NAD Glycohydrolase Activity in the Liver of Rats
Fed on Excess Leucine Diet and
Low or High Protein Diet

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Summary In rats fed for 2 weeks on a 10% casein diet supplemented
with 5% leucine, the hepatic NAD content was lowered when comparing
with the control rats pair-fed on the 10% casein diet. The hepatic NAD
glycohydrolase [EC 3.2.2.5] activity increased by 25% in the leucine-
supplemented diet-fed rats and by 40% in the nicotinic acid-free diet-fed
rats. The hepatic NAD glycohydrolase activity in the rats fed on the non-
protein diet freely for 1 week was elevated by 60% compared to the rats
fed on the 18% casein diet. The hepatic NAD content in the former
animals was significantly lower than that in the latter. The 70% casein diet
caused changes in neither hepatic NAD content nor NAD glycohydrolase
activity. These results indicated an inverse relationship between hepatic
NAD content and NAD glycohydrolase activity.

Key Words dietary excess leucine, dietary protein level, rat hepatic NAD
level, rat hepatic nicotinamide level, rat hepatic NAD glycohydrolase,
urinary N\textsuperscript{1}-methylnicotinamide

The authors have reported that the hepatic NAD and total nicotinamide
nucleotide levels decrease significantly when rats are fed on 10% or 20% casein diet
containing 5% L-leucine ad libitum (1). As the NAD-lowering effect of leucine was
more intense in the 10% casein diet-fed group, a possible explanation was that the
amino acid imbalance created by leucine supplementation of the low protein diet
resulted in a depression in the level of tryptophan in tissues available for conversion
to NAD. In our previous study (1) and in the report by Nakagawa and Sasaki (2),
however, plasma or serum tryptophan in the leucine-supplemented group was not
lowered but rather increased compared to the control group.

The reported effects of excess leucine on urinary excretion of nicotinic acid

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metabolites are not consistent. Excess leucine caused a significant increase in the excretion of \( N^1 \)-methyl nicotinamide in adult rats (3), a decrease in \( N^1 \)-methyl nicotinamide and quinolinic acid in female rats (2), had no effect on \( N^1 \)-methyl nicotinamide (4) and caused a decrease in \( N^1 \)-methyl nicotinamide and \( N^1 \)-methyl-2-pyridone-5-carboxamide excretion of experimental subjects (5). Changes in urinary excretion of nicotinic acid metabolites, if any, may suggest that the acceleration of NAD breakdown is also responsible for lowering the hepatic NAD level caused by excess dietary leucine.

Two enzymes, namely NAD glycohydrolase [EC 3.2.2.5] and NAD pyrophosphatase [EC 3.6.1.22], are known to participate in NAD degradation. Shimoyama et al. (6) studied these two enzyme activities in fasted rat liver, wherein the NAD concentration was decreased. They found that NAD glycohydrolase activity in the liver homogenate was elevated and that there was no change in NAD pyrophosphatase activity. The increase of NAD glycohydrolase activity resulting from starvation was also observed in rat liver microsomes (7).

In this report the possibility of stimulation of NAD glycohydrolase by dietary excess leucine was investigated. As diet intake was lowered by 20% in the excess leucine-supplemented diet-fed group in the previous experiment (1), animals in all groups were pair-fed in this study. The effect of dietary protein on the hepatic NAD glycohydrolase activity was also investigated, for the dietary protein level was responsible for the hepatic NAD content (1, 8).

**EXPERIMENTAL**

**Animals.** Male 4-week-old Sprague-Dawley rats (SPF) were purchased from Charles River Breeding Laboratories, Japan. Animals were kept in individual cages at 20°C in a room with a 12-hr light and 12-hr dark cycle.

**Diet.** Compositions of the experimental diets are shown in Table 1. The mineral mixture was made as per Hegsted et al. (9).

