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Tryptophan metabolism in vitamin B_6-deficient mice

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Vitamin B_6 deficiency was induced in mice by maintenance for 4 weeks on a vitamin B_6-free diet. Tryptophan metabolism was assessed by determining the urinary excretion of tryptophan metabolites, the metabolism of [^{14}C]tryptophan in vivo and the formation of tryptophan and niacin metabolites by isolated hepatocytes. The vitamin B_6-deficient animals excreted more xanthurenic acid and 3-hydroxykynurenine, and less of the niacin metabolites *N*^{1}-methyl nicotinamide and methyl-2-pyridone-4-carboxamide, than did control animals maintained on the same diet supplemented with 5 mg vitamin B_6/kg. After intraperitoneal injection of [^{14}C]tryptophan, vitamin B_6-deficient mice showed lower liberation of $^{14}CO_2$ from [methylene-^{14}C]tryptophan and [U-^{14}C]tryptophan than did controls, indicating impairment of kynureninase (*EC* 3.7.1.3) activity. There was no difference between the two groups of animals in the metabolism of [ring-2-^{14}C]tryptophan. Hepatocytes isolated from the vitamin B_6-deficient animals formed more 3-hydroxykynurenine and xanthurenic acid than did cells from control animals, but also formed more NADP and free niacin.

Tryptophan metabolism: Vitamin B_6 deficiency: Mice

Vitamin B_6 deficiency results in abnormal metabolism of tryptophan because the enzyme kynureninase (*EC* 3.7.1.3) in the oxidative pathway of tryptophan metabolism (see Fig. 1) is pyridoxal phosphate (vitamin B_6)-dependent. In deficiency its activity is severely impaired, so that, especially after a loading dose of tryptophan, there is increased accumulation of its substrates, kynurenine and 3-hydroxykynurenine, and increased formation of xanthurenic and kynurenic acids. This results in increased urinary excretion of these compounds (Lepkovsky & Nielson, 1942; Lepkovsky *et al.* 1943). The ability to metabolize a test dose of tryptophan has been used widely as a means of assessing vitamin B_6 nutritional status (Coursin, 1964; Allegri *et al.* 1978), although the validity and reliability of the tryptophan load test have been challenged (Coon & Nagler, 1969; Bender & Wynick, 1981; Bender, 1983*b*, 1987).

In vitro investigation of the kinetic variables of the enzymes involved in the oxidative pathway of tryptophan metabolism suggests that even under normal conditions the activities of kynureninase (Knox, 1953) and kynurenine hydroxylase (*EC* 1.14.13.9) (Bender & McCreanor, 1985) are so low that they are likely to provide (secondary) rate-limiting steps in the pathway. Significant amounts of kynurenine, 3-hydroxykynurenine, kynurenic acid and xanthurenic acid are excreted by animals fed on a diet providing adequate vitamin B_6, suggesting that in vivo the activities of kynurenine hydroxylase and kynureninase may be inadequate to cope with the metabolic flux through tryptophan dioxygenase (*EC* 1.13.11.11).

Studies of tryptophan metabolism in isolated hepatocytes do not support the view that kynureninase may be a rate-limiting step under normal conditions. Salter *et al.* (1986) determined metabolic flux through discrete sectors of the oxidative pathway and calculated that the control coefficient (that proportion of the total control of metabolic flux that can be attributed to an individual step) for kynureninase was $< 0{\cdot}004$. The over-whelming

