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The Mechanism of Inhibition of Rat Liver Tryptophan Pyrrolase Activity by 4-Hydroxypyrazolo[3,4-*d*]pyrimidine (Allopurinol)

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1. Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine) selectively inhibits the apotryptophan pyrrolase activity in homogenates of rat liver *in vitro* and after intraperitoneal administration. The inhibition is abolished by an excess of haematin. The allopurinol metabolite alloxanthine has no effect on the pyrrolase activity *in vitro* or after administration. Allopurinol also inhibits the activation of the enzyme *in vitro* by ascorbate, ethanol plus NAD^+, NADH, hypoxanthine or xanthine. It is suggested that these agents cause the conversion of a latent form of the pyrrolase into the apoenzyme, and that xanthine oxidase is not involved in this process. 2. The raised total pyrrolase activity observed after the administration of cortisol, cyclic AMP, tryptophan, salicylate or ethanol is lowered by allopurinol *in vitro* to the corresponding holoenzyme values. A similar effect is observed when allopurinol is administered shortly before cortisol or cyclic AMP. Pretreatment of rats with allopurinol completely prevents the enhancement of the pyrrolase activities by tryptophan, salicylate or ethanol. 3. It is suggested that allopurinol inhibits rat liver tryptophan pyrrolase activity *in vitro* and after administration by preventing the conjugation of the apoenzyme with its haem activator. The possible usefulness of combined allopurinol–tryptophan therapy of affective disorders is discussed.

It has been reported that allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine) inhibits the activity of rat liver tryptophan pyrrolase (L-tryptophan–O_2,oxidoreductase, EC 1.13.1.12) *in vitro* and after administration (Becking & Johnson, 1967). The administration of the drug or its addition *in vitro* also inhibits the activity of the cortisol or tryptophan-induced enzyme (Green & Curzon, 1968; Moon, 1971; Chytil, 1968). The activity measured in all these studies was that of either the holoenzyme or the total enzyme but not the two simultaneously. Chytil (1968) suggested that the inhibition of tryptophan pyrrolase by allopurinol is a consequence of a similar action on xanthine oxidase activity, and that the activation of the former by cyclic AMP and other purine derivatives is secondary to their enhancement of the latter enzyme activity. The present paper provides evidence suggesting that allopurinol inhibits the activity of rat liver tryptophan pyrrolase *in vitro* and after administration by preventing the conjugation of the apoenzyme with its haem activator, and that xanthine oxidase does not exert a regulatory effect on tryptophan pyrrolase activity.

Materials and Methods

Chemicals

Cortisol 21-acetate, cyclic AMP, dimethylformamide, haemin (haematin hydrochloride), NAD^+, NADH and L-tryptophan were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Allopurinol and its metabolite alloxanthine (oxypurinol) were a gift from the Wellcome Foundation Ltd., London NW1 2BP, U.K. Ethanol (99.7–100%) and all other chemicals (from BDH Chemicals Ltd., Poole, Dorset, U.K.) were of AnalaR grade, except for hypoxanthine, xanthine and sodium salicylate, which were of reagent grade.

Animals and injections

Male (180–290g) Wistar rats, maintained on MRC cube diet no. 41B, were killed between 12:00 and 15:00h by stunning and cervical dislocation. All the chemicals were given intraperitoneally 4h before death except alloxanthine, which was given 2h before death. Additional experiments were performed with allopurinol in which groups of rats were killed at between 15min and 6h after the injection. Cortisol acetate was dissolved in dimethylformamide and 0.1ml (2mg/100g body wt.) was injected; the corresponding control rats received an equal quantity of the solvent, which gave results identical with another control group of rats that had received 0.9% (w/v) NaCl. Tryptophan (20mg) was dissolved in the minimum amount of 1M-NaOH and diluted to 1ml with 0.9% NaCl after the pH had been adjusted to 7.3 with 1M-HCl; each rat received 1ml/100g body wt. Sodium salicylate (400mg/kg) was dissolved in 0.9% NaCl and ethanol (5ml/kg) was given as a 25% (v/v) solution in

Table 1. *Effects of various doses of allopurinol on rat liver tryptophan pyrrolase activity*

Each rat received a single intraperitoneal injection of either allopurinol (1.5–10mg/kg body wt.) or an equal volume of 0.9% (w/v) NaCl 2h before being killed. The enzyme activity was determined as described in the Materials and Methods section in either the absence (holoenzyme activity) or the presence (total enzyme activity) of added haematin (2μM). The apoenzyme activity was calculated by difference. The results are given as the mean±S.E.M. of each group of four rats.

