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EFFECTS OF BENSERAZIDE ON TRYPTOPHAN METABOLISM IN THE MOUSE

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Abstract—Benserazide [*N*-(seryl)-*N'*-(2,3,4-trihydroxybenzyl)-hydrazine, Ro4–4602] is known to inhibit aromatic amino acid decarboxylase. David [1] has shown that administration of [1-^{14}C]tryptophan to mice leads to a considerable evolution of $^{14}CO_2$, and that this is inhibited by prior administration of benserazide. This was interpreted as indicating that decarboxylation to tryptamine may be a major pathway of tryptophan metabolism in the mouse. In the present work, an alternative explanation is advanced. Evolution of $^{14}CO_2$ from [1-^{14}C]tryptophan could be due to metabolism of the alanine released by the action of kynureninase. In this case there would also be evolution of $^{14}CO_2$ following administration of [2-^{14}C]tryptophan. This has been demonstrated. It has further been shown that benserazide is a potent inhibitor of kynureninase and kynurenine aminotransferase in the mouse. The result of this inhibition is both a decrease in oxidative metabolism of tryptophan and an increase in the concentration of kynurenine in the liver. These effects are seen at levels of the drug similar to doses used clinically in treatment of Parkinson's disease. One effect of reduced oxidative metabolism of tryptophan would be reduced synthesis of nicotinamide, and it is possible that patients treated with benserazide may show some signs of niacin deficiency.

It has been suggested that a 'quantitatively significant' pathway of tryptophan metabolism in the mouse is by decarboxylation to tryptamine [1]. The evidence for this suggestion was primarily the evolution of $^{14}CO_2$ after administration of [1-^{14}C]tryptophan to mice, and inhibition of this by administration of benserazide [*N*-(seryl)-*N'*-(2,3,4-trihydroxybenzyl)-hydrazine (Ro4-4602)] a known inhibitor of aromatic amino acid decarboxylase (aromatic L-amino acid carboxy-lyase, EC 4.1.1.28).

An alternative explanation for the evolution of $^{14}CO_2$ following administration of [1-^{14}C]tryptophan can be advanced to account for the effect of benserazide. There are two established pathways of tryptophan metabolism in mammals by which the carboxyl carbon of tryptophan could be released as carbon dioxide:

(i) Decarboxylation of 5-hydroxytryptophan to 5-hydroxytryptamine. This is known to be catalysed by aromatic amino acid decarboxylase, and hence known to be inhibited by benserazide. However, under normal conditions it does not account for more than about 1 per cent of total body tryptophan metabolism, and therefore is unlikely to explain the findings of David [1].

(ii) The hydrolysis of hydroxykynurenine to hydroxyanthranilic acid in the oxidative pathway of tryptophan catabolism, catalysed by kynureninase (L-kynurenine hydrolase, EC 3.7.1.3), leads to the release of the side chain of the original tryptophan as alanine. It is to be expected that much of this alanine would undergo deamination to pyruvate, and then decarboxylation to acetyl-SCoA, releasing carbon dioxide from the carboxyl carbon of tryptophan. It is generally assumed that most of the tryptophan catabolised by mammals is by this pathway.

Hence if kynureninase were inhibited by benserazide the data reported by David [1] could be interpreted in terms of existing knowledge of metabolic pathways, without the need to postulate a novel pathway. Such inhibition would have the effect of reducing the production of carbon dioxide from the carboxyl

