Biochem. J. (1975) **150**, 511–520
Printed in Great Britain

The Regulation of Rat Liver Tryptophan Pyrrolase by Its Cofactor Haem

EXPERIMENTS WITH HAEMATIN AND 5-AMINOLAEVULINATE AND COMPARISON WITH THE SUBSTRATE AND HORMONAL MECHANISMS

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(*Received* 11 *April* 1975)

1. The administration of haematin or 5-aminolaevulinate to rats enhances the activity of liver tryptophan pyrrolase; both endogenous and newly formed apoenzymes become strongly haem-saturated. Haem activation does not stabilize tryptophan pyrrolase. 2. Actinomycin D, puromycin or cycloheximide prevent the activation of the enzyme by 5-aminolaevulinate but not that by haematin. The latter is inhibited by haem-destroying porphyrogens. 3. The combined injection of either haematin or 5-aminolaevulinate with cortisol does not produce an additive effect, whereas potentiation is observed when tryptophan is jointly given with either the cofactor or the haem precursor. 4. Further experiments on the substrate (tryptophan) mechanism of pyrrolase regulation are reported, and a comparison between this and the cofactor and hormonal mechanisms is made. 5. It is suggested that the substrate mechanism may also involve increased haem synthesis. 6. The role of tryptophan pyrrolase in the utilization of liver haem, and as a possible model for the exacerbation by drugs of human hepatic porphyrias, is discussed.

Tryptophan pyrrolase (L-tryptophan–O_2 oxidoreductase, EC 1.13.11.11) is the haem-dependent liver cytosol enzyme that catalyses the conversion of L-tryptophan into formylkynurenine. In rat (Feigelson & Greengard, 1961) or human (Altman & Greengard, 1966) liver, but not in livers of certain animal species (Badawy & Evans, 1974, and references therein), the pyrrolase exists in two forms. The already active holoenzyme does not require the addition of exogenous haematin for demonstration of its activity *in vitro*, whereas the haem-free predominant form or apoenzyme does. By using the catalytic property of the rat liver enzyme, labelled amino acid incorporation into its purified protein and immunological titration techniques (Knox, 1966; Schimke, 1969), it has been possible clearly and conclusively to distinguish two mechanisms by which the pyrrolase activity is regulated. Glucocorticoids cause a hormonal-type induction involving the synthesis of new apoenzyme, whereas tryptophan produces a substrate-type enhancement consisting of decreased degradation of pre-existing apoenzyme in the presence of the normal rate of its synthesis. The ratio of holoenzyme/apoenzyme activity in cortisol-treated rats resembles that of the basal enzyme (less than 1), thus suggesting that about 50% of the newly synthesized apoenzyme becomes conjugated with haem, whereas the tryptophan-activated enzyme exhibits a larger degree of haem saturation (see, e.g., Badawy & Evans, 1973). This action of tryptophan *in vivo*, together with its activation of the enzyme *in vitro*, have been suggested (Knox, 1966) to involve the promotion of the conjugation of the apoenzyme with haem and the subsequent reduction of the oxidized holoenzyme.

There is evidence suggesting that tryptophan pyrrolase activity may also be subject to regulation by its cofactor haem. Druyan & Kelly (1972) reported that the administration of repeated doses of the porphyrin and haem precursor 5-aminolaevulinate increases the saturation, in rat liver, of endogenous apo-(tryptophan pyrrolase) with its haem activator. We have subsequently confirmed this finding, and also demonstrated (Badawy & Evans, 1973) that the haem saturation of the apoenzyme can be modified by changes in the metabolism of porphyrins and haem. Thus the haem saturation of apo-(tryptophan pyrrolase) is decreased by agents that destroy haem or inhibit its synthesis, whereas the opposite is true for treatments increasing the utilization or the synthesis of this pigment.

In the present paper, the activation of rat liver tryptophan pyrrolase by the administration of haematin hydrochloride is demonstrated. The nature of this and of the activation of the enzyme by 5-aminolaevulinate are examined. Full time-courses of the effects on both forms of the pyrrolase of cortisol

and tryptophan administration are presented, further studies on the substrate mechanism are performed and a comparison of all three regulatory mechanisms is made. It is suggested that tryptophan may also act by increasing haem synthesis. It is also proposed that tryptophan pyrrolase may play a significant role in the utilization of liver haem, and that it may be considered as a model for the exacerbation by drugs of human genetic porphyria.

Materials and Methods

Chemicals

Actinomycin D and 2-allyl-2-isopropylacetamide were gifts respectively from Merck, Sharp and Dohme Ltd., Hoddesdon, Herts., U.K., and Roche Products Ltd., Welwyn Garden City, Herts., U.K. 5-Aminolaevulinate hydrochloride, cortisol 21-acetate, cycloheximide, dimethylformamide, griseofulvin (7-chloro-6-β-methylspiro-2',4,6-trimethoxybenzofuran-2(3H),1'[2]-cyclohexene-3,4'-dione), haemin (haematin hydrochloride), puromycin dihydrochloride and L-tryptophan were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (from Kodak Ltd., Kirkby, Liverpool, U.K.) was recrystallized from ethanol. All other chemicals (from BDH Chemicals Ltd., Poole, Dorset, U.K.) were of AnalaR grade.

