Effect of Supplementing Low Protein Diets with the Limiting Amino Acids on the Excretion of N^1-Methylnicotinamide and Its Pyridones in Rats

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ABSTRACT We have hypothesized that the ratio of the excreted by-products of niacin metabolism, N^1-methyl-2-pyridone-5-carboxamide (2-pyr) + N^1-methyl-4-pyridone-3-carboxamide (4-pyr)/N^1-methylnicotinamide (MNA), might be useful as an index to assess the adequacy of amino acid intake in rats. The experiment reported herein was performed to test this hypothesis. When a 10, 20 or 40% casein diet supplemented with 0.1, 0.2 or 0.4% L-methionine, respectively, was fed to rats, the urinary excretion of MNA decreased, and that of 4-pyr increased, as the level of dietary casein and methionine increased. Therefore, the ratio of (2-pyr + 4-pyr)/MNA increased with increasing dietary casein and methionine levels. When the limiting amino acids of casein or soy protein isolate were added to a low casein or low soy protein isolate diet, the urinary ratio of (2-pyr + 4-pyr)/MNA also increased. These results indicate that the increased urinary ratio of (2-pyr +4-pyr)/MNA can serve as a biological marker for adequate amino acid intake. *J. Nutr.* 119: 896–901, 1989.

INDEXING KEY WORDS:

• *protein adequacy* • *N^1-methyl-4-pyridone-3-carboxamide* • *N^1-methylnicotinamide* • *methionine* • *cystine* • *cysteine* • *threonine* • *glycine* • *nicotinamide methyltransferase* • *MNA oxidase* • *rat*

It is thought that increased excretion of N^1-methylnicotinamide (MNA) reflects an increased intake of niacin and/or tryptophan. However, we have reported that the urinary excretion of MNA decreased greatly when a 10% casein diet was fed to rats compared to values when a 20 or a 40% casein diet was fed (1). This effect might be due to the deficiency of L-methionine in casein, so we investigated the effect of supplementing a 10, 20 or 40% casein diet with 0.1, 0.2 or 0.4% L-methionine, respectively, on the urinary excretion of MNA and its pyridones. Furthermore, we have previously hypothesized that the increased ratio of N^1-methyl-2-pyridone-5-carboxamide (2-pyr) + N^1-methyl-4-pyridone-3-carboxamide (4-pyr)/MNA excretion reflects an increased intake of protein (1–3). In this report, we present evidence that the ratio of these excreted by-products of niacin metabolism increases not only with increasing dietary casein and methionine levels, but also with supplementation of low protein diets with the limiting amino acids. The present findings strengthen our hypothesis.

MATERIALS AND METHODS

Chemicals. MNA chloride was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 2-Pyr and 4-pyr were synthesized by the methods of Pullman and Colowick (4) and Shibata, Kawada and Iwai (5), respectively. L-Cysteine-HCl·H_2O and nicotinamide were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of the highest purity available from commercial sources.

Animal and diets. Rats of the Wistar strain (5 wk old) were purchased from Clea Japan (Tokyo, Japan). The rats were immediately placed in individual metabolic cages (CT-10; Clea Japan) and fed ad libitum a 20% casein diet supplemented with 0.2% methionine (**Table 1**) for 10 d. The rats were then fed diets containing 10, 20 or 40% casein supplemented with 0.1, 0.2 or 0.4% methionine (experiment 1, Table 1) or low protein casein- and soy protein isolate–based diets supplemented with limiting amino acids (experiment 2, **Table 2**) ad libitum for 9 d, and killed by decapitation at 0830–0930 h on d 10. The livers were removed, and a portion of liver (~ 1 g) immediately homogenized with a Teflon-glass homogenizer in five volumes of cold 50 mM potassium phosphate buffer, pH 7.0. The resulting homogenates were used as enzyme sources. Urine was collected for the last 2 d of the experimental period in aluminum-wrapped amber bottles containing 1 ml of 1 M HCl and 1 ml of 1% L-cysteine, and stored at −25°C.