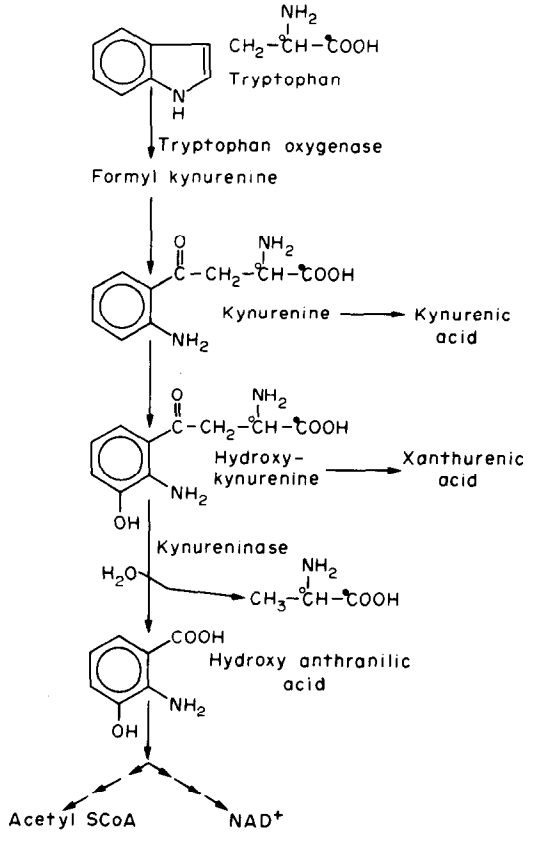

Fig. 1. The oxidative pathway of tryptophan metabolism.

group of tryptophan, since it would result in reduced formation of alanine from the side chain of hydroxykynurenine.

In this paper a number of investigations are reported demonstrating that kynureninase is indeed inhibited by benserazide, not only at the rather high dose used by David in his experiments [1], but also at levels approximating to the doses used clinically in therapy of Parkinson's disease. Such inhibition may have a serious effect not only on tryptophan metabolism, but also on niacin nutritional status in benserazide-treated patients, since a considerable amount of nicotinamide is normally synthesised from tryptophan [2, 3].

MATERIALS AND METHODS

Mice of two strains, NRMI and Schofield, were used in these studies. All animals weighed about 25 g. Reagents were all obtained from the usual commercial sources and were in general of analytical reagent grade; DL-[1-^{14}C]tryptophan and L-[2-^{14}C]tryptophan were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. The benserazide used was a generous gift of Roche Products Ltd, Welwyn Garden City, Herts, U.K.

(a) *[^{14}C]carbon dioxide release studies.* Mice were injected intraperitoneally with either 0.5 μCi L-[2-^{14}C]tryptophan or 1.0 μCi DL-[1-^{14}C]tryptophan, together with 50 mg/kg body weight non-radioactive L-tryptophan. They were placed in individual conical flasks through which carbon dioxide-free air was passed using a small compressor. Exhaled air was passed through 2 ml of 0.25 mole/l Hyamine hydroxide solution in *i*-propanol in a small tube, to trap carbon dioxide. The Hyamine hydroxide solution was changed at 10 min intervals for a total of 130 min; it was washed into scintillation counter vials with a further 2 ml of *i*-propanol, then 10 ml of scintillator solution (3 g PPO, 0.3 g POPOP per litre toluene) was added to each and radioactivity measured by liquid scintillation spectrometry.

(b) *Liver kynureninase assay.* The activity of kynureninase was assessed by its action on kynurenine to form anthranilic acid, which can be measured fluorimetrically. Liver was homogenised in 2 ml of 0.15 mole/l sodium chloride per gram of tissue; 0.5 ml of this homogenate and 0.5 ml of a sodium phosphate buffer (pH 7.0, 0.2 mole/l) were incubated together at 30° for 5 min. For inhibitor studies, benserazide was dissolved in the buffer, so that it was present with the enzyme during this pre-incubation period. The reaction was initiated by the addition of 0.2 ml of a solution of 10 m-mole/l DL-kynurenine. After incubation for a further 40 min the reaction was stopped by the addition of 1 ml of 1 mole/l trichloroacetic acid.

Precipitated protein was removed by centrifugation at 2000 *g* for 10 min and the supernatant was brought to approximate neutrality by the addition of 0.5 ml of 1 mole/l sodium hydroxide solution. The volume was then adjusted to 5 ml by the addition of 1 mole/l sodium phosphate buffer at pH 5.5, and the fluorescence due to anthranilic acid was measured (excitation 310 nm, emission 420 nm), using an Aminco–Bowman spectrophotofluorimeter. Under these conditions kynurenine did not significantly affect the fluorescence due to anthranilic acid, although addition of more than about 20 μmole of kynurenine did quench the fluorescence considerably. Preliminary kinetic studies showed that the K_m of kynureninase towards DL-kynurenine was about 790 μmole/l; this means that the concentration of substrate in the incubation mixture (16.7 m-mole/l) was considerably in excess of K_m. Assuming that the D-enantiomer is not a substrate, the value obtained for the K_m of the mouse liver enzyme towards kynurenine agrees well with the figure of 300 μmole/l L-kynurenine quoted by other workers [4].