Animals and injections

Inbred male Wistar rats (150–235g), maintained on cube diet MRC no. 41B and water *ad libitum*, were killed between 13:30 and 15:15h by stunning and cervical dislocation. Most chemicals were given intraperitoneally at various times before death as indicated in the Results section. 2-Allyl-2-isopropylacetamide (400mg/kg body wt., dissolved in dimethylformamide) was injected into the loose subcutaneous tissues of the neck; each rat received 0.1ml/100g body wt. Cortisol acetate (20mg/kg), 3,5-diethoxycarbonyl-1,4-dihydrocollidine (150mg/kg), griseofulvin (100mg/kg) and haematin hydrochloride (35mg/kg) were dissolved in dimethylformamide; each rat received 0.1ml/100g body wt. The injection of an equal volume of dimethylformamide has been shown (Badawy & Evans, 1973) to resemble that of 0.9% (w/v) NaCl in not significantly affecting tryptophan pyrrolase activity. Doses of dimethylformamide exceeding 1ml/kg body wt. increased the enzyme activity by a hormonal-type mechanism (A. A.-B. Badawy, unpublished work).

Actinomycin D (0.7mg/kg) and puromycin dihydrochloride (35mg/kg) were dissolved in 0.9% NaCl; each rat received 0.1–0.2ml/100g body wt. Cycloheximide (50mg) was dissolved in 0.4ml of 1M-NaOH and diluted to 2ml with 0.9% NaCl after the pH had been adjusted to 7.3 with 1M-HCl; each rat received 0.2ml/100g body wt. 5-Aminolaevulinate hydrochloride (dissolved in 0.9% NaCl) was given in up to four injections at 1h intervals (15mg/kg each) and the animals were killed at 1h after the last injection. Tryptophan (500mg) was dissolved in the minimum amount of 4M-NaOH and diluted to 20ml with 0.9% NaCl after the pH had been adjusted to 7.3 with 1M-HCl; each rat received 2ml/100g body wt. In some experiments, a smaller dose of tryptophan (5mg/100g body wt.) was given in a volume of 1ml/100g body wt.

Preparation of homogenates

The liver was removed within 10s of the death of the animal, and was homogenized for 1min at 1100rev./min in 6.5vol. of 140mM-KCl–2.5mM-NaOH at 0°C in a glass homogenizer with a loose-fitting Teflon pestle. The homogenates were incubated within 4–8min 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) in either the absence (holoenzyme activity) or the presence (total enzyme activity) of added haematin. The apoenzyme activity, calculated by difference, was then used to measure the ratio of holoenzyme/apoenzyme activity, which indicates the degree of haem saturation of the apoenzyme. 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 final concentration (2μM) that was optimum (Badawy & Evans, 1974) for enzyme activation. Samples (3ml) of the assay mixture were incubated at 37°C for 0, 15, 30, 45, 60 and 75min with shaking (120 oscillations/min) in stoppered 25ml conical flasks in an atmosphere of O_2. The reaction was stopped at each of the above time-intervals 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 by using $\varepsilon = 4540 \text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. A lag phase persisted for the first 30–45min of incubation of the basal enzyme and the enzyme induced by the administration of cortisol before the activity became linear with time. The enzyme activated by the administration of tryptophan, haematin or 5-aminolaevulinate, on the other hand, had the lag phase either abolished or shortened to 15min. Tryptophan pyrrolase activity was calculated

from the increase in the E_{365} with time during the linear phase. For an extinction range of 0.1–1.0, the line representing the linear phase exactly covered points at three to four consecutive time-intervals. The validity of this visual method was mathematically assessed in a number of experiments, and was found satisfactory (r not less than 0.97). In the experiments *in vitro*, cycloheximide was dissolved in the sodium phosphate buffer with the help of a little 0.1 M-NaOH, adjusted to pH7.3 with 0.1 M-HCl and added immediately before incubation.

Determination of the half-life of tryptophan pyrrolase

The enzyme activity was determined at 3 h after the administration of various compounds as indicated in the Results section. Cycloheximide (50 mg/kg body wt.) was then injected (i.e. at 3 h after the above treatments) and the activity was again determined 1 and 2 h later. The pyrrolase half-life was calculated from the rate of decline in enzyme activity, at 0, 1 and 2 h after cycloheximide administration, as plotted semi-logarithmically.

Results

Time-course of the effects of haematin, 5-aminolaevulinate, tryptophan and cortisol on rat liver tryptophan pyrrolase activity

The intraperitoneal administration of haematin hydrochloride enhanced the enzyme activity (Fig. 1). At 2 h, there was a small (28%) but significant ($P<0.05$) increase in the total pyrrolase activity, but more striking was the 200% increase ($P<0.001$) in that of the holoenzyme. The apoenzyme was therefore strongly saturated with its haem activator, as can also be judged from the increase in the ratio of holoenzyme/apoenzyme activity from a basal value of 0.56 at 0 h to 3.89 at 2 h after haematin administration. This

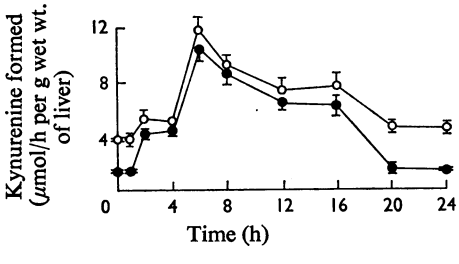

Fig. 1. *Time-course of the effect of haematin administration on rat liver tryptophan pyrrolase activity*

Haematin hydrochloride (35 mg/kg body wt.) was injected at zero time. Each point represents the mean value for four rats±S.E.M. The enzyme activity was determined as described in the Materials and Methods section. ○, Total enzyme activity (that measured in the presence of 2 μM-haematin); ●, holoenzyme activity (that in the absence of added cofactor).

