Tryptophan Pyrrolase in Haem Regulation

THE RELATIONSHIP BETWEEN THE DEPLETION OF RAT LIVER TRYPTOPHAN PYRROLASE HAEM AND THE ENHANCEMENT OF 5-AMINOLAEVULINATE SYNTHASE ACTIVITY BY 2-ALLYL-2-ISOPROPYLACETAMIDE

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1. Rat liver tryptophan pyrrolase haem is maximally depleted at 30 min after administration of a 400 mg/kg dose of 2-allyl-2-isopropylacetamide. This depletion lasts for 24 h, by which time 5-aminolaevulinate synthase activity becomes maximally enhanced. 2. though the above maximum depletion of pyrrolase haem (at 0.5 h) is also produced by a 100 mg/kg dose of the porphyrogen, this does not enhance synthase activity at 24 h. It and smaller doses, however, cause a smaller but earlier enhancement of synthase activity (maximum at 2 h) and produce a similarly short-lived depletion of pyrrolase haem. 3. The depletion of pyrrolase haem and the enhancement of synthase activity by the porphyrogen are inhibited by compound SKF 525-A and phenazine methosulphate, and are potentiated by nicotinamide but not by phenobarbitone. Phenazine methosulphate and nicotinamide also exert opposite effects on hexobarbital sleeping-time. 4. 2-Allyl-2-isopropylacetamide also depletes pyrrolase haem *in vitro*. It does so in liver homogenates of control rats in the presence, and in those of phenobarbitone-treated rats in the absence, of added NADPH. 5. A discussion of the present results in relation to previous work with other haemoproteins suggests that, whereas cytochrome *P*-450 (haem) is primarily involved in the production of the active (porphyrogenic) metabolite(s) of 2-allyl-2-isopropylacetamide, the haem pool used by tryptophan pyrrolase may play an important role in the effects of this compound on haem biosynthesis.

5-Aminolaevulinate synthase (EC 2.3.1.37), the rate-limiting enzyme of the haem-biosynthetic pathway, is the point at which haem is thought to regulate its own synthesis by a negative feedback mechanism(s) in avian (Granick, 1966; Granick *et al.*, 1975) and mammalian (De Matteis, 1972a) [including possibly also human (Jeelani Dhar *et al.*, 1975)] liver. This concept has arisen from work on the effects of several chemicals on synthase activity. Whereas avian liver does not discriminate between these chemicals, probably because of the greater sensitivity of its synthase and the lability of its haem-biosynthetic pathway (Granick, 1966; Creighton & Marks, 1972; De Matteis, 1973), mammalian liver responds to chemicals in two ways. Thus a number of chemically unrelated compounds (porphyrogens) stimulate the hepatic formation of porphyrins and markedly enhance the synthase activity (De Matteis, 1967), whereas several lipid-soluble drugs (including phenobarbitone) cause a moderate enhancement of synthase activity and an increase in

the concentration of microsomal haem, but not in that of porphyrins (see De Matteis, 1972b). When, however, one of these lipid-soluble drugs is administered together with a porphyrogen to intact animals (De Matteis & Gibbs, 1972) or to isolated perfused livers (Bock *et al.*, 1973), it potentiates the effects of the porphyrogen on synthase activity and porphyrin concentration, and the above two groups of workers suggested that this potentiation may explain the exacerbation by drugs of human hepatic porphyrias.

The mechanism(s) by which lipid-soluble drugs cause a moderate enhancement of mammalian liver synthase activity is not understood (see Badawy, 1978). By contrast, it is most likely that the marked enhancement of synthase activity caused by porphyrogens is produced by interference with the above negative-feedback mechanism involving the occurrence of an early depletion of liver haem. It is therefore reasonable to suggest that this haem belongs to the so-called regulatory free-haem pool that has

been suggested (Granick *et al.*, 1975; De Matteis, 1975) to be small and rapidly turning over. Since such a pool would be too small to be measured directly, the above depletion of haem has been demonstrated by indirect methods involving the determination of activity or concentration of, or haem utilization by, the hepatic haemoproteins catalase (EC 1.11.1.6), cytochrome *P*-450 and tryptophan pyrrolase (tryptophan 2,3-dioxygenase, EC 1.13.11.11). There is considerable evidence (see Badawy, 1978, and references cited therein) implicating this early depletion of mammalian liver haem in the production of experimental porphyria and its exacerbation by drugs.

Our work demonstrating the versatile role of tryptophan pyrrolase and its advantages over other hepatic haemoproteins in relation to haem utilization (for a review, see Badawy, 1979) suggests that, in addition to being a very sensitive marker for assessing delicate changes in liver haem concentration, pyrrolase may play an important role in the regulation of haem biosynthesis. As a first step in studying this latter possibility, we have examined in the present paper the relationship between the early depletion of rat liver tryptophan pyrrolase haem and the marked enhancement of 5-aminolaevulinate synthase activity caused by administration of the porphyrinogen 2-allyl-2-isopropylacetamide.