(c) *Urinary excretion of xanthurenic and kynurenic acids.* Six mice were placed in a metabolic cage for 6 hr daily (from 10.00 to 16.00 hr) and urine was collected. During this time they were allowed free access to water, but not food. They were given intraperitoneal injections of benserazide (50 mg/kg body weight), tryptophan (50 mg/kg body weight) or benserazide and tryptophan at the same doses, in a strict rotation with a treatment-free (control) day between each administration of drug. The same six mice were used throughout.

Urine was collected into 2 ml of 2 mole/l hydrochloric acid and was diluted to 10 ml with distilled water, then frozen and stored at −20° until required. Aliquots of the diluted urine were used for determination of creatinine by the following method: 1 ml of diluted urine was reacted with 5 ml of saturated picric acid solution and 2 ml of 100 g/l sodium hydroxide solution. After 15 min the absorbance at 520 nm was measured, relative to a reagent blank and a standard solution of creatinine.

The remainder of the urine was used for measurement of xanthurenic and kynurenic acids by the following modification of the method of Satoh and Price [5]. To one 4 ml sample of the diluted urine 1 ml of a solution containing 0.5 mole/l xanthurenic acid and 0.1 mole/l kynurenic acid was added; 1 ml of distilled water was added to another 4 ml sample. Both samples were then poured onto 2 cm long columns of Dowex 50-W ion exchange resin (acid form, 350 mg dry weight of resin per column). The columns were prepared prior to use by washing with 5 ml of 2 mole/l hydrochloric acid and 10 ml of water.

The eluate from the columns following the urine samples and a first 10 ml fraction of water was discarded; the columns were then washed with a further 25 ml of water and the eluate collected. This fraction contained essentially all the xanthurenic and kynurenic acids applied; the mean observed recovery of xanthurenic acid was 83 ± 3.5 per cent and kynurenic acid 79 ± 4 per cent.

Xanthurenic acid was measured by reacting 1 ml aliquots of the column eluate with 1 ml of saturated aqueous solution of sodium hydroxide; after centrifugation to clear the solution the fluorescence due to xanthurenic acid was measured (excitation 370 nm, emission 515 nm). Kynurenic acid was measured in 1.5 ml aliquots of the eluate, which were reacted with 1 ml of concentrated sulphuric acid; after cooling for a few minutes the fluorescence due to kynurenic acid was measured (excitation 365 nm, emission 440 nm). Under these conditions no cross-reaction was detectable between xanthurenic and kynurenic acids present

in the same solution. Especially for the measurement of kynurenic acid a very high background fluorescence was found, despite careful washing of the ion exchange resin; therefore a column blank, in which only water was passed through a column of ion exchange resin, was used, as well as the more usual reagent blank.

(d) *Liver tryptophan oxygenase assay.* Tryptophan oxygenase (L-tryptophan:oxygen 2,3-oxidoreductase (decyclising), EC 1.13.11.11) activity was measured by the method of Knox and co-workers [6].

(e) *Liver kynurenine aminotransferase assay.* Kynurenine aminotransferase [L-kynurenine:2-oxoglutarate aminotransferase (cyclising), EC 2.6.1.7] activity was measured by fluorimetric measurement of the kynurenic acid formed. Liver was homogenised in 4 ml of 0.15 mole/l sodium chloride solution per g tissue; 0.5 ml of this homogenate and 0.5 ml of 0.1 mole/l sodium phosphate buffer at pH 7.0 were incubated together for 5 min. As above, benserazide was dissolved in the buffer when required, so that it was present throughout the pre-incubation period.

The reaction was initiated by the addition of 0.2 ml of a solution containing 200 m-mole/l sodium 2-oxoglutarate and 10 m-mole/l DL-kynurenine. After 15 min the reaction was stopped by the addition of 1 ml of 1 mole/l trichloroacetic acid, and denatured protein was removed by centrifugation at 2000 *g* for 10 min. Kynurenic acid was measured fluorimetrically as above. To allow for the presence of endogenous kynurenic acid and other interfering materials an unincubated blank was carried through the same procedure.

