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EFFECTS OF BENSERAZIDE, CARBIDOPA AND ISONIAZID ADMINISTRATION ON TRYPTOPHAN–NICOTINAMIDE NUCLEOTIDE METABOLISM IN THE RAT

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Abstract—Weanling rats were maintained on a diet providing marginally adequate amounts of nicotinamide and vitamin B_6, with a considerable excess of tryptophan to allow endogenous synthesis of nicotinamide nucleotides. Groups of animals received Benserazide or Carbidopa at 0.9 mg/kg body wt/day or isoniazid at 1.3 mg/kg body wt/day. All three drugs led to a reduction in the excretion of N^1-methyl nicotinamide (the principal metabolite of nicotinamide nucleotides in the rat) after 2 and 3 days, compared with control animals receiving the same diet. After 7 days, excretion of this metabolite had returned to the control level. All three drugs are potent inhibitors of tryptophan oxygenase, kynureninase and 3-hydroxyanthranilate oxidase, but the effects of administration *in vivo* were not related to the apparent K_i values determined *in vitro*. All three drugs also caused an increase in the activity of nicotinamide deamidase, which may lead to increased efficiency of utilization of dietary nicotinamide in response to deficiency, and hence explain the recovery in N^1-methyl nicotinamide excretion towards the end of the experiment. Urinary excretion of tryptophan metabolites (xanthurenic and kynurenic acids and kynurenine) was not as expected. Benserazide and Carbidopa had no significant effect, while isoniazid caused an increase in kynurenine excretion and a decrease in xanthurenic acid excretion. It is suggested that this may be due to a reduced activity of kynurenine hydroxylase, although isoniazid had no effect on the activity of this enzyme after administration for 11 days, or when added *in vitro*. The results are discussed in relation to the mechanisms of isoniazid-induced pellagra and Benserazide- and Carbidopa-induced niacin depletion in man.

It is well established that administration of isoniazid (*iso*-nicotinic acid hydrazide) can lead to the development of pellagra in some patients. This may be the result of formation of a Schiff base between the hydrazide drug and the pyridoxal phosphate cofactor of the enzyme kynureninase (L-kynurenine hydrolase, EC 3.7.1.3). This results in reduced oxidative metabolism of tryptophan, and hence in reduced endogenous synthesis of nicotinamide nucleotides and increased reliance on dietary niacin. If the dietary intake is inadequate, pellagra (niacin deficiency) results. Administration of supplementary vitamin B_6 (pyridoxine) together with isoniazid overcomes this cofactor depletion and prevents the development of pellagra [1].

Bender and Russell-Jones [2] have reported on a patient in whom clinically and biochemically confirmed pellagra developed during treatment with isoniazid, despite apparently adequate supplementation with vitamin B_6. It is assumed that this was the result of inhibition by isoniazid of enzymes other than kynureninase in the tryptophan oxidative pathway (see Fig. 1). It has been shown previously [3] that isoniazid will also inhibit tryptophan oxygenase (L-tryptophan: oxygen oxido-reductase, EC 1.13.1.12), 3-hydroxyanthranilate oxidase (3-hydroxyanthranilate: oxygen 3,4-oxido-reductase (decyclizing), EC 1.13.11.6) and nicotinamide phosphoribosyltransferase (nicotinamide nucleotide: pyrophosphate phosphoribosyltransferase, EC 2.4.2.12) in rat liver homogenates. The inhibition of the latter two enzymes is only slight, having a very high apparent K_i, so it was assumed that neither inhibition would be important when the drug was administered to animals.

A previous report from this laboratory [4] showed that patients treated with Benserazide [Ro4-4602, *N*-seryl-N^1-(2, 3, 4-trihydroxybenzyl)-hydrazine] or Carbidopa (MK-486, α-hydrazino-3, 4-dihydroxyphenyl-α-methyl propionic acid) showed reduced excretion of N^1-methyl nicotinamide, indicative of niacin deficiency; N^1-methyl nicotinamide is the principal metabolite of nicotinamide nucleotides. However, pellagra has not been reported in Parkinsonian patients treated with dopa together with either of these inhibitors of dopa decarboxylase (L-aromatic amino acid carboxy-lyase, EC 4.1.1.28). Studies with rat liver homogenates [3] have shown that Benserazide and Carbidopa are considerably more potent inhibitors of tryptophan oxygenase and kynureninase *in vitro* than is isoniazid. Since about the same amounts of all three drugs are used clinically, it is surprising that the two decarboxylase inbibitors do not seem to lead to the development of pellagra, while isoniazid does.

