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TRYPTOPHAN METABOLISM IN THE ISOLATED PERFUSED LIVER OF THE RAT: EFFECTS OF TRYPTOPHAN CONCENTRATION, HYDROCORTISONE AND ALLOPURINOL ON TRYPTOPHAN PYRROLASE ACTIVITY AND KYNURENINE FORMATION

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1 The effect of tryptophan concentration on the rate of kynurenine appearance and tryptophan disappearance in the medium perfused through the isolated liver of the rat has been investigated. The effect of pretreatment of the rat with hydrocortisone or allopurinol was also examined, together with the effects of these treatments on liver tryptophan pyrrolase activity measured *in vitro* at the beginning and end of perfusion.

2 Hydrocortisone (5 mg/kg) injection 3 h before perfusion resulted in a four-fold increase in kynurenine production by the liver during perfusion with a medium containing either 0.1 mmol/l or 1.0 mmol/l tryptophan. Injection of allopurinol (20 mg/kg) together with hydrocortisone and addition of allopurinol (4 mg/100 ml) to the medium abolished the hydrocortisone-induced rise of kynurenine in the 0.1 mmol/l tryptophan medium but not the 1.0 mmol/l tryptophan medium.

3 Injection of cycloheximide (30 mg/kg) with hydrocortisone (5 mg/kg) 3 h before perfusion inhibited the hydrocortisone-induced rise of kynurenine production and the increase in pyrrolase activity measured *in vitro* both before and at the end of perfusion with 1.0 mmol/l tryptophan. This last result suggests that protein synthesis is involved not only in hydrocortisone induction of pyrrolase but also in substrate induction.

4 Kynurenine production in the 1.0 mmol/l tryptophan medium was less in both saline- and hydrocortisone-treated older rats (335–450 g) compared to younger rats (180–220 g). In agreement with a previous study, pyrrolase activity *in vitro* was also lower in both saline- and hydrocortisone-treated older rats at the beginning of the perfusion although activity had risen equally in both young and older rats at the end of perfusion.

5 There was little correlation between the rate of tryptophan disappearance from the medium and the activity of tryptophan pyrrolase either as measured *in vitro* or as indicated by the rate of kynurenine production.

6 In general, the production of kynurenine in the medium at the end of the 60 min perfusion was indicative of *in vitro* pyrrolase activity at the start of the perfusion.

7 It is concluded that while *in vitro* pyrrolase assay does not give a quantitative index of kynurenine production, it does provide a qualitative index. Furthermore, if kynurenine production in the isolated perfused liver of the rat is indicative of *in vivo* pyrrolase activity, then hydrocortisone must induce pyrrolase activity *in vivo*.

Introduction

The liver enzyme tryptophan pyrrolase (L-tryptophan-2, 3-dioxygenase, EC1.13.1.12) is the first enzyme on the major metabolic pathway degrading tryptophan (Figure 1). Its activity is increased by corticosteroids and tryptophan (Knox, 1951) and inhibited by allopurinol (Becking & Johnson, 1967; Green & Curzon, 1968; Badawy & Evans, 1973). Recent evidence has suggested that when the activity of the enzyme is increased by hydrocortisone treatment, plasma, liver and brain tryptophan concentrations

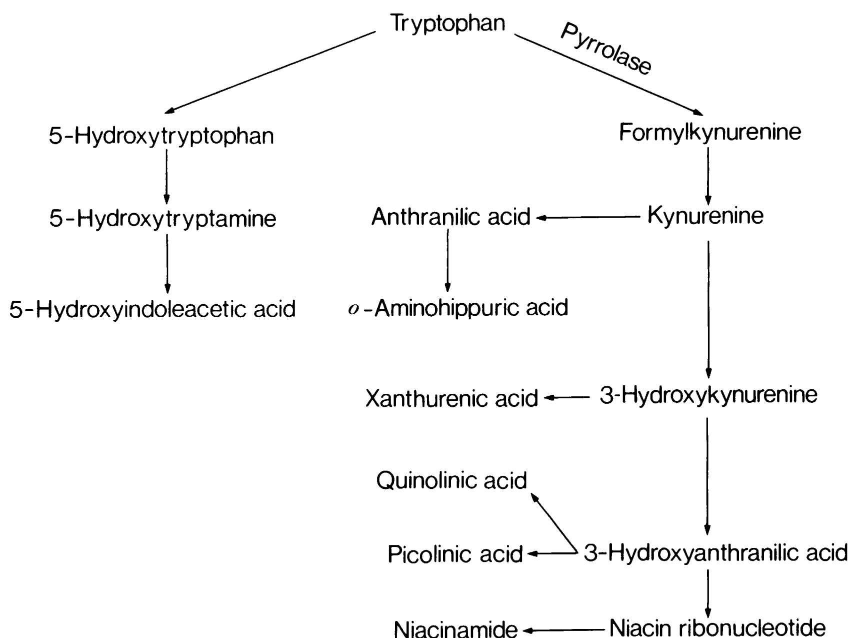

Figure 1 Tryptophan metabolism. A simplified diagrammatic metabolic pathway.

decrease and the brain tryptophan change leads to a decreased rate of synthesis of the neurotransmitter 5-hydroxytryptamine (Green, Sourkes & Young, 1975b; Green, Woods, Knott & Curzon, 1975c), the product of another metabolic pathway of tryptophan (Figure 1).