Materials and Methods

Chemicals

The sources of most chemicals have been described (Badawy & Evans, 1975a, 1976). 2-Allyl-2-isopropylacetamide and compound SKF 525-A (2-diethylaminoethyl 3,3-diphenylpropylacetate) were gifts respectively from Roche Products and Smith, Kline and French Laboratories, both of Welwyn Garden City, Herts., U.K. All other additional chemicals [from BDH Chemicals and Sigma (London) Chemical Co., both of Poole, Dorset, U.K.] were of the purest commercially available grades.

Animals and treatments

Locally bred male Wistar rats (100–150 g), maintained on cube diet 41B (Oxoid, Basingstoke, Hants., U.K.) and water, were starved for 48 h (unless otherwise stated) before being killed by stunning and cervical dislocation between 13:00 and 15:30 h.

2-Allyl-2-isopropylacetamide (12.5–500 mg/kg, in 1 ml of dimethylformamide) was injected into the loose subcutaneous tissues of the neck, and appropriate control rats received an equal volume of the solvent by the same route. Nicotinamide was freely administered in drinking water (100 mg/litre) for 3 days. The remaining compounds [dissolved in 1–2 ml of 0.9% (w/v) NaCl] were given intraperitoneally as follows: hexobarbital sodium

(100 mg/kg); phenazine methosulphate (*N*-methylphenazonium methosulphate, 10 mg/kg); compound SKF 525-A (45 mg/kg); phenobarbitone sodium (5 mg/kg once or 50, 50 and 80 mg/kg at 48, 42 and 24 h respectively before death). Control rats received the appropriate injection(s) of 0.9% (w/v) NaCl.

Chemical, enzymic and other determinations

Tryptophan pyrrolase activity was determined in liver homogenates (Badawy & Evans, 1975a) in either the absence (holoenzyme activity) or the presence (total enzyme activity) of added (2 μ M) haematin. The apoenzyme activity, obtained by difference, was used to calculate the haem-saturation ratio (that of holoenzyme/apoenzyme activity) which indicates the extent of the haem saturation of the apoenzyme (see also Badawy, 1978, 1979). When tested *in vitro*, 2-allyl-2-isopropylacetamide and NADPH were added in concentrations of 28.4 μ M and 2 mM respectively.

Hexobarbital sleeping-time was taken as the time elapsing between the onset of anaesthesia after intraperitoneal administration of a 100 mg/kg dose of the drug and the regaining of the righting reflex (Bond & De Matteis, 1969).

5-Aminolaevulinate synthase activity was determined by measuring the enzymically produced (Yoda *et al.*, 1974) 5-aminolaevulinate by the colorimetric procedure of De Matteis (1971) with some modifications of both methods. The details are given here for the first time. Fresh liver was homogenized in 4 vol. of 0.05 M-Tris/HCl/0.2 mM-pyridoxal 5'-phosphate, pH 8.0, by using a glass homogenizer with a loose-fitting Teflon pestle (at 0°C and at 1100 rev./min for 1 min). To each 25 ml conical flask (at 0°C) was added 0.2 ml of a solution of each of the following compounds in the above Tris/HCl buffer lacking the pyridoxal 5'-phosphate (the concentrations of solutions are given in parentheses): glycine (2.6 M), sodium succinate (0.26 M), ATP (0.26 M), CoA (0.26 mM), pyridoxal 5'-phosphate (0.26 mM), 2-mercaptoethanol (0.13 M), MgCl₂ (0.13 M), Na₂EDTA (0.104 M), KF (2.6 M), Tris/HCl buffer, pH 7.4 (0.05 M), succinyl-CoA synthetase (EC 6.2.1.4) [10 units/12 ml of 0.05 M-Tris/HCl buffer, pH 8.0; one unit will convert 1 μ mol of succinate into succinyl-CoA (3-carboxypropionyl-CoA)/min at pH 7.4 and at 30°C in the presence of CoA and GTP]. To this ice-cold mixture (2.2 ml) was added 3 ml of the liver homogenate. Incubation was under N₂ for 30 min at 37°C with shaking (150 oscillations/min). The reaction was stopped by the addition of 0.5 ml of 50% (w/v) trichloroacetic acid, and, after shaking for a further 2 min, the contents of the flasks were stored at –20°C for 17 h. Two liver samples per rat were treated in this manner. For tissue blanks, the remainder of the liver homogenates from each group of treatment were

pooled and two samples were treated as above, except that trichloroacetic acid was added before the homogenates. After thawing (at no more than 37°C), the mixture was centrifuged at 1000g for 10 min and the supernatant was analysed by the method of De Matteis (1971), with two modifications. These were: (1) the use of HCl instead of acetic acid to prepare the 1M-NaOH/1M-sodium acetate, pH 4.6; (2) the ether was shaken with and separated from the aqueous mixture representing the above incubation fluid. Each experiment included two samples (60 nmol each) of 5-aminolaevulinate hydrochloride and two water blanks, all of which were treated as the incubation supernatants.