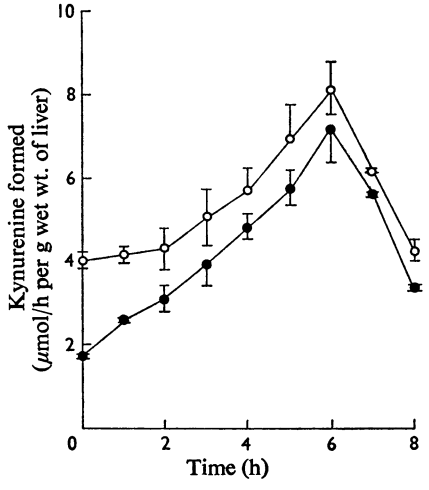

Fig. 2. *Time-course of the effect of repeated injections of 5-aminolaevulinate on rat liver tryptophan pyrrolase activity*

5-Aminolaevulinate hydrochloride was injected (at zero time) in up to four doses at 1 h intervals (15 mg/kg body wt. each) and the animals were killed at 1 h after the last injection. Each point represents the mean value for four rats±S.E.M. The enzyme activity was determined as described in the Materials and Methods section. ○, Total enzyme activity (that measured in the presence of 2 μM-haematin); ●, holoenzyme activity (that in the absence of added cofactor).

saturation remained unaltered for 2 h more, then both total enzyme and holoenzyme activities rose to a maximum (3.1- and 7.4-fold respectively) at 6 h and finally fell to normal values at 20–24 h after the injection. The ratio of holoenzyme/apoenzyme activity was increased (range 3.89–12.98) during the entire time-course of this enhancement.

The holo-(tryptophan pyrrolase) activity was significantly ($P<0.001$) increased (by 53%) at 1 h after a single intraperitoneal injection of 5-aminolaevulinate hydrochloride, whereas that of the total enzyme remained unaltered (Fig. 2). With repeated injections of the haem precursor at 1 h intervals, both pyrrolase activities continued to rise, with the apoenzyme being strongly saturated with its haem activator. Maximum increases (2.1–4.1-fold) were observed, as with haematin, at 6 h after the first of the 5-aminolaevulinate injections, and, although the total pyrrolase activity returned to the basal value at 8 h, that of the holoenzyme was still elevated, by twofold.

The time-course of the effects of an intraperitoneal injection of tryptophan on its pyrrolase activity is shown in Fig. 3. The total enzyme activity was not significantly altered at 1 or 2 h ($P>0.10$), whereas that of the holoenzyme was increased (by 94–100%, $P<0.001$). Both activities then rose to a maximum value (5.6–8.8-fold) at 4 h before returning to normal after

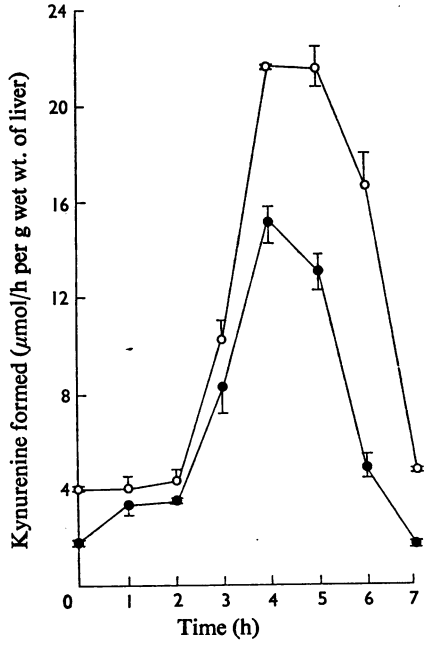

Fig. 3. *Time-course of the effect of tryptophan administration on rat liver tryptophan pyrrolase activity*

Tryptophan (500mg/kg body wt.) was injected at zero time. Each point represents the mean value for four rats ±S.E.M. The enzyme activity was determined as described in the Materials and Methods section. ○, Total enzyme activity (that measured in the presence of 2μM-haematin); ●, holoenzyme activity (that in the absence of added cofactor).

7h. The ratio of holoenzyme/apoenzyme activity was also increased (from a basal value of 0.74) to 5.18, 4.68, 4.00, 2.28 and 1.53 at 1, 2, 3, 4 and 5h after tryptophan administration.

When cortisol was administered (Fig. 4), the rise in the holoenzyme activity did not precede that in the total activity, and, whereas a 67% increase in the latter was significant ($P<0.025$) at 2h, the 47% rise in the former was not ($P>0.10$). The increase in the total pyrrolase activity observed at 3–8h (with a maximum 8.6-fold increase at 5h) was matched by a proportionate rise in that of the holoenzyme (with a maximum 7.7-fold increase at 5h). During the entire time-course of the cortisol induction of tryptophan pyrrolase activity, and before a return to normal at 12h, the ratio of holoenzyme/apoenzyme activity varied between 0.56 and 0.90 as compared with a basal ratio of 0.74.

