Effect of dietary protein level on some key enzymes of the tryptophan–NAD pathway

By U. SATYANARAYANA AND B. S. NARASINGA RAO

National Institute of Nutrition, Indian Council of Medical Research, Jamai-Osmania, Hyderabad-500 007, India

(*Received* 14 *July* 1976 – *Accepted* 25 *October* 1976)

1. Six groups of rats were given diets containing protein at three levels (50, 100 and 200 g/kg), with and without nicotinic acid. After 4 weeks on these diets some key enzymes of the tryptophan and nicotinic acid–NAD pathway, liver nicotinamide nucleotide concentration, and urinary metabolites of tryptophan and nicotinic acid were studied.

2. Liver nicotinamide nucleotide levels were lower in rats given the diet with 50 g protein/kg as compared to those in rats given diets with 100 and 200 g protein/kg. The addition of nicotinic acid to the diet resulted in a significant increase in the levels of nicotinamide nucleotides only in rats given 50 g protein/kg diet but not in those given either 100 or 200 g protein/kg diet.

3. Liver tryptophan oxygenase (*EC* 1.13.1.12) activity increased with increasing dietary protein level. Nicotinic acid in the diet had no effect on its activity.

4. Quinolinate phosphoribosyltransferase (*EC* 2.4.2.a) activity in liver was inversely related to dietary protein level, and nicotinic acid in the diet had no effect on its activity.

5. Liver nicotinate phosphoribosyltransferase (*EC* 2.4.2.11) activity and kidney picolinate carboxylase (*EC* 4.1.1.45) activity were not altered either by dietary protein level or nicotinic acid in the diet.

6. The addition of nicotinic acid to the diet resulted in increased excretion of *N'*-methylnicotinamide at all dietary protein levels.

7. The inverse relationship between protein level in the diet and liver quinolinate phosphoribosyltransferase activity, the rate-limiting enzyme of the tryptophan–NAD pathway suggests that the efficiency of conversion of tryptophan to NAD is related to protein level in the diet, the efficiency decreasing with an increase in the level of dietary protein.

It has been clearly established that tryptophan in the diet can replace nicotinic acid for the synthesis of nicotinamide nucleotides, the co-enzyme form of nicotinic acid (Gholson, Ueda, Ogasawara & Henderson, 1963; Nishizuka & Hayaishi, 1963). Horwitt, Harvey, Rothwell, Cutler & Haffron (1956), first reported that on average 60 mg dietary tryptophan is equivalent to 1 mg nicotinic acid (conversion ratio, 60:1). However, from more recent work, it would appear that this conversion ratio may not be constant and that it may be influenced by dietary factors. It was shown (Brown, Vivian, Reynolds & Price, 1958), that the tryptophan was converted to nicotinamide nucleotides, even when the tryptophan content of the diet was so low that the subjects were in negative nitrogen balance. A decrease in the conversion ratio from approximately 122 to approximately 75 was reported (Nakagawa, Takahashi, Suzuki & Masana, 1969) as the amount of added tryptophan and nicotinic acid in the diet increased. Such variations in efficiency of conversion of tryptophan to nicotinic acid may be mediated through alterations in enzymes concerned with tryptophan–nicotinamide nucleotide metabolism. Ghafoorunissa & Narasinga Rao (1974) have recently reported that the level of dietary protein and liver quinolinate phosphoribosyltransferase (*EC* 2.4.2.a) activity of rats are inversely related. A systematic study was therefore undertaken to determine the effects of dietary protein level on some important enzymes of the tryptophan and nicotinic acid–NAD pathway, liver nicotinamide nucleotide concentration and urinary tryptophan and nicotinic acid metabolites. The results of this study are reported here.

Table 1. *Composition of the diets (g/kg) given to groups of rats*

Ingredient	Group no. 1	2	3	4	5	6
Casein (vitamin-free)	61	61	122	122	244	244
(Protein)	(50)	(50)	(100)	(100)	(200)	(200)
Peanut oil	50	50	50	50	50	50
Salt mixture*	40	40	40	40	40	40
Vitamin mixture†	10	10	10	10	10	10
Nicotinic acid	0·04	—	0·04	—	0·04	—
L-cystine	2	2	2	2	2	2
Maize starch	837	837	776	776	654	654

* U.S. Pharmacoepia XVII (1965).
† National Academy of Sciences/National Research Council (1963) (without nicotinic acid).

