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Tryptophan Pyrrolase, the Regulatory Free Haem and Hepatic Porphyrias

EARLY DEPLETION OF HAEM BY CLINICAL AND EXPERIMENTAL EXACERBATORS OF PORPHYRIA

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1. The importance of the early depletion of liver haem in the production of porphyria is discussed and further supporting evidence is presented from experiments with tryptophan pyrrolase, under conditions of exacerbation of experimental porphyria by therapeutic and other agents. 2. In addition to the early depletion of pyrrolase haem by porphyrogens, a further depletion is produced when rats are given a porphyrogen plus an analogue or one of 19 drugs known to exacerbate the human disease. 3. Non-exacerbators of human porphyrias do not cause a further early depletion of pyrrolase haem and it is suggested that this system may be used as a screening test for possible exacerbation of the disease by new and existing drugs. 4. A similar further early depletion of haem is produced by combined administration of lead acetate plus phenobarbitone, thus suggesting that the depletion is a more general phenomenon in experimental porphyria. 5. The relationship between tryptophan pyrrolase and the regulatory free haem is discussed. It is suggested that pyrrolase may play an important role in the regulation of 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 regulates its own synthesis by a negative-feedback mechanism(s) in livers of chick embryos (Granick, 1966; Granick *et al.*, 1975) and mammals (see De Matteis, 1972*a*) including possibly also man (Jeelani Dhar *et al.*, 1975). In mammals, a number of chemically unrelated compounds (including 2-allyl-2-isopropylacetamide, 3,5-diethoxycarbonyl-1,4-dihydrocollidine and griseofulvin) stimulate the hepatic formation of porphyrins and enhance markedly the activity of the above synthase (De Matteis, 1967). Unlike these porphyrogens, several lipid-soluble drugs (including phenobarbitone and phenylbutazone) cause a moderate stimulation of synthase activity and an increase in the concentration of microsomal haem but not in that of porphyrins (see De Matteis, 1972*b*). When, however, one of these drugs is administered together with a porphyrogen to intact animals (De Matteis, 1973; 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 De Matteis (1973, 1975) suggested that this potentiation may explain the exacerbation by drugs of human hepatic porphyrias. That this potentiation is non-specific to either porphyrogens or drugs is suggested by the findings that it can also be produced by joint administration of lead acetate plus phenobarbitone (Maxwell & Meyer, 1976) or of a porphyrogen plus an analogue of it or of another porphyrogen (De Matteis & Gibbs, 1975).

The mechanism by which lipid-soluble drugs cause a moderate enhancement of synthase activity is not understood. Stabilization of synthase may be involved, as has been shown with phenobarbitone (Satyanarayana Rao *et al.*, 1972). By contrast, it is most likely that the marked enhancement of synthase activity by porphyrogens is produced by interference with the 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 regulatory free pool, which has been suggested (see De Matteis, 1975; Granick *et al.*, 1975) to be small and rapidly turning over. Since such a pool would be too small to be measured directly, the early depletion of haem has been demonstrated by indirect methods involving the determination of activity or concentration of, or haem utilization by, the liver haem enzymes catalase (EC 1.11.1.6), cytochrome *P*-450 and tryptophan pyrrolase (EC

1.13.11.11). There is considerable evidence (see Badawy & Evans, 1973; De Matteis, 1971, 1975; De Matteis & Gibbs, 1975; Badawy, 1977*a*) implicating this early depletion of haem in the production of experimental porphyria.