In the present study, the effects of all three drugs have been assessed *in vivo*. The changes in urinary excretion of tryptophan and niacin metabolites in response to drug treatment have been measured in animals receiving known intakes of these two

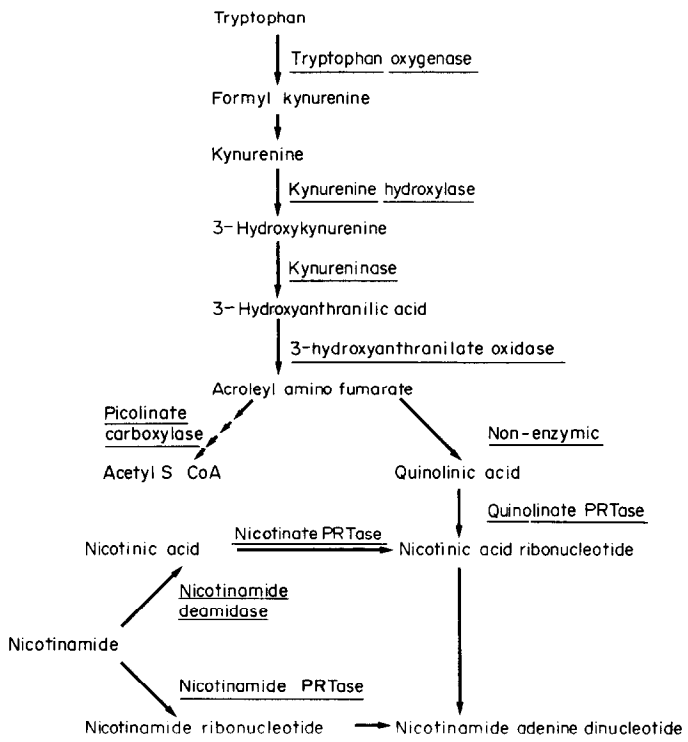

Fig. 1. The oxidative pathway of tryptophan metabolism and pathways for the incorporation of dietary niacin into nicotinamide nucleotides.

nutrients in their diet. The activities of the enzymes of the tryptophan–nicotinamide nucleotide pathway have been measured in liver homogenates following administration of the drugs to rats at doses equivalent to those used clinically, in an attempt to explain the differences between isoniazid and the dopa decarboxylase inhibitors.

METHODS

Animals and diets. The diet used in this study was based on that described by Carter *et al.* [5] for studies of tryptophan and niacin metabolism, consisting of maize meal and sucrose, with small amounts of vitamin-free casein and gelatin. A mixture of essential amino acids was also added to complement the protein of the diet. Additional energy was provided by corn oil, in which vitamins A,D,E and K were dissolved. Other vitamins and a mineral salt mixture were also included in the diet, to meet the National Research Council recommendations [6] for all nutrients except nicotinamide, which was added at only 0.75 mg/kg dry matter of the diet. The diet contained 1 g of tryptophan/kg dry matter, which would be more than adequate to permit synthesis of nicotinamide nucleotides to compensate for the nicotinamide deficiency, and 10 g ascorbic acid/kg dry matter, which was added to act as an anti-oxidant when the decarboxylase inhibitors were incorporated in the diet. Preliminary studies (B. I. Magboul and D. A. Bender, unpublished) showed that this diet was adequate to permit growth of weanling rats to at least the same extent as a standard animal house diet (Diet 86, Northern Farmers Ltd.). Benserazide and Carbidopa were incorporated in batches of the diet at 30 mg/kg dry matter, and isoniazid at 45 mg/kg.

For convenience, the major dry ingredients of the diet and the corn oil were mixed in the usual way, but the gelatin was dissolved in warm water (10 ml/g gelatin), and tryptophan, nicotinamide, ascorbic acid and drugs as appropriate were dissolved in the gelatin solution. This was then mixed with the dry ingredients to give a stiff paste, which set to a firm jelly on cooling, and could conveniently be stored at −20° until required. Feeding cubes of this jelly diet to animals minimized the wastage that occurs with powder diets, simplified measurement of food intake (and hence drug dosage), and prevented blockage of the urine/faeces separator of the metabolic cages by particles of spilled food.