EXPERIMENTAL

Animals

Young weanling albino rats (inbred strain of National Institute of Nutrition, Jamai-Osmania, Hyberabad-500 007), 21 d of age, were divided into six groups each of eight rats on the basis of body-weight and sex.

Diets

The composition of the diet fed to different groups is given in Table 1. Three protein levels were used, with vitamin-free casein as the protein source. Rats receiving a 50 g protein/kg, nicotinic acid-free diet were fed *ad lib.* and the remaining groups were individually pair-fed with these rats.

At the end of 4 weeks of feeding the experimental diets, urine was collected from each rat for 3 d under toluene (9 ml) and glacial acetic acid (3 ml, 17 M). Rats were then killed by decapitation.

Nicotinamide nucleotides concentration, the activities of tryptophan oxygenase (*EC* 1.13.1.12), quinolinate phosphoribosyltransferase and nicotinate phosphoribosyltransferase (*EC* 2.4.2.11) in liver, and picolinate carboxylase (*EC* 4.1.1.45) in kidney were determined. Urine samples were analysed for quinolinic acid, nicotinic acid and *N'*-methylnicotinamide contents.

MATERIALS

[Carboxyl-^{14}C]-nicotinic acid (1·8 μci/μmol) was obtained from Bhaba Atomic Research Centre, Trombay, India. Phosphoribosyl-1-pyrophosphate (sodium salt), quinolinic acid and ATP were purchased from Sigma Chemical Co. Ltd, St Louis, Missouri, USA. 3-Hydroxyanthranillic acid was obtained from California Co. for Biochemical Research, Los Angeles, California, USA.

Analytical methods

Liver nicotinamide nucleotides were determined fluorometrically by the alkali–acetone condensation method of Levitas, Robinson, Rosen, Huff & Perlzweig (1947).

Tryptophan oxygenase activity in liver homogenates was assayed by the method of Knox as described by Chiancone (1965).

Quinolinate phosphoribosyltransferase activity of liver was determined by the microbiological method of Nishizuka & Nakamura (1970), with *Lactobacillus arabinosus* (ATCC 8014) as described by Ghafoorunissa & Narasinga Rao (1973). The only modification was

that 600 nmol phosphoribosyl-1-pyrophosphate (sodium salt) was used in the assay mixture instead of 400 nmol corresponding magnesium salt.

Nicotinate phosphoribosyltransferase activity in liver was assayed by the method of Ikeda, Tsuji, Nakamura, Ichiyama, Nishizuka & Hayaishi (1965), and 40 μmol sodium fluoride was added to the incubation mixture to minimize the breakdown of phosphoribosyl-1-pyrophosphate (Ismande, 1964). The product formed, nicotinic acid mononucleotide, was separated from the substrate, nicotinic acid, using a Dowex 50x4-400 (Sigma Chemical Co. Ltd, St Louis, Missouri, USA) column as described previously by Anasuya & Narasinga Rao (1975).

Picolinate carboxylase activity in kidney was determined according to the method of Ikeda *et al.* (1965), as reported by Ghafoorunissa & Narasinga Rao (1973).

Protein in all enzyme preparations was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Urinary metabolites

Nicotinic acid in urine was estimated by the microbiological method using *L. arabinosus* as the test organism (Freed, 1966).

Quinolinic acid was estimated by the method of Henderson (1949). Quinolinic acid was decarboxylated to nicotinic acid by autoclaving with glacial acetic acid (17 M) for 2 h at 103·4 kNm^{-2}, and nicotinic acid was estimated by the microbiological method. The difference between nicotinic acid before and after decarboxylation was taken to represent the amount of quinolinic acid in the urine.

N'-methylnicotinamide was estimated fluorometrically using the alkali–acetone condensation method of Carpenter & Kodicek (1950).

Creatinine in the urine was estimated by Folin's method as described by Oser (1965). The levels of urinary metabolites were expressed as μgm metabolites/mg creatinine excreted.