Allopurinol (mg/kg)	Kynurenine formed (μmol/h per g wet wt. of liver)		Inhibition of apoenzyme activity (%)
	Holoenzyme activity	Total enzyme activity	
0	1.53±0.16	3.20±0.35	—
1.5	1.45±0.06	2.10±0.15	61
2.5	1.63±0.14	2.07±0.20	74
5.0	1.89±0.16	2.08±0.22	89
7.5	1.37±0.13	1.37±0.14	100
10.0	1.63±0.23	1.62±0.28	100

0.9% NaCl. Cyclic AMP (5mg/100g), alloxanthine (10mg/kg) and allopurinol (1.5–10mg/kg) were dissolved in the minimum amount of 1M-NaOH and diluted with 0.9% NaCl after the pH had been adjusted to 7.4–7.9 with 1M-HCl; each rat received 0.2ml/100g body wt. The corresponding control rats received an equal volume of 0.9% NaCl.

Preparation of homogenates

The liver was removed within 30s of the death of the animal and was homogenized for 1min at 1100rev./min in 7 volumes of a solution containing 140mM-KCl and 2.5mM-NaOH at 0°C in a glass homogenizer with a loose-fitting Teflon pestle. The homogenates were used within 4–9min of preparation.

Determination of tryptophan pyrrolase activity

The activity of the enzyme was determined in liver homogenates by measuring the formation of kynurenine from L-tryptophan (Feigelson & Greengard, 1961) either in the absence (holoenzyme activity) or in the presence (total enzyme activity) of added haematin. The apoenzyme activity was calculated by difference. Samples (15ml) of the homogenates were added to a solution containing 5ml of 0.03M-L-tryptophan, 15ml of 0.2M-sodium phosphate buffer, pH7.0 and 25ml of water at 0°C. Where necessary, haematin hydrochloride was dissolved in 0.1M-NaOH and 0.1ml was included in the overall mixture to give a concentration (2μM) that was optimum for enzyme activation. Samples (3ml) of the mixture were incubated with shaking at 37°C in stoppered 25ml conical flasks in an atmosphere of O_2 for appropriate time-intervals (15min each) up to 90min. The reaction was stopped by the addition of 2ml of 0.9M-trichloroacetic acid; the flasks and contents were shaken for a further 2min and then filtered on Whatman no. 1 filter paper. To a measured portion of the filtrate (2.5ml) was added 1.5ml of 0.6M-NaOH and the kynurenine present was determined by measuring the E_{365} with a Unicam SP.500 spectrophotometer and

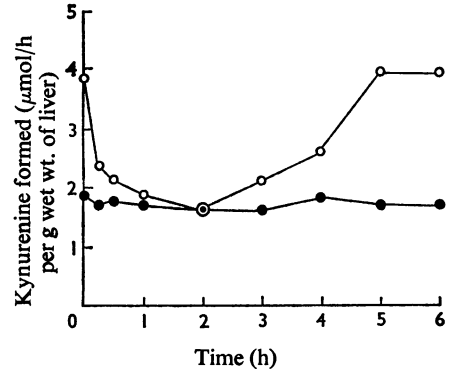

Fig. 1. *Time-course of the effects of intraperitoneal administration of allopurinol* (10*mg/kg body wt.*) *on the activity of rat liver tryptophan pyrrolase*

Allopurinol (10mg/kg body wt.) was injected at zero time. Each point represents the mean value for four rats except that the zero-time points are means for 30 animals. The enzyme activity was measured as described in the Materials and Methods section. ●, Holoenzyme activity (that measured in the absence of added haematin); ○, total enzyme activity (that measured in the presence of 2μM-haematin).

by using an ϵ of 4540 litre·mol^{-1}·cm^{-1}. Tryptophan pyrrolase activity was calculated from the increase in the E_{365} with time during the linear phase. The latter was preceded by a lag-phase that persisted for 30–45 min with the basal enzyme and the enzyme induced by the administration of either cortisol or cyclic AMP. The enzyme enhanced by the administration of tryptophan, salicylate or ethanol or by the addition of activators *in vitro* had the lag-phase either abolished or shortened to 15 min. For an extinction range (at 365 nm) of 0.10–0.75 the line representing the linear phase exactly covered three to four points 15 min apart. In the experiments *in vitro*, ascorbic acid, ethanol, NAD^+, NADH, hypoxanthine, xanthine, allopurinol and alloxanthine were dissolved in the sodium phosphate buffer with or without a little 1 M-NaOH, neutralized to pH 7.3 with 1 M-HCl and added immediately before incubation.