Male Wistar rats, bred in the Courtauld Institute, were weaned onto the control (drug-free) diet, and after 21 days they were randomly allocated to one of the three experimental diets (containing Benserazide, Carbidopa or isoniazid); a control group remained on the drug-free diet throughout the experiment. Food intake was measured daily, and urine was collected for 24-hr periods at intervals over 11 days. The animals were then killed by decapitation, and their livers were dissected out, frozen in liquid nitrogen and stored at −20° until required for analysis.

Analytical methods. Urine xanthurenic and kynurenic acids were determined fluorimetrically and kynurenine colorimetrically after ion exchange chromatography, as described previously [7], a modification of the methods of Satoh and Price [8] and Joseph and Risby [9].

Urine N^1-methyl nicotinamide was determined by the following small-scale modification of the alkali-ketone condensation method of Carpenter and Kodicek [10]: 0.5 ml aliquots of a 3-fold dilution of urine were mixed with an equal volume of either water or a standard solution of N^1-methyl nicotinamide (about 20 μmoles/l) as an internal standard, 0.5 ml of butanone and 0.25 ml of 5 M sodium hydroxide. This mixture was shaken vigorously for 5 min, then 0.325 ml of 5 M hydrochloric acid was added, and the mixture heated in a boiling water bath for 5 min. After rapid cooling to below 10°, 2 ml of 200 g/l potassium dihydrogen phosphate and 6 ml of water were added. After mixing, the fluorescence at 460 nm (excitation 430 nm) was measured using an Aminco-Bowman spectrophotofluorimeter. It was found that an internal standard was required for each sample of urine because of the presence of varying amounts of interfering materials which quenched the fluorescence of the N^1-methyl nicotinamide derivative. It was also found necessary to carry an unreacted sample of each urine through the same procedure, with water replacing the sodium hydroxide and hydrochloric acid, because of the presence of interfering materials that have a similar fluorescence spectrum to the N^1-methyl nicotinamide derivative.

Portions of liver were homogenized while still frozen in either 0.15 M sodium chloride or 0.25 M sucrose, depending on the enzyme to be measured. The following preparations were used for determination of enzyme activity by the methods that have been described previously [3]:

(a) unfractionated homogenate: tryptophan oxygenase and kynureninase;

(b) 100,000 *g* supernatant: 3-hydroxyanthranilate oxidase and picolinate carboxylase [amino-carboxymuconate semialdehyde decarboxylase (3′-oxoprop-2-amino-but-2-ene dioate carboxy-lyase), EC 4.1.1.45];

(c) charcoal-treated 100,000 *g* supernatant: quinolinate phosphoribosyl-transferase (nicotinate nucleotide:pyrophosphate phosphoribosyltransferase (carboxylating) EC 2.4.2.19) and nicotinate phosphoribosyltransferase (nicotinate nucleotide: pyrophosphate phosphoribosyltransferase, EC 2.4.2.11);

(d) protamine sulphate fractionated 100,000 *g* supernatant: nicotinamide phosphoribosyltransferase (nicotinamide nucleotide: pyrophosphate phosphoribosyltransferase, EC 2.4.2.12);

(e) deoxycholate lysed mitochondria: kynurenine hydroxlase (L-kynurenine; NADPH:oxygen oxidoreductase (hydroxylating), EC 1.14.12.9;

(f) microsomal pellet: nicotinamide deamidase (nicotinamide amidohydrolase, EC 3.5.1.19).

The activities of all enzymes were expressed per g wet wt of tissue.

RESULTS

Preliminary studies showed that the diet used in this experiment would permit the growth of weanling rats at least to the same extent as would a stock diet; Table 1 shows that none of the three drugs used had any effect on the growth rate of the animals from 21 days after weaning until the end of the experiment 11 days later. Similarly, none of the drugs had any significant effect on the food intake of the animals, so that tryptophan, nicotinamide and vitamin B_6 intakes were similar in all cases. The intake of the drugs per kg body wt are shown in Table 1.