Statistical analysis

Statistical analysis was carried out using analysis of variance and Fisher's *t* test.

RESULTS

Liver nicotinamide nucleotide content and enzyme activities in rats given diets containing different levels of protein are shown in Table 2.

In rats given a nicotinic acid-free diet, liver nicotinamide nucleotide levels were lower in those given 50 g protein/kg diet as compared with those given 100 and 200 g protein/kg diet, but there was no difference between the latter two groups. Addition of nicotinic acid to the diet resulted in a significant increase in the levels of nicotinamide nucleotides only in rats given 50 g protein/kg diet, but not in those given either 100 or 200 g protein/kg diet.

Liver tryptophan oxygenase activity increased with increasing dietary protein level. Although, the tryptophan oxygenase activity obtained with 50 g protein/kg diet was significantly different from that with 100 and 200 g protein/kg diet, the difference between values obtained with 100 and 200 g protein/kg diet was not statistically significant. Nicotinic acid had no effect on its activity at all protein levels.

Quinolinate phosphoribosyltransferase activity of liver was inversely related to the level of dietary protein. Nicotinic acid in the diet had no effect on its activity at any of the protein levels studied.

Neither picolinate carboxylase activity in kidney nor nicotinate phosphoribosyltransferase activity of liver was affected either by the level of dietary protein or by the presence of nicotinic acid in the diet.

Table 2. *Effect of protein level in the diet on nicotinamide nucleotides, tryptophan oxygenase* (EC 1.13.1.12), *quinolinate phosphoribosyltransferase* (EC 2.4.2.a) *and nicotinate phosphoribosyltransferase* (EC 2.4.2.11) *activities of liver and picolinate carboxylase* (EC 4.1.1.45) *activity of kidney of rats*

(Mean values with their standard errors; no. of rats in parentheses)

Group no.	Dietary regimen	Liver nicotinamide nucleotides (μg/g wet wt)		Tryptophan oxygenase (μmol kynurinine formed/h per g nitrogen)		Quinolinate phosphoribosyltransferase (nmol nicotinic acid mononucleotide formed/h per mg protein)		Nicotinate phosphoribosyltransferase (nmol nicotinic acid mononucleotide formed/h per mg protein)		Picolinate carboxylase (nmol substrate disappeared/min/ per mg protein)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	50 g protein/kg:										
1	With nicotinic acid	468·4 (8)	24·06	24 (8)	3·0	0·71 (6)	0·063	1·8 (6)	0·17	6·6 (8)	0·48
2	Without nicotinic acid	331·4 (8)	6·15	20 (8)	2·5	0·69 (6)	0·145	2·4 (6)	0·34	5·8 (8)	0·35
	100 g protein/kg:										
3	With nicotinic acid	519·5 (7)	21·02	76 (7)	15·2	0·47 (5)	0·058	2·2 (5)	0·13	5·7 (7)	0·39
4	Without nicotinic acid	529·7 (8)	39·21	80 (8)	12·1	0·47 (6)	0·069	1·9 (6)	0·16	5·8 (8)	0·32
	200 g protein/kg:										
5	With nicotinic acid	542·7 (7)	34·52	101 (7)	16·6	0·39 (6)	0·088	1·5 (5)	0·20	6·4 (7)	0·42
6	Without nicotinic acid	514·7 (8)	16·76	91 (8)	7·7	0·35 (6)	0·051	1·5 (6)	0·33	6·2 (8)	0·31

Statistical significance of differences between groups: for liver nicotinamide nucleotides: group 1 *v.* group 2, $P < 0{\cdot}001$; group 1 *v.* group 5, $P < 0{\cdot}05$; group 2 *v.* groups 4 and 6, $P < 0{\cdot}001$; for tryptophan oxygenase: group 1 *v.* groups 3 and 5 $P < 0{\cdot}001$; for quinolinate phosphoribosyltransferase: group 1 *v.* group 5, $P < 0{\cdot}05$; group 2 *v.* group 6, $P < 0{\cdot}02$.

