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INHIBITION *IN VITRO* OF THE ENZYMES OF THE OXIDATIVE PATHWAY OF TRYPTOPHAN METABOLISM AND OF NICOTINAMIDE NUCLEOTIDE SYNTHESIS BY BENSERAZIDE, CARBIDOPA AND ISONIAZID

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Abstract—The effects of three hydrazine derivatives on the enzymes of the tryptophan oxidative pathway and of nicotinamide nucleotide synthesis have been studied using preparations from rat liver. The compounds used were Benserazide and Carbidopa, two inhibitors of aromatic amino acid decarboxylase used together with dopa in the treatment of Parkinson's disease, and the anti-tubercular agent isoniazid.

All three drugs inhibited tryptophan oxygenase and kynureninase, at concentrations that are likely to be encountered *in vivo* following administration to patients or experimental animals. Isoniazid, but not Benserazide or Carbidopa, also inhibited 3-hydroxy-anthranilate oxidase and nicotinamide phosphoribosyltransferase. However, these two enzymes were only inhibited significantly at concentrations of the drug far in excess of those likely to be encountered *in vivo*.

On the basis of the *in vitro* enzyme inhibition studies, it is not possible to explain why patients treated with isoniazid (without supplementary vitamin B_6) develop clinical pellagra, while those treated with Benserazide or Carbidopa do not, despite biochemical evidence of niacin deficiency. It is suggested that this difference may be due either to differences in the intake of dietary niacin in these two groups of patients, or more probably to differences in the metabolism of the drugs and in their interactions with enzymes *in vivo* that are not apparent *in vitro*.

Two inhibitors of dopa decarboxylase (L-aromatic amino acid carboxy-lyase, EC 4.1.1.28) are used in the treatment of Parkinson's disease, to minimise the extra-cerebral metabolism of dopa, and so allow a lower dose of this amino acid to be used, in an attempt to avoid some of the unwanted side-effects of high doses of dopa. The two drugs, which do not cross the blood-brain barrier at the doses normally used, are Benserazide [Ro4-4602,*N*-seryl-*N'*-(2,3,4-trihydroxybenzyl)-hydrazine] and Carbidopa [MK 486, α-hydrazino-3,4-dihydroxyphenyl-α-methyl-propionic acid]. Previous studies [1] have shown that patients treated with these two drugs excrete considerably less N^1-methyl nicotinamide than normal; in many cases the excretion of this metabolite of nicotinamide was so low that patients could be classified as deficient in, or 'at risk of deficiency of', niacin by the generally accepted criteria [2]. The patients also showed abnormalities of tryptophan metabolite excretion compatible with, and indicative of, inhibition of the oxidative pathway of tryptophan metabolism (see Fig. 1).

Studies in mice [3] and with rat liver preparations [4] have shown that both Benserazide and Carbidopa are potent inhibitors of kynureninase (L-kynurenine hydrolase, EC 3.7.1.3). Since both drugs are hydrazine derivatives, and therefore would be expected to react with pyridoxal phosphate, this inhibition is predictable — kynureninase is a pyridoxal phosphate dependent enzyme. Presumably because of their hydroxy-aromatic substituents, both drugs are considerably more potent inhibitors of kynureninase than are other hydrazine derivatives [4], as they have a structural resemblance to kynurenine and hydroxykynurenine, the substrates of the enzyme.

It was reported previously [3] that Benserazide had no effect on the activity of tryptophan oxygenase (L-tryptophan: oxygen oxido-reductase, EC 1.13.1.12) from mouse liver. However, Young *et al.* [5] reported inhibition of this enzyme by the drug both *in vivo* and *in vitro*. In the present work, the effect of Benserazide and Carbidopa on the activity of tryptophan oxygenase has been measured by a new method. At the same time, the other enzymes involved in the synthesis of nicotinamide nucleotides from tryptophan and from dietary niacin (nicotinic acid and nicotinamide) have been tested for possible sensitivity to inhibition by these two hydrazine derivatives.

A further drug has also been included in this study, isoniazid (*iso*-nicotinic acid hydrazide). This is also a hydrazine derivative, used in the treatment of tuberculosis, and has long been known to cause clinical pellagra in some patients [6], apparently by inhibition of the endogenous synthesis of nicotinamide nucleotides from tryptophan. Since clinical pellagra has not been reported in patients treated with Benserazide or Carbidopa, despite their low excretion of N^1-methyl nicotinamide [1], it is of interest to compare the effects of these three drugs on the enzymes of tryptophan and niacin metabolism.

