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THE EFFECT OF DRUGS ON VITAMIN B_6 FUNCTION IN THE RAT

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Abstract—Some commonly used drugs were tested for an effect on vitamin B_6 function in Lister Hooded rats. An increase in urinary xanthurenic acid excretion after a tryptophan load was used as an indicator of impaired vitamin B_6 function. DL-Penicillamine, hydrallazine and phenelzine produced increases in xanthurenic acid excretion, and these were reversed by concomitant treatment of the animals with pyridoxine hydrochloride. DL-Penicillamine was the only drug which lowered the liver pyridoxal-5′-phosphate (PLP) content of these rats. DL-Penicillamine did not inhibit kynurenine aminotransferase (KAT) *in vitro*, whilst hydrallazine and phenelzine both inhibited the enzyme non-competitively with respect to the substrate. Hydrallazine produced mixed inhibition of KAT with respect to PLP; inhibition by phenelzine increased as the PLP concentration increased, indicating that the hydrazone formed between phenelzine and PLP was a more potent inhibitor of KAT than phenelzine itself. Isonicotinic acid hydrazide and its metabolites did not increase the xanthurenic acid excretion of tryptophan-loaded rats, and they were weak inhibitors of tryptophan pyrrolase *in vitro*. It is suggested that drugs may impair the normal function of vitamin B_6 by reducing tissue PLP levels, either through chemical reaction between drug and PLP or by inhibition of PLP synthesis. Drugs may also inhibit PLP-dependent enzymes without affecting systemic PLP levels.

Certain drugs interfere with the normal function of vitamin B_6 both in man and laboratory animals. For example, penicillamine [1–3], isonicotinic acid hydrazide (INH) [4], oral contraceptives [5, 6], hydrallazine [7] and cycloserine [8] have been reported to prevent the formation of pyridoxal-5′-phosphate (PLP) or to inhibit PLP-dependent enzymes, although the mechanisms underlying these actions have not been completely elucidated. Thus long-term therapy with such drugs may produce a vitamin B_6 deficiency, or may exacerbate an already existing, though unsuspected, vitamin B_6 deficiency.

We have therefore examined some commonly used drugs for their ability to interfere with vitamin B_6 function. The drugs were chosen on the basis of two criteria; either (a) their chemical structure suggested that they would react with PLP, or (b) many patients receiving them had shown symptoms of peripheral neuropathy or paraesthesia [9], adverse reactions which are frequently associated with vitamin B_6 deficiency [10].

Tryptophan metabolism has been extensively used in the detection of abnormalities of vitamin B_6 function, as it involves several PLP-dependent enzymes

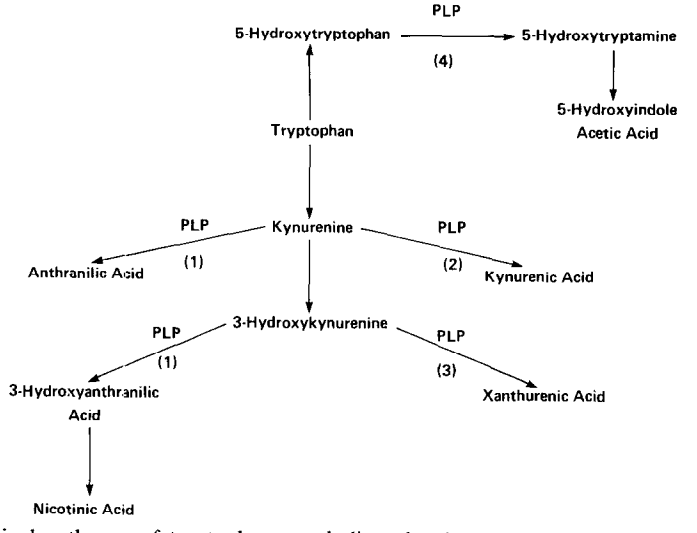

Fig. 1. Principal pathways of tryptophan metabolism showing the steps which require pyridoxal-5′-phosphate (PLP) as coenzyme. (1) Cytoplasmic kynureninase; (2) cytoplasmic kynurenine aminotransferase (KAT); (3) mitochondrial kynurenine aminotransferase; (4) aromatic L-amino acid decarboxylase.