This early depletion of haem has so far been demonstrated in experiments with porphyrogens administered alone. The possible occurrence of a similar effect by treatments causing potentiation of porphyria has not been examined, except in one experiment by De Matteis & Gibbs (1972), who found that the depletion of cytochrome *P*-450 observed at 5h after administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine is not further enhanced by joint administration of the potentiator phenylbutazone. This suggests either that the early depletion of haem is not enhanced by the above potentiator or that cytochrome *P*-450 haem is not involved in a possible further depletion. Rat liver tryptophan pyrrolase is sensitive to several experimental alterations of haem metabolism (see Badawy, 1977*a*; Badawy & Evans, 1973, 1975, 1976) and may therefore be useful in detecting a possible further early depletion of haem under conditions of potentiation of experimental porphyria. The experiments described and discussed in the present paper were designed to examine this possibility, and the results suggest that a further early depletion of pyrrolase haem is produced by exacerbators of experimental and human hepatic porphyrias. It is also proposed that the pyrrolase system showing this depletion may be used as a screening test for possible exacerbation by drugs of human hepatic porphyrias.

Materials and Methods

Animals

Male Wistar rats (150–200g), maintained on cube diet 41B (Oxoid, Basingstoke, Hants., U.K.) and water, were either locally bred or purchased from Anglia Laboratory Animals (Alconbury, Huntingdon, Cambs., U.K.). The animals were starved for either 24 or 48h before being killed.

Chemicals

The sources of various chemicals and the doses and methods of preparation and administration of some of them have been described (Badawy & Evans, 1973; Badawy, 1977*a*). The inhibitor of drug-metabolizing enzymes SKF 525-A (2-diethylaminoethyl 3,3-diphenylpropylacetate) was a gift from Smith, Kline and French (Welwyn Garden City, Herts., U.K.). In addition, 19 exacerbators and 10 non-exacerbators of human hepatic porphyrias were dissolved or suspended in arachis oil and were given (10ml/kg body wt.) by stomach tube. The sources and single doses (in mg/kg) of these drugs (the gifts are indicated by an asterisk) were as follows. Exacerbators: *chlordiazepoxide (Roche, Welwyn Garden City, Herts, U.K.; 1), chloroquine (Sigma, Kingston-upon-Thames, Surrey, U.K.; 5 of base), *chlorpropamide (Pfizer, Sandwich, Kent, U.K.; 2), *dichlorophenazone (Smith and Nephew, Welwyn Garden City, Herts., U.K.; 10), *diphenylhydantoin (Parke, Davis and Co., Pontypool, Gwent, U.K.; 10), ergotamine tartrate (Sigma; 0.5), ethanol (BDH, Poole, Dorset, U.K.; 800), *glutethimide (Ciba, Horsham, West Sussex, U.K.; 5), griseofulvin (Sigma; 50), *meprobamate (John Wyeth and Bros., Taplow, Maidenhead, Berks., U.K.; 5), α-methyldopa (Sigma; 2), oestradiol (Sigma; 0.3), phenobarbitone sodium (BDH; 2), *phenylbutazone (Geigy, Macclesfield, Cheshire, U.K.; 150), progesterone (Sigma; 2), *sedormid (Roche; 10), sulphanilamide (BDH; 50), thiopentone (Abbott, Queenborough, Kent, U.K.; 2) and *tolbutamide (Hoechst, Hounslow, Middlesex, U.K.; 30). Non-exacerbators: cortisol acetate (Sigma; 2), *glipizide (Pfizer; 0.5), *isocarboxazid (Roche; 0.5), morphine sulphate (Koch–Light, Colnbrook, Bucks., U.K.; 1), *oxypentifylline (Hoechst; 5), paracetamol or acetaminophen (Boots, Nottingham, U.K.; 10), pethidine hydrochloride (Roche; 1.5), *pheniramine maleate (Hoechst; 2), *propranolol (I.C.I., Macclesfield, Cheshire, U.K.; 5) and sodium salicylate (BDH; 10). For recent lists of exacerbators of human hepatic porphyrias, see Beattie & Goldberg (1972–1974) and Maxwell & Meyer (1976). Phenylbutazone is included among exacerbators, since it potentiates experimental porphyria (see De Matteis & Gibbs, 1972). The ten compounds listed above as non-exacerbators of porphyria are assumed to be so because of the absence of any reports to the contrary and also since some of them (particularly the sedatives) are actually prescribed in porphyrias.