(f) *Liver kynurenine concentration.* Kynurenine was measured fluorimetrically after homogenisation of liver in 10 ml of 0.4 mole/l perchloric acid per g of tissue. Denatured protein was removed by centrifugation at 2000 *g* for 10 min, and the supernatant volume was adjusted to 20 ml with 0.4 mole/l perchloric acid solution. Replicate 5 ml aliquots of this supernatant were then poured over 2 cm long columns of Dowex 50-W ion exchange resin, prepared as described above. To one sample 100 μg of DL-kynurenine in 1 ml of 0.1 mole/l hydrochloric acid was added; the same volume of hydrochloric acid alone was added to the other sample. The eluate from the application of the supernatant, and following washing with 10 ml of water, was discarded, and kynurenine was eluted by washing the columns with 5 ml of 0.5 mole/l trisodium orthophosphate solution. 1 ml of this eluate was then diluted with 6 ml of water and the fluoresence due to kynurenine was measured (excitation 370 nm, emission 470 nm). The mean observed recovery of the internal standard was 76 ± 7.6 per cent. Dilution of the eluate was found to be essential for fluorimetry of kynurenine, since at high concentrations a great deal of self absorption, and hence quenching of fluorescence, was observed.

RESULTS

As can be seen from Fig. 2, administration of [2-^{14}C]tryptophan to mice, together with a non-radioactive tryptophan load (50 mg/kg body weight) led to a considerable production of $^{14}CO_2$. The release of $^{14}CO_2$ following administration of [1-^{14}C]tryptophan (with the same nonradioactive tryptophan load) was somewhat greater.

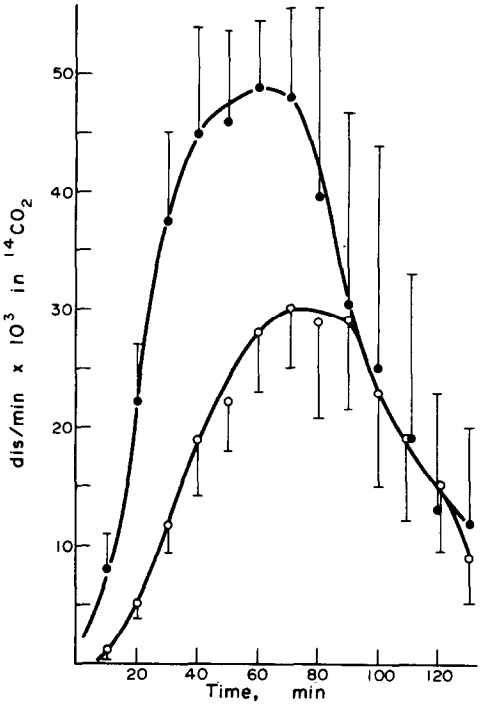

Fig. 2. Evolution of $^{14}CO_2$ following administration of DL-[1-^{14}C]tryptophan (closed circles) or L-[2-^{14}C]tryptophan (open circles) to mice. (Points show mean ± S.D. for 5 animals in each group).

The [1-^{14}C]tryptophan used was the racemic mixture and animals were each given 1 μCi so as to provide 0.5 μCi of the L-enantiomer; those animals treated with [2-^{14}C]tryptophan received 0.5 μCi of the L-enantiometer alone. This means that those animals treated with the DL-[1-^{14}C]tryptophan received a very small amount of very high specific radioactivity D-tryptophan, so that any trace metabolism of the D-enantiomer (for example by D-amino acid oxidase activity) would result in a disproportionate release of the radioactive label.

The effect of benserazide added *in vitro* to mouse liver homogenates on kynureninase activity is shown in Table 1. At 50 μg/g tissue (the same level as the dose given by David [1] to his animals) there was apparently total inhibition of kynureninase activity.