(Fig. 1). In vitamin B_6 deficiency, for example, urinary xanthurenic acid excretion of man and laboratory animals is increased after a loading dose of L-tryptophan [11, 30]. At first sight this observation seems paradoxical as xanthurenic acid is formed from 3-hydroxykynurenine by the enzyme kynurenine aminotransferase (KAT; L-kynurenine 2-oxoglutarate aminotransferase, EC 2.6.1.7) which is itself PLP-dependent (Fig. 1). Thus one would expect xanthurenic acid excretion to decrease rather than increase in vitamin B_6 deficiency. However, the other PLP-dependent enzymes involved in tryptophan metabolism (Fig. 1) occur in cytoplasm and are readily depleted of their coenzyme. The KAT, on the other hand, is located in mitochondria where it is protected from such depletion. The steps leading to kynurenic acid, anthranilic acid and 3-hydroxyanthranilic acid (Fig. 1) are therefore inhibited. More 3-hydroxykynurenine is thus available as substrate for the mitochondrial KAT, and this results in the increased formation and excretion of xanthurenic acid [12, 13].

The purpose of this study has been to examine the urine of drug-treated, tryptophan-loaded rats for an increase in xanthurenic acid content, and to ascertain whether such an increase could be reversed by concomitant treatment of the rats with pyridoxine. In addition, the liver PLP content of the drug-treated rats has been measured to determine if this parameter varies in parallel with changes in xanthurenic acid excretion. Finally, in order further to elucidate the mechanism of action of these drugs on a typical PLP-dependent enzyme of tryptophan metabolism, their effect has been studied *in vitro* on a partially purified preparation of KAT.

MATERIALS AND METHODS

Materials. The sources of the drugs used were as follows: DL-penicillamine, D-cycloserine, indomethacin, oxytetracycline and nalidixic acid (Sigma Chemical Co. Ltd.); nitrofurantoin (Biorex Laboratories); metronidazole (May & Baker Ltd.); ethambutol (Lederle Laboratories); ethionamide (May & Baker, Ltd.); hydrallazine (Ciba Laboratories Ltd.); phenelzine (William R. Warner Ltd.); INH and monoacetylhydrazine (Koch-Light Laboratories); ampicillin (Beecham Research Laboratories); procainamide (Roche Products Ltd.); phenytoin (Parke-Davis Ltd.). Acetylisonicotinic acid hydrazide was prepared by acetylation of isonicotinic acid hydrazide as described by Fox and Gibas [29] and converted to the hydrochloride, m.p. 220–222°.

Xanthurenic acid, PLP, L-kynurenine, α-oxoglutarate and kynurenic acid were obtained from Sigma Chemical Co. Ltd., L-tyrosine from British Drug Houses Ltd., Dowex 50W-X4 resin, H^+ form (100–200 mesh) from BioRad Laboratories and NCS from G.D. Searle Ltd. L-1-[^{14}C]tyrosine (sp. act. 59 mCi/mmole was obtained from the Radiochemical Centre, Amersham.

Tyrosine decarboxylase apoenzyme of *Streptococcus faecalis* was obtained from Sigma Chemical Co. Ltd. It was found necessary to remove residual PLP from the enzyme before use by dialysis overnight with several litres of 0.01 M semicarbazide in 0.01 M acetate buffer, pH 6.5, at 0°. The semicarbazide was then eliminated by dialysis overnight against 0.01 M acetate buffer, pH 5.5. All other chemicals used were of Analar grade.

Lister Hooded rats were obtained from OLAC Ltd., Bicester, Oxon, preliminary studies (unpublished) having confirmed an earlier report by Hope [31] that this strain is more susceptible than the Wistar strain to Vitamin B_6 deficiency.