Determination of tryptophan pyrrolase activity and the haem-saturation ratio

The enzyme activity was determined in liver homogenates (Badawy & Evans, 1975) in either the absence (holoenzyme activity) or the presence (total enzyme activity) of added haematin (2μM). 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 saturation of the apoenzyme with haem (see also Badawy, 1977*a*). The haem saturation was expressed by this ratio rather than as percentage saturation of the total enzyme because a 50% decrease in the former can be achieved by doses of porphyrogens smaller than those required to produce a similar decrease in the latter (see below).

Student's *t* test was used to assess the significance of differences between means.

Results

Conditions for assessing changes in the haem-saturation ratio of tryptophan pyrrolase

To assess the haem-saturation ratio as the sole variable, two factors must be considered. (1) To prevent changes in the ratio other than those caused by an altered haem availability, starved rats were used to raise the total pyrrolase (and therefore the holoenzyme) activity by a hormonal mechanism (see Badawy, 1977*b*), thus forestalling any enhancement by agents known or found to act by the same mechanism. 2-Allyl-2-isopropylacetamide and griseofulvin were still capable of enhancing the total pyrrolase activity in 24h-starved rats, and these were therefore used only in experiments with their analogues. The third porphyrogen, 3,5-diethoxycarbonyl-1,4-dihydrocollidine, did not alter the total enzyme activity and was therefore used in most experiments. (2) To test the possible further early depletion of haem in rats given joint doses of a porphyrogen plus either one of its analogues, or an exacerbator of human porphyria, it is important to administer the porphyrogen in a dose causing submaximum (preferably 50%) depletion of pyrrolase haem as expressed by the haem-saturation ratio. The results in Fig. 1 show that this is achieved by a 50mg/kg dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine in both fed and 24h-starved rats: half this dose decreased the ratio only in fed animals. As shown in Table 1, a 50mg/kg dose of either 2-allyl-2-isopropylacetamide or griseofulvin was also effective in decreasing the haem-saturation ratio by 50%. For the purposes of this work, we preferred to use the holoenzyme/apoenzyme ratio rather than percentage saturation of the total enzyme because a 50% decrease in the latter required doses of a porphyrogen that caused almost maximum depletion of holo-(tryptophan pyrrolase) haem.

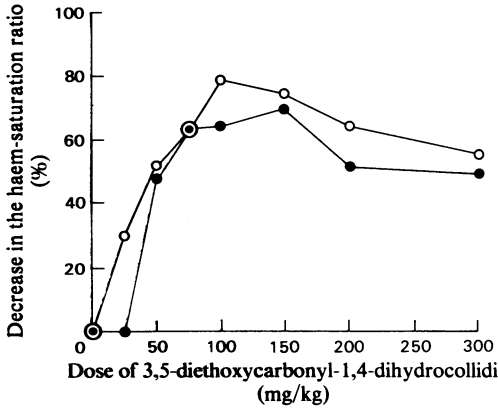

Fig. 1. *Effect of* 3,5-*diethoxycarbonyl*-1,4-*dihydrocollidine on the haem-saturation ratio of rat liver apo-(tryptophan pyrrolase)*

Both fed and 24h-starved rats were given an intraperitoneal injection of the above porphyrogen, in various doses, at 4h before death. The holoenzyme and total pyrrolase activities were then determined and the apoenzyme activity was calculated by difference. The haem-saturation ratio is the ratio of holoenzyme/apoenzyme activity. Each point represents the ratio calculated from the mean enzyme activities for four rats per group. ○, Fed rats; ●, starved rats.

Effects of haematin on rat liver tryptophan pyrrolase activity in vitro

These are shown in Fig. 2. Optimum activation of the enzyme was produced by 1 μM-haematin. However, 2 μM was used in all other experiments to ensure that minor changes in haem availability to the apoenzyme *in vitro* did not affect the activation.