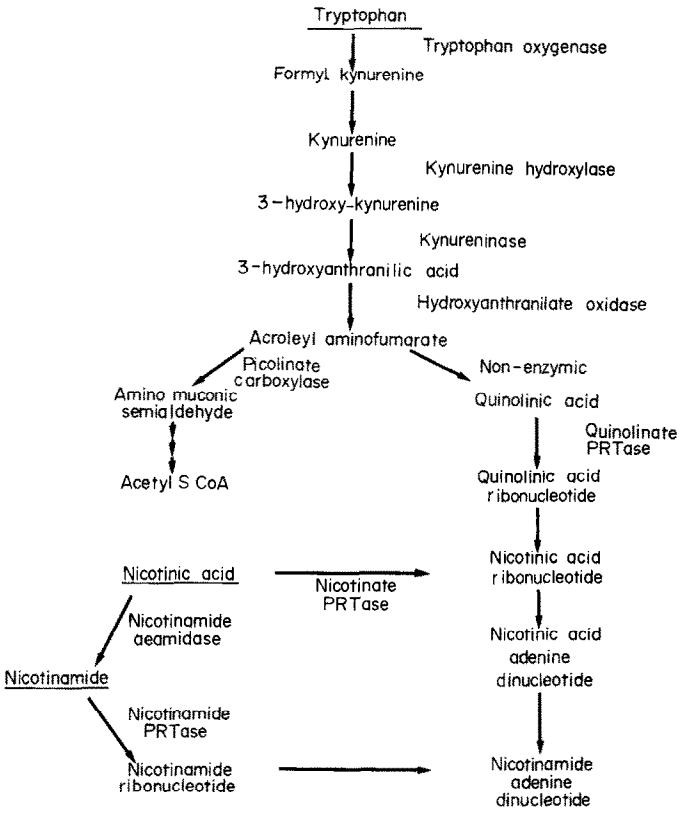

Fig. 1. The oxidative pathway of tryptophan metabolism, and pathways for the incorporation of dietary niacin into nicotinamide nucleotides.

METHODS

Male Wistar rats, bred in the Courtauld Institute and weighing between 150 and 200g, were used for all studies. They were fed on a standard animal house diet. Livers were dissected out immediately after killing the animals, and were either cooled in ice and homogenized in the appropriate medium for use that day, or were frozen in liquid nitrogen and stored at −20° until required for use, in which case tissue was homogenized in the appropriate medium while still frozen. Preliminary studies showed no difference in the activity of enzymes from livers treated in these two different ways.

For inhibitor studies, the drugs were dissolved in the incubation buffer, and were present together with the enzyme, and any cofactors, for a 10 min pre-incubation period before the reactions were initiated by the addition of substrate.

Tryptophan oxygenase. Liver was homogenized in 9 ml of 0.15 M NaCl per g of tissue, and the homogenate used as the source of the enzyme, without any fractionation. The incubation conditions for this enzyme were modified from those described by Knox *et al.* [7]; the buffer (0.2 M sodium phosphate, pH 7.0) contained sodium ascorbate (0.075 M) and haematin (0.2 M), so as to give expression of the full potential activity of the enzyme. The substrate was prepared by dissolving tryptophan at 40 mM in 0.3 M NaOH followed by neutralization with ascorbic acid. Both buffer and substrate were prepared freshly for each set of incubations.

Incubations were carried out in 25 ml conical flasks, with vigorous shaking, to ensure oxygenation of the reaction mixture. One millilitre of each of the buffer and enzyme preparation were incubated at 30° for 10 min, together with 100 μl of either a solution of 500 μM kynurenine or water, then the substrate was added, and the reaction allowed to continue for 40 min. The reaction was stopped by the addition of 1 ml of 1 M trichloroacetic acid and 3 ml of 1.5 M HCl. After mixing, denatured protein was removed by centrifugation.