Screening of drugs for effect on tryptophan metabolism. Groups of 4 male Lister Hooded rats (weight 100 g) were orally dosed with 40 mg L-tryptophan (dissolved in 5 ml water). From each group a pooled 24-hr urine sample was collected. The rats were then treated for 14 days by i.p. injection with the drug under test, a control group receiving the vehicle, either saline or arachis oil. They were then orally dosed as before with L-tryptophan, and a second 24-hr urine sample was collected from each group.

Xanthurenic acid was separated from the urine by ion-exchange chromatography according to the method of Satoh and Price [14] as modified by Heely [15] for use with small volumes of urine. The xanthurenic acid was then determined using an Aminco–Bowman spectrophotofluorimeter with slit arrangement 5, activation at 370 nm and fluorescence at 530 nm. Urinary creatinine was measured using Jaffe's reaction as described by Owens *et al.* [16]. Xanthurenic acid excretion was expressed as μg excreted/mg creatinine.

Experiments in which increased xanthurenic acid excretion occurred were repeated, an additional group of rats being included which received the drug together with pyridoxine hydrochloride (1 mg/day by i.p. injection).

Measurement of PLP in the livers of drug-treated rats. After the second 24-hr urine collection described above the rats were killed by cervical dislocation; their livers were then quickly removed and placed on ice. After extraction with metaphosphoric acid by the method of Bhagavan *et al.* [17], PLP was measured using the PLP-dependent enzymatic decarboxylation of L-1-[^{14}C]tyrosine as described by Bhagavan *et al.* [18], the $^{14}CO_2$ formed being then trapped and determined by the method of Mole and Shepherd [19].

Inhibition of KAT. KAT, partially purified from rat kidney by the method of Mason [20], was measured as described by Kilgallon and Shepherd [21] using L-kynurenine as substrate. The concentration of α-oxoglutarate was kept constant at 0.15 M, whilst the concentrations of PLP and L-kynurenine were varied (Fig. 2). A single enzyme preparation was used for all the assays. The velocity of the reaction was expressed as μmoles kynurenic acid formed during a 30-min incubation.

The data were plotted according to the method of Lineweaver and Burk [22], and the inhibitor constant K_i for each drug was calculated using the formula:

$$\text{intercept on ordinate} = \frac{1}{V_{max}}\left(1 + \frac{[I]}{K}\right).$$

Measurement of tryptophan pyrrolase activity. The method was that of Knox *et al.* [32] with methaemoglobin as cofactor. A rat was pretreated with an i.p.

Table 1. Effects of drugs on vitamin B_6 function

Drug	No. of reports of side effects in man*	Reaction with PLP	Xanthurenic acid excretion in rat† Dose of drug‡	Untreated	Drug-treated
INH	82	+	100	137	30
Acetyl-INH		−	130	123	13
Monoacetylhydrazine		+	30	97	16
1, 2-Diacetylhydrazine		−	45	80	47
DL-Penicillamine	9	+	100	77	481
Phenelzine	2	+	15	74	253
Hydrallazine	6	+	30	111	226
Indomethacin	22	−	10	46	37
Ampicillin	18	+	50	94	86
Procarbazine	5	−	200	164	110
Metronidazole	17	−	100	182	87
Nitrofurantoin	166	−	50	185	64
Oxytetracycline	11	−	150	63	28
Procainamide		+	200	54	32
D-Cycloserine		+	200	40	26
Ethambutol	20	−	100	76	36
Ethionamide		−	150	51	24
Phenytoin	14	−	100	68	63
Nalidixic acid	26	−	150	107	36

* Number of patients showing paraesthesia and neuropathy in period 1968–1978 [7].
† μg Xanthurenic acid per mg urinary creatinine after oral load of 40 mg L-tryptophan.
‡ mg per kg given i.p. twice daily, $\frac{1}{3}$ dose at 9 a.m., $\frac{2}{3}$ dose at 5 p.m.

injection of hydrocortisone acetate (25 mg/kg) to increase the pyrrolase content of the liver [32]. After 5 hr the rat was killed by cervical dislocation; the liver was quickly removed, washed, blotted and placed on ice. The liver was homogenized with 0.14 M KCl in 0.2 M phosphate buffer, pH 7.0. to give a 25% homogenate which was centrifuged for 1 hr at 100,000 *g* in a Beckman ultracentrifuge. The supernatant fraction was used for the enzyme assay.