Results

Effects of administration of allopurinol on rat liver tryptophan pyrrolase activity

Allopurinol, in single intraperitoneal doses of 1.5 mg/kg body wt. and above, significantly inhibited the total pyrrolase activity ($P < 0.05$ at least) without affecting that of the holoenzyme. The apoenzyme activity (obtained by difference) was inhibited by 61, 74 and 89% with doses of 1.5, 2.5 and 5 mg/kg body wt. respectively and by 100% with higher doses (Table 1).

The time-course of the effects of the injection of allopurinol (10 mg/kg) on the pyrrolase activity is shown in Fig. 1. The apoenzyme activity was inhibited by 66% at 15 min and by 100% at 2 h. The inhibition then decreased to 76% at 3 h, 62% at 4 h and finally disappeared after 5 h.

Table 2. *Effects of pretreatment of rats with allopurinol on the enhancement of rat liver tryptophan pyrrolase activity by cortisol, cyclic AMP, tryptophan, salicylate and ethanol*

Each rat received an injection of either 0.9% NaCl, cortisol acetate (20 mg/kg), cyclic AMP (50 mg/kg), tryptophan (200 mg/kg), salicylate (400 mg/kg) or ethanol (5 ml/kg) 4 h before being killed. Where necessary allopurinol (10 mg/kg body wt.) was injected 10 min before the inducers. The results are given as the means ± S.E.M. of each group of four rats.

	Kynurenine formed (μmol/h per g wet wt. of liver)			
	No addition		After allopurinol	
Injection	Holoenzyme activity	Total enzyme activity	Holoenzyme activity	Total enzyme activity
0.9% NaCl	1.82 ± 0.21	3.86 ± 0.39	1.84 ± 0.29	2.60 ± 0.40
Cortisol	5.58 ± 0.73	12.90 ± 1.04	5.55 ± 0.40	6.94 ± 0.32
Cyclic AMP	5.06 ± 0.17	9.20 ± 0.01	4.10 ± 0.68	4.55 ± 0.10
Tryptophan	5.67 ± 0.57	9.08 ± 0.38	1.69 ± 0.20	3.62 ± 0.46
Salicylate	6.61 ± 0.23	10.54 ± 0.28	1.98 ± 0.22	3.82 ± 0.59
Ethanol	6.77 ± 0.87	10.99 ± 1.13	1.94 ± 0.18	3.96 ± 0.54

Table 3. *Effects of allopurinol on rat liver tryptophan pyrrolase activity in vitro*

The results are given as the means ± S.E.M. of homogenates from four separate animals incubated with or without allopurinol (1.25–50 μM) in the absence (holoenzyme activity) or in the presence (total enzyme activity) of added haematin (2 μM). * represents a significant difference between the allopurinol and control values ($P = 0.02$–0.001).

	Kynurenine formed (μmol/h per g wet wt. of liver)			
	Holoenzyme activity		Total enzyme activity	
Concn. of allopurinol (μM)	+Allopurinol	Control	+Allopurinol	Control
1.25	1.40 ± 0.22	1.51 ± 0.29	2.71 ± 0.27	3.13 ± 0.28
2.50	1.61 ± 0.09	1.60 ± 0.27	2.44* ± 0.12	3.69 ± 0.31
5.00	1.04 ± 0.09	1.13 ± 0.13	1.25* ± 0.15	2.66 ± 0.16
10.00	1.49 ± 0.09	1.37 ± 0.07	1.29* ± 0.27	2.95 ± 0.17
50.00	1.28 ± 0.23	1.26 ± 0.23	1.53* ± 0.26	3.33 ± 0.31