The supernatant was poured down small columns of Dowex 50W (H^+) ion exchange resin (each column contained about 300 mg of dry resin, and had been pre-washed with 10 ml each of 2M HCl and water). The eluate from the columns was discarded, and the columns were washed with 30 ml of water and 5 ml of 1.5 M HCl. Kynurenine was then eluted with 5 ml of 5 M HCl and was measured by a modification of the method of Joseph and Risby [8]. Aliquots (2 ml) of the eluate were mixed with 1 ml of 7M NaOH and cooled in an ice bath. At 5 min intervals the following additions were made: 200 μl of 2.5 g/l $NaNO_2$ (prepared weekly); 200 μl of 100 g/l ammonium sulphamate and finally 200 μl of 10 g/l naphthyl ethylene diamine (NED) in 95% ethanol (prepared freshly for each set of samples). The absorbance at 555 nm, due to the NED adduct

of the diazonium salt of kynurenine, was measured after standing at room temperature for 90 min.

The addition of kynurenine to the incubation mixture allowed an estimation of the recovery of kynurenine not only through the column chromatography (routinely in excess of 90 per cent in preliminary experiments), but also through the incubation, when kynureninase might be expected to be active. The amount of kynurenine formed in the incubation was determined by comparison with an unincubated sample.

Kynurenine hydroxylase. The activity of kynurenine hydroxylase (L-kynurenine; NADPH: oxygen oxido-reductase (hydroxylating), EC 1.14.13.9) was measured by the method of Chiancone [9], using a washed suspension of liver mitochondria.

Kynureninase. The activity of kynureninase was measured by the fluorimetric method described previously [4], using an unfractionated liver homogenate.

3-Hydroxy-anthranilate oxidase. The activity of 3-hydroxy-anthranilate oxidase (3-hydroxy-anthranilate : oxygen 3,4-oxido-reductase (decyclising), EC 1.13.11.6) was measured using the 100,000 *g* supernatant of a homogenate of liver in 4 ml of 0.15 M NaOH per g of tissue. Samples (100 μl) of this supernatant were added to 2 ml of 0.2 M Tris–acetate buffer (pH 8.0), containing 1 M glutathione and 10^{-5} M Fe_2SO_4, and pre-incubated for 10 min at 30°. The reaction was initiated by the addition of 100 μl of 4.4 M 3-hydroxy-anthranilic acid in the same Tris–acetate buffer; the mixture was transferred to a fluorimeter cuvette and the rate of loss of fluorescence due to the substrate (excitation 320 nm, emission 410 nm) was followed for 2 min using a pen recorder attached to an Aminco–Bowman spectrophotofluorimeter. The sample chamber of the fluorimeter was maintained at 30° by circulating water from a thermostatically controlled reservoir.

Picolinate carboxylase. The activity of picolinate carboxylase [amino-carboxy-muconate semi-aldehyde decarboxylase (3′-oxo-prop-2-amino-but-2-ene dioate carboxy-lyase, EC 4.1.1.45] was measured using the 100,000 *g* supernatant from a homogenate of liver in 2 ml of 0.15 M NaCl per g of tissue, by the following modification of the method of Nishizuka *et al.* [10]. The substrate of the reaction, acroleyl aminofumarate, is unstable, and was prepared *in situ* from 3-hydroxy-anthranilic acid, using a partially purified preparation of 3-hydroxy-anthranilate oxidase [10], which was stable for 2–3 days at −20°. Samples (100 μl) of this preparation were incubated with 100 μl of 660 μM 3-hydroxy-anthranilic acid and 2 ml of the same Tris–acetate buffer containing glutathione and Fe_2SO_4 as was used for determination of 3-hydroxy-anthranilate oxidase (described above), in a spectrophotometer cuvette. A Beckman DB spectrophotometer was used, with the sample chamber maintained at 30° by circulating water from a thermostatically controlled reservoir; the reference cell contained buffer and enzyme preparation alone. The absorbance of the sample at 364 nm was followed using a pen recorder until it was maximal and constant, indicating that the oxidase reaction had proceeded essentially to completion. Samples (200 μl) of the picolinate carboxylase preparation, pre-incubated for 10 min at 30° together with any putative inhibitors, were then added to each cuvette, and the rate of loss of absorbance at 364 nm, due to acroleyl aminofumarate, was followed for a further 5 min. There was no detectable non-enzymic loss of acroleyl aminofumarate (by cyclisation to quinolinic acid) over 15–20 min under these conditions. In order to calibrate the recorder, it was necessary to assume that the whole of the 66 nmol of 3-hydroxy-anthranilic acid added initially was converted to acroleyl aminofumarate during the preliminary reaction.