Table 1. *Early effects of porphyrogens, their analogues or mixtures of both on rat liver tryptophan pyrrolase activity and the haem-saturation ratio*

Rats were starved for 24h before death. All compounds were dissolved in dimethylformamide and were injected (1ml/kg) intraperitoneally, except 2-allyl-2-isopropylacetamide, which was injected into the loose subcutaneous tissues of the neck. Porphyrogens were given at 15min before their respective analogues, which were administered at 4h before death. Control rats received two injections (15min apart) of dimethylformamide (1ml/kg); rats treated with either a porphyrogen or an analogue, but not the two together, also received an injection of the solvent. All compounds were given in a 50mg/kg dose each, except 2-propyl-2-isopropylacetamide (100mg/kg). Values are means±S.E.M. for each group of four rats (for enzyme activity) and a single determination of the ratio based on these means. Abbreviations: DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DC, 3,5-diethoxycarbonylcollidine; GF, griseofulvin; GF-TE, griseofulvin thioether analogue; AIA, 2-allyl-2-isopropylacetamide; PIA, 2-propyl-2-isopropylacetamide.

Treatment	Kynurenine formed (μmol/h per g wet wt. of liver) Holoenzyme activity	Total enzyme activity	Haem-saturation ratio
Control	3.4±0.11	8.2±0.5	0.71
DC	3.4±0.02	8.9±.12	0.62
DDC	2.6±0.17	8.1±0.2	0.47
DDC+DC	1.9±0.28	8.8±1.1	0.27
GF-TE	3.1±0.30	8.0±0.3	0.63
GF	4.0±0.26	14.8±1.0	0.37
GF+GF-TE	2.5±0.16	10.4±0.8	0.32
PIA	3.8±0.20	8.6±0.6	0.79
AIA	3.7±0.15	12.9±1.1	0.40
AIA+PIA	2.6±0.20	12.0±0.5	0.28

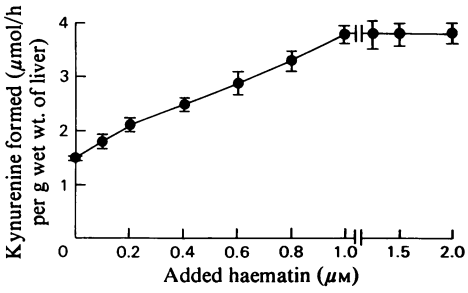

Fig. 2. *Effect of haematin on rat liver tryptophan pyrrolase activity in vitro*

Liver homogenates from fed rats were incubated *in vitro* with various concentrations of haematin. Each point represents the mean±S.E.M. for each group of four animals.

Table 2. *Late effects of porphyrogens, their analogues or mixtures of both on rat liver tryptophan pyrrolase activity and the haem-saturation ratio*

Experimental conditions and abbreviations are as described in Table 1, except that the rats were starved for 48h before death and the analogues of porphyrogens were given at 15min before their respective porphyrogens and at 24h before death. Values for the enzyme activity are means±S.E.M. for each group of four rats. Single ratio values are based on these means.

Treatment	Kynurenine formed (μmol/h per g wet wt. of liver) Holoenzyme activity	Total enzyme activity	Haem-saturation ratio
Control	2.3±0.14	8.4±0.8	0.38
DC	2.7±0.21	8.1±0.7	0.50
DDC	3.5±0.41	7.7±0.3	0.83
DDC+DC	5.6±0.66	9.1±1.6	1.60
GF-TE	2.4±0.14	6.5±0.3	0.58
GF	3.7±0.28	7.9±0.4	0.88
GF+GF-TE	4.7±0.32	7.7±0.9	1.56
PIA	2.5±0.19	8.1±0.4	0.45
AIA	3.7±0.26	7.8±0.6	0.90
AIA+PIA	5.2±0.53	8.4±0.4	1.62

Effects of porphyrogens, their analogues or mixtures of both on rat liver tryptophan pyrrolase activity and the haem-saturation ratio

The early (4h) effects of the above treatments are shown in Table 1. The three porphyrogens decreased the haem-saturation ratio from a control value of 0.71 to 0.37–0.47. The analogues of these porphyrogens did not cause any notable changes in the ratio or the enzyme activities. When each of these analogues was given with its corresponding porphyrogen, both the holoenzyme activity and the ratio were decreased to values below those observed with each individual porphyrogen; the decreases in activity were 27–37% ($P = 0.05$–0.025), whereas those in the ratio were 13–42%.