The incubation mixture consisted of 0.2 ml supernatant; 0.7 ml 0.2 M phosphate buffer, pH 7.0; 0.5 ml methaemoglobin (0.8 mg/ml water); 0.1 ml 0.3 M ascorbic acid in water; 1.3 ml water; 0.2 ml 0.05 M L-tryptophan in water. The reaction was started by adding the substrate L-tryptophan.

The reaction was followed for 45 min in a recording spectrophotometer at 25° and 360 nm (the absorption maximum for the kynurenine produced). The reference solution consisted of the complete incubation mixture with the L-tryptophan solution replaced by water. When the effect of inhibitors was studied, 1 ml water in the incubation medium was replaced by 1 ml of an aqueous solution of the inhibitor. The amount of product formed and hence the percentage inhibition of the enzyme reaction were calculated from the absorbance of a standard kynurenine solution.

Chemical reactions between drugs and PLP. The ability of the drugs to react with PLP *in vitro* was assessed spectrophotometrically as described by Mole and Shepherd [19]. The results are recorded in Table 1.

RESULTS

Of the drugs screened by the tryptophan load test for effects on vitamin B_6 function, DL-penicillamine, hydrallazine and phenelzine caused increased xanthurenic acid excretion in the rat (Table 1). These increases were reversed by concomitant treatment of the animals with pyridoxine (Table 2). Of these three drugs, only DL-penicillamine lowered the PLP level in rat liver (Table 3).

DL-Penicillamine at concentrations up to 0.1 mM did not inhibit partially-purified KAT. Phenelzine

Table 2. Reversal by pyridoxine of the drug induced increase in xanthurenic acid excretion of tryptophan-loaded rats

Drug*	Xanthurenic acid excretion† No pyridoxine Untreated	Drug-treated	With pyridoxine‡ Untreated	Drug-treated
Control	63	52	77	65
DL-Penicillamine	78	339	106	124
Phenelzine	74	253	57	73
Hydrallazine	74	256	81	105

* Doses as stated in Table 1.
† μg Xanthurenic acid per mg urinary creatinine after oral load of 40 mg L-tryptophan.
‡ Pyridoxine hydrochloride, 1 mg per rat per day, given i.p.

Table 3. Liver PLP content of drug-treated rats

Drug*	μg PLP per g liver (mean ± SEM)† No pyridoxine	With pyridoxine
Control	3.95 ± 0.29	3.73 ± 0.63
DL-Penicillamine	1.44 ± 0.12‡	3.08 ± 0.23
Hydrallazine	4.69 ± 0.65	4.03 ± 0.43
Phenelzine	3.93 ± 0.47	3.21 ± 0.41

* Doses as stated in Table 1.
† 4 animals in each group.
‡ $0.002 > P > 0.002$. $t = 7.56$.

and hydrallazine each inhibited the enzyme non-competitively with respect to substrate (Fig. 2a and 2b), the K_i values being 0.05 and 0.1 mM, respectively. When the PLP concentration was varied and the L-kynurenine concentration kept constant at 0.01 mM, hydrallazine produced mixed inhibition (Fig. 2c); under these conditions phenelzine caused increased inhibition with increasing coenzyme concentration (Fig. 2d).

Monoacetylhydrazine was the most potent of INH and its metabolites as an inhibitor of tryptophan pyrrolase, but the inhibition was of a low order (Table 4).

DISCUSSION

Of the drugs listed in Table 1, DL-penicillamine, hydrallazine and phenelzine produced a biochemical deficiency of vitamin B_6 in rats, this being assessed by the increased urinary xanthurenic acid excretion on tryptophan loading. The effect of the three drugs was reversed by concomitant treatment of the animals with pyridoxine (Table 2). No increase of xanthurenic acid excretion occurred in any of the control animals receiving the injection vehicle, i.e. either saline or arachis oil.