Mechanisms of action of haematin, 5-aminolaevulinate, tryptophan and cortisol on rat liver tryptophan pyrrolase activity

The effects of inhibitors of RNA and protein synthesis on the enhancement of rat liver tryptophan pyrrolase activity by haematin, 5-aminolaevulinate, tryptophan or cortisol are shown in Table 1. Pretreatment of rats with actinomycin D prevented the enhancement of the enzyme activity by 5-aminolaevulinate or cortisol but did not significantly affect that by haematin or tryptophan. The enhancement of the pyrrolase activity by 5-aminolaevulinate, tryptophan or cortisol, but not that by haematin, was also prevented by pretreatment of rats with the inhibitors of protein synthesis, puromycin and cycloheximide.

The effects of pretreatment of rats with the porphyrogens 2-allyl-2-isopropylacetamide, 3,5-diethoxycarbonyl-1,4-dihydrocollidine or griseofulvin on the activation of liver tryptophan pyrrolase by haematin administration are shown in Table 2. Given alone, each of the above three porphyrogens increased the total pyrrolase activity and simultaneously decreased the haem saturation of the newly formed apoenzyme (see also Badawy & Evans, 1973). Pretreatment of rats with 2-allyl-2-isopropylacetamide or 3,5-diethoxycarbonyl-1,4-dihydrocollidine decreased the haematin-induced rise in the holoenzyme activity by 100 and 79% respectively. Griseofulvin

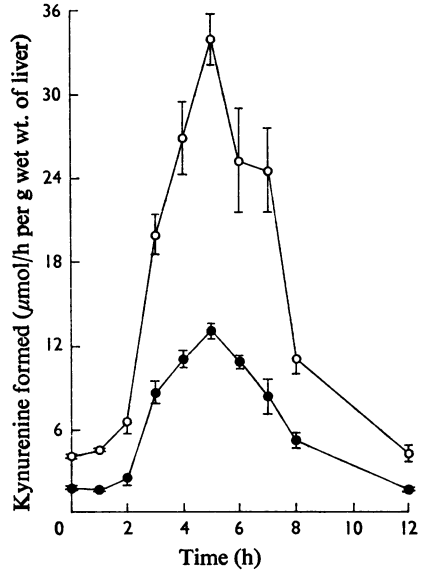

Fig. 4. *Time-course of the effect of cortisol administration on rat liver tryptophan pyrrolase activity*

Cortisol acetate (20mg/kg body wt.) was injected at zero time. Each point represents the mean value for four rats ±S.E.M. The enzyme activity was determined as described in the Materials and Methods section. ○, Total enzyme activity (that measured in the presence of 2μM-haematin); ●, holoenzyme activity (that in the absence of added cofactor).

Table 1. *Effects of pretreatment of rats with actinomycin D, puromycin or cycloheximide on the enhancement of liver tryptophan pyrrolase activity by haematin, 5-aminolaevulinate, tryptophan and cortisol*

Each rat received an intraperitoneal injection of either 0.9% (w/v) NaCl, haematin hydrochloride (35mg/kg), cortisol acetate (20mg/kg) or tryptophan (500mg/kg body wt.) 3h before being killed. 5-Aminolaevulinate hydrochloride was given in three injections at 1h intervals (15mg/kg body wt. each) and the animals were killed at 1h after the last injection. Where necessary, actinomycin D (0.7mg/kg), puromycin dihydrochloride (35mg/kg) or cycloheximide (50mg/kg body wt.) was administered 1h before any of the above treatments. The enzyme activity was determined, as described in the Materials and Methods section, in the absence (holoenzyme activity) as well as in the presence (total enzyme activity) of added haematin (2μM). Values are means±S.E.M. of each group of four rats.

Injection	Tryptophan pyrrolase activity (μmol of kynurenine formed/h per g wet wt. of liver)			
Pretreatment ...	Nil	Actinomycin D	Puromycin	Cycloheximide
0.9% NaCl				
Holoenzyme activity	1.6±0.1	1.7±0.2	1.5±0.1	1.3±0.1
Total enzyme activity	4.0±0.2	3.8±0.4	4.0±0.1	1.7±0.1
Haematin				
Holoenzyme activity	4.5±0.2	4.8±0.4	4.2±0.1	3.0±0.1
Total enzyme activity	4.5±0.1	4.9±0.5	4.3±0.4	3.0±0.1
5-Aminolaevulinate				
Holoenzyme activity	3.9±0.5	1.8±0.2	2.0±0.1	1.5±0.2
Total enzyme activity	5.1±0.6	2.8±0.3	3.5±0.3	1.9±0.2
Cortisol				
Holoenzyme activity	8.6±0.8	1.4±0.1	1.6±0.1	1.5±0.1
Total enzyme activity	19.9±1.4	3.4±0.2	4.3±0.3	1.9±0.1
Tryptophan				
Holoenzyme activity	8.2±1.2	7.4±0.8	1.8±0.2	1.9±0.3
Total enzyme activity	10.2±0.8	7.8±0.8	4.4±0.6	3.0±0.3

Table 2. *Effects of pretreatment of rats with the porphyrogens 2-allyl-2-isopropylacetamide, 3,5-diethoxycarbonyl-1,4-dihydrocollidine and griseofulvin on the activation of liver tryptophan pyrrolase by haematin administration*

Each rat received an injection of either 2-allyl-2-isopropylacetamide (400mg/kg body wt.), 3,5-diethoxycarbonyl-1,4-dihydrocollidine (150mg/kg) or griseofulvin (100mg/kg) 4h before being killed. In addition, some rats received any of the above three compounds 1h before an intraperitoneal injection of haematin hydrochloride (35mg/kg). 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). Values are means±S.E.M. of each group of four rats.

