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The metabolic origin of trigonelline in the rat

Jasbir S. SANDHU and D. R. FRASER
Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council, Cambridge CB4 1XJ, U.K.

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A hypothesis of Mason & Kodicek [(1970) *Biochem. J.* **120**, 515–521] that esterified nicotinic acid in niacytin from cereals is a precursor for trigonelline was investigated in rats. Single oral doses of niacytin resulted in the excretion of trigonelline in urine but only in rats that were niacin-deficient and were fed a cereal diet. These animals were found to have an abnormally permeable intestine, which allowed the uptake of molecules not usually absorbed. Orally administered synthetic [^{14}C]nicotinoyl[^{3}H]methylcellulose was shown to be absorbed by niacin-deficient rats on a cereal diet and [^{14}C]trigonelline was excreted in urine. These data indicate that dietary cereal induces a permeability defect in the intestinal mucosa of niacin-deficient rats, which allows the uptake of macromolecular niacytin. The nicotinoyl pyridine nitrogen atom is then methylated and slow hydrolysis releases trigonelline from the macromolecule.

The excretory products of nicotinic acid metabolism are mainly N^1-methyl-2-pyridone-5-carboxamide and N^1-methylnicotinamide. Trigonelline, the betaine of *N*-methylnicotinic acid, is also found in urine but, because it is a dietary constituent (e.g. in legumes and coffee) that is excreted unchanged (McKennis *et al.*, 1964) and because nicotinic acid is reported not to be converted into trigonelline *in vivo* (Hundley, 1954), this substance is thought not to be a metabolic derivative in mammals. However, when 'bound' nicotinic acid (niacytin) from cereals was given in single oral doses to niacin-deficient rats on a cereal diet, Mason & Kodicek (1970*b*) found that a prolonged excretion of trigonelline followed and this was equivalent to 30–40% of the administered nicotinic acid.

Niacytin is a heterogeneous mixture of polysaccharides and glycopeptides (mol.wt. 1500–17000) to which nicotinic acid is esterified (Mason & Kodicek, 1973; Mason *et al.*, 1973). As this nicotinic acid is nutritionally unavailable to rats (Mason & Kodicek, 1970*a*) the metabolic link between the nicotinic acid of niacytin and the observed excretion of trigonelline is uncertain. Mason & Kodicek (1970*b*) postulated that the small intestine was damaged in niacin-deficient rats, that it absorbed a macromolecular fragment of niacytin, that the nicotinic acid was then enzymically *N*-methylated while still attached to the macromolecule and that subsequent slow hydrolysis released metabolically inert trigonelline.

The various tenets of this hypothesis have now been further investigated and found to be essentially correct. During this study it was also discovered that the initiating permeability defect in niacin deficiency, which led to the metabolic production of trigonelline, is only apparent if the rats are fed a cereal-containing diet.

Experimental

Preparation of 'bound' nicotinic acid

Niacytin was extracted from wheat bran by the method of Mason & Kodicek (1970*a*), which yielded a product containing 3.3% (w/w) esterified nicotinic acid. Single doses of niacytin equivalent to 1 mg of nicotinic acid were given to rats by gastric intubation in 0.5 ml of aq. 10% (v/v) ethanol.

Synthetic 'bound' nicotinic acid was prepared by esterifying [*carboxy*-^{14}C]nicotinic acid (sp. radioactivity 58 mCi/mol) (The Radiochemical Centre, Amersham, Bucks., U.K.) to [^{3}H]methylcellulose (sp. radioactivity 34 mCi/mol) (mol.wt. approx. 100000) as described by Sandhu (1981). Approx. 50 mol of nicotinic acid was esterified/mol of methylcellulose. Single doses of this double-labelled material (2 μCi of ^{14}C; 0.025 μCi of ^{3}H) containing 1 mg of esterified nicotinic acid were given to rats in 0.5 ml of aqueous solution by gastric intubation. The identity of any [^{3}H]methylcellulose excreted in urine was determined by chromatography on Sephadex G-100 (Pharmacia, Uppsala, Sweden), and by testing for hexoses by the anthrone reaction (Roe, 1955).