Recovery of 5-aminolaevulinate hydrochloride was tested in separate experiments and was found to be 94% (93–95%) when concentrations of 30 and 60 nmol were added to the liver-homogenate incubation mixtures and treated as above. The results have been corrected for full recovery. In other experiments, a linear relationship was found between 5-aminolaevulinate hydrochloride concentration (range 0–100 nmol) and colour intensity (at 556 nm). From this graph, an absorption coefficient of $56.24 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ was calculated. This compared favourably with that ($56.80 \text{ mm}^{-1} \cdot \text{cm}^{-1}$) reported by De Matteis (1971).

Student's *t* test was used to assess the differences between control and test results.

Results

Assessment of changes in the haem-saturation ratio of tryptophan pyrrolase

An important requirement for this assessment (for a discussion, see Badawy, 1978) is that an administered agent (e.g. 2-allyl-2-isopropylacetamide in the present work) must not be allowed to modify pyrrolase activities by a mechanism(s) other than an altered haem availability. This requirement is met by the use of starved rats, in which the hormonally induced increases in both holoenzyme and total enzyme activities (see Badawy, 1977) forestall any stress-induced enhancement. Although the above porphyrinogen caused such an enhancement in 24 h-starved rats (Badawy, 1978), it did not do so in the 48 h-starved animals used in the present work.

Effects of administration of 2-allyl-2-isopropylacetamide on rat liver tryptophan pyrrolase activity and the haem-saturation ratio

The time-course of these effects of a 400 mg/kg dose is shown in Fig. 1. The total pyrrolase activity was not significantly altered ($P > 0.10$) at any of the time-intervals examined. By contrast, the holoenzyme activity was decreased by 59% ($P < 0.005$) as early as 15 min after the injection. The decrease reached a maximum (76%; $P < 0.001$) at 30 min and

was followed by a gradual but partial recovery of the holoenzyme to two-thirds of the basal value at 4 h. No further increase occurred until after 16 h, when the basal value was reached at 24–30 h. This was then followed by a 28% increase ($P < 0.05$) at 36 h and a final return to normal at 54 h. The haem-saturation ratio was also decreased by 2-allyl-2-isopropylacetamide from a control value (at zero time) of 1.00 to values of between 0.16 and 0.74 at 0.25–24 h. It then increased to up to 2.36 (at 36 h) and finally fell to the basal value at 48 h.

The effects of various doses of 2-allyl-2-isopropylacetamide were also examined at 0.5 h (Fig. 2). None of the doses tested exerted any significant effect on the total pyrrolase activity ($P > 0.10$). By contrast, the 12.5 mg/kg dose caused a small (17%) but significant ($P < 0.025$) decrease in that of the holoenzyme. The decrease was dose-dependent, with the 100 mg/kg dose causing the maximum effect (about 60%; $P < 0.001$). The haem-saturation ratio was also decreased (from a control value of 1.00) by 24–75%; the decreases were significant ($P = 0.05$ – 0.001), except that of the 12.5 mg/kg dose ($P > 0.10$).

The time-course of the effects of a 100 mg/kg dose of 2-allyl-2-isopropylacetamide on pyrrolase activity and the ratio is shown in Fig. 3. Whereas no significant changes in the total enzyme activity occurred ($P > 0.10$), that of the holoenzyme was decreased by 47% ($P < 0.001$) at 0.5 h. The decrease was 23% ($P < 0.005$) at 2 h. The holoenzyme activity remained unaltered for a further 1 h before returning to the basal value at 5 h.

The effects of pretreatment of rats with compound SKF 525-A, phenazine methosulphate, nicotinamide and phenobarbitone on the haem-depleting action of 2-allyl-2-isopropylacetamide (100 mg/kg; 0.5 h before death) are shown in Table 1. The above dose of the porphyrinogen decreased the holoenzyme activity and the ratio by 32–47 and 50–60% respectively ($P = 0.05$ – 0.001). None of the above four compounds exerted any significant effects on pyrrolase activities or ratio in control rats. Both compound SKF 525-A and phenazine methosulphate prevented the porphyrinogen-induced decreases in holoenzyme activity and the ratio. By contrast, nicotinamide enhanced the porphyrinogen effects on these two parameters by 23 and 28% respectively ($P = 0.05$ – 0.02). Phenobarbitone injections, on the other hand, did not modify the effects of 2-allyl-2-isopropylacetamide ($P > 0.10$). Similar results were obtained when only a single dose (5 mg/kg) of phenobarbitone sodium was given at 15 min before the porphyrinogen (100 mg/kg). The holoenzyme activity (in μmol of kynurenine formed/h per g wet wt. of liver) and the haem-saturation ratio (both means $\pm$ S.E.M. for each group of three to four rats) were as follows: control (5.0 ± 0.23 and 0.77 ± 0.04);

Fig. 1. Time-course of the effects of administration of 2-allyl-2-isopropylacetamide (400mg/kg) on rat liver tryptophan pyrrolase activity

Rats were starved for 48h before being killed and were given 2-allyl-2-isopropylacetamide (400mg/kg) subcutaneously at zero time. 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; O) of added haematin. The apoenzyme activity, obtained by difference, was used to calculate the haem-saturation ratio (that of holoenzyme/apoenzyme activity). Values are means $\pm$ S.E.M. for each group of four rats.