To accustom the animals to the powdery diet, all animals were prefed on the 18% casein diet for 3 days prior to administering the experimental diets. In all experiments, 5 rats per group were used. Water was supplied freely. In experiment I, the average amount of diet which the leucine-supplemented group consumed was fed to the rest of the animals. In experiment II, all rats were fed freely. After feeding experimental diets for certain days (for 2 weeks in experiment I and for 1 week in experiment II), rats were decapitated, and the liver was quickly excised and divided into two parts. One part was frozen and powdered finely in the frozen state for nucleotide and nicotinamide determinations. The other part was used for NAD glycohydrolase assay.

**Determination of nicotinamide nucleotides.** Both oxidized and reduced forms of nicotinamide nucleotides were extracted from the frozen tissue powder and determined according to Klingenberg (10) with an Eppendorf photometer 1101M.

**Determination of total nicotinic acid.** Total nicotinic acid content of the liver
### Table 1. Composition of experimental diets.

|                     | Experiment I | Experiment II |
|---------------------|--------------|--------------|
|                     | (A)          | (B)          | (C)          | (D)          | (E)          | (F)          | (G)          |
| Casein (g/kg)       | 110          | 110          | 110          | 110          | 110          | 100          | 110          |
| Corn oil (g/kg)     | 100          | 100          | 100          | 100          | 100          | 100          | 100          |
| Vitamin mix.        | 40           | 40           | 40           | 40           | 40           | 40           | 40           |
| Salt mix.           | 22           | 22           | 22           | 22           | 22           | 22           | 22           |
| Cellulose           | 20           | 20           | 20           | 20           | 20           | 20           | 20           |
| Nicotinic acid      | 99           | 99           | 99           | 99           | 99           | 99           | 99           |
| Dextrose            | 638          | 638          | 638          | 638          | 638          | 638          | 638          |
| L-Leucine           | 50           | 50           | 50           | 50           | 50           | 50           | 50           |

*Ingredients are expressed as g/kg, with the exception of nicotinic acid (mg).

a (mg/100 g diet); x-tocopherol, 11.0; ascorbic acid, 99.0; nisinol, 11.0; choline chloride, 165.0; menadione, 5.0; p-aminobenzonic acid, 11.0; thiamine•HCl, 2.2; riboflavin, 2.2; Ca-pantothenate, 6.6; biotin, 0.04; folax acid, 0.2; pyridoxine•HCl, 2.2; and vitamin B12, 0.003.

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was determined microbially using *Lactobacillus plantarum* (ATCC 8014) as a test organism after extracting nicotinic acid from the frozen liver powder with 1 N H$_2$SO$_4$ at 125°C for 30 min (11). Under this extraction condition, NADH and NADPH were found to be decomposed to the forms which could not be utilized by *L. plantarum*. Therefore, total nicotinic acid was obtained by adding the μmol of nicotinic acid from microbiological assay to the μmol of NADH and NADPH from enzymatic assay.

**Determination of nicotinic acid and nicotinamide.** One g of the frozen liver powder was homogenized with 10 ml of 70% ethanol. The homogenate was heated in a boiling-water bath for 2 min, cooled and centrifuged. The supernatant was evaporated and the residue was dissolved in a certain amount of water (sample). One ml of each sample was applied to a Dowex 1 formate column (X2; 200–400 mesh; diameter 7 mm; length 15 mm). Nicotinamide was washed through the column with 10 ml of water, and nicotinic acid was eluted with 10 ml of 0.05 N HCOOH. Nicotinamide and nicotinic acid contents of each fraction were determined microbiologically.

**Determination of urinary N1-methylnicotinamide.** Urinary N1-methylnicotinamide was measured fluorometrically according to Huff and Perlzweig (12).

**NAD glycohydrolase assay.** The liver NAD glycohydrolase activity was assayed according to the method of Ueda *et al.* (13) with the following modifications. Fresh liver was homogenized with 9 volumes of 0.25 M sucrose. The homogenate filtered through several layers of gauze was used as enzyme solution. The reaction mixture (total volume 1.1 ml) contained 0.65 ml of 0.1 M phosphate buffer, pH 7.2, 0.30 ml of 5 mM NAD solution containing 25 nCi of [carbonyl-14C]NAD, 0.125 ml of water and 0.025 ml of the enzyme solution. The reaction was started by adding the enzyme solution. The mixture was incubated for 10 min at 37°C, and the reaction was stopped by the addition of 0.25 ml of 35% HClO$_4$. The mixture was centrifuged for 10 min at 3,000 rpm. One ml of the supernatant was neutralized by the addition of 3 M KOH and the precipitate was removed by centrifugation. A half ml of the clear supernatant thus obtained was applied to a Dowex 1 formate column. The radioactivity in the flowthrough fraction (nicotinamide fraction) was determined with a Packard Tri-Carb liquid scintillation spectrometer with ACS II as scintillator cocktail.