	Kynurenine formed (μmol/h per g wet wt. of liver)			
	Untreated rats		Haematin-treated rats	
Pretreatment	Holoenzyme activity	Total enzyme activity	Holoenzyme activity	Total enzyme activity
Nil	1.6±0.1	4.0±0.2	4.5±0.2	4.5±0.1
2-Allyl-2-isopropylacetamide	2.2±0.1	10.8±0.7	2.1±0.2	4.0±0.3
3,5-Diethoxycarbonyl-1,4-dihydrocollidine	2.1±0.1	8.3±0.6	2.7±0.0	3.8±0.1
Griseofulvin	3.7±0.6	16.1±0.9	5.9±0.6	6.0±0.8

pretreatment did not prevent the increase in the holoenzyme activity observed in rats subsequently given haematin.

The effects on rat liver tryptophan pyrrolase activity of the administration of haematin, 5-aminolaevulinate, cortisol or tryptophan, either singly or in various combinations, are shown in Table 3. By subtracting the holoenzyme and total pyrrolase activities of 0.9%-NaCl-treated rats from the corresponding values of each single or double treatment, the following observations were made: (1) the administration of haematin, 5-aminolaevulinate or tryptophan rendered the cortisol-induced enzyme highly saturated with haem; (2) the combined injection of cortisol plus tryptophan gave an additive effect (the sum of the corresponding

Table 3. *Effects of administration of haematin, 5-aminolaevulinate, cortisol or tryptophan either individually or in various paired combinations on the activity of rat liver tryptophan pyrrolase*

Each rat received an intraperitoneal injection of either 0.9% (w/v) NaCl, haematin hydrochloride (35mg/kg), cortisol acetate (20mg/kg) or tryptophan (500mg/kg) 3h before being killed. 5-Aminolaevulinate hydrochloride was given in three injections at 1h intervals (15mg/kg body wt. each) and the animals were killed at 1h after the last injection. Some rats also received various combinations of the above compounds in the same doses and for the same duration of treatment as for single (individual) injections. 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). Values are means ±S.E.M. of each group of four rats.

Injection(s)	Kynurenine formed (μmol/h per g wet wt. of liver)	
	Holoenzyme activity	Total enzyme activity
0.9% NaCl	1.6±0.1	4.0±0.2
Haematin	4.5±0.2	4.5±0.1
5-Aminolaevulinate	3.9±0.5	5.1±0.6
Cortisol	8.6±0.8	19.9±1.4
Tryptophan	8.2±1.2	10.2±0.8
Cortisol+tryptophan	15.2±2.3	20.2±2.7
Cortisol+haematin	7.8±0.8	7.0±0.7
Cortisol+5-aminolaevulinate	7.5±0.8	9.7±1.1
Tryptophan+haematin	16.4±1.6	17.1±1.6
Tryptophan+5-aminolaevulinate	14.8±1.8	14.8±1.4

separate effects), whereas those of cortisol plus haematin or cortisol plus 5-aminolaevulinate did not; (3) the combined injection of tryptophan plus haematin or tryptophan plus 5-aminolaevulinate potentiated the individual effects caused by each treatment alone (i.e. was greater than the sum of the two separate effects), and caused the pyrrolase activity to be present almost exclusively as holoenzyme.

Effects of cortisol, haematin, 5-aminolaevulinate and tryptophan on the half-life of rat liver tryptophan pyrrolase

Cycloheximide (in a single intraperitoneal injection of 50mg/kg body wt.) was used to inhibit the synthesis of proteins, thus permitting the observation of the rate of tryptophan pyrrolase degradation. It was, however, necessary to ensure that cycloheximide did not exert an inhibitory action on the enzyme activity (other than that of inhibition of synthesis). The effect of cycloheximide on the enzyme activities

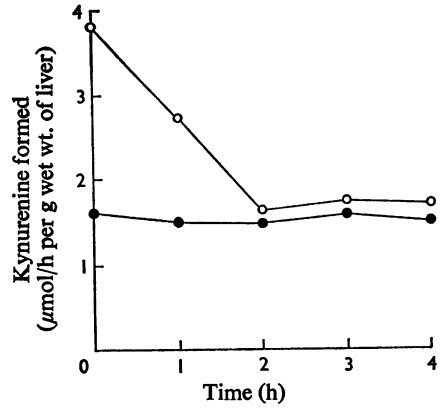

Fig. 5. *Time-course of the effect of cycloheximide administration on rat liver tryptophan pyrrolase activity*

Cycloheximide (50mg/kg body wt.) was injected at zero time. Each point represents the mean value for four rats. The enzyme activity was determined as described in the Materials and Methods section. ○, Total enzyme activity (that measured in the presence of 2μM-haematin); ●, holoenzyme activity (that in the absence of added cofactor). The apoenzyme activity was calculated by difference.