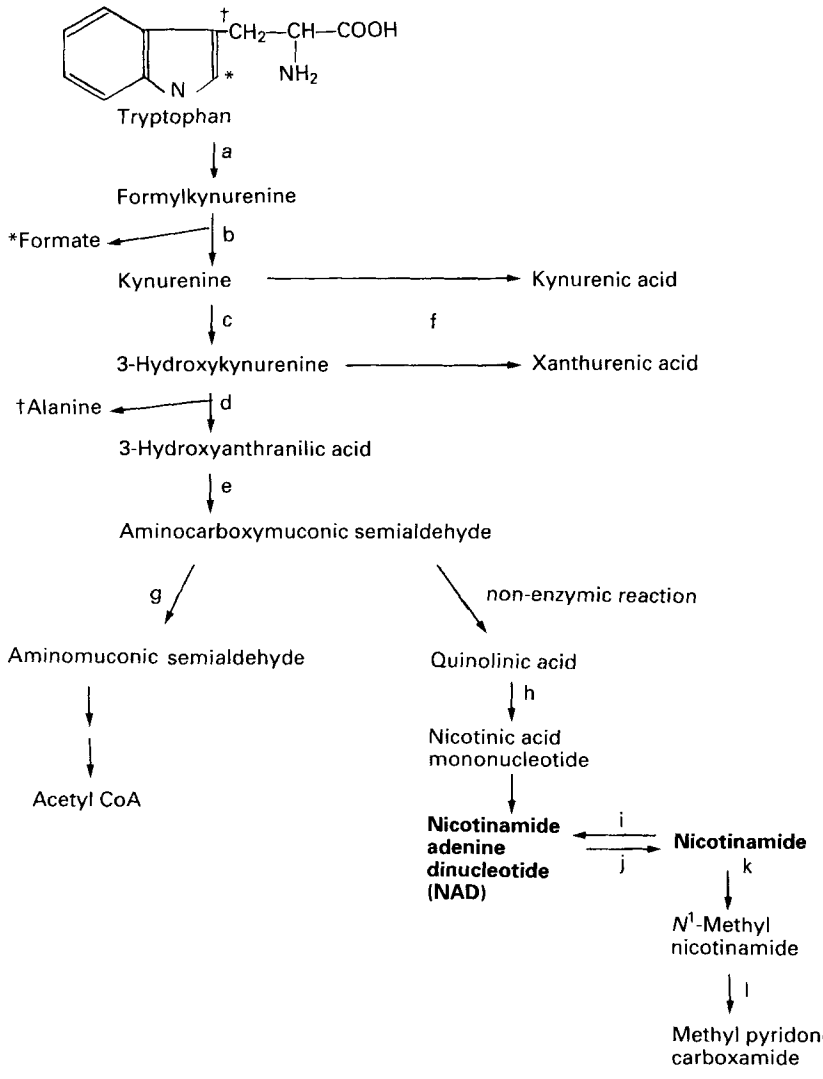

Fig. 1. The metabolism of tryptophan and nicotinamide nucleotides. a, Tryptophan dioxygenase(*EC* 1.13.11.11); b, formylkynurenine formamidase (*EC* 3.5.1.9); c, kynurenine hydroxylase (*EC* 1.14.13.9); d, kynureninase (*EC* 3.7.1.3); e, 3-hydroxyanthranilic acid oxidase (*EC* 1.13.11.6); f, kynurenine aminotransferase (*EC* 2.6.1.7); g, picolinate carboxylase (*EC* 4.1.1.45); h, quinolinic acid phosphoribosyltransferase (*EC* 2.4.2.19); i, nicotinamide phosphoribosyltransferase (*EC* 2.4.2.12); j, NAD glycohydrolase (*EC* 3.2.2.5) or poly(ADP-ribose) transferase (*EC* 2.4.2.30); k, nicotinamide *N*-methyl transferase (*EC* 2.1.1.1); l, aldehyde oxidase (*EC* 1.2.3.1). * The label from [ring-2-^{14}C]tryptophan is released as formate, and that from [methylene-^{14}C]tryptophan as alanine(†); both are metabolized onwards to $^{14}CO_2$.

control of metabolic flux was attributable to tryptophan dioxygenase (control coefficient 0·75) and the uptake of tryptophan into the cells (control coefficient 0·25). Induction of tryptophan dioxygenase by the previous administration of glucocorticoid hormones (the conditions under which Knox (1953) suggested that kynureninase would become rate-limiting) resulted in an increase in the control coefficient of transport to 0·75 and a decrease in the control coefficient of tryptophan dioxygenase to 0·25.

The present study was designed to investigate further the metabolism of tryptophan

in vitamin B_6-deficient animals; in hepatocytes from vitamin B_6-deficient animals, kynureninase does have a significant control coefficient (0·41) (Stanley *et al.* 1985).