One difficulty in interpreting previous data on the effect of drugs on pyrrolase activity is that the compounds were administered *in vivo* and subsequent measurement of pyrrolase activity was performed *in vitro*. To avoid this problem, Kim & Miller (1969) measured pyrrolase activity in the isolated perfused liver of the rat. However, when they perfused for 6 h, adding hydrocortisone to the medium, they found few significant changes of pyrrolase activity in terms of either tryptophan removal from the medium or appearance of kynurenine, the product of pyrrolase activity. This is at variance with *in vivo* studies which have demonstrated that steroids and tryptophan produce significant alterations in the rate of expired $^{14}CO_2$ from DL-[ring 2-^{14}C]-tryptophan (Moran & Sourkes, 1963; Green, 1969; Green *et al.*, 1975b; Young & Sourkes, 1975). Kim & Miller (1969) however were also unable to find a statistically significant increase in $^{14}CO_2$ excretion using these techniques.

We have now investigated the problem of the *in vivo* activity of this enzyme by observing the rate of disappearance of tryptophan and appearance of kynurenine in the medium circulating through an isolated perfused liver of the rat. The results are compared with the activity of the enzyme measured *in vitro* in a lobe obtained from the perfused liver before the start and at the end of the perfusion.

A preliminary account of some of this work has been given to the British Pharmacological Society (Green, Joseph & Woods, 1975a).

Methods

All experiments were performed on adult male Sprague-Dawley rats weighing 180–220 g (Anglia Laboratory Animals Ltd, Alconbury, Huntingdon) except those involved in the study on the effect of age, when rats weighing 335–450 g were used.

Rats were anaesthetized with pentobarbitone sodium (Abbott, Queensborough, Kent), 60 mg/kg intraperitoneally, and livers perfused by the method of Hems, Ross, Berry & Krebs (1966) using a semi-synthetic medium (Woods, Eggleston & Krebs, 1970) consisting of dialysed bovine serum albumin (2.6 g/100 ml), washed aged human red cells (2.5 g/100 ml) and bicarbonate buffered saline (Krebs & Henseleit, 1932) containing glucose (5 mmol/litre). During perfusion the medium was gassed with 95% O_2 and 5% CO_2 at a rate of 150–200 ml/minute. Before the start of perfusion, a small piece of liver was tied off

and removed for measurement of pyrrolase activity *in vitro*. A second piece was removed at the end of perfusion for further measurement of *in vitro* pyrrolase activity.

Pyrrolase activity was measured in the liver lobes by the method of Knox & Auerbach (1955) after the addition of 2 µM haematin to the reaction mixture (Kevitz & Wagner, 1965). *In vitro* pyrrolase activity is expressed in terms of units of activity, one unit being 1 µmol kynurenine formed h^{-1} g^{-1} liver (dry weight) at 37°C.

Livers were perfused at a rate of between 2 and 3 ml/min per g tissue with 100 ml of medium. A 5 ml sample was removed at zero time for determination of tryptophan and kynurenine and 5 ml samples removed at 15, 30, 45 and 60 min for further determinations. Results have been calculated allowing for the removal of these samples. The concentration of tryptophan is expressed in terms of µg compound per ml perfusate plasma. Plasma refers to the medium after centrifugation to remove red blood cells.

Tryptophan was measured both in medium and liver by the method of Denckla & Dewey (1967). A new method was developed to measure kynurenine in the medium as it was found that tryptophan seriously interfered with the method of Spiera & Vallarino (1969). The method developed is based on the principle that after deproteinization, kynurenine can be diazotized and coupled with napthyl ethylene diamine, the coloured product then being extracted into amyl alcohol and back-extracted into dilute sulphuric acid to be read at 560 nM on a spectrophotometer (Joseph, 1973).

The perfusion medium (5 ml) was centrifuged and 3 ml of the 'plasma' supernatant deproteinized with 3 ml trichloroacetic acid (20% v/v). After centrifugation, 5 ml of the supernatant was pipetted into a 25 ml stoppered tube, and 0.1 ml sodium nitrite (0.5 M) added and mixed. After 5 min, 0.1 ml ammonium sulphamate (2.5%) was added and mixed and after a further 5 min, 0.2 ml napthyl ethylene diamine (0.25%) in ethanol (95% v/v) was also added and mixed. One hour later amyl alcohol (5 ml) was added, and the tubes stoppered, shaken for 1 min and centrifuged briefly; 4 ml of the upper organic phase was transferred to a 25 ml stoppered tube containing 4 ml sulphuric acid (1N). The tube was shaken for 1 min, centrifuged briefly and 3 ml of the lower aqueous phase pipetted into a test tube and clarified with acetone (0.2 ml). Optical density was read on a Unicam SP1800 spectrophotometer. Standards of 5 ml containing 1 µg/ml kynurenine sulphate in 10% (v/v) trichloroacetic acid and blanks of 5 ml trichloroacetic acid (10% v/v) were carried through the procedure in place of deproteinized 'plasma' supernatant.

No interference resulted when tryptophan, or other amino acids, 3-hydroxykynurenine, kynurenic acid, 3-hydroxyanthranilic acid or *p*-aminobenzoic acid were

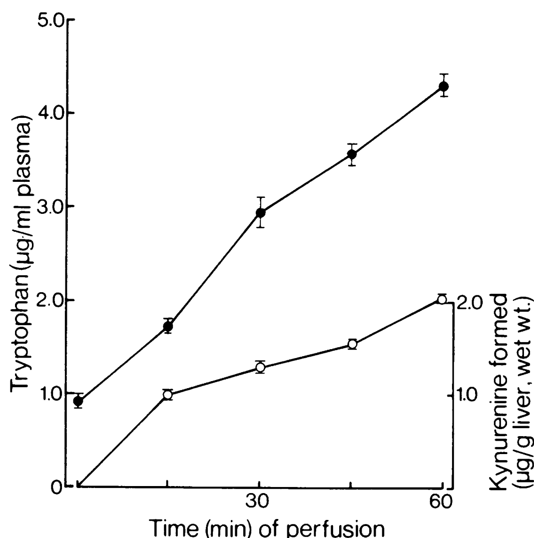

Figure 2 Effect of perfusion of basal medium on the production of kynurenine and concentration of tryptophan in the medium. Control rat livers were perfused with the basal medium and tryptophan (●) and kynurenine (○) production measured in the medium. Points show mean of determinations on 4 animals. Vertical bars show s.e. mean.

added to 'plasma'. Acetylkynurenine and *o*-aminohippuric acid did interfere, giving 80% and 50% respectively of the reading obtained with an equimolar solution of kynurenine (Joseph & Risby, 1975; Friedel & Curzon, unpublished observations).