Table 3. *Effect of protein level in the diet on the urinary excretion (μg/mg creatinine) of quinolinic acid, nicotinic acid and* N′*-methylnicotinamide*

(Mean values with their standard errors; no. of rats in parentheses)

Group no.	Dietary regimen	Quinolinic acid		Nicotinic acid		*N′*-methylnicotinamide	
		Mean	SE	Mean	SE	Mean	SE
	50 g protein/kg:						
1	With nicotinic acid	5·5 (8)	1·14	34·2 (7)	5·31	54·0 (8)	9·35
2	Without nicotinic acid	6·3 (8)	1·55	7·4 (8)	0·76	8·8 (8)	0·98
	100 g protein/kg:						
3	With nicotinic acid	13·7 (7)	3·19	25·7 (7)	3·95	26·2 (7)	3·42
4	Without nicotinic acid	18·3 (8)	2·46	9·5 (8)	0·84	12·8 (8)	2·44
	200 g protein/kg:						
5	With nicotinic acid	12·8 (7)	2·61	19·0 (7)	2·28	27·4 (6)	2·62
6	Without nicotinic acid	17·0 (8)	2·98	8·8 (8)	0·92	12·1 (7)	2·22

Statistical significance of differences between groups: for quinolinic acid: group 1 *v.* groups 3 and 5, $P < 0{\cdot}05$; group 2 *v.* group 4, $P < 0{\cdot}001$; group 2 *v.* group 6, $P < 0{\cdot}01$; for nicotinic acid: group 1 *v.* group 2, $P < 0{\cdot}001$; group 1 *v.* group 3, $P < 0{\cdot}01$; group 1 *v.* group 5, $P < 0{\cdot}001$, group 3 *v.* group 4, $P < 0{\cdot}001$; group 5 *v.* group 6, $P < 0{\cdot}001$; for *N′*-methylnicotinamide: group 1 *v.* group 2, $P < 0{\cdot}001$; group 1 *v.* groups 3 and 5, $P < 0{\cdot}001$; group 3 *v.* group 4, $P < 0{\cdot}05$; group 5 *v.* group 6, $P < 0{\cdot}01$.

Values for the amounts of metabolites of tryptophan and nicotinic acid excreted in urine are given in Table 3. Urinary excretion of quinolinic acid was higher in rats given 100 and 200 g protein/kg diet as compared to those given 50 g protein/kg diet. However, there was no difference in quinolinic acid excretion between rats given 100 and 200 g protein/kg diet. The quinolinic acid excretion was slightly higher at each protein level when the diet was free from nicotinic acid than when the diet contained added nicotinic acid.

The trend of urinary excretion of nicotinic acid and *N′*-methylnicotinamide was more or less similar at different levels of protein. There were no significant differences in the excretion of nicotinic acid and *N′*-methylnicotinamide at the three protein levels when the diet contained no nicotinic acid. Excretion of both metabolites was higher with each dietary protein level when nicotinic acid was added to the diet. Inclusion of nicotinic acid in the diet resulted in a significantly greater increase in the excretion of nicotinic acid and *N′*-methylnicotinamide in rats given 50 g protein/kg diet as compared to those of rats given 100 and 200 g protein/kg diet.

DISCUSSION

Studies in recent years have suggested that the efficiency of conversion of tryptophan to nicotinic acid is not constant, but may depend on nutritional status (Brown *et al.* 1958; Nakagawa *et al.* 1969). In the conversion of tryptophan to nicotinamide nucleotides, the activities of enzymes of the tryptophan–NAD pathway may be expected to play a crucial role. Although tryptophan oxygenase, the first enzyme in the pathway, is known to be regulated by dietary and hormonal factors, little is known about the regulation of the other enzymes of the pathway by nutritional factors.

In the present study the effect of dietary protein level on the important enzymes of the

tryptophan–NAD pathway was studied. To determine the effect of dietary nicotinic acid on these enzymes, a nicotinic acid-free diet was prepared for each of the three protein levels studied. It must be pointed out, however, that the rats given the nicotinic acid free diet might conceivably receive some amount of nicotinic acid through coprophagy. This was prevented in this study by housing the rats in raised wire-mesh bottomed cages with no bedding mesh inside the cage. With this type of arrangement, very few faeces were observed to remain at the bottom of the cage and hence coprophagy was considered to be negligible. Therefore, the rats on the nicotinic acid-free diet can be considered to be truly nicotinic acid deficient.