METHODS

Male BK albino mice were purchased as weanlings from Banting & Kingman (Hull, Humberside) and were assigned randomly to receive either a vitamin B_6-free diet or the same diet supplemented with 5 mg pyridoxine hydrochloride/kg, as described previously (Symes *et al.* 1984). The animals were maintained in groups of five per cage, and received food and water *ad lib.*

Once each week the animals were transferred to individual metabolism cages, and urine was collected for 24 h. Each urine sample was diluted to 20 ml with distilled water and then frozen and stored at −20° until required for the determination of tryptophan metabolites, as described later. They were allowed free access to food and water during this period.

After the animals had been receiving the diets for 3–4 weeks, they received 0·1 μCi [^{14}C]tryptophan by intraperitoneal injection at 09.00 hours, and were transferred to separate sealed glass jars, through which air was passed slowly from a compressor. Exhaled air was bubbled through 1 ml 2-methoxyethylamine in a narrow tube to trap $^{14}CO_2$. The 2-methoxyethylamine was changed at 10 min intervals for 2 h and was then mixed with 3 ml Ecoscint-A water-miscible scintillation fluid (National Diagnostics, Aylesbury, Bucks) and radioactivity was determined in a liquid-scintillation spectrometer. Each animal received an intraperitoneal injection of [ring-2-^{14}C]tryptophan (CEA, Gif-sur-Yvette, France; specific activity 50 Ci/mol) to assess metabolic flux through tryptophan dioxygenase and formylkynurenine formamidase (*EC* 3.5.1.9) on the first day, [methylene-^{14}C]tryptophan (Amersham International plc, Amersham, Bucks; specific activity 59 Ci/mol) to assess metabolic flux through kynurenine hydroxylase and kynureninase on the next day, and [U-^{14}C]tryptophan (NEN; Du Pont (UK) Ltd, Stevenage, Herts; specific activity 556 Ci/mol) to assess overall oxidative metabolism on the third day. (In previous studies (Bender, 1983*a*), [benzene ring-U-^{14}C]tryptophan was used to give a more precise estimate of metabolic flux through picolinate carboxylase (*EC* 4.1.1.45) and the total oxidative branch of the pathway; this positional isomer of [^{14}C]tryptophan is no longer available.) On the day the animals received [ring-2-^{14}C]tryptophan, they received a second intraperitoneal injection, of 0·1 μCi [^{14}C]formate (Amersham International plc, Amersham, Bucks; specific activity 59 Ci/mol), 2 h after the injection of tryptophan, in order to ensure that any changes in the liberation of $^{14}CO_2$ from [ring-2-^{14}C]tryptophan were not due to changes in the onward metabolism of the formate released by formylkynurenine formamidase. Similarly, after the injection of [methylene-^{14}C]tryptophan the animals received an injection of 0·1 μCi [U-^{14}C]alanine (Amersham International plc, Amersham, Bucks; specific activity 171 Ci/mol), in order to exclude artifacts due to changes in the onward metabolism of the alanine released by kynureninase. In each case the collection of $^{14}CO_2$ was continued for a further 2 h.

During the fourth and fifth weeks after weaning, isolated hepatocytes were prepared from one animal each day by perfusion of the liver with collagenase (*EC* 3.4.24.3) from the superior vena cava to the hepatic portal vein, as described previously (Bender & Olufunwa, 1988); the viability of the cells was assessed by Trypan blue exclusion. After the superior vena cava had been cannulated, a sample of blood (0·2–0·5 ml) was drawn from the inferior vena cava, which was then ligated. The hepatic portal vein was then cut, and perfusion commenced.

The hepatocytes were incubated for 30 min at 37° in a final volume of 10 ml (5–10 mg dry weight of cells/incubation) with 60 μmol tryptophan/l. The formation of kynurenine,

3-hydroxykynurenine, xanthurenic acid, total nicotinamide nucleotides (NAD and NADP, oxidized and reduced forms), total niacin (nicotinic acid plus nicotinamide), N^1-methyl nicotinamide, and methyl-2-pyridone-4-carboxamide, was determined as described previously (Bender & Olufunwa, 1988).