Table 4. *Half-life of rat liver tryptophan pyrrolase after various treatments*

Each rat received, 3h before being killed, an intraperitoneal injection of either 0.9% NaCl, cortisol acetate (20mg/kg), haematin hydrochloride (35mg/kg), or tryptophan (500mg/kg). 5-Aminolaevulinate hydrochloride was given in three doses at 1h intervals (15mg/kg body wt. each) and the animals were killed at 1h after the last injection. Some rats also received, 3h before death, an injection of tryptophan (50mg/kg) either alone or combined with either cortisol or haematin in the doses described above. The pyrrolase activities were determined with or without added haematin (2μM), as described in the Materials and Methods section, immediately after death. At 3h after each of the above treatments, cycloheximide (50mg/kg) was injected, and the enzyme activities were determined 1 and 2h later. The decline in enzyme activity at 0, 1 and 2h after cycloheximide administration was plotted on semi-logarithmic paper and the plots were used to obtain the half-life of the enzyme. Each value has been calculated from the mean of four rats used in each time-interval for each treatment.

Treatment	Tryptophan pyrrolase half-life (h)	
	Holoenzyme	Total enzyme
0.9% NaCl	7.7	2.3
Cortisol	2.2	2.3
Haematin	2.3	2.3
5-Aminolaevulinate	2.2	2.2
Tryptophan	11.4	6.7
Tryptophan*	7.5	4.9
Tryptophan*+cortisol	7.3	4.4
Tryptophan*+haematin	4.7	3.2

* Dose of tryptophan, 50mg/kg.

Table 5. *Comparison of various aspects of the hormonal, substrate and cofactor mechanisms of regulation of rat liver tryptophan pyrrolase activity*

(+) → (++++), Arbitrary scale of increase; +, positive effect or presence; −, negative effect or absence; (?), indirect evidence.

Aspect ... Mechanism	Hormonal	Substrate	Cofactor
Principal effector	Corticosteroids	L-Tryptophan	Haem, 5-aminolaevulinate
Other agents	Corticosteroid-releasing agents	α-Methyl-DL-tryptophan, salicylate, ethanol, catecholamines, long-chain fatty acids	Porphyrogens (late action), phenobarbitone, morphine (?)
Early saturation of apoenzyme with haem	−	+	+
General saturation of apoenzyme with haem	−	+	+
Holoenzyme/apoenzyme ratio	<1	>1	>1
Increased haem synthesis or concentration	−	+, (?)	+
Stabilization of enzyme	−	+	−
Synthesis of new apoenzyme	(++++)	(++)	(+)
Prevention of synthesis by actinomycin D	+	−	+ (5-Aminolaevulinate), − (haem)
Prevention of synthesis by puromycin or cycloheximide	+	+	+ (5-Aminolaevulinate), − (haem)

was therefore investigated both after administration and *in vitro*. The time-course of the effect of cycloheximide administration (50mg/kg body wt.) on the activity of rat liver tryptophan pyrrolase is shown in Fig. 5. During the 4h observation period, the holoenzyme activity was not significantly altered, whereas that of the total enzyme was decreased by 29% ($P<0.05$) at 1h, and by 55–58% ($P<0.005$) at 2–4h. The apoenzyme activity, calculated by difference, was therefore decreased by 45% ($P<0.005$) at 1h, and by 91–100% ($P<0.005$) at 2–4h. To ascertain that this decrease in the apoenzyme activity caused by cycloheximide was not produced by prevention of the conjugation of the apoenzyme with its haem activator, homogenates from livers of rats treated with cycloheximide (50mg/kg body wt.) 2h previously were incubated with excess of haematin *in vitro*. The enzyme activities in the absence or presence of 2, 4, 6 and 8 μM-haematin were (in μmol of kynurenine formed/h per g wet wt. of liver, ±S.E.M. of each group of four rats) 2.0±0.1, 2.1±0.1, 2.2±0.1, 2.2±0.1 and 2.1±0.1 respectively. Cycloheximide added *in vitro* (1mM) to normal rat liver homogenates did not significantly alter the basal pyrrolase activities. The mean control holoenzyme and total enzyme activities (expressed as above) were 1.4±0.1 and 4.0±0.2 respectively, and in the presence of 1mM-cycloheximide there were no significant differences (1.5±0.1 and 3.8±0.4 respectively).

The results of the half-life measurements are summarized in Table 4. The half-life of the total apoenzyme (2.3h) was not altered by the administration of cortisol, haematin or 5-aminolaevulinate. Only tryptophan increased the half-life of the holoenzyme and total enzyme, to 11.4 and 6.7h respectively. The administration of an inactive dose of tryptophan (50mg/kg body wt.) did not alter the extent of the pyrrolase enhancement by cortisol or haematin, but prolonged the half-life of the enzyme from rats treated with these two agents, and also increased the half-life of the basal total pyrrolase.

The present and other findings (Badawy & Evans, 1973, 1975; Badawy & Smith, 1971, 1972; Greengard & Feigelson, 1961; Greengard *et al.*, 1963; Knox, 1951, 1966; Marver *et al.*, 1966*a*,*b*; Schimke, 1969; Schimke *et al.*, 1965; Sourkes, 1971) are summarized (Table 5) in the form of a comparison of various aspects of the hormonal, substrate and cofactor mechanisms regulating the activity of rat liver tryptophan pyrrolase.