Fig. 2. Effects of administration of various doses of 2-allyl-2-isopropylacetamide on rat liver tryptophan pyrrolase activity

Rats were starved for 48h before being killed and were given various doses of 2-allyl-2-isopropylacetamide or an equal volume (1 ml/kg) of the solvent dimethylformamide at 30 min before death. 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; O) of added haematin. The apoenzyme activity, obtained by difference, was used to calculate the haem-saturation ratio (that of holoenzyme/apoenzyme activity). Values are means $\pm$ S.E.M. for each group of four rats.

Fig. 3. Time-course of the effects of administration of 2-allyl-2-isopropylacetamide (100mg/kg) on rat liver tryptophan pyrrolase activity

Experimental details are as described in Fig. 1, except that the dose of 2-allyl-2-isopropylacetamide was 100mg/kg. Values are means $\pm$ S.E.M. for each group of four rats. Symbols: ●, holoenzyme activity; O, total enzyme activity.

The above effects of phenazine methosulphate and nicotinamide were also confirmed with various doses of 2-allyl-2-isopropylacetamide in 24h- or 48h-starved rats (results not shown).

Effects of phenazine methosulphate and nicotinamide on hexobarbital sleeping time

Both agents caused significant ($P < 0.001$) changes in sleeping time. The latter was prolonged by

phenobarbitone (4.6 ± 0.35 and 0.77 ± 0.06); 2-allyl-2-isopropylacetamide (2.9 ± 0.22 and 0.35 ± 0.03); phenobarbitone plus 2-allyl-2-isopropylacetamide (2.7 ± 0.18 and 0.34 ± 0.04).

Table 1. *Effects of pretreatment of rats with various agents on the 2-allyl-2-isopropylacetamide-induced depletion of liver tryptophan pyrrolase haem*

Rats were starved for 48h before being killed and were given (at 0.5h before death) a subcutaneous injection of either 2-allyl-2-isopropylacetamide (100mg/kg) or an equal volume (1ml/kg) of the solvent dimethylformamide. Pretreatments consisted of giving compound SKF 525-A (45 mg/kg) 45 min, phenazine methosulphate (10mg/kg) 10min, nicotinamide (100mg/litre of drinking water) 72h, or phenobarbitone sodium (50, 50 and 80mg/kg) respectively 48, 42 and 24h, before the above treatments. Control rats received the appropriate pretreatments with 0.9% NaCl or drinking water (Nil) for the same durations. 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. The apoenzyme activity, obtained by difference, was used to calculate the haem-saturation ratio (that of holoenzyme/apoenzyme activity). Values are means $\pm$ S.E.M. for each group of four rats. The effects of 2-allyl-2-isopropylacetamide (in columns 4 and 6) are compared with those of dimethylformamide (columns 1 and 3), whereas only one difference was found in dimethylformamide-treated rats (in the total activity after nicotinamide administration). Significance of differences is indicated as follows: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; † $P < 0.005$; †† $P < 0.001$.

Haem-saturation ratio or tryptophan pyrrolase activity (μ mol of kynurenine formed/h per g wet wt. of liver)

Pretreatment	Dimethylformamide-treated rats			2-Allyl-2-isopropylacetamide-treated rats		
	Holoenzyme activity (1)	Total enzyme activity (2)	Haem-saturation ratio (3)	Holoenzyme activity (4)	Total enzyme activity (5)	Haem-saturation ratio (6)
0.9% NaCl	5.0 $\pm$ 0.57	9.0 $\pm$ 0.9	1.25 $\pm$ 0.11	3.3 $\pm$ 0.15*	9.9 $\pm$ 1.3	0.50 $\pm$ 0.08†
Compound SKF 525-A	5.2 $\pm$ 0.17	9.3 $\pm$ 0.6	1.27 $\pm$ 0.15	6.1 $\pm$ 0.20**	9.6 $\pm$ 0.6	1.74 $\pm$ 0.28
0.9% NaCl	5.0 $\pm$ 0.12	9.2 $\pm$ 0.4	1.19 $\pm$ 0.08	3.4 $\pm$ 0.19††	9.2 $\pm$ 0.8	0.59 $\pm$ 0.05††
Phenazine methosulphate	5.2 $\pm$ 0.17	9.6 $\pm$ 0.7	1.18 $\pm$ 0.29	5.0 $\pm$ 0.35	9.4 $\pm$ 0.6	1.14 $\pm$ 0.10
Nil	5.5 $\pm$ 0.75	10.0 $\pm$ 0.5	1.22 $\pm$ 0.27	3.5 $\pm$ 0.22*	9.3 $\pm$ 0.8	0.60 $\pm$ 0.08*
Nicotinamide	4.6 $\pm$ 0.35	8.7 $\pm$ 0.3*	1.12 $\pm$ 0.17	2.7 $\pm$ 0.12†	9.0 $\pm$ 0.5	0.43 $\pm$ 0.03***
0.9% NaCl	5.5 $\pm$ 0.42	13.1 $\pm$ 1.0	0.72 $\pm$ 0.06	2.9 $\pm$ 0.10††	13.0 $\pm$ 0.4	0.29 $\pm$ 0.01††
Phenobarbitone	5.1 $\pm$ 0.26	12.6 $\pm$ 1.0	0.68 $\pm$ 0.09	3.0 $\pm$ 0.27†	12.5 $\pm$ 1.0	0.32 $\pm$ 0.02***

56% by phenazine methosulphate, but was shortened by 33% by nicotinamide.