The blood which was withdrawn from the inferior vena cava before the beginning of the perfusion was centrifuged at 2000 *g* for 15 min. Plasma tryptophan was determined by the norharman fluorimetric method (Denckla & Dewey, 1967). The activation of aspartate aminotransferase (*EC* 2.6.1.1) by pyridoxal phosphate was determined in the lysed washed erythrocyte pellet as an index of vitamin B_6 nutritional status, by the modification of the [2,3-^{3}H]aspartate method of Schuster *et al.* (1978) described previously (Bender *et al.* 1982), with and without pre-incubation in 0·25 mmol pyridoxal phosphate/l. The results have been expressed as the activation coefficient, the ratio of activity after activation of apo-enzyme with pyridoxal phosphate: basal activity of native holoenzyme.

Urine xanthurenic and kynurenic acids and kynurenine were determined after chromatography of 10 ml samples of the diluted urine on small columns of Dowex 50W (H^+) ion-exchange resin, as described by Satoh & Price (1958). Xanthurenic and kynurenic acids were determined fluorimetrically (Satoh & Price, 1958) and kynurenine colorimetrically after diazotization and coupling to naphthyl ethylenediamine (Joseph & Risby, 1975). 3-Hydroxykynurenine was determined fluorimetrically after reaction with *p*-toluene sulphonyl chloride in acetone (Watanabe *et al.* 1970), N^1-methyl nicotinamide by the small-scale modification of the alkali–ketone fluorimetric method of Carpenter & Kodicek (1950) that has been described previously (Bender, 1980), and methyl-2-pyridone-4-carboxamide colorimetrically by hypobromite-catalysed rearrangement to the amine, diazotization and coupling to naphthyl ethylenediamine (Holman, 1954), after removal of potentially interfering substances with MB-5113 mixed-bed ion-exchange resin (Bender, 1983*b*).

RESULTS

As shown in Table 1, the vitamin B_6-deficient animals grew more slowly than those fed on the control diet, showing a mean gain in weight of 11% over the 4 weeks of the experiment, compared with a gain of 31% in the control animals. After 4 weeks on the diet, the erythrocyte aspartate aminotransferase activation coefficient was 1·44 (SE 0·103) in the deficient animals, and 1·09 (SE 0·066) in the control animals ($0·05 > P > 0·01$, *t* test). There was no significant difference in the plasma concentration of tryptophan (control 72·7 (SE 5·27) μmol/l, vitamin B_6-deficient 76·4 (SE 8·54); $P > 0·1$, *t* test).

Table 1 shows the urinary excretion of tryptophan metabolites at weekly intervals through the study. 3-Hydroxykynurenine excretion was significantly higher in the deficient animals throughout the experiment. Urinary kynurenic acid showed no consistent difference between the two groups of animals; xanthurenic acid excretion was slightly higher throughout, significantly so after 3 and 4 weeks on the deficient diet. N^1-methyl nicotinamide and methyl pyridone carboxamide excretion were consistently lower in the deficient animals; the differences were significant in weeks 3 and 4 for methyl pyridone carboxamide, and in week 4 for N^1-methyl nicotinamide.

Fig. 2 shows the effects of vitamin B_6 deficiency for 4 weeks on the metabolism of [^{14}C]tryptophan, formate and alanine in intact mice. There was no significant effect of diet on the liberation of $^{14}CO_2$ from [ring-2-^{14}C]tryptophan or [^{14}C]formate ($P > 0·1$, analysis of variance), although there was a suggestion of an early greater rate of tryptophan metabolism in the deficient animals ($^{14}CO_2$ liberation at 10 min: 7·34 (SE 0·724) counts/min per 10^3 cpm injected in deficient animals, 4·47 (SE 0·364) in controls; $P < 0·001$, *t* test). As shown in Table 2, there was no significant difference in the total amount of $^{14}CO_2$ recovered

Table 1. *Body-weight and urinary tryptophan metabolites in mice maintained for 1–4 weeks from weaning on control or vitamin B_6-deficient diets*

(Values are means with their standard errors for five animals in each group)