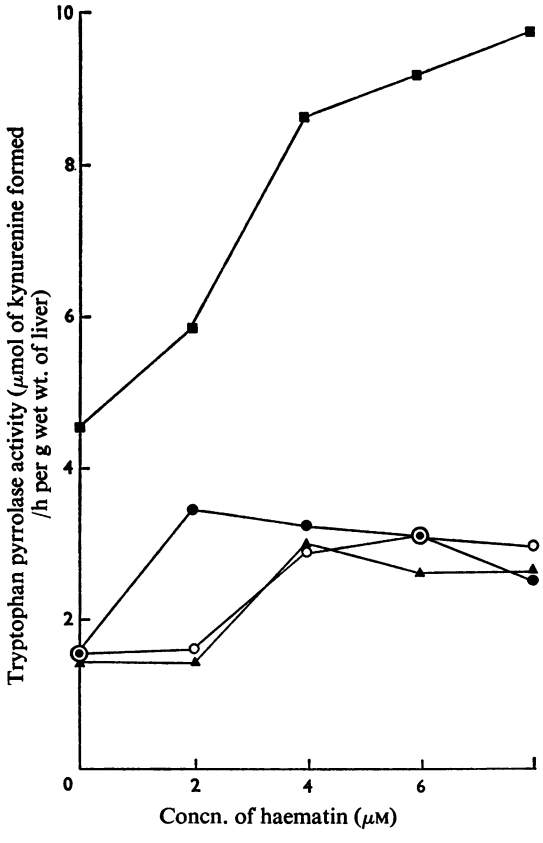

Fig. 2. *Effects of haematin on tryptophan pyrrolase activity in normal rats and in those treated with cortisol plus allopurinol in vivo, or allopurinol in vivo and in vitro*

The enzyme activity was determined, as described in the Materials and Methods section, in the absence or in the presence of various concentrations of added haematin. Allopurinol was either added *in vitro* (0.05mM), or injected (10mg/kg body wt.) alone 2h previously, or 10min before cortisol (20mg/kg), and the animals were killed 4h after administration of the hormone. Each point represents the mean value for four rats. ●, No addition; ○, allopurinol (0.05mM); ▲, allopurinol injected alone; ■, allopurinol injected before cortisol.

The effects of the administration of allopurinol on the enhancement of the pyrrolase activity by various treatments *in vivo* is shown in Table 2. The drug had no significant effect on the rise in the holoenzyme activity caused by either cortisol or cyclic AMP whereas that in the total activity was decreased to the corresponding holoenzyme values. Allopurinol completely prevented the increase in both holoenzyme and total pyrrolase activities produced by tryptophan, salicylate or ethanol.

Effects of allopurinol on rat liver tryptophan pyrrolase activity in vitro

The results (Table 3) show that allopurinol in concentrations of 2.5μM and above significantly inhibited the total pyrrolase activity in homogenates of rat liver *in vitro*. The holoenzyme activity was not altered by any of the concentrations of allopurinol tested.

The inhibition of the apoenzyme activity by allopurinol *in vitro* or at 2h after intraperitoneal administration was abolished by the addition to the incubation mixture of an excess of haematin (Fig. 2). The inhibition of the cortisol-induced rise in the apoenzyme activity by allopurinol pretreatment of rats was also reversed by haematin. Allopurinol added *in vitro* decreased the total pyrrolase activity in livers of rats treated with cortisol, cyclic AMP, tryptophan, salicylate or ethanol to the corresponding holoenzyme values (Table 4) without affecting the latter.

The stimulation of both holoenzyme and total pyrrolase activities by ascorbate, ethanol plus NAD^+, NADH, hypoxanthine or xanthine *in vitro* (Table 5) was completely inhibited by the addition of allopurinol (Table 6).

The allopurinol metabolite alloxanthine did not alter the pyrrolase activity *in vitro* or *in vivo*. At 0.05mM, it slightly (9–11%) inhibited the basal enzyme activities *in vitro*. By 2h after the administration of alloxanthine (10mg/kg body wt.) in the rat, the holoenzyme and total pyrrolase activities were (in μmol of kynurenine formed/h per g wet wt. of liver ±S.E.M. of each group of four rats) 1.86±0.04 and 3.63±0.19 compared with the control (0.9% NaCl) values of 1.53±0.16 and 3.20±0.35 respectively.