Penicillamine is already known to cause increased excretion of xanthurenic acid in tryptophan-loaded rats of other strains [25, 30]. The drug produced a highly significant reduction in the PLP content of the rat liver (Table 3). These effects are consistent with the depletion of PLP by penicillamine with which it reacts rapidly to form a thiazolidine derivative [30].

Hydrallazine caused an increase (Table 1), reversible by pyridoxine, (Table 2), in the xanthurenic acid excretion of tryptophan-loaded rats, but it did not lower liver PLP levels (Table 3). In man hydrallazine causes peripheral neuropathy and produces increased xanthurenic acid excretion after tryptophan loading; both of these effects are reversed by pyridoxine [28], and they have been assumed to be due to inactivation of PLP by its chemical reaction with hydrallazine to form the hydrazone. However, the failure of hydrallazine to lower PLP levels in the liver of rats suggests that other mechanisms may be involved.

Phenelzine reacted with PLP to form the hydrazone (Table 1), and it produced an increase, reversible by pyridoxine (Table 2), in the xanthurenic acid excretion of tryptophan-loaded rats. However, it did not lower the PLP content of the liver (Table 3). There are very few reports of peripheral neuropathy produced by phenelzine in man (Table 1); presumably this is because the therapeutic dose of this drug

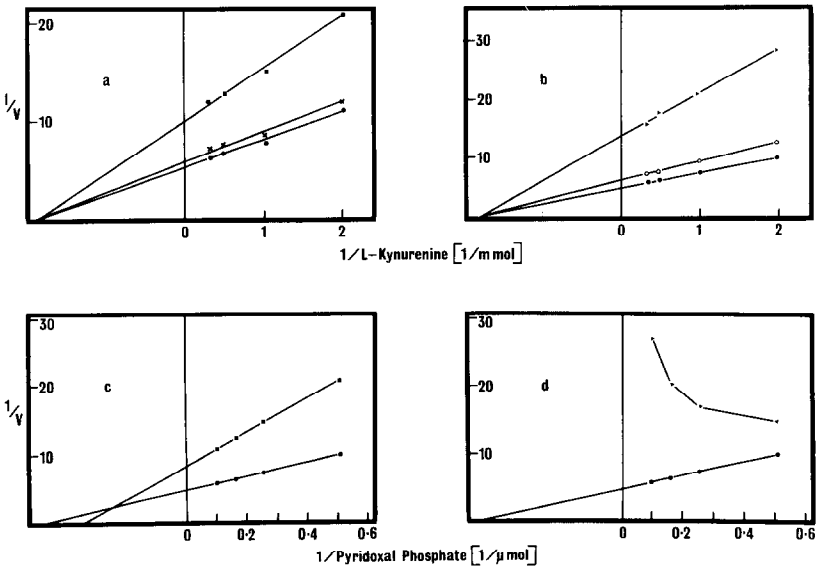

Fig. 2. Double reciprocal plots for the inhibition of rat kidney KAT by hydrallazine and phenelzine. (a) and (b) with L-kynurenine as substrate at a constant PLP concentration of 40 μM. (c) and (d) with varying PLP concentration at a constant L-kynurenine concentration of 1.0 mM.

●: No inhibitor.
×: 10 μM hydrallazine.
■: 100 μM hydrallazine.
○: 10 μM phenelzine.
▲: 100 μM phenelzine.

Table 4. Per cent inhibition of tryptophan pyrrolase by isonicotinic acid hydrazide (INH) and derivatives *in vitro**

Inhibitor concentration (M)	Inhibitor		
	INH	Acetyl-INH	Monoacetylhydrazine
10^{-2}	0	28	45
10^{-3}	0	22	29
10^{-4}	0	0	15
10^{-5}	0	0	17
10^{-6}	0	0	17

* L-Tryptophan concentration 3×10^{-3} M. All results are the mean of duplicate determinations.

is relatively low compared with that of penicillamine or hydrallazine. Phenelzine has not previously been reported to cause increased excretion of xanthurenic acid in man or the rat.