Period on diet (weeks)...	1				2				3				4			
Diet	Control		Deficient		Control		Deficient		Control		Deficient		Control		Deficient	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Body-wt (g)	23·6	0·21	25·8**	0·71	28·0	0·42	27·0	0·81	30·2	0·72	28·0	0·81	30·8	0·73	28·6	1·00
Urine metabolites (/24 h):																
Kynurenine (nmol)	659	195·8	642	166·3	731	115·8	1018	174·8	827	66·9	1268	231·6	806	57·7	963	81·6
Hydroxykynurenine (nmol)	66	5·2	139**	13·3	64	5·8	149***	11·9	68	5·7	151***	13·3	72	5·4	158***	8·7
Kynurenic acid (μmol)	123	9·3	142	27·4	129	16·3	98	11·0	162	19·2	146	25·4	135	10·4	128	11·0
Xanthurenic acid (μmol)	55	8·5	80	21·4	49	6·9	67	6·3	41	4·8	98*	21·4	50	3·9	93*	17·4
N^1-methyl nicotinamide (nmol)	226	57·3	147	55·8	178	61·1	69	7·1	152	31·5	128	25·1	240	29·9	160*	25·7
Methyl pyridone carboxamide (nmol)	337	93·9	172	42·0	1737	400·8	250*	85·3*	2018	443·9	527*	99·5	3133	392·6	576***	147·8

Values for the control group were significantly different from those of the deficient group at the same time-point (t test): *$0·05 > P > 0·01$, **$0·01 > P > 0·001$, ***$P < 0·001$. The same five animals in each group were studied each week.