Table 1. The effect of benserazide on liver kynureninase activity *in vitro*

Benserazide (μg/g liver)	Kynureninase activity nmoles anthranilic acid formed/min/g liver	% inhibition
0	43.6 ± 3.2	—
2.5	16.4 ± 0.7*	65
25.0	2.2 ± 0.2*	95
50.0	undetectable	100

Benserazide at the concentrations shown was added to liver homogenate and pre-incubated for 5 min before the reaction was initiated by addition of substrate as described in the Methods section. (Figures show mean of 5 determinations ± S.E.M.)

* Significantly different from control, $P < 0.001$.

Table 2. Urinary xanthurenic acid excretion following benserazide and tryptophan administration

Treatment	Urinary xanthurenic acid μmole/μmole creatinine
Control	0.7 ± 0.2
Tryptophan 50 mg/kg body weight	2.1 ± 1.0*
Benserazide 50 mg/kg body weight	0.7 ± 0.4
Benserazide + tryptophan	1.0 ± 0.2*†

(Figures show mean of 5 determinations ± S.D.)
* Significantly different from control, $P < 0.02$.
† Significantly different from tryptophan group, $P < 0.05$.

Benserazide at concentrations as low as 2.5 μg/g tissue, which is of the same order as doses used clinically in treatment of Parkinson's disease, also led to very significant inhibition of kynureninase.

The results obtained for the urinary excretion of xanthurenic acid are shown in Table 2. Following administration of a tryptophan load (50 mg/kg body weight) there was a considerable increase in xanthurenic acid excretion, as would be expected. Benserazide alone (at 50 mg/kg body weight) had no effect on the excretion of xanthurenic acid. However, administration of benserazide together with tryptophan led to a significantly lesser increase in xanthurenic acid excretion than that observed after tryptophan alone. Basal excretion of kynurenic acid by the mice was below the limit of reliable detection by the method used, possibly partly because of the high fluorescence blank caused by some unidentified material eluted from the ion exchange resin, referred to above. No data for kynurenic acid are therefore reported here.

The observation that benserazide apparently prevents the formation of xanthurenic acid following a tryptophan load suggests the possibility that the drug may act by preventing the entry of tryptophan into the oxidative pathway. However, as can be seen from Table 3, even at concentrations up to 200 μg/g tissue, benserazide had no effect on tryptophan oxygenase activity *in vitro*. The activity of the enzyme was also measured in livers from six mice that had received 50 mg benserazide/kg body weight 3 hr before death. The mean tryptophan oxygenase activity in these animals, 312 ± 20 nmole kynurenine formed/min/g liver, was not significantly different from that of a group

Table 3. The effect of benserazide on liver tryptophan oxygenase activity *in vitro*

Benserazide (μg/g liver)	Tryptophan oxygenase activity (nmole kynurenine formed/min/g liver)
0	309 ± 24
25	344 ± 20 (N.S.D.)
50	318 ± 2 (N.S.D.)
100	324 ± 25 (N.S.D.)
200	341 ± 20 (N.S.D.)

Benserazide at the concentrations shown was added to liver homogenate and pre-incubated for 5 min before the reaction was initiated by addition of substrate as described in the Methods section. (Figures show mean of 5 determinations ± S.E.M.)

Table 4. The effect of benserazide on liver kynurenine aminotransferase activity *in vitro*

Benserazide (μg/g liver)	Kynurenine aminotransferase activity (nmole kynurenic acid formed/min/g liver)	% Inhibition
0	3.21 ± 0.17	—
2.5	1.64 ± 0.25*	49
25.0	1.25 ± 0.46*	61
50.0	undetectable	100

Benserazide at the concentrations shown was added to liver homogenate and pre-incubated for 5 min before the reaction was initiated by addition of substrate as described in the Methods section. (Figures show mean of 5 determinations ± S.E.M.).
* Significantly different from control, $P < 0.001$.

of control animals examined at the same time, 307 ± 26 nmole kynurenine formed/min/g liver.

The effect of benserazide on kynurenine aminotransferase (the enzyme that catalyses the conversion of kynurenine to kynurenic acid and hydroxykynurenine to xanthurenic acid) was also investigated. As can be seen from Table 4, benserazide at 50 μg/g tissue led to apparently total inhibition of the enzyme. Even at low concentrations (2.5 μg/g liver) benserazide was a potent inhibitor of kynurenine aminotransferase.