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Estimation of nicotinic acid and its derivatives

Niacytin (20mg) or maize meal (1g) were hydrolysed with 5M-NaOH for 15min at 100°C. The pH was then adjusted to 1–2 and the samples further treated by ion-exchange chromatography and permanganate oxidation by the method of Tyler & Shrago (1980). Nicotinic acid and trigonelline were assayed by high-pressure liquid chromatography (Sandhu & Fraser, 1981). Trigonelline N^1-methylnicotinamide and N^1-methyl-2-pyridone-5-carboxamide in urine were also assayed by the same chromatographic method.

The amounts of trigonelline detected in both niacytin and maize meal are slight underestimates as the alkali treatment was found to convert 10–15% of added standard trigonelline into nicotinic acid by demethylation.

Assessment of intestinal permeability in rats

To determine whether niacin-deficient rats had an abnormal permeability of the mucosa of the small intestine, the uptake of two non-metabolisable sugars was measured. This method was derived from a test for intestinal function in man (Cobden *et al.*, 1980) and used [^{3}H]cellobiotol and [^{14}C]mannitol (J. S. Sandhu & D. R. Fraser, unpublished work). With a permeability defect, the 5-h urinary excretion of [^{3}H]cellobiotol was increased, whereas that of [^{14}C]mannitol decreased. Hence an increase in ^{3}H/^{14}C ratio in urine indicated an abnormality in intestinal function.

Animals

Male Norwegian hooded rats of the Dunn Nutritional Laboratory strain were weaned at body weights of 45–55g. Niacin deficiency was induced by feeding deficient diets either based on casein and free of cereals (Nakashima *et al.*, 1978) or based on maize meal (Harris & Kodicek, 1950). Control animals were given single oral doses of 20mg of nicotinamide at weekly intervals. Metabolic studies were done after 28 days by which time the growth rates of the deficient animals were much slower than those of the controls. The rats were then housed individually in metabolic cages. Total urine was collected under toluene, pooled daily from each group of rats, then filtered, made up to 15ml with water, and stored at −25°C.

Experimental protocol

Groups of five deficient and five control animals on both cereal-free and maize-meal diets (i.e. 20 rats in total) were used to study the metabolism of each test substance. Natural or synthetic 'bound' nicotinic acid was given as a single dose by gastric intubation. Futher groups of five niacin-supplemented rats on the cereal-free and maize-meal diets received the same quantity of natural or synthetic 'bound' nicotinic acid by intraperitoneal injection. Total urine collections were made for each group at 24h intervals for 2 days before dosing and at 5, 10, 24h and every subsequent 24h for 7 days afterwards.

Results

Urinary trigonelline after niacytin administration

Animals on the maize-meal diet, before receiving niacytin, excreted 17–20μg of trigonelline per day irrespective of whether or not they were niacin-deficient (Table 1). In contrast, trigonelline was not detected in urine of rats on the casein diet (Table 1). As maize meal contained trigonelline at about 15μg/g (Table 2) and as the rats were consuming up to 3g of maize meal per day, the urinary output of trigonelline on this diet could be readily attributed to this source. The absence of urinary trigonelline from rats on the trigonelline-free casein diet again indicated that trigonelline was not a product of endogenous nicotinic acid metabolism. This finding is in agreement with another study where a different strain of rats was used (Carter, 1980).

After a single oral dose of niacytin, the niacin-deficient rats on the maize-meal diet showed an increased output of trigonelline, which reached a maximum of over 80μg/day at 3 days after dosing and was still above the background level by 7 days (Table 1). The control animals had little change in output after niacytin administration.