0022-3166/89 $3.00 Received 31 October 1988. Accepted 6 March 1989.

TABLE 1

Experiment 1: Composition of the experimental diets

	10% Casein + 0.1% Met	20% Casein + 0.2% Met	40% Casein + 0.4% Met
		g/kg diet	
Vitamin-free casein[1]	100	200	400
L-Methionine[1]	1	2	4
Sucrose[1]	264	230	163
α-Cornstarch	525	458	323
Corn oil	50	50	50
Mineral mixture[2]	50	50	50
Vitamin mixture[3]	10	10	10

[1]Obtained from Wako Pure Chemical Industries, Osaka, Japan.
[2]Provided the following (g/kg of diet): $CaHPO_4 \cdot 2H_2O$, 7.28; KH_2PO_4, 12.85; $NaH_2PO_4 \cdot H_2O$, 4.675; NaCl, 2.33; Calcium lactate, 17.545; ferrous citrate, 1.59; $MgSO_4$, 3.585; $ZnCO_3$, 0.055; $MnSO_4 \cdot 6H_2O$, 0.060; $CuSO_4 \cdot 5H_2O$, 0.015; and KI, 0.005. Obtained from Oriental Yeast Kogyo Co., Toyko, Japan.
[3]Provided the following (mg/kg of diet except as indicated): retinyl acetate, 5000 IU; cholecalciferol, 1000 IU; tocopheryl acetate, 50; menadione, 52; thiamin-HCl, 12; riboflavin, 40; pyridoxine-HCl, 8; cyanocobalamin, 0.005; ascorbic acid, 300; D-biotin, 0.2; folate, 2; calcium pantothenate, 50; *para*-aminobenzoic acid, 50; nicotinic acid, 60; inositol, 60; choline chloride, 2000; and made up to 10 g with cellulose powder. Obtained from Oriental Yeast Kogyo Co.

The room temperature was kept at 22 ± 2°C, the humidity was about 60% and a 12-h light/dark cycle was maintained. Body weight and food intake were measured daily at around 0900 h.

Analyses. The methods used for measuring liver activity of nicotinamide methyltransferase (EC 2.1.1.1) (6), 2-pyr–forming MNA oxidase [EC 1.2.3.1: MNA + H_2O + A (unknown acceptor) → 2-pyr + AH_2 + H^+] (3) and 4-pyr–forming MNA oxidase [MNA + H_2O + A (unknown acceptor) → 4-pyr + AH_2 + H^+] (3) have been described. In rats, 2-pyr–forming MNA oxidase and 4-pyr–forming MNA oxidase have been reported to be different enzymes (7). Urine values for nicotinamide, 2-pyr and 4-pyr were simultaneously measured by the high performance liquid chromatographic (HPLC) method of Shibata, Kawada and Iwai (5). Urine values for MNA were measured by the HPLC method of Shibata (8).

Statistical analysis. The significance of differences was determined by analysis of variance and evaluated by Bartlett's test and Duncan's new multiple range test (9).

RESULTS

Body weight gain, food intake and food efficiency ratio. *Experiment 1.* Body weight gain, food intake and the food efficiency ratio (g gain/g fed) were not different among the three groups fed 10, 20 or 40% casein diets supplemented with 0.1, 0.2 or 0.4% methionine, respectively (**Table 3**).

Experiment 2. Body weight gain and the food efficiency ratio increased when the low protein diets (9% casein or 10% soy protein isolate) were supplemented with the limiting amino acids (**Table 4**). Food intake was not different between the groups fed the two casein diets, but for soy protein isolate diets, food intake was higher in rats fed the supplemented diet than in rats fed the unsupplemented diet (Table 4).