D-Cycloserine did not cause increased xanthurenic acid excretion in our rats; however, the L- and DL-forms have been reported as causing other symptoms of vitamin B_6 deficiency in man and the rat [8, 23], while D-cycloserine inhibits rat kidney KAT *in vitro* [24]. It is possible, therefore, that, as found with the stereoisomers of penicillamine [1, 25], the D-isomer of cycloserine is less potent than the L-isomer as an antagonist of vitamin B_6 function.

Our procarbazine-treated rats did not show increased xanthurenic acid excretion on tryptophan loading. Nevertheless, it is known that procarbazine lowers the plasma level of PLP in mice, and its neurological toxic effects in the mouse and man are consistent with an impairment of PLP function [26].

The failure of INH to increase xanthurenic acid excretion in rats confirms an earlier report [27]. However, INH produces pyridoxine-responsive neuropathy accompanied by an increase in urinary xanthurenic acid after a tryptophan load in man [4, 33]. INH is metabolized in man to monoacetylhydrazine and acetyl-INH [34], but in the rat INH is not acetylated and so does not form these metabolites [33]. Of the two metabolites monacetylhydrazine inactivates PLP by reacting with it *in vitro* to form the hydrazone (Table 1). However, monoacetylhydrazine and acetyl-INH, like INH itself, did not increase the xanthurenic acid excretion of tryptophan-loaded rats (Table 1). The different effects of INH in man and the rat cannot therefore be attributed to the different routes of metabolism of this drug in these species.

It is possible that INH or its metabolites could cause depletion of vitamin B_6 and, at the same time, inhibit tryptophan pyrrolase, the first enzymic step in the metabolic pathway from tryptophan to xanthurenic acid (Fig. 1). This inhibition of the pyrrolase would then prevent the overloading of subsequent PLP-dependent steps which is the basis of the tryptophan load test, and it would consequently prevent the increase in xanthurenic acid excretion which would otherwise be observed. However, INH and its metabolites were insufficiently potent as inhibitors of rat liver tryptophan pyrrolase *in vitro* (Table 4) to produce significant inhibition of the enzyme *in vivo*.

These results suggest that the different susceptibility of man and the rat to INH-induced B_6-deficiency and increased xanthurenic acid excretion on tryptophan loading arises from differences in the metabolism of tryptophan or PLP in these species rather than from differences in their metabolism of the drug. Further evidence for this is that human subjects who are slow acetylators of INH are particularly susceptible to pyridoxine-responsive peripheral neuropathy [10]. Dogs are also poor acetylators of hydrazines [35], and when treated with INH they show increased urinary excretion of xanthurenic acid after tryptophan loading [36]. In these species, therefore, the increased excretion of xanthurenic acid is due to the INH itself and not to its metabolites.

Hydrallazine (Fig. 2a) and phenelzine (Fig. 2b) each inhibited KAT non-competitively with respect to substrate. Hydrallazine produced mixed inhibition with respect to PLP (Fig. 2c), while the inhibition produced by phenelzine increased with increasing coenzyme concentration (Fig. 2d). Thus these inhibitors appear to influence the binding of PLP by the enzyme. In particular, the hydrazone formed between PLP and phenelzine is more potent than phenelzine itself as an inhibitor of KAT.

The results of this study suggest that drugs may interfere with vitamin B_6 function in at least two ways: (a) by lowering the concentration of functional PLP owing to chemical inactivation, or to inhibition of PLP synthesis, by the drug; (b) through an inhibition of PLP-dependent enzymes which is not accompanied by changes in tissue PLP levels. Thus if PLP levels only were measured, some drug-induced effects on PLP-dependent pathways could be overlooked. Moreover, species differences may occur in tryptophan metabolism, and different metabolic pathways may have different sensitivities to PLP deficiency.

While the tryptophan load test in rats is undoubtedly a useful preliminary screen for drug-induced vitamin B_6 deficiency, it does not provide a reliable guide as to whether a particular drug will produce a similar deficiency in man. As far as possible, therefore, the tryptophan load test in drug-treated rats should be repeated in patients receiving the same drug.

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