Discussion

Allopurinol, a xanthine oxidase inhibitor both *in vivo* (Elion *et al.*, 1963) and *in vitro* (Watts *et al.*, 1965), has been shown to inhibit non-competitively the activity of rat liver tryptophan pyrrolase (Becking & Johnson, 1967). The drug also inhibits the activation of the pyrrolase by cyclic AMP and other purines which must be converted into hypoxanthine before exerting their action *in vitro* (Chytil, 1968). This author suggested that the H_2O_2 formed by the action of xanthine oxidase on hypoxanthine causes the conversion of a latent form of the pyrrolase into the apoenzyme. The participation of xanthine oxidase in the inhibitory action of allopurinol on tryptophan pyrrolase activity was further suggested (Chytil,

Table 4. *Effect of allopurinol* (0.05 mM) *on the total activity of rat liver tryptophan pyrrolase induced by cortisol, cyclic AMP, tryptophan, salicylate and ethanol*

The total pyrrolase activity was determined 4 h after the injection of various inducers as described in Table 2 in the presence of added (2 μM) haematin and with or without allopurinol (0.05 mM). Values for the holoenzyme activity are included for reference. The results are given as the means ± S.E.M. of each group of four rats.

	Kynurenine formed (μmol/h per g wet wt. of liver)		
	Total enzyme activity		Holoenzyme activity
Injection	Control	+Allopurinol	Control
0.9% NaCl	3.61 ± 0.30	1.60 ± 0.20	1.65 ± 0.10
Cortisol	12.90 ± 1.04	6.16 ± 0.61	5.58 ± 0.73
Cyclic AMP	9.20 ± 0.01	4.67 ± 0.07	5.16 ± 0.17
Tryptophan	9.08 ± 0.38	5.15 ± 0.45	5.67 ± 0.57
Salicylate	10.54 ± 0.28	4.23 ± 0.40	6.61 ± 0.23
Ethanol	10.99 ± 1.13	4.95 ± 0.23	6.77 ± 0.87

Table 5. *Effects of ascorbate, ethanol plus NAD$^+$, NADH, hypoxanthine and xanthine on rat liver tryptophan pyrrolase activity in vitro*

The results are given as the means ± S.E.M. of homogenates from four separate animals incubated in the absence (holoenzyme activity) or in the presence (total enzyme activity) of added haematin (2 μM). All substances tested were added at 5 mM except ethanol (10 mM) and NAD$^+$ (2 mM). All test values were significantly different from controls ($P = 0.05$–0.005).

	Kynurenine formed (μmol/h per g wet wt. of liver)			
	Holoenzyme activity		Total enzyme activity	
Addition	Control	Test	Control	Test
Ascorbate	1.49 ± 0.24	2.97 ± 0.56	3.08 ± 0.37	5.69 ± 0.47
NADH	1.51 ± 0.26	3.16 ± 0.12	3.63 ± 0.61	7.97 ± 0.47
Ethanol+NAD$^+$	1.47 ± 0.23	2.99 ± 0.22	3.31 ± 0.58	6.70 ± 0.66
Hypoxanthine	1.36 ± 0.08	2.72 ± 0.35	2.88 ± 0.24	5.65 ± 0.50
Xanthine	1.76 ± 0.15	3.38 ± 0.23	3.76 ± 0.57	6.30 ± 0.86

Table 6. *Effect of allopurinol on the activation of rat liver tryptophan pyrrolase by ascorbate, ethanol plus NAD$^+$, NADH, hypoxanthine and xanthine in vitro*

The results are given as the means ± S.E.M. of homogenates from four separate animals incubated in the absence (holoenzyme activity) or in the presence (total enzyme activity) of added haematin (2 μM). Test flasks had each of the activators in the concentrations listed in Table 5, together with allopurinol (0.05 mM) whereas other (nil) flasks had no activators or allopurinol. There was no significant difference between test and nil flasks except for the total activity in the presence of xanthine plus allopurinol.

	Kynurenine formed (μmol/h per g wet wt. of liver)			
	Holoenzyme activity		Total enzyme activity	
Addition	Nil	Test	Nil	Test
Ascorbate	1.83 ± 0.28	2.11 ± 0.41	3.97 ± 0.24	4.31 ± 0.22
NADH	2.07 ± 0.31	2.24 ± 0.17	4.22 ± 0.51	4.35 ± 0.51
Ethanol+NAD$^+$	2.69 ± 0.23	2.47 ± 0.41	5.29 ± 0.54	6.01 ± 0.12
Hypoxanthine	1.43 ± 0.07	1.31 ± 0.11	2.96 ± 0.22	2.77 ± 0.18
Xanthine	1.56 ± 0.23	1.64 ± 0.21	3.52 ± 0.28	2.64 ± 0.06

