the purified enzyme yielded an elution volume slightly larger than that for bovine serum albumin. On this basis the enzyme from rat small intestine has a molecular weight of approx. 85 000.

Intestinal NAD glycohydrolase activity in embryonic and germ-free rats

It is widely recognized that NAD glycohydrolase activity during fetal development is lower than that during adulthood[37–39]. To see the difference between the enzyme activity in the fetus and that in the adult, respective assays were carried out. As shown in Table IV, the embryo 3 days before birth presented a lower enzyme activity than the adult, but the germ-free adult displayed no difference from the conventional

TABLE IV

COMPARISON OF THE INTESTINAL NAD GLYCOHYDROLASE ACTIVITY OF VARIOUS RATS

The assay conditions were described in the legend of Table I. The number of separate experiments is shown in parentheses.

Rats	*Activity* (%)*
Conventional (7)	100
Embryo** (3)	75.0
Germ-free (1)	96.8

* The value for the conventional rat was settled at 100%.
** 3 days before birth.

adult in this respect. These results also indicate that NAD glycohydrolase activity in the small intestine is not due to microorganisms present in the small intestine, although bacilli present in the mucous membrane of the pars preventricularis of the conventional rat act upon nicotinamide deamidation in rat stomach[40,41].

Fate of NAD in isolated small intestine

To see the fate of NAD in the small intestine and the permeability of NAD into

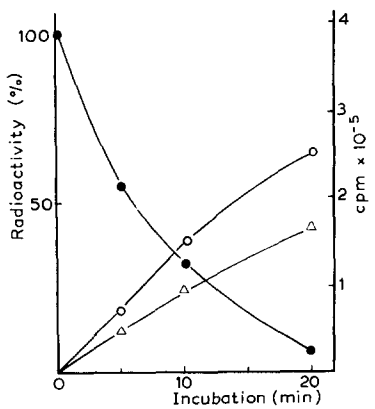

Fig. 5. Fate of NAD in isolated small intestine. The rat small intestine was removed, rinsed out and everted. An ileal segment of 8 cm was incubated with 0.8 ml saline soltuion on the serosal side and 8 ml of the same solution containing [^{14}C]NAD (0.25 μCi, 0.25 μmole) on the mucosal side, according to the method of Crain and Wilson[28]. At the time indicated, aliquots of the mucosal side solution were pipetted to the same volume of 0.1 M acetate solution to stop enzyme action and then chromatographed in Solvent II for separation of the ^{14}C-labeled compound produced during incubation. ●——●, NAD; ○——○, nicotinamide. Aliquots of the serosal side solution were also pipetted and the radioactivity was measured. △——△, radioactivity in the serosal side solution (note that these values are expressed as 40 × scale).

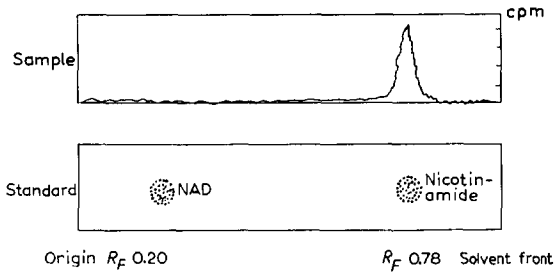

Fig. 6. Radioactive scan of chromatogram of the ^{14}C-labeled compound in the serosal side. A portion of the solution in the serosal side obtained from the experiment described in Fig. 5 was chromatographed in Solvent II. Authentic standards were chromatographed at the same time. The instrument was set at 0.3 K.

the mucosal membrane, the metabolism of [^{14}C]NAD in isolated everted small intestine was investigated. As shown in Fig. 5, a decrease of [^{14}C]NAD following an increase of [^{14}C]nicotinamide was observed in the mucosal side following incubation at 37 °C. Furthermore, no other detectable compounds such as NMN or nicotinic acid were observed, and the radioactivity in the serosal side increased linearly during incubation. The radioactive compound in the serosal side was analyzed by paper chromatography 5 and 20 min after incubation in Solvent systems I, II and III, as described under Materials and Methods. Fig. 6 shows that nearly all this compound is present as nicotinamide and that neither NAD nor its derivatives could be detected. These results indicate that the NAD present in the small intestine had been cleaved to nicotinamide and had entered the portal vein.