Urinary excretion of nicotinamide, MNA, 2-pyr and 4-pyr, and the resulting ratio of (2-pyr + 4-pyr)/MNA. *Experiment 1.* The urinary excretion of nicotinamide and 2-pyr remained constant even when the level of dietary casein and methionine increased (**Table 5**). On

TABLE 2

Experiment 2: Composition of the experimental diets

	9% Casein	9% Casein + amino acids	10% Soy protein isolate	10% Soy protein isolate + amino acids
			g/kg of diet	
Vitamin-free casein[1]	90	90	0	0
Soy protein isolate[2]	0	0	100	100
Glycine[1]	0	20	0	0
L-Threonine[1]	0	0.78	0	2.5
L-Cystine[1]	0	2	0	0
L-Methionine[1]	0	0	0	3.1
α-Cornstarch	533	518.11	527	522.9
Sucrose[1]	267	259.11	263	261.5
Corn oil	50	50	50	50
Mineral mixture[3]	50	50	50	50
Vitamin mixture[3]	10	10	10	10

[1]Obtained from Wako Pure Chemical Industries (Osaka, Japan).
[2]A gift from Fuji Oil (Izumisano, Osaka, Japan).
[3]For composition, see footnote to Table 1.

TABLE 3

Experiment 1: Effect of dietary casein and methionine levels on body wt gain, food intake and food efficiency ratio (FER)[1]

	10% Casein + 0.1% Met	20% Casein + 0.2% Met	40% Casein + 0.4% Met
Body wt gain, *g/9 d*	52.8 ± 4.4	50.4 ± 2.8	53.5 ± 4.7
Food intake, *g/9 d*	148.0 ± 8.3	140.5 ± 8.2	144.4 ± 9.2
FER, *g gain/ g fed*	0.357 ± 0.016	0.359 ± 0.022	0.371 ± 0.031

[1]Values are means ± SD for five rats.

the contrary, the urinary excretion of 4-pyr increased, and that of MNA decreased, with increasing dietary casein and methionine. In particular, this effect was observed when the 10% casein and 0.1% methionine diet was fed to rats compared to feeding rats the 20% casein and 0.2% methionine diet. The total urinary excretion of nicotinamide, MNA, 2-pyr and 4-pyr remained constant regardless of the dietary casein and methionine level (Table 5). The urinary ratio of (2-pyr + 4-pyr)/MNA increased with increasing casein and methionine levels (Table 5).

Experiment 2. The urinary excretion of nicotinamide and 2-pyr did not change even when the limiting amino acids were added to the low casein diet or low soy protein isolate diet (**Table 6**). On the other hand, the urinary excretion of 4-pyr increased, while that of MNA decreased, when the casein or soy protein isolate diet was supplemented with limiting amino acids (Table 6). The total urinary excretion of nicotinamide, MNA, 2-pyr and 4-pyr was significantly lower in the groups of rats fed the supplemented casein and soy protein isolate diets compared to rats fed the unsupplemented diets (Table 6). However, the urinary ratio of (2-pyr + 4-pyr)/MNA was significantly higher in the groups fed the supplemented casein and soy protein isolate diets than in the groups fed unsupplemented diets (Table 6).

Nicotinamide methyltransferase, 2-pyr–forming MNA oxidase and 4-pyr–forming MNA oxidase activity. *Experiment 1.* Nicotinamide methyltransferase activity increased with increasing dietary casein and methionine levels (**Table 7**). The activity of 2-pyr–forming MNA oxidase and 4-pyr–forming MNA oxidase in the groups fed either 20% casein and 0.2% methionine or 40% casein and 0.4% methionine was higher than that of the group fed the 10% casein and 0.1% methionine diet (Table 7).

Experiment 2. Nicotinamide methyltransferase activity was lower in the groups fed the amino acid–supplemented casein and soy protein isolate diets than in the groups fed the unsupplemented diets (**Table 8**). The activity of 2-pyr–forming MNA oxidase and 4-pyr–forming MNA oxidase was higher in the groups fed the supplemented casein and soy protein isolate diets than in the groups fed the unsupplemented diets (Table 8).