**RESULTS**

In experiment I, as shown in Table 2, dietary excess leucine suppressed body weight gain in both the nicotinic acid-devoid and -supplemented groups. The NAD content per g liver tissue was reduced in the animals fed on the excess leucine diet containing nicotinic acid. The hepatic NAD level tended to decrease in the animals fed on the diets devoid of nicotinic acid, and addition of leucine caused no further decrease in the NAD content. In the animals fed freely on the 10% casein diet devoid of nicotinic acid, diet intake increased by 10% compared to the nicotinic
Table 2. Effect of dietary excess leucine on body weight gain and hepatic nicotinamide adenine dinucleotide levels (Experiment I).

| Diet    | Body wt gain (g/2wks/rat) | Nicotinamide adenine dinucleotide |
|---------|---------------------------|----------------------------------|
|         |                           | NAD | NADH | NADP (μmol/g wet liver) | NADPH | Total |
| (A) + NA  | 26.0 ± 3.4*               | 0.69 ± 0.08 | 0.19 ± 0.03 | 0.09 ± 0.02 | 0.30 ± 0.05 | 1.27 ± 0.11 |
| (B) + NA + Leu | 14.3 ± 9.1*           | 0.55 ± 0.03** | 0.22 ± 0.08 | 0.07 ± 0.03 | 0.25 ± 0.04 | 1.09 ± 0.14 |
| (C) − NA | 25.8 ± 2.8               | 0.60 ± 0.08 | 0.22 ± 0.03 | 0.08 ± 0.03 | 0.28 ± 0.03 | 1.18 ± 0.20 |
| (D) − NA + Leu | 16.0 ± 5.5***    | 0.57 ± 0.05* | 0.18 ± 0.03 | 0.10 ± 0.01 | 0.28 ± 0.05 | 1.12 ± 0.11 |

* Means ± SD. ** Indicate significant difference from (A) at the 5, 2 and 1% levels respectively by *t*-test.
Table 3. Effect of dietary excess leucine on hepatic NAD glycohydrolase activity.

| Diet          | NAD glycohydrolase ( Nicotinamide formed, μmol/min/g wet liver) |
|---------------|------------------------------------------------------------------|
| (A) +NA       | 1.79 ± 0.31*                                                     |
| (B) +NA + Leu | 2.24 ± 0.33*                                                     |
| (C) -NA       | 2.50 ± 0.17*                                                     |
| (D) -NA + Leu | 2.53 ± 0.29*                                                     |

* Means ± SD. * Indicates significant difference from (A) at the 5% level by t-test.

Table 4. Effect of dietary excess leucine on hepatic total nicotinate and nicotinamide contents and urinary excretion of N1-methylnicotinamide.

| Diet          | Hepatic content | Urinary excretion of N1-methylnicotinamide (nmol excreted/day) |
|---------------|-----------------|-----------------------------------------------------------------|
|               | Total nicotinate | Nicotinamide |                                                             |
|               | (μmol/g wet liver) |           |                                                             |
| (A) +NA       | 1.92 ± 0.15      | 0.52 ± 0.07 | 1,976 ± 155                                                  |
| (B) +NA + Leu | 1.84 ± 0.25      | 0.65 ± 0.17 | 1,771 ± 658                                                  |
| (C) -NA       | 2.11 ± 0.30      | 0.61 ± 0.07 | 375 ± 181*                                                   |
| (D) -NA + Leu | 1.91 ± 0.05      | 0.56 ± 0.11 | 281 ± 139*                                                   |

* Indicates significant difference from (A) at the 1% level by t-test.

acid-containing diet-fed group (1). This was probably why the hepatic NAD level could be maintained at the normal level in the rats fed on the nicotinate-free diet ad libitum. Other nicotinamide nucleotides and total nicotinamide nucleotides were not affected at all by the dietary excess leucine.