Effects in vitro of 2-allyl-2-isopropylacetamide, NADPH or both on rat liver tryptophan pyrrolase activity and the haem-saturation ratio

The above effects in 48h-starved rats were examined in untreated rats and in those given multiple injections of phenobarbitone (Table 2). In untreated rats, neither the porphyrin alone nor NADPH alone exerted any significant effects ($P > 0.01$) on pyrrolase activities or the ratio. Addition of the two together, however, decreased the holoenzyme activity and the ratio by 42 and 54% respectively ($P = 0.02-0.001$). In phenobarbitone-treated rats, NADPH alone exerted no significant effects, whereas 2-allyl-2-isopropylacetamide alone decreased the holoenzyme activity by 21% ($P < 0.05$), but the decrease in the ratio was not significant. Addition of both agents together caused further decreases in holoenzyme activity and the ratio; the combined decreases (compared with control values) were 43 and 57% respectively ($P = 0.05-0.005$).

The control pyrrolase activities in Table 2 are lower than those observed after administration of

phenobarbitone. This difference did not affect the haem-saturation ratio and may be explained by the absence of treatment of control rats with multiple injections of 0.9% NaCl, because, when this was performed, both control and phenobarbitone-treated rats had similar enzyme activities (Table 1). This also suggests that phenobarbitone injection(s) does not influence tryptophan pyrrolase activities, a finding that has previously been reported (Badawy & Evans, 1973b).

Effects of administration of 2-allyl-2-isopropylacetamide on rat liver 5-aminolaevulinate synthase activity

The time-course of the effects of a 400 mg/kg dose is shown in Fig. 4. At 1h, synthase activity was increased by 95% ($P < 0.05$). The activity was further increased by 142, 178 and 354% ($P = 0.005-0.001$) at 2, 3 and 4h respectively. No further increase occurred during the following 3h, but at 8, 12, 16 and 24h the activity was elevated by 550, 737, 866 and 1054% respectively ($P < 0.001$). It then fell to the basal value at 48h.

The time-course of the effects on synthase activity of a 50 mg/kg dose of 2-allyl-2-isopropylacetamide is shown in Fig. 5. A 69% increase ($P < 0.001$)

Table 2. *Effects in vitro of 2-allyl-2-isopropylacetamide, NADPH or both on rat liver tryptophan pyrrolase activity and the haem-saturation ratio*

Rats were starved for 48 h before being killed. Some rats received injections of phenobarbitone sodium (50, 50 and 80 mg/kg) respectively at 48, 42 and 24 h before death. Liver homogenates were incubated in the absence or the presence of 2-allyl-2-isopropylacetamide (28.4 μ M), NADPH (2 mM) or both. 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. The apoenzyme activity, obtained by difference, was used to calculate the haem-saturation ratio (that of holoenzyme/apoenzyme activity). Values are means $\pm$ S.E.M. for each group of four rats. All differences are in relation to the results in column (1) and their significance is indicated as follows: * $P < 0.05$; ** $P < 0.02$; † $P < 0.005$; †† $P < 0.001$. Pyrrolase activities are expressed in μ mol of kynurenine formed/h per g wet wt. of liver. Abbreviation: AIA, 2-allyl-2-isopropylacetamide.

Injection(s)	Activities or ratio after additions <i>in vitro</i>			
	Nil (1)	AIA (2)	NADPH (3)	AIA + NADPH (4)
None				
Holoenzyme activity	2.60 $\pm$ 0.05	2.60 $\pm$ 0.10	2.71 $\pm$ 0.06	1.50 $\pm$ 0.11††
Total enzyme activity	6.20 $\pm$ 0.63	6.00 $\pm$ 0.60	6.32 $\pm$ 0.80	6.11 $\pm$ 0.90
Haem-saturation ratio	0.72 $\pm$ 0.11	0.76 $\pm$ 0.09	0.75 $\pm$ 0.12	0.33 $\pm$ 0.03**
Phenobarbitone				
Holoenzyme activity	4.22 $\pm$ 0.22	3.30 $\pm$ 0.18*	3.94 $\pm$ 0.30	2.40 $\pm$ 0.12†
Total enzyme activity	9.14 $\pm$ 1.26	9.22 $\pm$ 1.13	9.00 $\pm$ 1.10	8.94 $\pm$ 0.81
Haem-saturation ratio	0.86 $\pm$ 0.22	0.56 $\pm$ 0.07	0.76 $\pm$ 0.07	0.37 $\pm$ 0.02*

Fig. 4. *Time-course of the effects of administration of 2-allyl-2-isopropylacetamide (400 mg/kg) on rat liver 5-aminolaevulinate synthase activity*

Rats were starved for 48 h before being killed and were given 2-allyl-2-isopropylacetamide (400 mg/kg) subcutaneously at zero time. The enzyme activity was determined as described in the Materials and Methods section. Values are means $\pm$ S.E.M. for each group of four rats.

occurred at 1 h and was followed by maximum enhancement (390%; $P < 0.001$) 1 h later. Activity then fell, reaching 12.23-fold the basal value at 5 h.