Plasma 5-hydroxytryptamine and 5-hydroxyindoleacetic acid were measured essentially by the method of Curzon & Green (1970).

Drugs were administered 3 h before the perfusion. They were given intraperitoneally, dissolved in 0.9% w/v NaCl solution (saline) or, in the case of allopurinol, suspended in saline containing 1% carboxymethyl cellulose. They were obtained from the following sources: L-tryptophan from Sigma Chemical Co., hydrocortisone sodium succinate (Solu-cortef) from Upjohn Ltd and allopurinol from Burroughs Wellcome.

Results

Effects of perfusion of basal medium on kynurenine and tryptophan concentration in the medium and on pyrrolase activity

Rats were anaesthetized and the livers perfused with basal medium with no tryptophan added to it. There was a small but significant appearance of kynurenine

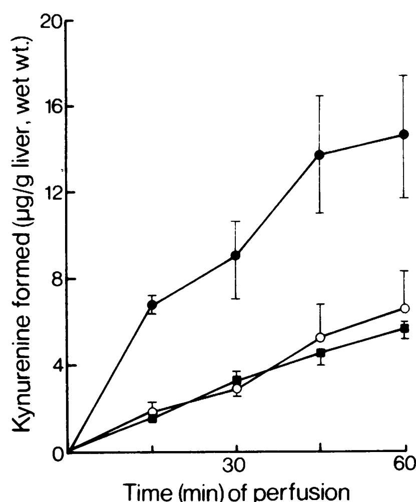

Figure 3 Effect of perfusion of 0.1 mmol/l tryptophan medium on the production of kynurenine in perfusate from livers of rats injected 3 h previously with saline (○), hydrocortisone 5 mg/kg (●) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg) with allopurinol (4 mg/100 ml) also added to the perfusate (■). Points show mean of determinations performed on at least 3 animals. Vertical bars show s.e. mean. When allopurinol was injected with hydrocortisone as above but not added to the medium the final kynurenine formation was 10.13 ± 1.33 (3).

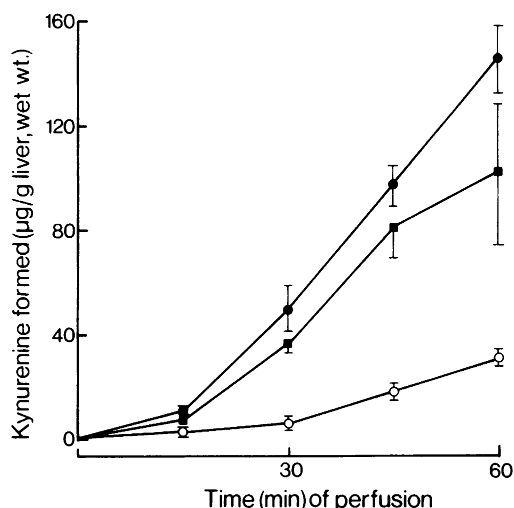

Figure 4 Effect of hydrocortisone and hydrocortisone plus allopurinol on kynurenine production in the perfusion medium. Effect of perfusion of 1.0 mmol/l tryptophan medium on the production of kynurenine in perfusate from livers of rats injected 3 h previously with saline (○), hydrocortisone 5 mg/kg (●) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg) with allopurinol (4 mg/100 ml) also added to the perfusate (■). Points show mean of determinations performed on at least 3 animals. Vertical bars show s.e. mean. When allopurinol was injected with hydrocortisone as above but not added to the medium the final kynurenine formation was 99.3 ± 15.06 (3).

over the 60 min perfusion period (Figure 2). The basal medium contained 0.95 µg/ml tryptophan and, during perfusion, its tryptophan concentration rose linearly to reach a concentration of 4.34 µg/ml (Figure 2). *In vitro* pyrrolase activity was essentially unchanged at the end of the 60 min perfusion compared with activity at the start of perfusion (Table 1).

Effects of hydrocortisone and allopurinol on the production of kynurenine by the isolated perfused liver

Rats were injected with saline, hydrocortisone (5 mg/kg), allopurinol (20 mg/kg) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg). Three hours later they were anaesthetized and the livers perfused with the medium containing either 0.1 mmol/l (20.4 µg/ml) or 1.0 mmol/l (204 µg/ml) tryptophan.

Table 1 Effect of perfusion of basal medium on *in vitro* pyrrolase activity

Treatment	*Pyrrolase activity (units)*
Control	3.62 ± 1.78 (4)
60 min perfusion with basal medium	3.96 ± 0.55 (4)

Results expressed as mean ± s.e. mean with number of animals shown in brackets. Pyrrolase activity expressed as units; for details, see methods section.

When 0.1 mmol/l tryptophan was perfused kynurenine appearance in the medium increased during the second half of the perfusion (Figure 3). A similar pattern was observed when 1.0 mmol/l tryptophan was perfused (Figure 4). When hydrocortisone (5 mg/kg) had been injected 3 h prior to perfusion, considerably more kynurenine appeared in the medium during perfusion with either 0.1 mmol/l or 1.0 mmol/l tryptophan.