DISCUSSION

We have previously hypothesized that the ratio of (2-pyr + 4-pyr)/MNA is a useful criterion for assessing the adequacy of amino acid intake, based on data showing that the urinary excretion of 4-pyr increased concomitantly with a decrease in MNA when rats were fed a diet containing 20% casein compared to those fed 10% casein (1–3). We have also suggested that the increased urinary ratio of (2-pyr + 4-pyr)/MNA is mainly attributed to increased 4-pyr–forming MNA oxidase activity (3). Furthermore, we have reported that the total urinary excretion of nicotinamide, MNA, 2-pyr and 4-pyr was not different among groups of rats fed 10, 20 or 40% casein diets (1).

In the conversion of tryptophan into nicotinamide, vitamin B-6, riboflavin and niacin are needed. Therefore, there is the possibility that a lack of increase in the total urinary excretion of nicotinamide, MNA, 2-pyr and 4-pyr with an increasing level of dietary casein is attributed to deficiencies of these vitamins that result from the additional protein intake. However, this

TABLE 4

Experiment 2: Effect of supplementing the low protein diets with amino acids on body wt gain, food intake and food efficiency ratio (FER)[1]

	9% Casein	9% Casein + amino acids	10% Soy protein isolate	10% Soy protein isolate + amino acids
Body wt gain, *g/9 d*	20.5 ± 3.1[a]	30.3 ± 2.1[b]	14.5 ± 5.5[a]	51.3 ± 4.9[c]
Food intake, *g/9 d*	131.9 ± 6.8[a]	127.6 ± 1.9[a]	127.1 ± 12.4[a]	157.0 ± 5.1[b]
FER, *g gain/g fed*	0.155 ± 0.021[a]	0.237 ± 0.015[b]	0.112 ± 0.035[a]	0.326 ± 0.026[c]

[1]Values are means ± SD for five rats; values having a different superscript letter in the same row are significantly different, $P < 0.05$.

TABLE 5

Experiment 1: Effect of dietary casein and methionine levels on the urinary excretion of nicotinamide and its metabolites, and the urinary ratio of (2-pyr + 4-pyr)/MNA[1]

	10% Casein + 0.1% Met	20% Casein + 0.2% Met	40% Casein + 0.4% Met
		nmol/d	
Nicotinamide	231 ± 49	243 ± 118	213 ± 38
MNA	2671 ± 539[a]	953 ± 213[b]	836 ± 291[b]
2-Pyr	429 ± 76	420 ± 66	424 ± 91
4-Pyr	4858 ± 1084[a]	6980 ± 1201[b]	7116 ± 1626[b]
Sum[2]	8189 ± 881	8596 ± 1433	8589 ± 1895
(2-Pyr + 4-Pyr)/ MNA	2.03 ± 0.91[a]	7.95 ± 1.65[b]	9.67 ± 2.80[b]

[1]Values are means ± SD for five rats; values having a different superscript letter in the same row are significantly different, $P < 0.05$.

[2]Sum of the urinary excretion of nicotinamide, MNA (N^1-methylnicotinamide), 2-pyr (N^1-methyl-2-pyridone-5-carboxamide) and 4-pyr (N^1-methyl-4-pyridone-3-carboxamide).

is unlikely for the following reasons. The diets used in these experiments contained 0.8 mg of pyridoxine-HCl, 4 mg of riboflavin and 6 mg of nicotinic acid per 100 g of diet; Harper (10) has reported that 0.25 mg of pyridoxine-HCl, 0.5 mg of riboflavin and 2.5 mg of nicotinic acid per 100 g of diet are sufficient to meet the dietary requirements of rats. In this connection, we have reported that even when a diet containing five times the normal level of vitamin mixture was fed to rats (2), the urinary ratio of (2-pyr + 4-pyr)/MNA increased about tenfold in rats fed 20% casein compared to those fed a 10% casein diet (2.57 ± 0.44 vs. 0.27 ± 0.06). In addition, in rats fed five times the normal level of vitamin mixture, the ratio of (2-pyr + 4-pyr)/MNA decreased compared to rats fed a diet containing a normal dietary vitamin level when both 10% and 20% casein diets were fed (1.35 ± 0.22 vs. 0.27 ± 0.06 for rats fed a 10% vs. a 20% casein diet, respectively; 13.23 ± 2.71 vs. 2.57 ± 0.44 for rats fed a low vs. a high level of vitamin mixture, respectively). Thus, this excretion ratio might decrease when excess nicotinamide is administered.