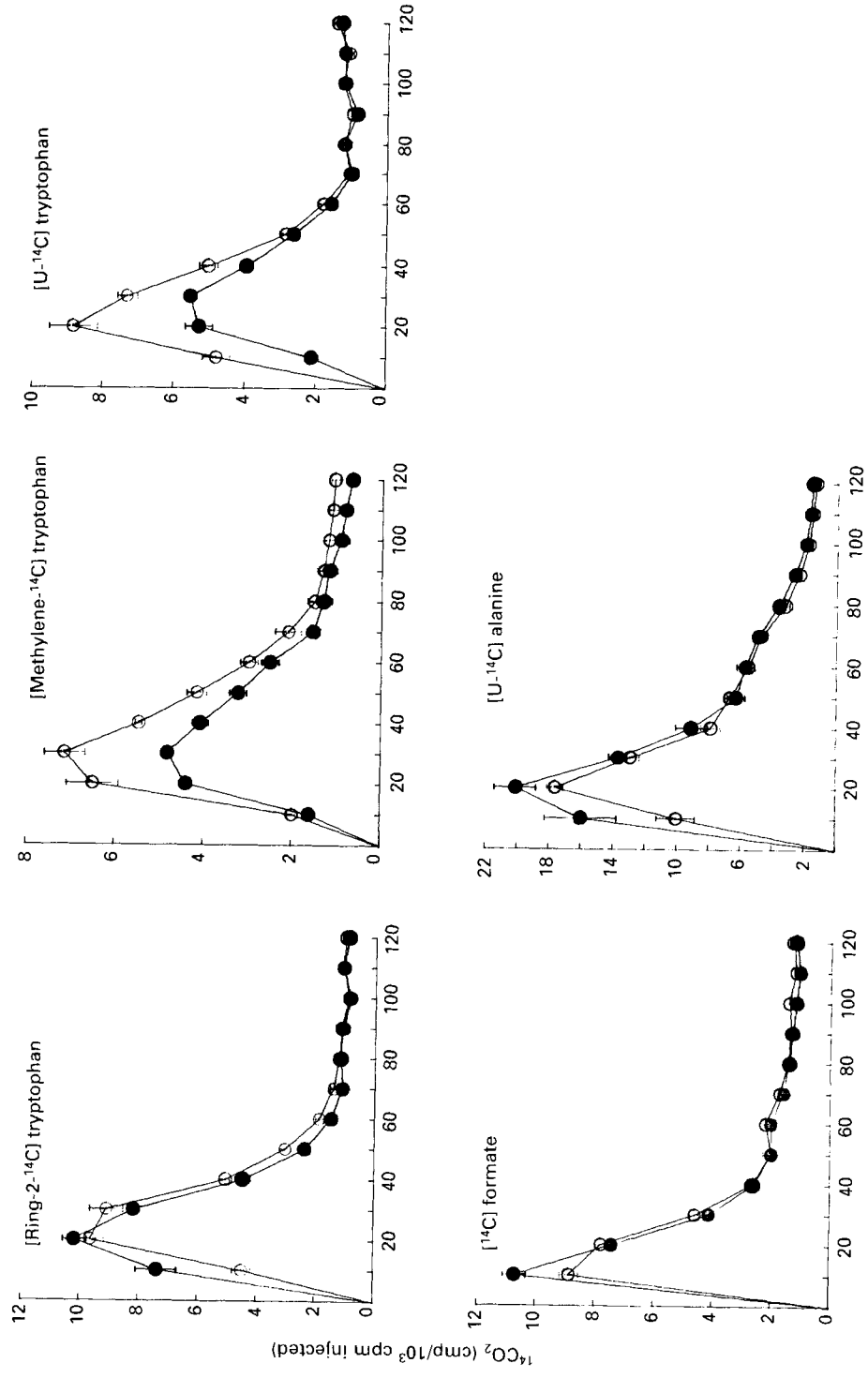

Fig. 2. The liberation of $^{14}CO_2$ (counts/min (cpm) per 10^3 cpm injected) from [^{14}C]tryptophan, [^{14}C]formate and [U-^{14}C]alanine in vitamin B_6-deficient and control mice in vivo after intraperitoneal injection. Points are means with their standard errors represented by vertical bars for five animals/group. (○), Control; (●), vitamin B_6-deficient mice. The total recovery of $^{14}CO_2$ in these studies is shown in Table 2.

Table 2. *Total recovery of $^{14}CO_2$ from [^{14}C]tryptophan, [^{14}C]formate and [U-^{14}C]alanine in vitamin B_6-deficient and control mice in vivo after intraperitoneal injection*

(Values are means with their standard errors for five animals in each group)

	$^{14}CO_2$ (cpm/10^3 cpm injected)			
	Control		Vitamin B_6-deficient	
	Mean	SE	Mean	SE
[Ring-2-^{14}C]tryptophan	39·7	3·09	40·2	3·49
[^{14}C]formate	36·5	1·49	36·6	1·85
[Methylene-^{14}C]tryptophan	36·5	3·08	27·1*	2·42
[U-^{14}C]alanine	75·1	5·15	86·6	6·56
[U-^{14}C]tryptophan	37·6	2·58	27·8**	1·58

cpm, counts/min.

Values for the control group were significantly different from those for the deficient group (*t* test): *$0·1 > P > 0·05$, **$0·05 > P > 0·01$.

Table 3. *The formation of tryptophan metabolites (nmol formed /30 min per mg dry wt of cells) by isolated hepatocytes incubated for 30 min with 60 μmol tryptophan/l*

(Values are means with their standard errors for cells isolated from five animals in each group)

	Control		Vitamin B_6-deficient	
	Mean	SE	Mean	SE
Kynurenine	2·9	0·38	2·3	0·16
3-Hydroxykynurenine	0·82	0·027	1·46**	0·134
Xanthurenic acid	7·7	0·67	9·3*	0·60
NADP	0·65	0·103	1·22**	0·21
Niacin	15·2	0·96	23·4**	2·17
N^1-methyl nicotinamide	0·07	0·009	0·07	0·015
Methyl pyridone carboxamide	1·3	0·34	1·5	0·12

Values for the deficient group were significantly different from those for the control group (*t* test): *$0·1 > P > 0·05$; **$0·05 > P > 0·01$.

from [ring-2-^{14}C]tryptophan or [^{14}C]formate over 2 h. The deficient animals showed significantly reduced liberation of $^{14}CO_2$ from [methylene-^{14}C]tryptophan ($P = 0·0069$, analysis of variance), but slightly increased liberation of $^{14}CO_2$ from [^{14}C]alanine. The reduced flux through kynureninase suggested by the reduction in $^{14}CO_2$ liberation from [methylene-^{14}C]tryptophan was reflected in a reduced liberation of $^{14}CO_2$ from [U-^{14}C]tryptophan ($P = 0·047$, analysis of variance).