DISCUSSION

Microsomal NAD glycohydrolase has been investigated in various organs, but the presence of this enzyme at a relatively high activity level in the small intestine has remained unnoticed and the investigation of its properties has been neglected. Recently, particulate NAD glycohydrolase from various tissues has been solubilized by trypsin digestion[10,42] and with lipase[9,43]. We were also able to solubilize NAD glycohydrolase from rat small intestine with porcine pancreatic lipase. As reported here, a comparison of the properties of rat small intestinal enzyme with those of other mammalian tissues demonstrates that they are similar types of NAD glycohydrolase. The K_m values for NAD and NADP in the small intestine were $2.7 \cdot 10^{-5}$ and $3.8 \cdot 10^{-6}$ M, respectively. Dickerman *et al.*[10] reported the Michaelis constants for NAD and NADP from rat spleen to be about $2.3 \cdot 10^{-5}$ and $6.0 \cdot 10^{-6}$ M, respectively, and that NAD hydrolysis could be reduced by the addition of NADP into the reaction mixture. From the data described in Fig. 4 and ref. 10, it is possible to conclude that NADP may influence the NAD levels through the inhibition of NAD glycohydrolase activity *in vivo*, because the K_m value for NADP is lower than that for NAD.

The physiological role of microsomal NAD glycohydrolase is yet unknown. The points thus far reported are: (a) the NAD level may be influenced by NAD glycohydrolase[22,43,44] and (b) the nicotinamide normally released by NAD glycohydrolase could be incorporated into NAD[45]. The data offered in the present report would sug-

gest that NAD contained in the diet may be cleaved into nicotinamide by NAD glycohydrolase in the mucous membrane of the small intestine and then enter the portal vein. Since it has been reported that nicotinamide may enter the NAD biosynthetic pathways *via* nicotinamide mononucleotide without deamidation to nicotinic acid in perfused rat liver[46], in contrast to the previous observations of NAD biosynthesis in mammals[47,48], the cleavage of the glycoside linkage of NAD present in the diet should produce a powerful effect on NAD formation in mammals. Furthermore, it is interesting to speculate that the stringent control of the NAD glycohydrolase by NADP would point to the biological relevance of this problem.

Although Everse *et al.*[19] expressed the possibility that pyridine nucleotides may enter the animal cells intact, the present observations along with the data described in ref. 20 would not support such a supposition. However, this disagreement in results may be due to the differences in pyridine nucleotide amounts used by the different investigators. The amount of labeled pyridine nucleotide used by Everse *et al.*[19] in their experiments was 1000 mg per kg body weight. This value is much greater than that used here, and most of the pyridine nucleotide injected into the intraperitoneal cavity may have been absorbed without cleavage. These apparently opposite results may, because of differences in pyridine nucleotide concentration, complement rather than contradict each other.

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REFERENCES

1 *Report of the Commission of Enzymes of the International Union of Biochemistry, Oxford, London, New York, Paris*, Pergamon Press, New York, 1961, p. 112.
2 P. Handler and J. R. Klein, *J. Biol. Chem.*, 143 (1942) 49.
3 L. J. Zatman, N. O. Kaplan and S. P. Colowick, *J. Biol. Chem.*, 200 (1953) 1917.
4 L. J. Zatman, N. O. Kaplan, S. P. Colowick and M. M. Ciotti, *J. Biol. Chem.*, 209 (1954) 453.
5 L. J. Zatman, N. O. Kaplan, S. P. Colowick and M. M. Ciotti, *J. Biol. Chem.*, 209 (1954) 467.
6 H. McIlwain, *Biochem. J.*, 46 (1950) 612.
7 H. McIlwain and R. Rodnight, *Biochem. J.*, 44 (1949) 470.
8 D. Stathakos and K. Wallenfels, *Biochem. Z.*, 346 (1966) 346.
9 N. I. Swislocki and N. O. Kaplan, *J. Biol. Chem.*, 242 (1967) 1083.
10 H. W. Dickerman, A. San Pietro and N. O. Kaplan, *Biochim. Biophys. Acta*, 62 (1962) 230.
11 E. C. G. Hofmann, *Biochem. Z.*, 329 (1957) 428.
12 E. C. G. Hofmann and S. Rapoport, *Biochem. Z.*, 329 (1957) 437.
13 E. Leone and L. Bonaduce, *Biochim. Biophys. Acta*, 31 (1959) 292.
14 H. G. Windmueller and N. O. Kaplan, *Biochim. Biophys. Acta*, 56 (1962) 388.
15 I. Windman, A. Bekienkunst and A. Artman, *Biochim. Biophys. Acta*, 32 (1964) 405.
16 N. I. Swislocki, M. I. Kalish, F. I. Chasalow and N. O. Kaplan, *J. Biol. Chem.*, 242 (1967) 1089.
17 R. Apitz, K. Mickelson, K. Shriver and E. H. Cordes, *Arch. Biochem. Biophys.*, 143 (1971) 359.
18 F. N. Minard and C. H. Hann, *J. Biol. Chem.*, 238 (1963) 2474.
19 J. Everse, N. O. Kaplan and S. Schichor, *Arch. Biochem. Biophys.*, 136 (1970) 106.
20 T. Deguchi, A. Ichiyama, Y. Nishizuka and O. Hayaishi, *Biochim. Biophys. Acta*, 158 (1968) 382.
21 N. O. Kaplan, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. 2, Academic Press, New York, 1955, p. 660.