Niacin-deficient rats on the casein diet excreted only traces of trigonelline after an oral dose of niacytin and there was no difference in response between the control and deficient groups (Table 1). Thus trigonelline excretion after niacytin administration was not just dependent on niacin deficiency but required also the presence of a dietary factor, which appeared to be in maize meal. Although it was possible that niacytin administration had improved the niacin status of the deficient animals and had induced them to consume more of the maize-meal diet and hence more trigonelline, food-intake and weight-gain measurements showed this not to be the case. Furthermore, the excretion of nicotinic acid metabolites did not change after the niacytin dose (Table 3), so no improvement in niacin status had occurred. With other preparations of niacytin about 10–20% of the bound nicotinic acid was found to be biologically available (Mason & Kodicek, 1970*a*). The reason for the apparently complete unavailability of nicotinic acid in the niacytin used in the current study is unknown. It is possible that with this preparation either the nicotinoyl ester bonds were particularly resistant to hydrolysis or with extreme niacin deficiency conditions for hydrolysis in the gut were minimal. Both the maize-meal and casein diets induced severe niacin deficiency as assessed by

Table 1. *Urinary excretion of trigonelline after administration of niacytin to rats on the maize-meal and cereal-free diets*
Values are expressed as μg of trigonelline excreted/rat per 24 h, calculated from the amount in pooled urine from five rats in each group. Niacytin was given on day 0. Trace amounts of trigonelline indicate less than 4 μg/rat per 24 h. Trigonelline was undetectable at less than 1 μg/rat per 24 h.

	Trigonelline excretion (μg)					
	Maize-meal diet			Cereal-free diet		
Time (days)	Niacin-deficient rats (oral niacytin)	Niacin-supplemented rats (oral niacytin)	Niacin-supplemented rats (intraperitoneal niacytin)	Niacin-deficient rats (oral niacytin)	Niacin-supplemented rats (oral niacytin)	Niacin-supplemented rats (intraperitoneal niacytin)
−1	19	20	17	0	0	0
0	18	18	19	0	0	0
1	63	30	97	Trace	Trace	68
2	81	18	154	Trace	Trace	102
3	85	19	159	Trace	Trace	140
4	72	16	142	0	0	126
5	62	20	106	0	0	86
6	49	18	68	0	0	58
7	30	20	31	0	0	18

Table 2. *Concentrations of total nicotinic acid and total trigonelline in niacytin and maize meal*
Values are expressed as μg/g and are means ± S.E.M. from four estimations.

	Nicotinic acid	Trigonelline
Niacytin	33 340 ± 28	33.8 ± 3.5
Maize meal	28.8 ± 3.0	14.6 ± 1.5

suppression of growth and decreased excretion of nicotinic acid metabolites in urine (Table 4).

When niacytin was given by intraperitoneal injection to niacin-supplemented rats a large increase in trigonelline excretion occurred in both the maize meal and casein diet groups (Table 1). The time course of excretion followed the pattern of the oral dose to niacin-deficient rats on the maize-meal diet with a peak at 3 days after dosing. Again, as the injected niacytin contained only 1 μg of trigonelline (Table 2) and the rats in both groups excreted about 600 μg over 7 days, the trigonelline could not have come from an exogenous source. It is therefore concluded that if niacytin is given parenterally to rats, neither niacin deficiency nor dietary maize meal are necessary for metabolic production of trigonelline to occur. These findings support the hypothesis of Mason & Kodicek (1970*b*) that an intestinal permeability defect in niacin-deficient rats on a maize-meal diet allows niacytin to gain access to an *N*-methylating enzyme process.

Assessment of intestinal permeability

The urinary excretion of [^{3}H]cellobiotol and [^{14}C]mannitol over 5 h after their oral administration to rats on the various dietary regimes is shown in Table 5. With the casein diet, niacin deficiency produced a slight decrease in mannitol excretion but no change in cellobiotol excretion compared with niacin-supplemented controls. However, with niacin deficiency on the maize-meal diet there was an increased output of cellobiotol contrasting with a decrease in that of mannitol. This was reflected in a clear rise in the urinary ^{3}H/^{14}C ratio.