Quinolinate phosphoribosyltransferase. The activity of quinolinate phosphoribosyltransferase [nicotinate nucleotide: pyrophosphate phosphoribosyltransferase (carboxylating) EC 2.4.2.19] was measured by the following modification of the method of Pinder *et al.* [11]. The source of the enzyme was 100,000 *g* supernatant from a homogenate of rat liver in 5 ml of 0.25 M sucrose per g of tissue, which had been stirred with 50 mg of activated charcoal per ml of homogenate for 15 min at 0° before centrifugation. Samples (200 μl) of this supernatant were incubated with 0.1 ml of 0.6 M sodium phosphate (pH 7.5) and 100 μl of a solution of 3 mg of 5-phosphoribosyl pyrophosphate in a solution containing 6.6 mM $MnCl_2$ and 12 mM $MgCl_2$, for 10 min at 37°. The reaction was initiated by the addition of 100 μl of a solution of 20 mM sodium [^{14}C]quinolinate. After 30 min the reaction was stopped by the addition of 100 μl of glacial acetic acid, the samples were transferred to a boiling water bath for 2 min, then cooled in ice and centrifuged to remove denatured protein. Aliquots (50 μl) of the supernatant were applied to the origins of paper chromatograms, together with a small amount of non-radioactive nicotinic acid ribonucleotide (the product of the reaction) as a marker for chromatography. The chromatograms were developed overnight in ascending *iso*-butyric acid:water:ammonium hydroxide (61:33:1.7), and after drying in air the nicotinic acid ribonucleotide was located by its absorbance under u.v. illumination (254 nm). Regions of the chromatograms containing the product were excised and radioactivity measured as described previously [12].

Radioactive quinolinic acid was synthesised from [U-^{14}C]aniline by means of a Skraup condensation with glycerol, in the presence of nitrobenzene, to form quinoline, followed by oxidation to quinolinate by H_2O_2 in the presence of Cu^{II} ions, as described by Gholson *et al.* [13]. This procedure gave an 8 per cent recovery of radioactivity as [$^{14}C_{2,3,7,8}$] quinolinate, and a product with the same i.r. absorption spectrum and m.p. as commercially available quinolinic acid. It was dissolved in equimolar NaOH for use as substrate in the enzyme reaction.

Nicotinate phosphoribosyltransferase and nicotinamide phosphoribosyltransferase. The activity of nicotinate phosphoribosyltransferase (nicotinate nucleotide: pyrophosphate phosphoribosyltransferase, EC 2.4.2.11) was measured using the 100,000 *g* supernatant from a charcoal treated homogenate of liver in 3 ml of 0.25 M sucrose per g of tissue, prepared as described for quinolinate phosphoribosyltransferase above. The activity of nicotinamide phosphoribosyltransferase (nicotinamide nucleotide:

Table 1. The effects of Benserazide, Carbidopa and isoniazid on the activity of enzymes from rat liver, *in vitro**

Enzyme	No addition (control)	Benserazide (a) 250 μM (b) 500 μM	Carbidopa (a) 250 μM (b) 500 μM	Isoniazid (a) 2.5 mM (b) 10 mM
Tryptophan oxygenase	9.9 ± 0.2	(a) 3.8 ± 0.3†	(a) 3.6 ± 0.4†	(b) 3.7 ± 0.4†
Kynurenine hydroxylase	20.3 ± 1.5	(a) 22.2 ± 0.8	(a) 19.6 ± 0.9	(a) 20.5 ± 2.3
Kynureninase	24.5 ± 2.9	(a) 2.3 ± 0.6†	(a) 0.58 ± 0.07†	(b) 10.3 ± 0.4†
3-Hydroxy-anthranilate oxidase	113 ± 10	(b) 114 ± 12	(b) 108 ± 12	(b) 72 ± 6†
Picolinate carboxylase	1.38 ± 0.14	(b) 1.38 ± 0.13	(b) 1.24 ± 0.14	(b) 1.52 ± 0.13
Quinolinate phosphoribosyltransferase	0.52 ± 0.02	(b) 0.59 ± 0.05	(b) 0.52 ± 0.08	(b) 0.46 ± 0.07
Nicotinate phosphoribosyltransferase	0.63 ± 0.06	(b) 0.58 ± 0.05	(b) 0.63 ± 0.11	(b) 0.63 ± 0.06
Nicotinamide phosphoribosyltransferase	2.7 ± 0.13	(b) 2.66 ± 0.18	(b) 2.72 ± 0.26	(b) 2.39 ± 0.10†
Nicotinamide deamidase	12.8 ± 1.2	(b) 11.9 ± 0.7	(b) 11.8 ± 0.05	(b) 12.6 ± 1.6