When allopurinol (20 mg/kg) was injected with the hydrocortisone (5 mg/kg), it brought about only a slight reduction in the increased kynurenine production which had been caused by the hydrocortisone. This was observed following perfusion with either 0.1 mmol/l or 1.0 mmol/l tryptophan (see legends to Figures 3 and 4). However, if rats that had been pretreated with hydrocortisone plus allopurinol were perfused with a medium containing 0.1 mM

tryptophan plus allopurinol (4 mg/100 ml medium), the concentration of kynurenine in the medium was no greater than in the control animals (Figure 3). When 1.0 mmol/l tryptophan was perfused through livers of rats pretreated with hydrocortisone plus allopurinol, there was little inhibition of the hydrocortisone-induced rise of kynurenine production even if allopurinol (4 mg/100 ml medium) had been added to the medium (Figure 4).

When livers from rats pretreated with allopurinol (20 mg/kg) were perfused with 0.1 mmol/l tryptophan plus allopurinol (4 mg/100 ml medium) kynurenine production was no lower than that seen in animals not given allopurinol.

Effect of hydrocortisone and allopurinol on rat liver pyrrolase activity measured in vitro *before and after the 60 min perfusion*

As stated in the methods section, a lobe of liver was tied off both at the beginning and the end of the perfusion and pyrrolase activity measured *in vitro* on these lobes. This section gives the results of these measurements performed on rats treated as described in the last section.

When control (untreated) rat livers were perfused with 0.1 mmol/l tryptophan there was a small increase in pyrrolase activity (50%) at the end of the 60 min perfusion (Table 2). When 1.0 mmol/l tryptophan was present, activity had increased 300% at the end of perfusion (Table 2).

Injection of hydrocortisone (5 mg/kg) 3 h before the perfusion, as expected, increased initial pyrrolase activity. There was no further increase in enzyme activity at the end of the 60 min perfusion with 0.1 mmol/l tryptophan but a considerable further increase when 1.0 mmol/l tryptophan had been perfused.

If rats had been injected with hydrocortisone (5 mg/kg) and allopurinol (20 mg/kg) 3 h previously, there was a significant inhibition of the rise of activity seen after hydrocortisone alone (Table 2). While this was overcome at the end of the perfusion with 0.1 mmol/l tryptophan alone, addition of allopurinol to the medium prevented this increase. Allopurinol (4 mg/100 ml) in the medium did not, however, prevent the increase seen when 1.0 mmol/l tryptophan was perfused (Table 2).

Injection of animals with allopurinol (20 mg/kg) produced a 30% inhibition of enzyme activity 3 h later and there was a further small inhibition of activity at the end of perfusion when allopurinol (4 mg/100 ml medium) was added to the 0.1 mmol/l tryptophan medium (Table 2).

Table 2 Pyrrolase activity before and at the end of a 60 min perfusion with 0.1 mmol/l or 1.0 mmol/l tryptophan medium through livers of rats pretreated with either hydrocortisone, allopurinol or hydrocortisone plus allopurinol

Injected	*Pyrrolase activity (units) before perfusion*	*Added to medium*	*Pyrrolase activity (units) at end of 60 min perfusion with:*	
			0.1 mmol/l tryptophan	*1.0 mmol/l tryptophan*
Saline	6.32 ± 0.75 (13)	—	9.54 ± 3.16 (5)	20.7 ± 6.75 (3)
Hydrocortisone (5 mg/kg)	20.15 ± 2.20 (21)*	—	22.9 ± 3.54 (6)*	48.7 ± 3.52 (4)*
Hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg)	10.54 ± 1.85 (10)†	—	19.0 ± 6.76 (3)	35.2 ± 6.7 (3)
		Allopurinol	9.91 ± 3.64 (3)†	43.0 ± 6.2 (3)
Allopurinol (20 mg/kg)	4.04 ± 0.40 (3)‡	Allopurinol	3.41 ± 1.03 (3)‡	ND

Rats were injected with saline, hydrocortisone (5 mg/kg), allopurinol (20 mg/kg) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg), 3 h before perfusion of the livers with 0.1 mmol/l or 1.0 mmol/l tryptophan. At the start and end of the perfusion, pyrrolase activity was determined and activity is given in units (see methods section). Allopurinol (4 mg/100 ml) was added to some of the perfusion media as shown above. Results expressed as mean ± s.e. mean with number of observations in brackets. * Different from saline-injected controls, $P<0.001$. † Different from hydrocortisone-injected animals, $P<0.001$. ‡ Different from saline-injected controls, $P<0.05$. ND not determined.

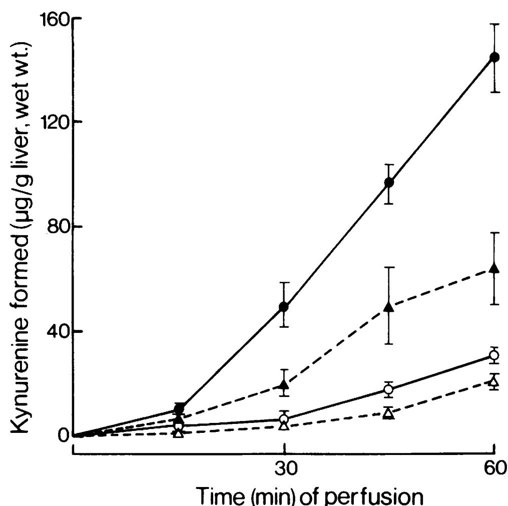

Figure 5 Effect of hydrocortisone and age of the rats on production of kynurenine in the liver perfusion medium. Rats were injected with either saline or hydrocortisone (5 mg/kg) 3 h prior to perfusion with 1.0 mmol/l tryptophan medium. Young animals approx. 35 days (solid lines) and older rats approx. 100 days (broken lines) were used. Open symbols show kynurenine production in perfusate of control animals. Solid symbols show concentration of kynurenine in perfusate of animals injected with hydrocortisone (5 mg/kg) 3 h previously. Points show mean of determinations performed on at least 3 animals with ±s.e.m. shown as vertical bars. At 60 min, old rats have a lower production of kynurenine than younger rats injected with saline ($P < 0.05$) or hydrocortisone ($P < 0.01$).