In the present study, tryptophan oxygenase, quinolinate phosphoribosyltransferase, picolinate carboxylase and nicotinate phosphoribosyltransferase activities were studied. Of these four enzymes, only two, tryptophan oxygenase and quinolinate phosphoribosyltransferase, were found to be influenced by the protein level in the diet.

With increasing level of protein in the diet, the liver tryptophan oxygenase activity was found to increase. This was obviously related to the increase in tryptophan intake with increasing protein level in the diet. This observation is consistent with earlier reports (Knox & Mehler, 1951; Knox, 1966) that tryptophan oxygenase is a substrate-inducible enzyme. Tryptophan oxygenase activity was not affected by nicotinic acid in the diet.

Quinolinate phosphoribosyltransferase is the rate-limiting enzyme in the conversion of tryptophan to NAD and catalyses the conversion of quinolinic acid to nicotinic acid ribonucleotide (Nishizuka & Hayaishi, 1963). There is little information available about the factors that regulate this enzyme. It was shown recently by Ghafoorunissa & Narasinga Rao (1973) that inclusion of excess leucine in the diet reduced the activity of this enzyme in liver and kidney of rats. The results of the present study demonstrate an inverse relationship between dietary protein level and liver quinolinate phosphoribosyltransferase activity. This finding confirms a preliminary observation reported by Ghafoorunissa & Narasinga Rao (1974) that liver quinolinate phosphoribosyltransferase activity of rats given a diet containing 20 g protein/kg was significantly higher than that in rats given 180 g protein/kg diet. As with tryptophan oxygenase, quinolinate phosphoribosyltransferase activity was not found to be influenced by nicotinic acid in the diet at any of the dietary protein levels studied. However, there seemed to be an inverse relationship between quinolinate phosphoribosyltransferase activity and liver NAD levels. Livers of rats given 50 g protein/kg diet had higher quinolinate phosphoribosyltransferase activities and lower NAD levels compared to those of rats given 100 and 200 g protein/kg diet. However, these results do not indicate conclusively whether NAD level has any feedback control of quinolinate phosphoribosyltransferase activity. Further studies of this relationship are obviously required. The mechanism by which protein level in the diet regulates quinolinate phosphoribosyltransferase activity is not clear. Studies are underway to elucidate this mechanism.

Neither picolinate carboxylase nor nicotinate phosphoribosyltransferase activities was found to be affected by protein level in the diet. Picolinate carboxylase is an important enzyme of the glutarate pathway of tryptophan metabolism, and has been shown to control the amount of NAD formed from tryptophan in alloxan-diabetic rats (Ikeda *et al.* 1965). Picolinate carboxylase activity has been reported to be increased in rats given excess leucine in their diet (Ghafoorunissa & Narasinga Rao, 1973) and to be inversely related to quinolinate phosphoribosyltransferase activity. There was no inverse relationship in rats given different levels of protein; quinolinate phosphoriboysltransferase activity changed without any change in picolinate carboxylase activity. This would suggest that the two enzymes may be regulated by independent mechanisms.

The finding in the present study that liver nicotinamide neucleotide levels were lower with 50 g protein/kg diet than with 100 or 200 g protein/kg diet is in agreement with the report

of Bhan & Venkitasubramanian (1965). These authors also reported that changing the dietary protein level from 100 to 200 g/kg diet did not alter the liver nicotinamide nucleotide levels. Except with 50 g protein/kg diet, the addition of nicotinic acid to the diet had no effect on liver nucleotides. However, the presence of nicotinic acid at all three dietary protein levels resulted in increased excretion of *N'*-methylnicotinamide, the increase being considerably greater with 50 g protein/kg diet.

Since the urinary source of *N'*-methylnicotinamide is nicotinamide nucleotides, its increased excretion may be taken as a reflection of the greater turnover of nucleotides. Thus, it would appear that the nicotinamide nucleotide turnover is increased when nicotinic acid is included in the diet.