As hepatic NAD glycohydrolase activity was localized in microsomes, nuclear membrane and plasma membrane (14), whole homogenate was used as an enzyme solution to measure total NAD degrading activity. An approximate 70% increase in the enzyme activity was observed after sonication or freeze-thawing and therefore the enzyme solutions used in the following experiments were sonicated with a Tomy UR-200P sonicator (20 kHz, 15 sec x 3) at 0°C. As shown in Table 3, NAD glycohydrolase activity was elevated by supplementation of leucine in the nicotinic acid-containing diet. Elimination of nicotinic acid from diets caused significant elevation of the enzyme activity. Addition of leucine to the nicotinic acid-free diet, however, caused no further increase in the enzyme activity. From the data in Tables 2 and 3, NAD glycohydrolase activity tended to increase when the hepatic NAD level decreased.

Free nicotinic acid, total nicotinate and nicotinamide levels were not significantly different in any dietary treatments (Table 4). Urinary excretion of N1-
methylnicotinamide during the last 3 days of the experiment was significantly lower in the nicotinic acid-devoid diet-fed groups (Table 4). The effect of dietary excess leucine, however, was not observed. It was quite interesting that the level of nicotinamide, a direct substrate for nicotinamide methyltransferase [EC 2.1.1.1], was not lowered at all in the nicotinic acid-devoid diet-fed animals, the urinary N1-methylnicotinamide of which was significantly reduced.

According to Ghafoorunissa and Narasinga Rao (15), the hepatic NAD content was decreased in the 2% casein diet-fed rats, of which the activity of quinolinate phosphoribosyl transferase, one of the rate-limiting enzymes in the tryptophan-NAD pathway, was elevated. Therefore, under this condition the NAD-degrading activity might possibly be elevated. In experiment II, after feeding the diets shown in Table 1 for 7 days ad libitum, animals were killed by decapitation and their hepatic NAD, nicotinamide and NAD glycohydrolase activity were determined. Diet intake and body weight gain are shown in Table 5. The hepatic NAD content was significantly lowered in the non-protein diet-fed group (Table 6), the NAD glycohydrolase activity of which was significantly elevated. The hepatic nicotinamide content measured as a pooled sample did not differ, however, in any protein level.

Table 5. Effect of dietary protein levels on body weight gain and dietary intake (Experiment II).

| Diet     | Body wt gain (g/wk/rat) | Diet intake (g/wk/rat) |
|----------|-------------------------|------------------------|
| (E) Non protein | −22.0 ± 4.0** | 39.2 ± 4.4** |
| (F) 18% casein     | 27.0 ± 9.7       | 77.1 ± 13.9           |
| (G) 70% casein     | 18.2 ± 8.9       | 56.2 ± 4.9*           |

* ** Indicate significant difference from (F) at the 2 and 0.1% levels respectively by t-test.

Table 6. Effect of dietary protein levels on hepatic NAD and nicotinamide contents and NAD glycohydrolase activity.

| Diet     | Content | NAD glycohydrolase activity (Nicotinamide formed, µmol/min/g wet liver) |
|----------|---------|---------------------------------------------------------------------|
|          | NAD     | Nicotinamide |                                                                  |
|          | (µmol/g wet liver) | (µmol/g wet liver) |                                                                 |
| (E) Non protein | 0.46 ± 0.05* | 0.76 | 4.02 ± 0.73** |
| (F) 18% casein     | 0.56 ± 0.08 | 0.73 | 2.53 ± 0.42 |
| (G) 70% casein     | 0.53 ± 0.07 | 0.76 | 2.54 ± 1.01 |

* ** Indicate significant difference from (F) at the 5 and 1% levels respectively by t-test.
DISCUSSION