The excretion of these sugars is directly related to the quantity absorbed from the small intestine (J. S. Sandhu & D. R. Fraser, unpublished work). It is therefore concluded that niacin deficiency on the cereal diet affects the permeability of the small intestine, whereas niacin deficiency in the absence of dietary maize meal had little effect. As the permeability defect enhances uptake of the non-metabolized disaccharide cellobiotol, this finding is again compatible with the postulate that abnormal absorption of intact or partially digested niacytin results in trigonelline formation.

The nature of the maize-meal component that induces the permeability defect in niacin deficiency is not reported in the present paper, but preliminary results indicate that this property resides in the glutelin or prolamin proteins of cereals. When rats were fed the niacin-free casein diet supplemented either with zein from maize or with wheat gluten, the cellobiotol/mannitol absorption test revealed that the

Table 3. *Excretion of nicotinic acid metabolites after oral administration of niacytin to rats on the maize-meal diet*
N^1-Methylnicotinamide (N^1MN) and N^1-methyl-2-pyridone-5-carboxamide (MPC) are expressed as μg excreted/rat per 24 h, calculated from the amount in pooled urine from five rats in each group. Niacytin was given on day 0. The last oral dose of 20 mg of nicotinamide had been given to the niacin-supplemented rats 7 days before urine collection was begun.

	Excretion (μg)					
	Niacin-deficient rats			Niacin-supplemented rats		
Time (days)	N^1MN	MPC	N^1MN/MPC	N^1MN	MPC	N^1MN/MPC
−1	15	18	0.83	85	34	2.50
0	14	19	0.75	83	36	2.31
1	13	14	0.93	80	33	2.42
2	16	16	1.00	86	33	2.61
3	12	15	0.80	81	32	2.53
4	15	21	0.71	84	35	2.40
5	14	18	0.78	88	36	2.44
6	13	15	0.87	83	34	2.44
7	16	20	0.80	80	33	2.42

Table 4. *Assessment of niacin deficiency state by growth rate and excretion of nicotinic acid metabolites for rats on niacin-deficient and niacin-supplemented diets*
Each group of five weanling rats was fed the appropriate diet for 4 weeks before urine was collected for individual analysis. Values are means ± S.E.M.

Diet	Body weight (g)	Metabolite excretion (μg)*		
		N^1-Methylnicotinamide (*a*)	2-Pyridone (*b*)	(*a*)/(*b*)
Niacin-supplemented (maize-meal)	78 ± 4.8	86 ± 9.1	35 ± 2.0	2.46 ± 0.30
Niacin-deficient (maize-meal)	58 ± 3.2	13.5 ± 3.0	16 ± 1.1	0.84 ± 0.20
Niacin-supplemented (casein)	83 ± 3.7	106 ± 16.4	38 ± 2.1	2.79 ± 0.24
Niacin-deficient (casein)	51 ± 5.2	11.3 ± 5.1	22 ± 1.8	0.61 ± 0.13

* Metabolite excretion is expressed as μg/rat per 24 h. For all results the deficient values were significantly less than the controls ($P < 0.001$).

Table 5. *Assessment of intestinal permeability in niacin-deficient rats*
A mixture of 4 mg of [^{3}H]cellobiotol (sp. radioactivity 154 mCi/mol) and 1 mg of [^{14}C]mannitol (sp. radioactivity 82 mCi/mol)/100 g body wt. was given by gastric intubation to rats previously starved for 12–16 h. Total urinary output of [^{3}H]cellobiotol and [^{14}C]mannitol was measured during the 5 h after administration. Values, given in μg, are means ± S.E.M. for five rats in each group.