The effects of various doses of 2-allyl-2-iso-

propylacetamide were also examined at 2 and 24 h (Fig. 6). At 2 h, the 12.5 mg/kg dose caused a 156% increase ($P < 0.001$), whereas the 25 mg/kg dose caused maximum enhancement (422%; $P < 0.001$).

By contrast, doses of the porphyrin of 100 mg/kg and below failed to alter synthase activity at 24 h. Enhancement at this time-interval was maximum (850%; $P < 0.001$) with the 300 mg/kg dose.

The effects of pretreatment of rats with compound SKF 525-A, phenazine methosulphate, nicotinamide and phenobarbitone on the enhancement of synthase activity observed at 2 h after administration of a 100 mg/kg dose of 2-allyl-2-isopropylacetamide are shown in Table 3. None of the above four compounds exerted any significant effects ($P > 0.10$) on synthase activity of control rats. Synthase enhancement by 2-allyl-2-isopropylacetamide was de-

creased by compound SKF 525-A and phenazine methosulphate. Direct comparison of results suggests that the decreases by these two agents were 32 and 51% respectively ($P = 0.02-0.05$), but a comparison of the net effects of 2-allyl-2-isopropylacetamide shows that the decreases were 65 and 87% respectively. By contrast, nicotinamide enhanced the effect of the porphyrin on synthase activity by

Fig. 5. Time-course of the effects of administration of 2-allyl-2-isopropylacetamide (50 mg/kg) on rat liver 5-aminolaevulinate synthase activity

Experimental details are as described in Fig. 4, except that the dose of 2-allyl-2-isopropylacetamide was 50 mg/kg. Values are means $\pm$ S.E.M. for each group of four rats.

Fig. 6. Effects of administration of various doses of 2-allyl-2-isopropylacetamide on rat liver 5-aminolaevulinate synthase activity

Rats were starved for 48 h before being killed and were given various doses of 2-allyl-2-isopropylacetamide or an equal volume (1 ml/kg) of the solvent dimethylformamide at 2 (O) or 24 h (●) before death. The enzyme activity was determined as described in the Materials and Methods section. Values are means $\pm$ S.E.M. for each group of four rats.

Table 3. Effects of pretreatment of rats with various agents on the 2-allyl-2-isopropylacetamide-induced enhancement of liver 5-aminolaevulinate synthase activity

Experimental details are as described in Table 1, except that the animals were killed at 2 h after administration of 2-allyl-2-isopropylacetamide or the solvent dimethylformamide. Synthase activity was determined as described in the Materials and Methods section. Values are means $\pm$ S.E.M. for each group of four rats. The results in column (2) are compared with those in column (1) and significance of differences is indicated as follows: * $P < 0.025$; *** $P < 0.01$; †† $P < 0.001$.

Pretreatment	5-Aminolaevulinate synthase activity (nmol of 5-aminolaevulinate formed/min per g wet wt. of liver)	
	Dimethylformamide-treated rats (1)	2-Allyl-2-isopropylacetamide-treated rats (2)
0.9% NaCl	1.24 $\pm$ 0.20	3.32 $\pm$ 0.45***
Compound SKF 525-A	1.36 $\pm$ 0.08	1.64 $\pm$ 0.28
0.9% NaCl	1.08 $\pm$ 0.10	3.05 $\pm$ 0.16††
Phenazine methosulphate	1.39 $\pm$ 0.14	2.08 $\pm$ 0.08***
Nil	1.56 $\pm$ 0.06	3.48 $\pm$ 0.19††
Nicotinamide	1.61 $\pm$ 0.11	4.36 $\pm$ 0.19††
0.9% NaCl	1.91 $\pm$ 0.09	3.69 $\pm$ 0.58*
Phenobarbitone	2.24 $\pm$ 0.23	2.87 $\pm$ 0.25

25% ($P < 0.02$) (direct comparison) or by 44% (of net effect). Phenobarbitone injections, on the other hand, caused a small (22%) decrease ($P > 0.10$) in the porphyrinogen-induced enhancement of synthase activity. A single phenobarbitone dose (5 mg/kg) given 15 min before the porphyrinogen (100 mg/kg) did not influence synthase enhancement either. The enzyme activities (in nmol of 5-aminolaevulinate formed/min per g wet wt. of liver, $\pm$ S.E.M. for each group of four rats) were as follows: control (1.36 ± 0.17); phenobarbitone (1.37 ± 0.16); 2-allyl-2-isopropylacetamide (2.83 ± 0.33); 2-allyl-2-isopropylacetamide plus phenobarbitone (2.87 ± 0.55).