It is possible that our previously reported results (1–3) are due to the deficiency of methionine in casein, because it is known that the urinary excretion of tryptophan metabolites, such as MNA, is changed by the dietary amino acid balance other than tryptophan (11). So, in this report we investigated the effect of supplementing the 10, 20 or 40% casein diets with 0.1, 0.2 or 0.4% methionine, respectively, on the urinary excretion of MNA and its pyridones. The results were similar to those observed in the previous experiment using casein diets without added methionine (1). However, the percentage of MNA in the total urinary metabolites was slightly lower, and that of 4-pyr was slightly higher, in the group of rats fed the 10% casein and 0.1% methionine diet (Table 5) than in the group fed the 10% casein diet (1) [MNA, 33% vs. 57%, 4-pyr, 59% vs. 34% for rats fed the 10% casein and 0.1% methionine diet (calculated from Table 5) vs. the 10% casein diet (1), respectively]. So, the urinary ratio of (2-pyr + 4-pyr)/MNA was higher in the group fed the 10% casein and 0.1% methionine diet (Table 5) than in the group fed the 10% casein diet (1) [2.03 ± 0.91 vs. 0.70 ± 0.04 for rats fed the 10% casein and 0.1% methionine diet (Table 5) vs. 10% casein diet (1)] and the gain in body weight was also higher in the group of rats fed the 10% casein and 0.1% methionine diet (Table 3) than in the group fed the 10% casein diet (1) [5.9 g/d (Table 3) vs. 2.8 g/d for rats fed the 10% casein and 0.1% methionine diet vs. the 10% casein diet (1)]. These changes would be the result of the improved amino acid intake by supplementing the 10% casein diet with 0.1% methionine.

We also investigated how supplementing the low protein diet with the limiting amino acids affected the growth of rats and the proportion of the urinary excretion of nicotinamide and its metabolites. Supplementing the low protein diets with the limiting amino acids

TABLE 6

Experiment 2: Effect of supplementing the low protein diets with amino acids on the urinary excretion of nicotinamide and its metabolites, and the ratio of (2-pyr + 4-pyr)/MNA[1]

	9% Casein	9% Casein + amino acids	10% Soy protein isolate	10% Soy protein isolate + amino acids
			nmol/d	
Nicotinamide	320 ± 36	296 ± 36	461 ± 280	262 ± 52
MNA	2640 ± 661[a]	357 ± 65[b]	5565 ± 1182[c]	535 ± 85[d]
2-Pyr	289 ± 38[a]	223 ± 36[a]	441 ± 62[b]	389 ± 81[b]
4-Pyr	2623 ± 595[a]	3532 ± 392[b]	2617 ± 222[a]	5064 ± 608[c]
Sum[2]	5905 ± 406[a]	4409 ± 495[b]	9084 ± 1610[c]	6262 ± 735[a]
(2-Pyr + 4-Pyr)/MNA	1.20 ± 0.048[a]	10.68 ± 1.36[b]	0.56 ± 0.08[a]	10.33 ± 1.60[b]

[1]Values are means ± SD for five rats; values having a different superscript letter in the same row are significantly different, $P < 0.05$.

[2]Sum of the urinary excretion of nicotinamide, MNA, 2-pyr and 4-pyr. The key to abbreviations is given in a footnote to Table 5.

TABLE 7

Experiment 1: Effect of dietary casein and methionine levels on the activity of nicotinamide methyltransferase, 2-pyr–forming MNA oxidase and 4-pyr–forming MNA oxidase in liver[1]

	10% Casein + 0.1% Met	20% Casein + 0.2% Met	40% Casein + 0.4% Met
	nmol/(h·g liver)		
Nicotinamide methyltransferase	208 ± 44[a]	412 ± 89[b]	599 ± 97[c]
2-Pyr–forming MNA oxidase[2]	119 ± 51[a]	487 ± 46[b]	511 ± 57[b]
4-Pyr–forming MNA oxidase[2]	418 ± 189[a]	3425 ± 799[b]	3043 ± 952[b]

[1]Values are means ± SD for five rats; values having a different superscript letter in the same row are significantly different, $P < 0.05$.