1968) by the finding that the drug does not inhibit the activation of the latter by ascorbate. The present results indicate that the pyrrolase activation by ascorbate as well as other agents including purines (Table 5) is completely prevented by allopurinol *in vitro* (Table 6). Whereas hypoxanthine and xanthine are substrates for xanthine oxidase, ascorbate is not, and NADH inhibits the enzyme activity (Della Corte & Stirpe, 1970). Thus NADH, whether produced during the action of xanthine oxidase on hypoxanthine and/or xanthine or of alcohol dehydrogenase on ethanol plus NAD^+ (Badawy & Evans, 1973*a*), may be the pyrrolase activating agent. The activation of tryptophan pyrrolase by the agents listed in Table 5 may be caused by the conversion into the active enzyme of a form(s) that, by using ascorbate as activator, has been described as latent (Knox, 1966) and as monomer (Cho-Chung & Pitot, 1967). The above findings (Tables 5 and 6), the direct inhibition of the pyrrolase activity by allopurinol administration and *in vitro* (Tables 1 and 3) and the absence of inhibition by the allopurinol metabolite alloxanthine, which also inhibits xanthine oxidase activity (Elion, 1966), suggest that the latter enzyme does not exert a regulatory effect on the pyrrolase activity.

The selective inhibition by allopurinol of rat liver apotryptophan pyrrolase activity after administration (Fig. 1 and Table 1) and *in vitro* (Table 3) and the reversal of this by excess of haematin (Fig. 2) suggest that the drug acts by interfering with the conjugation of the apoenzyme with its haem activator. In this respect, allopurinol resembles the alkaloid yohimbine (Sourkes *et al.*, 1969) but differs from 2,2-bipyridyl or salicylate (Badawy & Smith, 1971) which only inhibit the holoenzyme activity. That allopurinol is a specific apoenzyme inhibitor is also indicated by the finding that pretreatment of the rats with it lowers the cortisol- or cyclic AMP-induced rise in the total pyrrolase activity to the corresponding holoenzyme values without affecting these values (Table 2), and that added *in vitro* it also decreases the total activity of the enzyme induced by cortisol, cyclic AMP, tryptophan, salicylate or ethanol (Table 4) to values that are either similar to, or slightly lower than, those of the corresponding holoenzyme.

The inhibition of the pyrrolase activity of cortisol-treated rats by allopurinol pretreatment (Table 2) is not due to decreased apoenzyme synthesis, since excess of haematin is effective in restoring the total activity (Fig. 2). Activation of pre-existing apoenzyme by tryptophan (Schimke, 1969), salicylate which acts by displacing protein-bound tryptophan (Badawy & Smith, 1972), and ethanol (Badawy & Evans, 1973*a*) is completely prevented by pretreatment of the rats with allopurinol (Table 2), presumably because the apoenzyme there is already inactive (Fig. 1). Inhibition of the apoenzyme activity in livers of rats chronically treated with ethanol also blocks the activation of the pyrrolase by tryptophan, salicylate or ethanol (Badawy & Evans, 1973*b*).

It has been reported (Curzon, 1971) that allopurinol does not prevent the cortisol-induced rise in $^{14}CO_2$ expired by rats given a tracer dose of DL-2[-^{14}C]-tryptophan. This finding may be due to endogenous haem stores counteracting the inhibition *in vivo*. The complete prevention of the tryptophan-induced rise in pyrrolase activity in rats pretreated with allopurinol (Table 2) is consistent with the reported impairment of the hepatic metabolism of a tryptophan load and the enhanced rise in brain tryptophan after the injection of the amino acid by allopurinol (Curzon, 1971). The usefulness of L-tryptophan in the treatment of depressive illness is believed to be due to its conversion in the brain into 5-hydroxytryptamine, the metabolism of which is impaired in such illness (Curzon, 1969; Lapin & Oxenkrug, 1969). Although the synthesis of 5-hydroxytryptamine in the brain represents a very small fraction of the overall body metabolism of tryptophan, relatively large doses of the amino acid (5–7 g daily) are therapeutically effective. This may be explained by the ability of plasma proteins to bind large amounts of the amino acid (Badawy & Smith, 1972) and that most of the plasma free tryptophan is removed by the liver via the quantitatively more important kynurenine pathway. The impairment of the hepatic metabolism of tryptophan discussed above (Curzon, 1971) and reported here (Table 2) suggests that the anti-depressant action of the amino acid may be enhanced by joint allopurinol administration.

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