* Figures show mean nmole of product formed per min per g wet weight of tissue, ± S.D. for 5 determinations.
† Significantly different from control activity, $P<0.02$, *t*-test.

pyrophosphate phosphoribosyltransferase, EC 2.4.2.12) was measured using a protamine sulphate fractionated 100,000 *g* supernatant from a homogenate of liver in 10 ml of 0.25 M sucrose per g of tissue, as described by Pinder *et al.* [11].

Incubations for both activities were carried out as described by Pinder *et al.* [11], but an alternative chromatographic system was used to separate substrate and product; papers were developed in ascending 1 M ammonium acetate (adjusted to pH 5.0): 95 per cent ethanol (30:70). Location of the product by inspection under u.v. illumination and counting of radioactivity were carried out as described above.

Nicotinamide deamidase. The activity of nicotinamide deamidase (nicotinamide amidohydrolase, EC 3.5.1.19) was measured using the microsomal pellet from a charcoal homogenate of liver in 3 ml of 0.25 M sucrose per g of tissue, and the incubations were carried out as described by Pinder *et al.* [11], in the presence of 20 mg/ml bovine serum albumin to adsorb the endogenous inhibitor of the enzyme, and so allow expression of its full potential activity. Substrate and product were separated by paper chromatography, using ascending acetone:propan-2-ol:water: NH_4OH (50:40:7:3), and again the product was located by its u.v. absorbance; radioactivity was measured as described above.

RESULTS

As can be seen from Table 1, only tryptophan oxygenase and kynureninase were inhibited significantly by all three of the drugs tested, Benserazide, Carbidopa and isoniazid. 3-Hydroxy-anthranilate oxidase and nicotinamide phosphoribosyltransferase were significantly inhibited by isoniazid, but not by the other two drugs. None of the other enzymes was inhibited by any of the drugs tested, at concentrations up to 500 μM for Benserazide and Carbidopa and 10 mM for isoniazid.

Table 2 shows the apparent K_i values obtained for these inhibitors of the affected enzymes.

DISCUSSION

Young *et al.* [5] have reported that tryptophan oxygenase is inhibited by Benserazide, both *in vitro* and *in vivo* in experimental animals. However, a previous report from this laboratory [3] showed no inhibition of mouse liver tryptophan oxygenase by Benserazide. The activity of the enzyme was determined then by the method of Knox *et al.* [7], which depends on the increase in absorbance at 357.5nm due to the formation of kynurenine. This method is subject to interference from oxidation products of Benserazide, which have a considerable absorbance at this wavelength [14]. Furthermore, this method of assay takes no account of the further metabolism of kynurenine by kynureninase in crude tissue preparations; since kynureninase is inhibited by Benserazide, it is to be expected that there would be a greater recovery of kynurenine under these conditions, and hence any inhibition of tryptophan oxygenase might well be masked by this inhibition of further metabolism of the product. The assay method described here is more tedious than that of Knox *et al.* [7], but it overcomes these problems in that it allows measurement of kynurenine in the presence of polyphenolic oxidation products of Benserazide, and also measures recovery of kynurenine through the incubation. An additional benefit of this assay is that one of the essential control samples is an unincubated tissue blank, which allows determination of the endogenous tissue concentration of kynurenine, a measurement that would be useful in studies with these and other drugs *in vivo* in experimental animals.

The demonstration of inhibition of tryptophan oxygenase by Benserazide, Carbidopa and isoniazid confirms and extends the report by Young *et al.* [5], and resolves the conflict between their results and those reported previously from this laboratory [3]. There is no evidence that tryptophan oxygenase is a pyridoxal phosphate dependent enzyme, and there appear to be no previous reports of its inhibition by hydrazine derivatives.