Discussion

Cofactor mechanism of regulation of rat liver tryptophan pyrrolase activity

We have presented evidence (Badawy & Evans, 1973) suggesting the existence of a cofactor (haem) mechanism regulating the activity of rat liver tryptophan pyrrolase. In an attempt to study the possible existence of a relationship between hepatic porphyria and tryptophan metabolism, it was then found that the saturation of apo-(tryptophan pyrrolase) with its haem activator could be modified by treatments causing destruction, inhibition of synthesis, increased utilization and enhanced synthesis of liver haem. In the present work, the administration of haematin (Fig. 1) or of its precursor 5-aminolaevulinate (Fig. 2) enhances the activity of rat liver tryptophan pyrrolase. The initial action of these two agents involves an increased saturation of the endo-

genous apoenzyme with haem, thus resembling the activation of the enzyme by haematin *in vitro* that has been demonstrated by Feigelson & Greengard (1961). The delay (up to 2h) in the appearance of this saturation after haematin but not 5-aminolaevulinate administration may be explained by the former being slowly taken up by the liver, presumably because of its binding to plasma proteins. The above saturation caused by the administration of haematin or its precursor is then followed by an overall increase in the activity of the apoenzyme, the majority of which is also haem-saturated.

The haematin-induced increase in holo-(tryptophan pyrrolase) activity is inhibited by pretreatment of rats with 2-allyl-2-isopropylacetamide or 3,5-diethoxycarbonyl-1,4-dihydrocollidine but not by that with griseofulvin (Table 2). These findings are consistent with the former two porphyrogens destroying liver haem, and with griseofulvin acting only by inhibiting its synthesis (De Matteis, 1971, 1972, 1973*a*; De Matteis & Gibbs, 1972). As expected, the activation of tryptophan pyrrolase by haematin administration is not prevented by actinomycin D, puromycin or cycloheximide (Table 1). All three agents block the pyrrolase activation by 5-aminolaevulinate, thus suggesting that a step(s) in the haem-biosynthetic pathway beyond 5-aminolaevulinate synthetase requires, for pyrrolase activation at least, the continued synthesis of mRNA and protein. There is evidence from glucose experiments (Marver *et al.*, 1966*a*) that one such step may involve 5-aminolaevulinate dehydratase. Further work is, however, required to investigate the relationship between RNA and/or protein synthesis and enzymes of the haem-biosynthetic pathway beyond 5-aminolaevulinate synthetase.

The unexpected absence of an additive effect on tryptophan pyrrolase activity of the combined injection of cortisol (hormonal-type inducer) plus either haematin or 5-aminolaevulinate (Table 3) suggests that exogenous as well as newly synthesized haem partially inhibits the cortisol induction of apo-(tryptophan pyrrolase) synthesis. This suggestion is consistent with the reported inhibition by haematin of the synthesis of other hepatic enzymes, such as 5-aminolaevulinate synthetase and cytochrome *P*-450 (Marver *et al.*, 1968; Tyrrell & Marks, 1972) and NADPH–cytochrome *c* reductase and *p*-nitrophenol–UDP-glucuronyltransferase (Bock *et al.*, 1973).

An opportunity of dissociating the phenomena of enzyme activation, stabilization and synthesis is provided by the inability of haematin, 5-aminolaevulinate or cortisol administration to prolong the the half-life of tryptophan pyrrolase (Table 4). The activation of the enzyme observed early after the administration of haematin or 5-aminolaevulinate may, however, lead to increased synthesis, as is suggested by the subsequent increase in the apoenzyme activity (Figs. 1 and 2). A similar situation with tryptophan has been explained (Greengard & Feigelson, 1961) by the decreased free apoenzyme (caused by haem saturation) providing a stimulus for further apoenzyme synthesis. Further experiments are required to clarify this point.

Substrate and hormonal mechanisms of regulation of rat liver tryptophan pyrrolase activity

The initial increase in the haem saturation of endogenous apo-(tryptophan pyrrolase) and the subsequent production of a largely haem-saturated apoenzyme caused by tryptophan administration (Greengard & Feigelson, 1961) are confirmed here (Fig. 3), and suggest the existence of a mechanism of action common to tryptophan and haematin. The increase in the total pyrrolase activity caused by tryptophan (Fig. 3) is, however, larger than that observed after haematin (Fig. 1) or 5-aminolaevulinate (Fig. 2) administration. This suggests that tryptophan may increase the pyrrolase activity by an additional mechanism not shared by haematin. By contrast with the cofactor and substrate effects, cortisol (Fig. 4) does not cause an initial saturation of the apoenzyme with haem, but produces a large increase in the total pyrrolase activity while maintaining a ratio of holoenzyme/apoenzyme activity similar to that of the basal enzyme (less than 1). The cortisol-induced increase in the holoenzyme activity represents the conjugation of about 50% of the newly formed apoenzyme with haem whose origin is probably endogenous, since cortisol does not enhance haem synthesis (Marver *et al.*, 1966*c*).

It has been suggested (Schimke *et al.*, 1965; Knox, 1966; Schimke, 1969) that the accumulation of tryptophan pyrrolase after the administration of tryptophan represents the combined results of stabilization plus synthesis at the normal rate. The present results, showing stabilization of the pyrrolase by tryptophan but not by cortisol (Table 4), confirm previous findings (Garren *et al.*, 1964; Schimke *et al.*, 1965; Shore *et al.*, 1971), and also suggest that simple activation (by haematin or 5-aminolaevulinate) does not lead to a more stable enzyme. The mechanism by which tryptophan stabilizes the pyrrolase is not clearly understood. Knox (1966) suggested that after promoting the conjugation of haem with the apoenzyme and the subsequent reduction of the oxidized holoenzyme, tryptophan sequesters the active form and protects it against degrading enzymes. Knox (1966) also suggested, from experiments *in vitro*, that these effects could be achieved *in vivo* by moderate increases in the hepatic concentrations of tryptophan. The finding (Table 4) that a non-activating dose of tryptophan (50mg/kg body wt.) stabilizes the basal, cortisol-induced and haematin-activated enzyme is consistent with the above suggestion.