Table 2 shows the urinary excretion of N^1-methyl nicotinamide and three tryptophan metabolites, xanthurenic and kynurenic acids and kynurenine.

During the early part of the experiment (days 2 and 3 after the initiation of the experimental diets) all three drugs led to a significant decrease in the excretion of N^1-methyl nicotinamide, compared with the control animals. However, by days 8 and 9 of the experiment the excretion of this metabolite was the same in the drug-treated animals as in the control group.

A sample of 48 hr urine collection for each animal was used for determination of xanthurenic and kynurenic acids and kynurenine, to allow adequate sensitivity after ion exchange chromatography. The figures in Table 2 are corrected to show excretion per 24 hr. Benserazide and Carbidopa had no significant effect on the excretion of these metabolites, while isoniazid led to a significant reduction in the excretion of xanthurenic acid and an increase in the excretion of kynurenine.

Table 3 shows the effects of administration of the drugs on the activities of the enzymes of the tryptophan–nicotinamide nucleotide pathway. Tryptophan oxygenase, kynureninase and 3-hydroxyanthranilate oxidase were all inhibited significantly by Carbidopa and isoniazid; the inhibition of tryptophan oxygenase and 3-hydroxyanthranilate oxidase by Benserazide was not statistically significant at this

Table 1. Weight gain of animals, food intake and drug dosage through experimental period*

	Weight gain (%)	Food intake (g wet wt/kg body wt/day)		Drug dose (mg/kg body wt)	
		Day 2 †	Day 9	Day 2	Day 9
Control	62.1 ± 9.4	142 ± 14	130 ± 6	—	—
Benserazide	58.9 ± 7.2	136 ± 11	127 ± 24	0.9 ± 0.1	0.9 ± 0.2
Carbidopa	65.2 ± 11.4	137 ± 25	126 ± 8	0.9 ± 0.2	0.9 ± 0.1
Isoniazid	60.4 ± 21.3	137 ± 23	125 ± 17	1.4 ± 0.2	1.3 ± 0.2

* Results expressed as mean ± S.D. of five animals per group.

† Days refer to number of days since introduction of experimental diet at 21 days after weaning onto control diet.

Table 2. Urinary excretion of tryptophan and niacin metabolites*

	N^1-methyl nicotinamide (μmoles/24 hr)				On days 5 + 6 (pooled sample) (μmoles/24 hr)		
	Day 2†	Day 3	Day 7	Day 8	Xanthurenic acid	Kynurenic acid	Kynurenine
Control	2.15 ± 0.20	2.06 ± 0.27	2.17 ± 0.25	2.21 ± 0.29	1.59 ± 0.66	0.48 ± 0.25	0.14 ± 0.03
Benserazide	1.34 ± 0.16§	1.70 ± 0.25‡	2.13 ± 0.42	2.74 ± 0.35	1.27 ± 0.47	0.26 ± 0.14	0.16 ± 0.06
Carbidopa	1.39 ± 0.33§	1.51 ± 0.19§	2.12 ± 0.21	2.12 ± 0.31	1.39 ± 0.92	0.29 ± 0.14	0.14 ± 0.05
Isoniazid	1.52 ± 0.41§	1.38 ± 0.16§	2.24 ± 0.42	2.16 ± 0.51	0.89 ± 0.23‡	0.25 ± 0.17	0.25 ± 0.11‡

* Results expressed as mean ± S.D. of five animals per group.
† Days refer to number of days since introduction of experimental diet, at 21 days after weaning onto control diet.
‡ Significantly different from control, $0.1 > P > 0.05$, unpaired *t*-test.
§ Significantly different from control, $P < 0.05$, unpaired *t*-test.

dose of the drug. None of the other enzymes investigated was significantly inhibited.

There was a significant increase in the activity of nicotinamide deamidase in response to all three drugs.