An inverse relationship between protein level in the diet and the activity of quinolinate phosphoribosyltransferase, the rate-limiting enzyme of the tryptophan–NAD pathway, suggests that the efficiency of conversion of tryptophan to NAD is related to protein level in the diet, the efficiency decreasing with increasing levels of dietary protein. It is not possible to draw a definite conclusion concerning the efficiency of this conversion, either from the liver nucleotide levels or urinary excretion of quinolinic acid and *N'*-methylnicotinamide. The liver nicotinamide nucleotide levels and the urinary *N'*-methylnicotinamide excretion are determined by the rate of turnover of nucleotides, which appear to be influenced by both protein and nicotinic acid level in the diet. Quinolinic acid excretion is influenced by the level of tryptophan intake and tryptophan oxygenase activity, both of which increased with increased protein intake. Hence it appears that a direct demonstration of the efficiency of conversion of tryptophan to NAD in vivo with labelled substrate will be necessary to answer this question.

We are grateful to Dr S. G. Srikantia, Director, National Institute of Nutrition, Jamai-Osmania, Hyderabad-500 007, for his keen interest and helpful suggestions during this study. One of us (U.S.) is a recipient of ICMR Fellowship.

REFERENCES

Anasuya, A. & Narasinga Rao, B. S. (1975). *Biochem. Med.* **12**, 365.
Bhan, A. K. & Venkitasubramanian, T. A. (1965). *Indian J. Biochem.* **2**, 41.
Brown, R. R., Vivian, V. M., Reynolds, M. S. & Price, J. M. (1958). *J. Nutr.* **66**, 599.
Carpenter, K. J. & Kodicek, E. (1950). *Biochem. J.* **46**, 421.
Chiancone, F. M. (1965). In *Newer Methods in Nutritional Biochemistry*, vol. 2, p. 249 [A. Albanese, editor]. New York and London: Academic Press.
Freed, M. (1966). In *Methods of Vitamin Assay*, 3rd ed., p. 169 [Association of Official Vitamin Chemists, editors]. New York: Interscience Publishers.
Ghafoorunissa & Narasinga Rao, B. S. (1973). *Biochem. J.* **134**, 425.
Ghafoorunissa & Narasinga Rao, B. S. (1974). *Life Sci.* **15**, 1597.
Gholson, R. K., Ueda, I., Ogasawara, N. & Henderson, L. M. (1963). *J. biol. Chem.* **239**, 1208.
Henderson, L. M. (1949). *J. biol. Chem.* **181**, 677.
Horwitt, M. K., Harvey, C. C., Rothwell, W. S., Cutler, J. L. & Haffron, D. (1956). *J. Nutr.* **60**, Suppl. 1, 1.
Ikeda, M., Tsuji, H., Nakamura, S., Ichiyama, A., Nishizuka, Y. & Hayaishi, O. (1965). *J. biol. Chem.* **240**, 1395.
Ismande, J. (1964). *Biochim. biophys. Acta* **85**, 255.
Knox, W. E. (1966). In *Advances in Enzyme Regulation*, vol. 4, p. 287 [G. Weber, editor]. Oxford: Pergamon Press.
Knox, W. E. & Mehler, A. H. (1951). *Science, N.Y.* **113**, 237.
Levitas, M., Robinson, J., Rosen, F., Huff, J. W. & Perlzweig, W. A. (1947). *J. biol. Chem.* **167**, 169.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Nakagawa, I., Takahashi, T., Suzuki, T. & Masana, Y. (1969). *J. Nutr.* **99**, 325.
National Academy of Sciences/National Research Council (1963). *Rep. natn. Acad. Sci., Wash.* no. 1100.
Nishizuka, Y. & Hayaishi, O. (1963). *J. biol. Chem.* **238**, 3369.
Nishizuka, Y. & Nakamura, S. (1970). *Meth. Enzym.* **17A**, 491.
Oser, B. L. (editor) (1965). In *Hawk's Physiological Chemistry*, 14th ed., p. 1233. New York: McGraw-Hill Book Company.
U.S. Pharmacoepia XVII (1965). p. 862.

Printed in Great Britain