Effect of age of the rats on appearance of kynurenine in medium during perfusion with 1.0 mmol/l tryptophan medium and on in vitro *pyrrolase activity*

The previous observation that older rats have a lower *in vitro* basal pyrrolase activity and show a smaller increase in activity following hydrocortisone (Green & Curzon, 1975) was confirmed (Table 3). At the end of the 60 min perfusion with 1.0 mmol/l tryptophan, there was a rise in *in vitro* pyrrolase activity both in control and hydrocortisone-treated rats. which was similar in young and older animals (Table 3).

During perfusion with 1.0 mmol/l tryptophan medium, there was a small, but statistically significant, decrease in the amount of kynurenine appearing in the medium from older animals compared with younger animals (Figure 5) and a much larger decrease when both groups had been pretreated with 5 mg/kg hydrocortisone (Figure 4).

Effect of cycloheximide on hydrocortisone-induced increase of pyrrolase activity

We wished to observe what would happen if a protein synthesis inhibitor was given with the hydrocortisone to confirm that hydrocortisone induction of pyrrolase activity is the result of new enzyme protein being formed. Rats were injected with saline, hydrocortisone (5 mg/kg) or hydrocortisone (5 mg/kg) plus cycloheximide (30 mg/kg) 3 h before perfusion with the 1 mmol/l tryptophan medium. *In vitro* pyrrolase activity was measured, as before, at the beginning and end of perfusion.

Cycloheximide totally abolished the hydrocortisone-induced rise in pyrrolase activity observed at the beginning of perfusion. Furthermore, there was little increase in *in vitro* pyrrolase activity at the end of 1 h perfusion (Table 4). The cycloheximide pretreatment also abolished the increase of kynurenine in the medium observed when hydrocortisone alone had been injected (Table 4).

Concentration of tryptophan in the medium during liver perfusion of rats pretreated with hydrocortisone, allopurinol, hydrocortisone plus allopurinol and hydrocortisone plus cycloheximide

The concentration of tryptophan in the perfusion medium used during the experiments already

Table 3 Effect of hydrocortisone and age of rats on pyrrolase activity before and after a 60 min perfusion with 1.0 mmol/l tryptophan medium

Age (days)	*Injected*	*Pyrrolase activity (units): before perfusion*	*after perfusion*
35	Saline	6.32±0.75 (13)	20.7±6.75 (3)
100	Saline	4.12±1.60 (3)	21.4±0.44 (3)
35	Hydrocortisone	20.15±2.20 (21)	48.7±3.52 (4)
100	Hydrocortisone	10.03±2.96 (3)*	42.8±5.7 (3)

Rats were injected with saline or hydrocortisone (5 mg/kg) 3 h before perfusion. Pyrrolase activity determined at start of perfusion and at end of 60 min perfusion with 1.0 mmol/l tryptophan medium. Pyrrolase activity expressed in units (see methods section). Results expressed as mean±s.e. mean with number of observations in brackets. * Different from 35 day hydrocortisone-treated rats, $P < 0.01$.

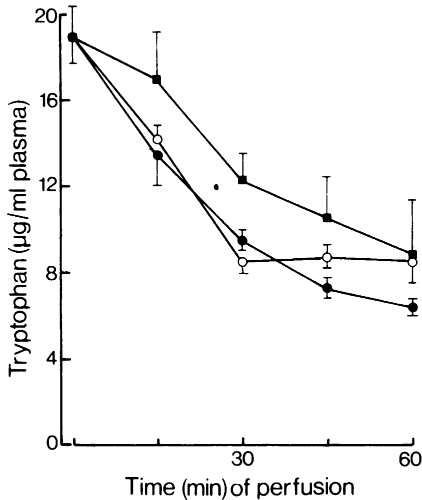

Figure 6 Concentration of tryptophan in medium during a 60 min incubation; effect of hydrocortisone or hydrocortisone plus allopurinol pretreatment. The change in tryptophan concentration during perfusion with 0.1 mmol/l tryptophan medium is shown. Rats were injected 3 h before the start of perfusion with saline (○), hydrocortisone (5 mg/kg) (●) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg) with allopurinol (4 mg/100 ml) added to the perfusion medium (■).

described was also studied. The decline in tryptophan concentration in the 0.1 mmol/l tryptophan medium was very similar in the perfusate from livers of rats pretreated either with hydrocortisone (5 mg/kg) or saline. There was a slower initial decline in tryptophan concentration in the perfusate from livers of rats pretreated with hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg) provided that allopurinol (4 mg/100 ml) had also been added to the medium (Figure 6). However, the tryptophan concentration at 1 h was no different from that found in the control animals.

When 1.0 mmol/l tryptophan was perfused through livers of rats pretreated with hydrocortisone (5 mg/kg), there was an initial rapid drop in tryptophan concentration which was also seen in saline-injected controls. However, while the tryptophan concentration in the perfusate of hydrocortisone-treated rats continued to decline steadily, that of the control animals decreased only slightly during the remaining 45 min (Figure 7). The decrease in tryptophan concentration in the perfusate from livers of rats pretreated with hydrocortisone (5 mg/kg) and allopurinol (20 mg/kg), even with allopurinol (4 mg/100 ml) in the medium, showed little difference from the perfusate of rats injected with hydrocortisone only (Figure 7).