Discussion

Depletion of tryptophan pyrrolase haem by 2-allyl-2-isopropylacetamide

Preferential depletion of rat liver tryptophan pyrrolase haem is a very early phenomenon in the sequence of events leading to the production of porphyria by 2-allyl-2-isopropylacetamide. Thus, whereas decreases in concentrations of cytochrome *P*-450 and catalase of only 9–27% (under basal conditions) occur at 1–2 h (Satyanarayana Rao *et al.*, 1972), the depletion of pyrrolase haem is maximum (76%) at 30 min (Fig. 1). Moreover, the finding (Fig. 1) that the partial recovery of pyrrolase haem (at 5 h) is interrupted for 11 h, whereas the recovery of cytochrome *P*-450 is not (see also De Matteis, 1971), suggests that pyrrolase haem remains a constant target for most of the time-course of enhancement of 5-aminolaevulinate synthase activity by the above porphyrinogen (see Fig. 4). These changes, together with the sensitivity of pyrrolase haem to very small doses of 2-allyl-2-isopropylacetamide (Fig. 2), strongly suggest that depletion of pyrrolase haem may play an important role in the action(s) of this porphyrinogen on liver haem metabolism, in view of the importance of haem depletion in synthase enhancement (see the introduction).

2-Allyl-2-isopropylacetamide depletes liver haem by causing its destruction to green and other unidentified pigments by an ill-defined process mediated by a metabolite(s) of the porphyrinogen produced by the microsomal NADPH-dependent mixed-function oxidase system. This conclusion is based on the findings (see De Matteis, 1971) that compound SKF 525-A, an inhibitor of the above oxidase system (Fouts & Brodie, 1955), prevents both the loss of cytochrome *P*-450 and the production of green pigments, whereas the mixed-function-oxidase inducer phenobarbitone potentiates both effects. It should be pointed out here that phenobarbitone achieves its effects only at the expense of newly synthesized cytochrome *P*-450 (see also Unseld & De Matteis, 1978). We have previously

shown (Badawy & Evans, 1973b) that the 2-allyl-2-isopropylacetamide-induced depletion of pyrrolase haem in fed rats is prevented by compound SKF 525-A. The results in Table 1 show that this latter compound is also effective in 48 h-starved rats. By contrast, pretreatment with phenobarbitone in the doses used by De Matteis (1971) to induce cytochrome *P*-450 synthesis does not modify the action of 2-allyl-2-isopropylacetamide on pyrrolase haem. The following conclusions may be drawn from this finding: (1) the failure of phenobarbitone is not surprising, because it does not alter haem concentration of the basal pyrrolase; (2) the fact that this is so suggests that tryptophan pyrrolase assayed in whole homogenates does not utilize the increase in microsomal haem concentration produced by this phenobarbitone treatment.

The 2-allyl-2-isopropylacetamide-induced depletion of tryptophan pyrrolase haem can be modified in opposite directions by pretreatment of rats with phenazine methosulphate and nicotinamide (Table 1). It remains to be seen if these two agents are also capable of acting on other haemoproteins. Since the above porphyrinogen depletes haem via a metabolite(s) produced by the NADPH-dependent mixed-function-oxidase system, it may be suggested that the above two agents exert their effects by altering the activity of this system. Support to this suggestion is provided by the ability of the two compounds to alter hexobarbital sleeping-time in opposite directions (see the text). The mechanism(s) by which phenazine methosulphate and nicotinamide alter drug metabolism is not clearly understood at present. They may do so via their opposite effects on liver [NADPH] (see Badawy & Evans, 1976) or by another mechanism(s). Further work is required to examine these possibilities.

2-Allyl-2-isopropylacetamide depletes cytochrome *P*-450 (De Matteis, 1971) and destroys both endogenous microsomal haem and that administered exogenously (Unseld & De Matteis, 1978) *in vitro* only in the presence of an added NADPH-generating system. The results in Table 2 show that pyrrolase haem is also depleted *in vitro* when both the porphyrinogen and NADPH are added together to liver homogenates from control and phenobarbitone-treated rats. Compared with the findings of Unseld & De Matteis (1978), the present changes are achieved by the use of one-quarter the amount of liver and a considerably lower concentration of the porphyrinogen. Moreover, this latter concentration is capable of depleting pyrrolase haem in homogenates from phenobarbitone-treated rats in the absence of added NADPH. This finding may be explained by phenobarbitone enhancing not only the synthesis of cytochrome *P*-450, but also the [NADPH] (see Badawy & Evans, 1973a, 1975b), and by suggesting that pyrrolase haem is preferentially affected *in*

vitro in the presence of a small but sufficient amount of the metabolite(s) of 2-allyl-2-isopropylacetamide.

Relationship between depletion of tryptophan pyrrolase haem and enhancement of 5-aminolaevulinate synthase activity by 2-allyl-2-isopropylacetamide

The marked enhancement of 5-aminolaevulinate synthase activity by the above porphyrinogen (Fig. 4) exhibits two important features: (1) it is preceded by the depletion of pyrrolase haem (Fig. 1); (2) it is interrupted at 4 h after porphyrinogen administration. This interruption is not apparent from the less-detailed time-course reported by Marver *et al.* (1966a,b). It may be of interest that the onset of this interruption (at 4 h) coincides with the interruption of the recovery of pyrrolase haem (Fig 1), although the latter interruption persists for 12 h more. These findings and that showing that the depletion of cytochrome *P*-450 continues unhindered for the whole 8 h observation period (Unsold & De Matteis, 1978) suggest that the depletion of pyrrolase haem is more closely associated with synthase enhancement by the above porphyrinogen.