[2]The key to abbreviations is given in a footnote to Table 5.

caused an increase in body weight gain and in the urinary ratio of (2-pyr + 4-pyr)/MNA (Table 4, 6). This increase was attributed to the decrease in MNA excretion and the concomitant increase in 4-pyr excretion by supplementing the low protein diets with the limiting amino acids. Similar results were obtained when rats were fed the 10% casein and 0.1% methionine diet compared to the 20% casein and 0.2% methionine diet (Table 4), and when rats were fed a diet containing the 10% compared to 20% casein (1) (due to the increase in total protein). These results further strengthen our hypothesis that the urinary ratio of (2-pyr + 4-pyr)/MNA is useful as a criterion for the adequacy of amino acid intake.

The activity of liver nicotinamide methyltransferase decreased slightly when the low protein diets were supplemented with the limiting amino acids (Table 8); the urinary excretion of methylated compounds such as MNA, 2-pyr and 4-pyr also decreased (Table 6). Methyltransferase activity increased with increasing the casein and methionine levels (Table 7), but the urinary excretion of methylated compounds remained constant (Table 5). We have reported that a similar finding was observed when the 10, 20 and 40% casein diets were fed (1). Furthermore, we have reported that the urinary excretion of MNA could very likely be controlled by the nicotinamide concentration, rather than by the activity of nicotinamide methyltransferase (6).

Regarding the present results, it is unlikely that the change in methyltransferase activity is associated with the change in the urinary ratio of (2-pyr + 4-pyr)/MNA. The 2-pyr–forming MNA oxidase activity increased with increasing casein and methionine levels and by supplementing the low protein diets with the limiting amino acids (Table 7, 8), but the urinary excretion of 2-pyr did not increase (Table 5, 6). A similar finding was observed when the 10, 20 and 40% casein diets were fed (1). It is therefore also considered unlikely that the change in 2-pyr–forming MNA oxidase activity is associated with the change in the ratio of (2-pyr + 4-pyr)/MNA. The 4-pyr–forming MNA oxidase activity and the concomitant urinary excretion of 4-pyr significantly increased when rats fed the 10% casein and 0.1% methionine diet were compared to those fed the 20% casein and 0.2% methionine diet (Table 5, 7) and when the low protein diets were supplemented with limiting amino acids (Table 6, 8). These results are similar to previously reported results for 10 and 20% casein diets (1); increased body weight gains were also observed (Table 2, 3). The significant decrease in MNA excretion along with the significant increase in 4-pyr excretion were observed when amino acid intake was improved with increasing the dietary protein level (Table 5) or by supplementing the low protein diets with the limiting amino acids (Table 6).

ACKNOWLEDGMENT

The authors express their sincere thanks to Fuji Oil Co., Ltd. (Izumisano, Osaka, Japan) for the supply of soy protein isolate.

TABLE 8

Experiment 2: Effect of supplementing the low protein diets with amino acids on the activity of nicotinamide methyltransferase, 2-pyr–forming MNA oxidase and 4–pyr forming MNA oxidase in liver[1]

	9% Casein	9% Casein + amino acids	10% Soy protein isolate	10% Soy protein isolate + amino acids
			nmol/(h·g liver)	
Nicotinamide methyltransferase	421 ± 64[a]	286 ± 59[b]	439 ± 52[a]	229 ± 36[b]
2-Pyr–forming MNA oxidase	109 ± 51[a]	299 ± 52[b]	36 ± 13[c]	632 ± 156[d]
4-Pyr–forming MNA oxidase	182 ± 78[a]	1934 ± 401[b]	65 ± 33[c]	2379 ± 799[b]

[1]Values are means ± SD for five rats; values having a different superscript letter in the same row are significantly different, $P < 0.05$. The key to abbreviations is given in a footnote to Table 5.

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