Injection of cycloheximide (30 mg/kg) with hydrocortisone (5 mg/kg) was effective in preventing the additional tryptophan decrease in the 1 mmol/l tryptophan perfusate produced by hydrocortisone rather than saline (Table 5).

The concentration of tryptophan at the end of perfusion through livers of 100 day old rats was lower than when 35 day rats were used (Table 5). The further decrease in tryptophan concentration produced by pretreatment with hydrocortisone (5 mg/kg) compared to saline-injected rats was also greater in older rats (Table 5).

Concentration of tryptophan in the liver 30 min after the start of perfusion with a medium containing 0.1 mmol/l or 1.0 mmol/l tryptophan

A lobe of liver was tied off at the start of perfusion and the tryptophan concentration measured. Thirty

Table 4 Effect of cycloheximide on the hydrocortisone-induced rise of pyrrolase activity and production of kynurenine in the perfusion medium of livers perfused with 1.0 mmol/l tryptophan

Injected	*Kynurenine in medium at end of 60 min perfusion (µg ml⁻¹ g⁻¹ liver, wet wt)*	*Pyrrolase activity (units):* before perfusion	at end of perfusion
Saline	31.1 ± 3.1 (3)	6.32 ± 0.75 (13)	20.7 ± 6.75 (3)
Hydrocortisone (5 mg/kg)	145.0 ± 13.3 (8)*	20.15 ± 2.20 (21)†	48.7 ± 3.52 (4)†
Hydrocortisone (5 mg/kg) plus Cycloheximide (30 mg/kg)	32.4 ± 8.7 (4)‡	6.53 ± 0.67 (4)§	10.2 ± 2.8 (4)§

Results show production of kynurenine at end of 60 min perfusion and pyrrolase activity measured *in vitro* at beginning and end of perfusion with 1.0 mmol/l tryptophan. Rats were injected with saline, hydrocortisone or hydrocortisone plus cycloheximide 3 h before perfusion. Results are expressed as mean ± s.e. mean with number of determinations in brackets. *Different from saline, $P<0.05$. †Different from saline, $P<0.01$. ‡ Different from hydrocortisone, $P<0.05$. § Different from hydrocortisone, $P<0.01$.

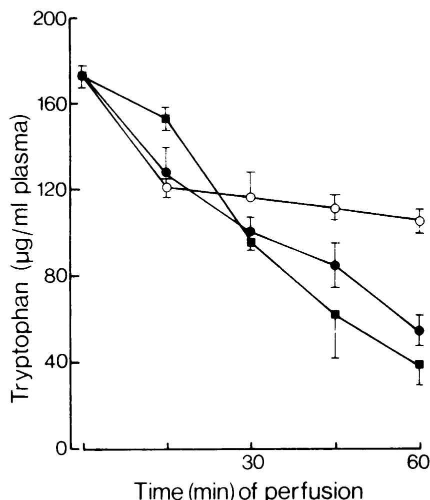

Figure 7 Concentration of tryptophan in medium during a 60 min incubation; effect of hydrocortisone or hydrocortisone plus allopurinol pretreatment. The change in tryptophan concentration during perfusion with 1.0 mmol/l tryptophan medium is shown. Rats were injected 3 h before the start of perfusion with saline (O), hydrocortisone (5 mg/kg) (●) or hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg) with allopurinol (4 mg/100 ml) added to the perfusion medium (■).

minutes later following perfusion with either 0.1 mmol/l or 1.0 mmol/l tryptophan a further lobe of liver was taken and tryptophan concentration in these lobes also measured. While tryptophan was not concentrated by the liver during perfusion with 0.1 mmol/l tryptophan, there was some tryptophan uptake during perfusion with 1.0 mmol/l tryptophan (Table 6).

Discussion

The first major point to be clarified is whether the appearance of kynurenine in the perfusion medium is indicative of tryptophan pyrrolase activity. Tryptophan pyrrolase is the first enzyme on the kynurenine pathway (Figure 1) converting L-tryptophan to L-formylkynurenine (Knox & Mehler, 1950) which is metabolized rapidly to kynurenine by formylase, an enzyme present in excess in the liver (Mehler & Knox, 1950). The normal metabolite of pyrrolase activity detected in the *in vitro* assay therefore is kynurenine.

Kynurenine is metabolized to other metabolites detectable in urine after a tryptophan load to various species, including rat and man (Brown & Price, 1956; McDaniel, Hundley & Sebrell, 1956; Korbitz, Price & Brown, 1963; Leklem, Woodford & Brown, 1969; Leklem, 1971).

In our experiments, the mean tryptophan loss from

Table 5 Concentration of tryptophan in medium at end of 60 min perfusion (with 1.0 mmol/l or 0.1 mmol/l tryptophan) of livers from rats of different ages pretreated with hydrocortisone, hydrocortisone plus allopurinol, allopurinol or hydrocortisone plus cycloheximide

Age (days)	*Injected (3 h previously)*	*Added to medium*	*Tryptophan conc (μg/ml plasma) 60 min after start of perfusion with:* 0.1 mmol/l tryptophan	1.0 mmol/l tryptophan
35	Saline	—	8.5 ±0.6 (3)	106.0 ± 5.6 (3)
35	Hydrocortisone (5 mg/kg)	—	6.32 ± 0.34 (5)	54.0 ± 7.0 (6)*
35	Hydrocortisone (5 mg/kg)	—	6.71 ± 1.5 (3)	50.0 ± 6.0 (3)
	plus allopurinol (20 mg/kg)	Allopurinol	8.8 ± 2.5 (3)	39.0 ± 10.0 (3)
35	Allopurinol (20 mg/kg)	Allopurinol	10.0 ± 1.8 (3)	ND
35	Hydrocortisone (5 mg/kg) plus cycloheximide (30 mg/kg)	—	ND	116.0 ± 15.0 (3)†
100	Saline	—	ND	34.0 ± 6.0 (3)
100	Hydrocortisone (5 mg/kg)	—	ND	8.6 ± 3.0 (3)