As shown in Table 3, hepatocytes isolated from vitamin B_6-deficient animals formed more 3-hydroxykynurenine, xanthurenic acid, NADP and niacin than did cells from animals fed on the control diet. There was no significant difference in the formation of kynurenine, N^1-methyl nicotinamide or methyl pyridone carboxamide.

DISCUSSION

Previous studies with rats (Symes *et al.* 1984; Bowden *et al.* 1986; Bender *et al.* 1989) have shown that maintenance for 3–4 weeks from weaning on the vitamin B_6-free diet used in the present study results in a significant degree of vitamin B_6 depletion, as assessed by the liver content of pyridoxal phosphate. In the present study, in mice, the same diet led to the development of significant vitamin B_6 deficiency, as assessed by the erythrocyte aspartate aminotransferase activation coefficient.

The deficient mice showed the expected abnormalities of excretion of tryptophan metabolites: an elevation of urinary 3-hydroxykynurenine and xanthurenic acid, even in the absence of a loading dose of tryptophan. There was also a reduction in the excretion of N^1-methyl nicotinamide and methyl pyridone carboxamide, the two end-products of the pathway. The metabolic fate of aminocarboxymuconic semialdehyde (see Fig. 1) will depend on the balance between the (saturable) enzymic reaction of picolinate carboxylase (leading to total oxidation) and non-enzymic cyclization to quinolinic acid (the precursor of NAD), which has linear kinetics. Hence, the formation of NADP changes considerably as the rate of formation of aminocarboxymuconic semialdehyde changes. This in turn depends on the rate of metabolic flux through the pathway from tryptophan (Mehler *et al.* 1964; Ikeda *et al.* 1965). Reduced activity of kynureninase resulting from vitamin B_6 deficiency would, therefore, be expected to result in the reduced formation of NADP and the niacin metabolites N^1-methyl nicotinamide and methyl pyridone carboxamide shown in Table 1.

The production of $^{14}CO_2$ from [^{14}C]tryptophan in intact animals, shown in Fig. 2 and Table 2, is also in agreement with expectations. There was no effect of vitamin B_6 deficiency on the production of $^{14}CO_2$ from [ring-2-^{14}C]tryptophan, which reflects the activity of tryptophan dioxygenase. There was clear impairment of the activity of kynureninase in vivo in vitamin B_6 deficiency, with a significant reduction in the peak height and total production of $^{14}CO_2$ from [methylene-^{14}C]tryptophan in the deficient animals. This was not an artifact of reduced metabolism of the alanine released by kynureninase; indeed there was a slight increase in the production of $^{14}CO_2$ from [U-^{14}C]alanine in the deficient animals. The production of $^{14}CO_2$ from [U-^{14}C]tryptophan showed the same effect as did that from [methylene-^{14}C]tryptophan, suggesting that the activity of picolinate carboxylase, and hence total oxidation of tryptophan via acetate, is not affected by vitamin B_6 deficiency.

The results with isolated hepatocytes are at variance with those in intact animals. As shown in Table 3, there was indeed increased formation of 3-hydroxykynurenine and xanthurenic acid in hepatocytes from vitamin B_6-deficient animals, but this was accompanied by increased formation of NADP and free niacin, whereas urinary excretion of niacin metabolites was reduced in the deficient animals.

It is difficult to reconcile the results in intact animals, which show the expected increase in urinary excretion of kynurenine metabolites and reduced excretion of niacin metabolites in vitamin B_6 deficiency, with those obtained in hepatocytes isolated from the same animals, which show increased formation of both kynurenine and niacin metabolites. It is possible that vitamin B_6 deficiency affects either the catabolism of NADP or the methylation of nicotinamide to N^1-methyl nicotinamide, although there is no evidence that the enzymes involved are vitamin B_6-dependent. It may be that an artifact is induced during the isolation of hepatocytes by perfusion of the liver with collagenase, so that the isolated hepatocyte is not a useful model for such studies. Alternatively, it is possible that studies of whole-body tryptophan metabolism reflect not only hepatic, but also extra-hepatic, metabolism of tryptophan and kynurenine.

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