DISCUSSION

Previous studies of the effects of Benserazide, Carbidopa and isoniazid on the activity of enzymes of the tryptophan–nicotinamide nucleotide pathway in rat liver homogenates [3] showed that all three drugs inhibited kynureninase, presumably by formation of a metabolically inactive complex between the hydrazine derivative and the pyridoxal phosphate cofactor of the enzyme. All three drugs also inhibit tryptophan oxygenase, and hence would be expected to reduce the entry of tryptophan into the pathway of nicotinamide nucleotide synthesis [3]. Inhibition of tryptophan oxygenase by Benserazide [11] and Carbidopa [12] has been reported previously, and Frieden *et al.* [13] showed that a number of phenolic compounds will inhibit this enzyme, apparently interacting with the haem binding site. This would explain the action of Benserazide and Carbidopa on tryptophan oxygenase, since both are phenolic compounds, but would not account for the inhibition by isoniazid, which is not phenolic. There is no evidence that this enzyme is pyridoxal phosphate dependent, so interaction with this cofactor is unlikely to explain the action of isoniazid. However, it is possible that isoniazid is capable of interacting with the inhibitory nicotinamide nucleotide binding site of tryptophan oxygenase [14]. Madras *et al.* [12] have shown that a number of hydrazine derivatives of tryptophan analogues are more potent inhibitors of tryptophan oxygenase than are the parent compounds, and suggested that the hydrazines can interact with the haem binding site of the enzyme, thus preventing activation of apo-tryptophan oxygenase.

In studies *in vitro* [3] it was shown that isoniazid, but not the other two drugs, also inhibited 3-hydroxyanthranilate oxidase and nicotinamide phosphoribosyltransferase, although the apparent K_i values for these two enzymes were so high that it was considered unlikely that either enzyme would be significantly inhibited following administration of the drug at normal levels. However, as can be seen from Table 3, all three drugs, when given for 11 days, led to significant inhibition of 3-hydroxyanthranilate oxidase. Neither isoniazid nor the two decarboxylase inhibitors had any effect on the activity of nicotinamide phosphoribosyltransferase.

A further difference between the apparent K_i values determined *in vitro* and effective inhibitory potency following administration is shown by the effects on tryptophan oxygenase and kynureninase. As can be seen from Table 3, all three drugs are approximately equipotent *in vivo,* yet the apparent K_i of isoniazid is 20-fold higher for tryptophan oxygenase that are the K_i values of Benserazide and Carbidopa, and about 100-fold higher for kynureninase [3]. These differences suggest that pharmacokinetic factors are important.

The diet used in these experiments provided considerably less nicotinamide than the recommended

Table 3. Liver enzyme activities following administration of drugs for 11 days*

	Enzyme activity (nmoles product formed/min/g wet wt)			
	Control	Benserazide	Carbidopa	Isoniazid
Tryptophan oxygenase	49.6 ± 12.4	44.1 ± 9.9	17.6 ± 3.5†	30.9 ± 1.7†
Kynurenine hydroxylase	25.5 ± 2.0	22.7 ± 5.7	29.6 ± 9.6	24.9 ± 4.5
Kynureninase	25.6 ± 1.9	22.1 ± 1.6†	19.9 ± 2.7†	20.2 ± 1.2†
3-Hydroxyanthranilate oxidase	113 ± 30	87 ± 21	51 ± 22†	43 ± 15†
Quinolinate phosphoribosyltransferase	0.48 ± 0.09	0.62 ± 0.16	0.65 ± 0.15	0.52 ± 0.22
Nicotinate phosphoribosyltransferase	0.89 ± 0.09	0.84 ± 0.13	0.83 ± 0.03	0.77 ± 0.12
Nicotinamide phosphoribosyltransferase	1.63 ± 0.25	1.74 ± 0.39	1.69 ± 0.17	1.54 ± 0.24
Nicotinamide deamidase	17.3 ± 1.1	28.1 ± 2.5‡	21.4 ± 3.2†	26.6 ± 3.8†
Picolinate carboxylase	5.7 ± 0.7	5.5 ± 0.4	5.3 ± 0.5	5.4 ± 0.4

* Results expressed as mean ± S.D. of five animals per group.
† Significantly different from control, $0.1 > P > 0.05$, unpaired t-test.
‡ Significantly different from control, $P < 0.05$, unpaired t-test.