Further work is clearly required to examine the role tryptophan may play in modifying the activity of the pyrrolase-degrading enzyme(s). The pyrrolase-stabilizing action of tryptophan may also explain, at least in part, the potentiation observed after the combined injection of tryptophan plus either haematin or 5-aminolaevulinate (Table 3).

The cortisol induction is blocked by pretreatment of rats with actinomycin D, puromycin or cycloheximide, whereas only the latter two agents are capable of preventing the tryptophan activation of tryptophan pyrrolase (Table 1). These findings with both forms of the enzyme confirm those of previous experiments in which either the holoenzyme or the total enzyme activity was measured (Greengard *et al.*, 1963; Badawy & Smith, 1971). Schimke *et al.* (1965) reported that the RNA-dependent cortisol induction of the pyrrolase activity is associated with an enhanced synthesis of the apoenzyme, whereas after tryptophan administration pyrrolase synthesis proceeds at normal rates. This latter finding cannot be reconciled with the ability of inhibitors of protein synthesis to prevent the pyrrolase enhancement by tryptophan. This controversy can simply be explained by inhibitors of protein synthesis exerting unknown effects, or by tryptophan activating the pyrrolase by an unrecognized mechanism. Although the former possibility cannot be ignored, the results in Fig. 5 and supporting evidence in the text suggest that, as far as pyrrolase is concerned, cycloheximide does not inhibit functions other than synthesis. It may therefore be useful to consider the possibility that cycloheximide or puromycin inhibits the tryptophan-mediated synthesis of a substance(s) other than apo-(tryptophan pyrrolase). That this substance may be haem itself is suggested by both direct and indirect evidence. Tryptophan enhances the activity of rat liver 5-aminolaevulinate synthetase (Marver *et al.*, 1966*b*), which is the rate-limiting enzyme of the biosynthetic pathway of porphyrins and haem (Granick, 1966). At 2h after the administration of tryptophan, Greengard & Feigelson (1961) observed a 20% increase in the microsomal concentration of rat liver haem. Whether this increase is confined to the haem of cytochrome *P*-450 and/or cytochrome b_5 (which together account for total microsomal haem under normal conditions) or extends to the so-called free haem pool, which is increased by 5-aminolaevulinate (Druyan & Kelly, 1972, and references therein), remains to be determined. The indirect evidence suggesting an effect of tryptophan on haem synthesis is provided by the following findings: (1) the haem used for saturation of endogenous apo-(tryptophan pyrrolase) early after tryptophan administration (Fig. 3) may not be of an endogenous (preformed) origin, since cortisol, which does not enhance haem synthesis (Marver *et al.*, 1966*c*), causes conjugation of (presumably preformed) haem only after apo-(tryptophan pyrrolase) synthesis has started (Fig. 4); (2) the tryptophan enhancement of the holoenzyme activity is inhibited by the inhibitors of haem synthesis 3,5-diethoxycarbonyl-1,4-dihydrocollidine and griseofulvin, but not by 2-allyl-2-isopropylacetamide, which does alter haem synthesis (Badawy & Evans, 1973). The effects of tryptophan on haem metabolism have not been studied beyond the above aspects.

Role of tryptophan pyrrolase in the utilization of liver haem, and as a possible model for the exacerbation by drugs of human porphyria

Its short half-life (Table 4) and its rapid response to various stressful stimuli (Table 5) and to experimental alterations in haem metabolism (Badawy & Evans, 1973) suggest that tryptophan pyrrolase may play a significant role in the utilization of liver haem. Any increase in the pyrrolase activity and any drug causing such increase could therefore be considered as a means of increasing haem utilization. De Matteis (1973*b*) reported that the administration of agents such as phenobarbitone, thiopentone or lindane, which increase the utilization of liver haem by cytochrome *P*-450, together with either 2-allyl-2-isopropylacetamide or 3,5-diethoxycarbonyl-1,4-dihydrocollidine, enhances the stimulation of 5-aminolaevulinate synthetase activity caused by the latter two porphyrogens. De Matteis (1973*b*) suggested that this potentiation provides a model for the exacerbation by drugs of human genetic porphyria by two mechanisms (increased haem utilization and decreased haem concentration) which together cause a stronger decline in liver haem, which exercises a negative feedback inhibition of its own synthesis at the 5-aminolaevulinate synthetase step. Increased haem utilization by apo-(tryptophan pyrrolase) could also provide a similar model for the exacerbation by drugs of human porphyria. This pyrrolase model may also explain the potentiation of the effects on tryptophan pyrrolase activity observed after the combined injection of tryptophan plus either haematin or 5-aminolaevulinate (Table 3).

We are very grateful to the Wellcome Trustees for support, Dr. F. De Matteis for helpful discussions, Mr. C. J. Morgan, Mr. C. G. Brook and Miss Jill C. Davis for technical assistance and Mr. A Dacey for animal maintenance.

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