The late (24h) effects of the above treatments are shown in Table 2. The holoenzyme activity and the ratio were both increased by all three porphyrogens, the rises in the former being 52–61% ($P = 0.025$–0.0025). None of the analogues, given alone, caused any significant changes in these parameters, but when given jointly with its corresponding porphyrogen it increased both the holoenzyme activity and the ratio to values above those observed with each individual porphyrogen, the further rises in the holoenzyme activity being 27–60% ($P = 0.05$–0.025).

Effects of 3,5-diethoxycarbonyl-1,4-dihydrocollidine with or without exacerbators of human hepatic porphyrias on rat liver tryptophan pyrrolase activity and the haem-saturation ratio

The early effects of these treatments are shown in Table 3. At 4h, 3,5-diethoxycarbonyl-1,4-dihydrocollidine decreased the ratio by 40–54% and the holoenzyme activity by 28–39% ($P = 0.05$–0.005). None of the exacerbators listed caused any significant changes in the ratio or in the activity, except phenylbutazone, which decreased both activities equally. However, when each of these exacerbators was given with the porphyrogen, it decreased the holoenzyme activity (below that observed with the porphyrogen alone) by 16–47% ($P = 0.05$–0.005) and the haem-saturation ratio by 22–58%. The following additional exacerbators of porphyria exerted similar effects: chlordiazepoxide, chloroquine, chlorpropamide, dichlorophenazone, diphenylhydantoin, ergotamine, glutethimide, griseofulvin, meprobamate, α-methyldopa, oestradiol, sedormid, sulphanilamide, thiopentone and tolbutamide (results not shown). By contrast, the non-exacerbators of porphyria used in Table 3 did not exert any significant effects on the pyrrolase activity or the haem-saturation ratio whether they were given alone or in combination with 3,5-diethoxycarbonyl-1,4-dihydrocollidine. The following additional non-exacerbators also had no effect: glipizide, isocarboxazid, morphine sulphate, oxypentifylline, pheniramine maleate and sodium salicylate (results not shown).

The late (24h) effects of some of these compounds are shown in Table 4. None of the compounds tested alone exerted any effects on the pyrrolase activities or the haem-saturation ratio. The increased haem saturation of the pyrrolase caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine was not further enhanced by the non-exacerbators tested, but was increased by the exacerbators. These increased the holoenzyme activity by 33–42% ($P = 0.05$–0.025) and the haem-saturation ratio by 50–73% (in comparison with the values observed with the porphyrogen alone).

Table 3. *Early effects of clinical exacerbators and non-exacerbators of porphyria (with or without the combined administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine) on rat liver tryptophan pyrrolase activity and the haem-saturation ratio*

Rats were starved for 24h before death. Drugs were either dissolved or finely suspended in arachis oil (10ml/kg) and were given by stomach tube (in the doses listed in the Materials and Methods section) at 4h before death and at 15min after an intraperitoneal injection of either dimethylformamide (1ml/kg) or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC; 50mg/kg in 1ml of dimethylformamide). Control rats received both solvents (15min apart), whereas those treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine only were given arachis oil 15min later. Values for the enzyme activity are means±S.E.M. for each group of four rats. Single ratio values are based on these means.