Although the maximum depletion of pyrrolase haem (at 0.5 h) is achieved by a 100 mg/kg dose of 2-allyl-2-isopropylacetamide (Fig. 2), this dose, unlike larger ones, does not alter synthase activity at 24 h (Fig. 6). This may be explained by other factors being involved in synthase enhancement by larger doses of the porphyrinogen, or by smaller doses (100 mg/kg and below) exerting their effects at earlier time-intervals. The latter possibility is supported by the following findings: (1) the depletion of pyrrolase haem by the 100 mg/kg dose is no longer evident at 5 h (Fig. 3); (2) maximum enhancement of synthase activity by the 50 mg/kg dose is observed at as early as 2 h (Fig. 5); (3) this maximum enhancement (at 2 h) is achieved by the 25 mg/kg dose (Fig. 6); (4) it is also known (White & Muller-Eberhard, 1977) that agents such as norethindrone and ethinyloestradiol cause a maximum enhancement of synthase activity (by a mechanism resembling that by 2-allyl-2-isopropylacetamide) at 2 h, and their depletion of (cytochrome *P*-450) haem is also short-lived. It should be pointed out here that the depletion of cytochrome *P*-450 shown by the above authors is only significant at 2 h, but not earlier. It therefore remains to be seen whether these steroids exert an earlier effect on tryptophan pyrrolase haem.

The findings discussed so far suggest that the enhancement of 5-aminolaevulinate synthase activity by 2-allyl-2-isopropylacetamide need only be explained by the depletion of tryptophan pyrrolase haem. This is also because, although the effects of small doses of 2-allyl-2-isopropylacetamide on cytochrome *P*-450 haem have not been examined under basal conditions, it is unlikely that these doses will

cause a significant change, because only a moderate depletion of cytochrome *P*-450 is observed early after administration of doses of 300–400 mg/kg (De Matteis, 1971; Satyanarayana Rao *et al.*, 1972). The importance of the very small haem pool used by tryptophan pyrrolase in the effects of the above porphyrinogen on synthase activity is further suggested by the now accepted view that the regulatory free-haem pool [which is utilized by tryptophan pyrrolase (Badawy, 1979)] is small and rapidly turning over (De Matteis, 1975; Granick *et al.*, 1975). By adopting this concept, a number of findings can now be more readily explained. (1) The failure of multiple injections of phenobarbitone to potentiate the effect of 2-allyl-2-isopropylacetamide on 5-aminolaevulinate synthase activity at 5 (De Matteis, 1971) or 2 h (Table 3), despite the greater depletion of cytochrome *P*-450 haem (De Matteis, 1971), and the finding (Table 1) that tryptophan pyrrolase haem is not further depleted under these conditions provide further support to the possible involvement of this (pyrrolase) haem pool in the action of the above porphyrinogen on synthase activity, because when the porphyrinogen-induced depletion of pyrrolase haem is influenced by compound SKF 525-A, phenazine methosulphate and nicotinamide (Table 1), parallel changes in synthase activity are observed (Table 3). The inability of phenazine methosulphate completely to inhibit synthase enhancement at 2 h after porphyrinogen administration may be explained by the short-lived effect of the former compound on liver [NADPH]. (2) Non-involvement of cytochrome *P*-450 haem in the potentiation of the enhancement of 5-aminolaevulinate synthase activity by the porphyrinogen 3,5-diethoxycarbonyl-1,4-dihydrocollidine is suggested by the failure of the potentiator phenylbutazone to cause a further early depletion of the above haem (De Matteis & Gibbs, 1972), whereas under these and several other conditions involving potentiation of experimental porphyria, tryptophan pyrrolase haem is further depleted (Badawy, 1978). (3) The findings of the present paper and those discussed above provide an alternative to the suggested (De Matteis, 1978, and references cited therein) involvement of lipid solubility as one of two basically different mechanisms causing synthase enhancement by porphyrinogens. This is also because administration of a small single dose or large multiple doses of phenobarbitone (a lipid-soluble drug) together with a dose of 2-allyl-2-isopropylacetamide sufficient to cause maximum depletion of pyrrolase haem, thus satisfying the specific mechanism, does not influence synthase enhancement by this porphyrinogen at 2 h (see the text and Table 3), nor does it render the above dose of the porphyrinogen (100 mg/kg) capable of enhancing synthase activity at 24 h (results not shown) as do larger doses (Fig. 6).

In conclusion, the present results are compatible with the hypothesis that tryptophan pyrrolase haem plays an important role in the enhancement of 5-aminolaevulinate synthase activity by 2-allyl-2-isopropylacetamide, and that cytochrome *P*-450 haem is primarily involved in the production from the above porphyrigen of the active metabolite(s) responsible for the changes in haem destruction and biosynthesis.

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