22 M. Shimoyama, M. Ohota, K. Kakehi and I. Ueda, *Biochim. Biophys. Acta*, 215 (1970) 207.
23 A. Kornberg and B. L. Horecker, in S. P. Colowick and N. O Kaplan, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 323.
24 Y. Tanigawa, M. Shimoyama, J. Asamizu and I. Ueda, *Biochim. Biophys. Acta*, 230 (1971) 510.
25 O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
26 B. M. Anderson and C. D. Anderson, *J. Biol. Chem.*, 238 (1963) 1475.
27 C. H. Hogeboom and W. C. Schneider, in E. Chargaff and J. N. Davidson, *The Nucleic Acid*, Vol. 2, Aacdemic Press, New York, 1955, p. 105.
28 R. K. Crane and T. H. Wilson, *J. Appl. Physiol.*, 12 (1958) 145.
29 K. B. Jacobson and N. O. Kaplan, *J. Biophys. Biochem. Cytol.*, 3 (1957) 31.
30 M. Dixon, *Biochem. J.*, 55 (1953) 170.
31 R. Apitz, K. Mickelson, K. Shriver and E. H. Cordes, *Arch. Biochem. Biophys.*, 143 (1971) 359.
32 A. A. Abdel-Latif and S. G. A. Alvisatos, *J. Biol. Chem.*, 237 (1962) 500.
33 ibid., *J. Biol. Chem.*, 260 (1961) 2710.
34 K. Nakazawa, K. Ueda, T. Honjo, K. Yoshihara, Y. Nishizuka and O. Hayaishi, *Biochem. Biophys. Res. Commun.*, 32 (1968) 143.
35 C. Ben-Haygim, A. Hochman and Mordhay Avron, *J. Biol. Chem.*, 242 (1967) 2837.
36 P. Andrews, *Biochem. J.*, 91 (1964) 222.
37 A. M. Nemeth and H. Dickerman, *J. Biol. Chem.*, 235 (1960) 1761.
38 R. M. Burton, *J. Neurochem.*, 2 (1957) 15.
39 M. Shimoyama, H. Sakamoto, T. Takeuchi, K. Yamaguchi and I. Ueda, *Biochim. Biophys. Acta*, 184 (1969) 204.
40 Y. Tanigawa, M. Shimoyama, R. Murashima, T. Ito, K. Yamaguchi and I. Ueda, *Biochim. Biophys. Acta*, 201 (1970) 394.
41 M. Shimoyama, Y. Tanigawa, T. Ito, R. Murashima, I. Ueda and T. Tomoda, *J. Bacteriol.*, 108 (1971) 191.
42 H. G. Windmueller and N. O. Kaplan, *Biochim. Biophys. Acta*, 56 (1962) 388.
43 S. Green and O. Bodanski, *J. Biol. Chem.*, 280 (1965) 2574.
44 P. Scriba, S. Schneider and H. Holzer, *Z. Krebsforsch.*, 63 (1960) 547.
45 R. K. Gholson, *Nature*, 212 (1966) 943.
46 J. Keller, M. Liesch and H. Grunicke, *Eur. J. Biochem.*, 22 (1971) 263.
47 H. Ijichi, A. Ichiyama and O. Hayaishi, *J. Biol. Chem.*, 241 (1966) 3701.
48 Y. Hagino, S. J. Lan, C. Y. Ng and L. M. Henderson, *J. Biol. Chem.*, 243 (1968) 4900.