Body weight gain was suppressed in the leucine-supplemented group in spite of higher nitrogen intake, compared to the leucine-unsupplemented diet-fed group. Rogers et al. (16) reported that 5% leucine added to 9% casein diet caused growth depression and, for complete restoration to the growth rate of the control group, additional supplementation with tryptophan, phenylalanine and threonine was necessary besides isoleucine and valine. Lojkin (17) also found that the addition of isoleucine and valine to the high-leucine diet was not sufficient to restore the $N^1$-methylnicotinamide level, the nitrogen balance and the condition of the fetuses to the levels obtained for the rats fed on the unsupplemented diet. These studies suggested that the growth-depressing effect of a high leucine intake was the result of a more complex amino acid interrelationship than the mere leucine-isoleucine and valine antagonism.

In the previous paper (1) the authors reported that supplementation of leucine at the 5% level to the 10 or 20% casein diet caused a significant decrease in the hepatic nicotinamide nucleotide level in rats fed freely. The hepatic NAD-lowering effect of leucine was also observed, though somewhat weakened, in the pair-fed rats. A decrease in the hepatic NAD level could be brought about by either inhibiting conversion of tryptophan or nicotinic acid to NAD or accelerating NAD degradation. Shastri and Nath (18) reported that acetoacetate administered to tryptophan-fed rats led to lower levels of pyridine nucleotides in the liver, but that no such effect was found in animals supplemented with nicotinic acid. According to Anasuya and Narasinga Rao (19) the erythrocytes of pellagrins had lower levels of NAD and NADP and exhibited impaired synthesis of NAD from nicotinic acid in vitro. In this paper the possibility that the enhanced NAD degradation might cause a decrease in the hepatic NAD level was investigated.

The method described by Colowick et al. (20) has been widely used for the assay of NAD glycohydrolase activity. In their method, the remaining NAD after termination of the enzyme reaction was converted to a complex, with the cyanide therein having an absorption maximum at 340 nm. Even under the optimum condition, the difference of absorbance at 340 nm obtained by the hepatic enzyme was very small, possibly because of an exchange reaction, and therefore it was difficult to get an exact enzyme activity. On the contrary, in the method using [carbonyl-14C]NAD, the enzymatically formed nicotinamide was separated and the radioactivity in the nicotinamide fraction was determined. With this method even a very low enzyme activity could be precisely determined by elevating the specific activity of NAD.

NAD glycohydrolase activity was elevated by supplementing the 10% casein diet with 5% l-leucine, which might be one of the causes of the lowered NAD level in the liver. In the animals fed on the nicotinic acid-devoid diet, NAD glycohydrolase activity was significantly elevated compared to the nicotinic acid-fed control animals, but leucine supplementation caused no further increase in the enzyme activity.

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activity. When the nicotinic acid-free diet was fed, NAD synthesized from tryptophan in the liver might be converted to nicotinamide by the increased NAD glycohydrolase, and the extrahepatic tissues might be provided with the thus-formed nicotinamide. The mechanism by which NAD glycohydrolase activity was raised in the nicotinic acid-devoid diet-fed animals and the reason why the effect of leucine appeared only when nicotinic acid was included in the diet have to be elucidated in the future.

Under various physiological and nutritional conditions, such as hyperthyroidism, diabetes mellitus, X-ray irradiation, low and high dietary protein levels and so on, the hepatic NAD level has been known to fluctuate. Though quinolinate phosphoribosyltransferase activity was elevated in the protein deficiency status (15), the hepatic NAD level was reduced (8). The decrease in hepatic NAD was considered to be due to the decrease in kynurenine 3-hydroxylase activity in hyperthyroidism (21) and to the decreased efficiency of NAD biosynthesis from tryptophan in X-ray irradiated mice (22). On the other hand, streptozotocin induced NAD degradation in mouse pancreatic islets (23) which was considered to be due to an increase in poly(ADP-ribose) synthetase activity (24). Taking our present result into consideration along with the data reported by Shimoyama et al. (6), Blake et al. (7) and Yamamoto and Okamoto (24), not only NAD synthesizing activity but also NAD degrading activity might contribute considerably to the control of NAD levels.

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