intake for the rat [6], so it might be expected that the animals would be dependent on endogenous synthesis of nicotinamide nucleotides from tryptophan. Hence, any inhibition of the pathway night be expected to lead to some degree of niacin deficiency. The data for urinary excretion of N^1-methyl nicotinamide shown in Table 2 reflect this to some extent. During the early stages of the experiment (after receiving the drug for 2 and 3 days), the animals receiving the drugs excreted significantly less of this metabolite than did the drug-free control animals. However, by days 7 and 8 of the experiment they had apparently recovered, in that their excretion of N^1-methyl nicotinamide was no different from that of control animals. When the animals were killed after 11 days of drug treatment, their livers had a significantly greater activity of nicotinamide deamidase than did those of the control animals. Any change in the activity of an enzyme that is due to change in the amount of enzyme present (i.e. due to changes in the rate of synthesis or degradation) would be expected to develop relatively gradually, perhaps over several days, unlike an effect on the activity of pre-existing enzyme, such as simple inhibiton or activation, which would be expected to be apparent immediately after initiation of treatment. Therefore, either there was an increase in the amount of nicotinamide deamidase present in the tissue under these conditions, or the enzyme was activated by some metabolite of the drugs that accumulates only gradually during administration. These results provide evidence for the hypothesis that the major pathway of utilization of dietary nicotinamide is by deamidation to nicotinic acid and incorporation into nicotinic acid nucleotide, rather than by direct incorporation into nicotinamide nucleotides by the action of nicotinamide phosphoribosyl transferase [15]. An increase in the activity of nicotinamide deamidase, as seen in this experiment, would lead to an increase in the efficiency of utilization of dietary nicotinamide, and hence compensate to some extent for inhibition of endogenous synthesis of nicotinamide nucleotides from tryptophan. This would explain the changes in N^1-methyl nicotinamide excretion between days 2 and 3 and 7 and 8 shown in Table 2.

The excretion of xanthurenic and kynurenic acids and kynurenine in response to tryptophan administration is a standard test of vitamin B_6 nutritional status [16], reflecting the activity of kynureninase, which is a pyridoxal phosphate dependent enzyme. In this experiment the animals were provided with an adequate amount of vitamin B_6 [6], and were effectively in a state of continuous tryptophan load, as the diet provided 1 g of tryptophan/kg dry matter (about 1.2 nmoles of tryptophan/kg body wt/day). Therefore, if inhibition of kynureninase were a major effect of the drugs, the excretion of all three metabolites would be higher in the drug-treated than in the control animals. However, as can be seen from Table 2, this is not so. Benserazide and Carbidopa had no significant effect on tryptophan metabolite excretion; isoniazid led to a significant fall in xanthurenic acid excretion, and a corresponding increase in kynurenine excretion, with no effect on kynurenic acid. This effect is similar to that reported in a patient treated with isoniazid who had developed pellagra [2], in whom administration of 2 g of tryptophan led to the expected increase in kynurenic acid and kynurenine excretion, with only a small increase in xanthurenic acid. Both in the case of this patient and in the present study, this effect could be explained by inhibition of kynurenine hydroxylase. As can be seen from Fig. 1, inhibition of this enzyme would lead to a relatively greater formation of kynurenic acid than xanthurenic acid in response to an increased flux of metabolites through the oxidative pathway. However, as can be seen from Table 3, none of the drugs had any effect on the activity of kynurenine hydroxylase, as measured by the method of Chiancone [17] when there is an excess of NADPH in the incubation medium. The enzyme has been reported to have a relatively high K_m for NADPH (0.25×10^{-4} M) [18], and it is therefore possible that it is especially sensitive to small changes in the liver concentration of NADPH, such as might be expected to occur during the early stages of this experiment. It is also possible that isoniazid may compete with

NADPH for the catalytic site of the enzyme, an effect that would be masked by the saturating amount of NADPH used in the incubation.

The present study thus confirms to some extent the results obtained with Benserazide, Carbidopa and isoniazid *in vitro* as inhibitors of the enzymes of tryptophan oxidative metabolism in the rat [3], but shows that pharmacokinetic factors are important, in that the apparent K_i values determined *in vitro* are not reflected in relative inhibitory potency following administration of the drugs. Nevertheless, it is still not possible to explain why pellagra is observed in patients treated with isoniazid (even when supplementary vitamin B_6 is given), yet has not been reported in Parkinsonian patients treated for long periods with Benserazide or Carbidopa.

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