The inhibition of kynureninase by all three enzymes has been reported previously [4,6], and since the enzyme is pyridoxal phosphate dependent, this was expected.

It was reported previously that bacterial nicotinamide deamidase was inhibited by isoniazid, but not by either Benserazide or Carbidopa [1]. In the present study there was no such inhibition of the mam-

Table 2. Inhibition *in vitro* of enzymes of tryptophan and niacin metabolism by Benserazide, Carbidopa and isoniazid*

	Benserazide	Carbidopa	Isoniazid
Tryptophan oxygenase	41.8 ± 1.2	26.3 ± 0.6	540 ± 20
Kynureninase	26.4 ± 6.2	4.7 ± 1.3	480 ± 30
3-Hydroxy-anthranilate oxidase	†	†	$10.1 \pm 1.6 \times 10^3$
Nicotinamide phosphoribosyltransferase	†	†	$60 \pm 14 \times 10^3$

* Figures show apparent K_i (μmole/l) ± S.D. for 5 determinations.
† Indicates no significant inhibition observed, see Table 1.

malian liver enzyme. The extent to which inhibition of intestinal bacterial nicotinamide deamidase may be physiologically important depends on the relative importance *in vivo* of deamidation of dietary nicotinamide in the intestinal lumen, followed by incorporation of nicotinic acid into nucleotides, similar deamidation in the liver, and direct incorporation of nicotinamide into nucleotides — an area of unresolved controversy [15–17].

It is important to consider whether the inhibitions of enzymes of tryptophan metabolism reported here are likely to be of any significance physiologically when the drugs are administered to patients or experimental animals. Schwartz *et al.* [14,18] have studied the pharmacokinetics of radioactive Benserazide. Their data indicate that following administration to rats of a dose of 10 mg/kg body weight (approximately the dose used in clinical practice), the maximum concentration that would be expected to occur in the liver would be about 30 nmole/g, and for up to 24 hr after a single dose a liver concentration of 5–10 nmole/g could be expected. The concentrations of Carbidopa in the liver would probably be similar to those reported for Benserazide after a similar dose. This means that the inhibition of tryptophan oxygenase and kynureninase by these two drugs would be expected to be significant *in vivo*, assuming that the apparent K_i values shown in Table 2 (determined *in vitro*) are also applicable *in vivo*.

It is known that following a clinical dose of isoniazid, kynureninase is significantly inhibited [6], and since the K_i of tryptophan oxygenase is similar to that of kynureninase, it is to be assumed that this enzyme will also be inhibited *in vivo*. However, in view of the extremely high apparent K_i values determined for 3-hydroxy-anthraniliate oxidase and nicotinamide phosphoribosyltransferase, it is doubtful if these enzymes would be significantly affected by the concentrations of isoniazid that would be expected in the liver following a dose of the order of 10 mg/kg body weight.

From the data reported here on the inhibition of isolated enzymes, it is not possible to account for the relatively common occurrence of clinical pellagra (niacin deficiency) in tuberculous patients treated with isoniazid without supplementary vitamin B_6 [6], while similar doses of Benserazide or Carbidopa, (both of which are more potent inhibitors of tryptophan oxygenase and kynureninase than is isoniazid), do not cause so severe a deficiency as to precipitate clinical pellagra. This is despite the fact that a significant proportion of patients treated with these two decarboxylase inhibitors have significantly reduced urine concentrations of N^1-methyl nicotinamide [1]. It must be assumed that Parkinsonian patients treated with Benserazide or Carbidopa receive a dietary intake of niacin that is at least marginally adequate to meet their requirements, while tuberculous patients treated with isoniazid presumably do not receive sufficient niacin to compensate for the inhibition of endogenous synthesis of nicotinamide nucleotides from tryptophan. It is at least traditionally established that tuberculosis is associated with a poor diet, which may well be deficient in niacin.

It is also probable that the interactions between the drugs and the enzymes of tryptophan and niacin metabolism are more complex *in vivo* than the simple inhibitions shown *in vitro*, so that comparison of probable tissue concentrations of the drugs and their inhibitory potencies are not valid under physiological conditions. Animal studies *in vivo* with all three drugs, under conditions of controlled tryptophan, niacin and vitamin B_6 intake, are in progress.

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