	[^{3}H]Cellobiotol (μg)	[^{14}C]Mannitol (μg)	^{3}H/^{14}C
Maize-meal diet			
Niacin-deficient	67.6 ± 4.1	31.1 ± 2.7	2.17†
Control	50.3 ± 3.1	43.1 ± 3.1	1.17
Casein diet			
Niacin-deficient	59.5 ± 5.2	39.6 ± 4.8	1.50
Control	63.8 ± 6.5	44.3 ± 3.9	1.44
Casein diet + zein*			
Niacin-deficient	80.7 ± 6.1	36.4 ± 3.2	2.22†
Control	51.3 ± 5.0	44.6 ± 5.1	1.15
Casein diet + gluten*			
Niacin-deficient	77.8 ± 6.5	31.1 ± 3.2	2.50†
Control	61.1 ± 5.1	50.7 ± 4.1	1.21

* The casein diet designed by Nakashima *et al.* (1978) was diluted here with either 10% (w/w) zein or 10% (w/w) wheat gluten.
† Significantly different from control ($P < 0.001$).

Table 6. *Urinary excretion of trigonelline after administration of [^{14}C]nicotinoyl [^{3}H]methylcellulose to rats on the maize-meal and cereal-free diets*

Values are expressed as μg of trigonelline excreted/rat per 24h, calculated from the amount in pooled urine from five rats in each group. [^{14}C]Nicotinoyl[^{3}H]methylcellulose was given on day 0 by the routes indicated. Limits for trigonelline detection are as in Table 1.

	Trigonelline excretion (μg)					
	Maize-meal diet			Cereal-free diet		
Time (days)	Niacin-deficient rats (oral niacytin)	Niacin-supplemented rats (oral niacytin)	Niacin-supplemented rats (intraperitoneal niacytin)	Niacin-deficient rats (oral niacytin)	Niacin-supplemented rats (oral niacytin)	Niacin-supplemented rats (intraperitoneal niacytin
−1	19	20	18	0	0	0
0	18	22	20	0	0	0
1	47	28	98	Trace	Trace	76
2	63	21	163	Trace	Trace	135
3	74	22	190	Trace	Trace	168
4	58	20	170	0	0	148
5	45	19	102	0	0	83
6	38	23	60	0	0	46
7	36	22	45	0	0	21

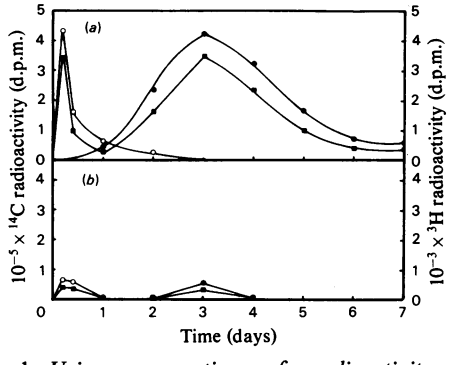

Fig. 1. *Urinary excretion of radioactivity after administration of [^{14}C]nicotinoyl[^{3}H]methylcellulose to rats on the maize-meal diet*

Plotted values represent the radioactivity per rat excreted over 24h, calculated from the amount in pooled urine from five niacin-deficient rats (*a*) and from five niacin-supplemented rats (*b*). Symbols: ○, [^{14}C]nicotinic acid; ●, [^{14}C]trigonelline; ■, [^{3}H]-methylcellulose.

same abnormal permeability had been produced (Table 5). Rats on these diets when supplemented with niacin showed no evidence of this intestinal abnormality.

Metabolism of synthetic 'bound' nicotinic acid

Observations reported so far only suggest that niacytin is taken up by the intestine of niacin-deficient rats on the cereal diet and that the esterified nicotinic acid is metabolized to trigonelline. No direct evidence had been obtained that a macromolecule was absorbed by these animals and that the 'bound' nicotinyl residues were in fact precursors of the urinary trigonelline. By administering synthetic non-digestible [^{3}H]methylcellulose esterified with [^{14}C]nicotinic acid, both the absorption and metabolic questions could be answered.

When single oral doses of the double-labelled macromolecule were given to rats, similar patterns of trigonelline excretion were found to those after niacytin administration (Table 6). Niacin-deficient rats on the maize-meal diet gave an increased excretion of trigonelline with a peak at 3 days, whereas only traces were detected in rats on the casein diet. With intraperitoneal injection, niacin-supplemented rats on both diets produced about 680μg of trigonelline over 7 days.