Rats were injected 3 h before the start of perfusion with either saline, hydrocortisone (5 mg/kg), hydrocortisone (5 mg/kg) plus allopurinol (20 mg/kg), allopurinol (20 mg/kg) or hydrocortisone (5 mg/kg) plus cycloheximide (30 mg/kg). In some experiments allopurinol (4 mg/100 ml) was added to the medium as shown above. In another group of experiments, 100 day old rats were pretreated with saline or hydrocortisone (5 mg/kg) for comparison with younger rats. Results show the concentration of tryptophan in the medium at the end of a 60 min perfusion with either 0.1 mmol/l or 1.0 mmol/l tryptophan medium. Results expressed in μg tryptophan/ml plasma and show mean s.e. mean with number of determinations in brackets. ND = not determined. * Different from saline-injected, $P < 0.01$. † Different from hydrocortisone-injected, $P < 0.01$.

the medium containing 1 mmol/l tryptophan in the first 30 min of perfusion was 6700 μg; 292 μg had accumulated in liver (Table 6) and a further 63 μg was recovered as kynurenine in the medium. Thus out of 20.4 mg of tryptophan added to the medium 5.345 mg is unaccounted for (25%). Kynurenine is only a small proportion of the total metabolites of the kynurenine pathway found in urine after a tryptophan load (see review by Leklem, 1971) so it seems reasonable to assume that the tryptophan not accounted for is present as other metabolic products. No evidence was found for the presence of 5-hydroxytryptamine or 5-hydroxyindoleacetic acid in the medium under our conditions. When similar calculations were applied to our studies using 0.1 mmol/l tryptophan it was found that there was a recovery of somewhat more than 50% of the tryptophan after 30 minutes.

Clearly kynurenine can only reflect the state of flux down the pathway and is not the sole product of pyrrolase activity. Nevertheless, it seems to be an index of tryptophan degradation, a view strengthened by the fact that little kynurenine appears in the medium in the absence of tryptophan in the perfusate (basal medium).

The method for kynurenine determination is not completely specific (see methods section). However, the known interfering compounds are other products on the pyrrolase pathway, so that the compounds being measured as kynurenine almost certainly represent an index of pyrrolase activity. During the final stages of this investigation, a more specific method for determining plasma kynurenine was developed (Joseph & Risby, 1975). It produced qualitatively similar results when applied to some of the samples in this study, although the actual kynurenine values were about 50% lower.

The observation that the tryptophan in the medium rose during perfusion with the basal medium suggests that the liver releases tryptophan either from endogenous stores or protein degradation when the circulating amino acid concentration is low. This also occurs with some other amino acids (Schimassek & Gerok, 1965). It is not known however whether a similar release occurs when tryptophan is present in the medium. Exchange diffusion of tryptophan has certainly been observed in brain tissue (Parfitt & Grahame-Smith, 1974).

Kynurenine concentration increased to some extent during the second half of the perfusion with 0.1 mmol/l tryptophan and this observation, taken together with the increase in enzyme activity at the end of the perfusion measured *in vitro* (Table 2) and the rapid drop in plasma tryptophan during the first 30 min suggests some degree of enzyme induction. This is not too surprising if one assumes that plasma free tryptophan only is directly available to the liver (for a discussion of the role of free and bound tryptophan, see Curzon & Knott, 1974). A perfusate tryptophan concentration of 0.1 mmol/l is somewhat higher than that found under physiological conditions and since albumin is only at half physiological concentration more free tryptophan is available than normal to induce the enzyme. It may be that plasma tryptophan concentration becomes steady at 8 μg/ml which would now correspond to a physiological free tryptophan concentration. This further suggests that tryptophan is only metabolized by pyrrolase when the concentration of unbound amino acid is greater than normal, thereby assisting tryptophan economy when necessary.

Although Kim & Miller (1969) were unable to observe any increase in kynurenine concentration in the perfusion media of rats treated with hydrocortisone compared with control animals, we observed a four-fold increase both during perfusion with 0.1 mmol/l and 1.0 mmol/l tryptophan. This difference is probably attributable to the different technique employed. We began to perfuse when the enzyme had already been induced but Kim & Miller perfused the liver for several hours and then added hydrocortisone to the medium. Since steroid induction of pyrrolase involves the formation of new enzyme protein (Feigelson & Greengard, 1962) it is not, perhaps, surprising that little new enzyme protein was formed in livers perfused with a rabbit blood medium (John & Miller, 1966) which may have been deficient

Table 6 Concentration of tryptophan in the isolated perfused rat liver 30 min after start of perfusion

Time after start of perfusion (min)	*Concentration of tryptophan in medium (mmol/l)*	*Concentration of tryptophan in liver (μg/g liver, wet wt)*	*Total tryptophan accumulated by liver (μg)**
0	—	8.65 ± 0.92 (6)	—
30	0.1	9.80 ± 1.20 (3)	13.8 ± 3.2 (3)
30	1.0	48.93 ± 8.68 (4)	292 ± 53.4 (4)

* Calculated on basis of initial and final concentration of tryptophan in liver of individual experiments multiplied by the liver weight of that experiment. Results show mean ± s.e. mean with number of observations in brackets. Concentration of tryptophan in liver at 0 min was performed before the start of experiment. There is some error in these results deriving from plasma tryptophan trapped in the blood vessels of the liver. Extracellular space in the liver has been calculated at 22.2% (Woods, Eggleston & Krebs, 1970).

in some of the factors required for synthesizing new enzyme. The present results demonstrate clearly that hydrocortisone increases enzyme activity in the intact organ. This effect is apparent during subsequent perfusion and is consistent with previous observations demonstrating an increase in pyrrolase activity *in vivo* by measurement of the rate of degradation of labelled tryptophan (Green *et al.*, 1975b; Young & Sourkes, 1975).