The effect of benserazide administration on the liver kynurenine concentration was investigated in 5 mice that had received 50 mg/kg body weight benserazide, together with a tryptophan load (50 mg/kg body weight) 1.5 hr before killing, and in a control group of animals that received the tryptophan load alone. In the control animals, the liver kynurenine concentration was 4.76 ± 0.55 μmole/g tissue, while in those animals that had received benserazide as well as tryptophan it had risen to 7.47 ± 0.79 μmole/g tissue, a highly significant difference.

DISCUSSION

It has been demonstrated that tryptamine is a normal constituent of mammalian brain [8], and that various drug treatments can alter its concentration [9]. However, the origin of this tryptamine is unclear. It has been claimed that tryptophan is a substrate for mammalian aromatic amino acid decarboxylase [10], but the activity is barely detectable even using extremely high concentrations of tryptophan, and at the unphysiological pH of 9.0 [10, 11]. Weil-Malherbe [12] has claimed to have demonstrated tryptamine formation from tryptophan in brain slices, but the separation methods he used were unable to differentiate between tryptamine and 5-methoxy-tryptamine, a compound known to be formed *in vivo* from 5-hydroxy-tryptamine.

David [1] has proposed that decarboxylation to tryptamine is a 'quantitatively significant' pathway of tryptophan metabolism in the mouse. His evidence was based mainly on the production of $^{14}CO_2$ from [1-^{14}C]tryptophan, and inhibition of this by benserazide, a known inhibitor of aromatic amino acid decarboxylase. In order to minimise interference from gastro-intestinal bacteria, many of which are known

to have an active tryptophan decarboxylase, David treated his animals with neomycin and sulfasuxidine for 24 hr before each experiment. However, it is doubtful whether this is a sufficient time to permit gut sterilisation, and this treatment would not be expected to deplete intestinal yeasts and fungi which might also have tryptophan decarboxylase activity. Thus, although David did detect tryptamine in his animals (by homogenisation of the entire carcase less the gastro-intestinal tract) following oral administration of [3-^{14}C]tryptophan (in which the label would be retained in tryptamine), and a monoamine oxidase inhibitor, this could have been a result of residual gastro-intestinal bacterial action.

The present work proposes an alternative explanation for the evolution of $^{14}CO_2$ from [1-^{14}C]tryptophan, and inhibition of this reaction by benserazide Benserazide has been shown to inhibit kynureninase, and this would prevent release during oxidative metabolism of the side chain of tryptophan as alanine. Alanine would normally be expected to undergo deamination to pyruvate, followed by decarboxylation to acetyl-SCoA, and thence total oxidation to carbon dioxide. Evolution of $^{14}CO_2$ would therefore be expected to be essentially the same following administration of [1-^{14}C] or [2-^{14}C]tryptophan. This has been demonstrated. At the same time benserazide has been shown to inhibit kynurenine aminotransferase, and as a result of the inhibition of two enzymes involved in its onward metabolism, to increase the liver concentration of kynurenine.

It is not surprising that benserazide should inhibit enzymes other than aromatic amino acid decarboxylase. The drug is a hydrazide, and it is well established that hydrazides inhibit a great many pyridoxal phosphate-dependent enzymes, by formation of hydrazones with the cofactor. As well as acting as an inhibitor of pyridoxal phosphate-dependent enzymes by this mechanism, it might be expected that benserazide would have a greater potency as an inhibitor of enzymes whose substrates contain a hydroxyphenyl group. There might be an affinity between the inhibitor and the substrate binding site, as well as the irreversible reaction with the cofactor. This remains to be further investigated.

One potentially important implication of the present work is that inhibition of kynureninase, which has been demonstrated to occur even at relatively modest levels of benserazide, will lead to inhibition of the synthesis of nicotinamide from tryptophan. It is not known how important this synthesis is relative to dietary intake of niacin, but it is known that following administration of isoniazid, which similarly inhibits kynureninase by cofactor blockage, signs of niacin deficiency and even frank clinical pellagra have been reported [13]. It is therefore possible that prolonged treatment of Parkinsonian patients with benserazide may lead to some degree of niacin deficiency.

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