Analysis of the radioactivity in urine showed that in the first 10h after dosing all the ^{14}C in the chromatographic elution profile was associated with nicotinic acid. By 24h [^{14}C]trigonelline had begun to appear in urine and over 7 days this radioactivity followed the same pattern as trigonelline estimated by chromatographic analysis (Fig. 1*a*). As oral administration of non-esterified [^{14}C]nicotinic acid did not produce any [^{14}C]trigonelline in urine, the labelled trigonelline found with [^{14}C]nicotinoyl-methylcellulose must have been a metabolic consequence of the nicotinoyl ester link with the macromolecule. Only small amounts of both [^{14}C]-

nicotinic acid and [^{14}C]trigonelline were detected in urine from niacin-supplemented rats (Fig. 1*b*).

On Sephadex G-100 chromatography, ^{3}H in urine was eluted in one peak that corresponded to the relative retention time of methylcellulose. No ^{14}C was associated with this methylcellulose peak from urine. The carbohydrate identity of the ^{3}H-containing fractions was confirmed by a positive anthrone reaction for hexose. Again, the excretion of [^{3}H]methylcellulose closely resembled the ^{14}C excretion pattern in the niacin-deficient rats (Fig. 1*a*). Over 7 days about 19% of the orally administered methylcellulose had appeared in urine. As more than 89% of parenterally administered [^{3}H]methylcellulose was excreted over 7 days, that found in urine after an oral dose closely reflects the quantity absorbed by the intestine. Only traces of [^{3}H]-methylcellulose were present in urine from the niacin-supplemented rats.

Thus [^{3}H]methylcellulose of mol.wt. approx. 100000 was absorbed and excreted by niacin-deficient maize-meal-fed rats, and the [^{14}C]nicotinoyl residues were cleaved off and appeared in urine mainly as [^{14}C]trigonelline. As this model compound is handled exactly as would be predicted by the Mason–Kodicek hypothesis, this observation is strong evidence that trigonelline production after niacytin administration occurs in the same way.

Discussion

A metabolic origin for trigonelline in rat urine has been demonstrated by using substrate nicotinoyl residues esterified to a polysaccharide macromolecule. An essential requirement for the natural production of this metabolite is the abnormal absorption of 'bound' nicotinic acid by the small intestine. Because the transit of unabsorbed niacytin along the small intestine would have been completed by 12 h after dosing, the persistent slow excretion of trigonelline over several days must have resulted from a gradual hydrolysis of *N*-methylated nicotinic acid at some location remote from the point of absorption. Neither the anatomical site of *N*-methylation nor the enzyme responsible have yet been determined.

The permeability defect in the intestinal mucosa is the result of an interaction of dietary maize meal with cells damaged by deficiency of niacin. There is, however, no specific requirement for niacin deficiency as the only precondition for development of this defect. Administration of triparanol (Merrell Co., Cincinnatti, OH, U.S.A.), an inhibitor of 24-dehydrocholesterol reductase, will also sensitize the cells to dietary maize meal, allowing the uptake of macromolecules and the formation of trigonelline (J. S. Sandhu & D. R. Fraser, unpublished work). It is possible that a range of noxious stimuli will cause intestinal cells to respond to maize meal in this way. Similarly, there is no absolute specificity for maize meal to induce this permeable state. Other milled cereals, including wheat, oats and barley, have also been shown to have this effect (J. S. Sandhu & D. R. Fraser, unpublished work). From preliminary studies (Table 4) the active component appears to be present in glutelin or prolamin preparations from cereals.

A variety of small molecules, such as amino phenols and cinammic acids, are known to be linked to the niacytin type of macromolecule in cereals (Mason *et al.*, 1973). Because these would also be absorbed under conditions where trigonelline is produced it is likely that other xenobiotic metabolites are formed by similar mechanisms to that for trigonelline.

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