Cycloheximide blocked the hydrocortisone-produced increase in kynurenine formation; other protein synthesis inhibitors have been shown to have similar effects (Gray, Camiener & Bhuyan, 1964; Valyi-Nagy & Doroczy, 1967). While nogalamycin does not alter substrate induction (Gray *et al.*, 1964), Korner & Labrie (1967) showed that actinomycin inhibited substrate induction. Cycloheximide also inhibited substrate induction occurring during the perfusion (Table 5) which argues against the view that tryptophan induces pyrrolase solely through substrate stabilization (Shimke, Sweeney & Berlin, 1964). Perfusion with 1.0 mmol/l tryptophan increased *in vitro* enzyme activity at the end of perfusion, both in control and hydrocortisone-pretreated animals. This increase was not affected by age, a finding consistent with the fact that substrate induction is not altered in older rats (Correll, Turner & Haining, 1965; Haining & Correll, 1966).

The mechanism by which allopurinol produces its inhibitory action on tryptophan pyrrolase *in vivo* and *in vitro* has been the subject of some debate (see Chytil, 1968; Becking & Johnson, 1969; Julian & Chytil, 1970). Badawy & Evans (1973) demonstrated that allopurinol prevented conjugation of the apoenzyme with its haem activator. In our study allopurinol inhibited the hydrocortisone-induced rise of kynurenine formation but was only really effective when allopurinol was also added to the medium (see Figures 3 and 4, and results in legends), suggesting that it was otherwise being 'washed out' of the liver and inhibition occurred at physiological concentrations of tryptophan (0.1 mmol/l) but not under load conditions (1.0 mmol/l).

There has been discussion as to whether allopurinol might prove useful in preventing breakdown of tryptophan in the liver when the amino acid is given to depressed patients (Badawy & Evans, 1973, 1974; Young & Sourkes, 1974; Fernando, Joseph & Curzon, 1975). To find out whether allopurinol was not inhibiting pyrrolase at higher tryptophan concentrations because its concentration was too low we injected rats with 40 mg/kg allopurinol with hydrocortisone, also adding 10 mg/100 ml to the 1.0 mmol/l tryptophan medium. Even at this high dose of allopurinol, there was no significant decrease in kynurenine production by the isolated perfused liver. While these results indicate that allopurinol may be ineffective as an inhibitor of pyrrolase when a tryptophan load is given, it should be pointed out that Fernando *et al.* (1975) did observe that allopurinol administration increased the concentration of tryptophan in the brain following a tryptophan load. Further, 1 mmol/l is considerably higher than the plasma tryptophan concentration observed in subjects given a 50 mg/kg load (Green & Woods, unpublished observations).

In agreement with Kim & Miller (1969), the rate of tryptophan disappearance from the medium had little connection with previous drug treatment. However, the rate of tryptophan disappearance from the 1.0 mmol/l tryptophan medium was about 10 times faster than from the 0.1 mmol/l tryptophan medium, which is consistent with the fact that this 10-fold increase in tryptophan concentration also resulted in a 10-fold increase in the appearance of kynurenine. The rate of tryptophan disappearance from the 0.1 mmol/l tryptophan medium up to 30 min and the subsequent levelling off is very similar to that observed by Ng, Hagino, Swan & Henderson (1970). Both Kim & Miller (1969) and Ng *et al.* (1970) reported that under load conditions the liver initially removed around 2 mg tryptophan/g liver in one hour. Our results show an initial rate of removal of 2.4 mg tryptophan/g liver in one hour.

The liver perfusion experiments described, clearly do not represent an *in vivo* situation; for example, the medium lacks the presence of other amino acids that compete with tryptophan for uptake into the liver. Nevertheless as a perfused whole organ it is nearer to the *in vivo* situation than a liver homogenate preparation.

In general, there was good correlation between kynurenine appearance in the medium and enzyme activity measured *in vitro* at the beginning of the perfusion. For example, hydrocortisone caused a rise in enzyme activity and kynurenine production by the same factor (four-fold) and allopurinol and cycloheximide reduced the increase in enzyme activity and blocked the increase in kynurenine formation.

The *in vitro* results following hydrocortisone show a kynurenine production of around 20 μmol h^{-1} g^{-1} (dry weight). Conversion of this figure to μg kynurenine h^{-1} g^{-1} (wet weight) using the wet/dry wt ratio of 3.45 (Woods & Krebs, 1971) reveals a value of 1205 μg h^{-1} g^{-1}. Kynurenine production by the isolated perfused liver was 20 μg h^{-1} g^{-1}. Even allowing for the fact that *in vitro* the tryptophan concentration is 3.0 mmol/l whereas it is 1.0 mmol/l in the perfusion, and the fact that the kynurenine is perhaps metabolized further in the perfusion system, these figures reveal that kynurenine production in the 'ideal' situation of the *in vitro* assay with added co-factors bear little relationship in quantitative terms to the *in vivo* situation although as discussed earlier it appears to give a qualitative index of *in vivo* tryptophan pyrrolase activity.

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