Treatment	Kynurenine formed (μmol/h per g wet wt. of liver) Holoenzyme activity	Total enzyme activity	Haem-saturation ratio
A. Exacerbators:			
Control	5.2±0.22	11.7±0.2	0.80
DDC	3.4±0.16	11.0±1.1	0.45
Phenylbutazone	4.0±0.36	8.1±0.3	0.97
DDC+phenylbutazone	1.8±0.13	8.4±0.8	0.27
Control	4.6±0.50	9.2±0.3	1.00
DDC	2.9±0.34	8.3±0.8	0.53
Ethanol	4.5±0.14	9.4±0.5	0.92
DDC+ethanol	2.1±0.20	9.7±0.9	0.22
Control	4.4±0.53	8.5±0.2	1.07
DDC	3.1±0.15	9.3±0.7	0.50
Phenobarbitone	4.5±0.40	9.2±0.2	0.96
DDC+phenobarbitone	1.8±0.11	8.4±0.5	0.27
Control	5.1±0.49	10.6±1.3	0.93
DDC	3.5±0.29	10.5±0.3	0.50
Progesterone	5.1±0.49	10.5±1.5	0.94
DDC+progesterone	2.4±0.18	9.8±0.3	0.32
B. Non-exacerbators			
Control	5.1±0.49	10.6±1.3	0.93
DDC	3.5±0.29	10.5±0.3	0.50
Cortisol	5.2±0.23	12.9±0.2	0.68
DDC+cortisol	3.5±0.16	10.9±0.17	0.47
Control	4.5±0.06	10.4±0.8	0.76
DDC	3.0±0.23	9.6±0.3	0.45
Paracetamol	3.9±0.39	10.2±1.0	0.62
DDC+paracetamol	3.0±0.23	10.3±1.0	0.41
Control	4.7±0.17	9.8±0.7	0.92
DDC	3.2±0.16	9.9±0.8	0.48
Pethidine	4.3±0.42	9.7±0.7	0.80
DDC+pethidine	3.3±0.29	9.2±0.7	0.56
Control	4.6±0.37	10.1±1.1	0.84
DDC	3.1±0.14	9.5±0.2	0.48
Propranolol	4.8±0.09	11.2±0.5	0.75
DDC+propranolol	3.4±0.32	10.0±0.5	0.51

Table 4. *Late effects of clinical exacerbators and non-exacerbators of porphyria (with or without the combined administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine) on rat liver tryptophan pyrrolase activity and the haem-saturation ratio*

Experimental conditions are as described in Table 3 (and the doses of drugs as in the Materials and Methods section), except that the rats were starved for 48h before death and the three drugs of each group were given at 15min after the porphyrogen 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC, 50mg/kg) or an equal volume (1ml/kg) of dimethylformamide and at 24h before death. Values for the enzyme activity are means±S.E.M. for each group of four rats. Single ratio values are based on these means.

Treatment	Kynurenine formed (μmol/h per g wet wt. of liver) Holoenzyme activity	Total enzyme activity	Haem-saturation ratio
Exacerbators:			
Control	2.6±0.08	7.3±0.2	0.55
DDC	3.3±0.09	6.5±0.4	1.03
Ethanol	2.9±0.28	7.0±0.4	0.71
DDC+ethanol	4.5±0.41	7.4±0.2	1.55
Meprobamate	2.6±0.21	7.0±0.5	0.59
DDC+meprobamate	4.4±0.51	6.9±0.2	1.76
Phenobarbitone	2.6±0.05	7.1±0.6	0.58
DDC+phenobarbitone	4.7±0.60	7.2±0.6	1.88
Non-exacerbators:			
Control	2.9±0.18	7.6±0.3	0.62
DDC	4.1±0.37	7.4±0.8	1.24
Morphine	2.7±0.23	6.8±0.3	0.66
DDC+morphine	3.9±0.32	7.1±0.7	1.22
Paracetamol	2.5±0.07	6.2±0.4	0.67
DDC+paracetamol	4.0±0.18	7.0±0.1	1.33
Pheniramine	2.8±0.23	7.9±0.4	0.55
DDC+pheniramine	4.1±0.20	7.9±0.7	1.08

The effects of pretreatment of rats with compound SKF 525-A on the early depletion of pyrrolase haem caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine with or without joint ethanol administration are shown in Table 5. Compound SKF 525-A prevented the depletion of pyrrolase haem (i.e. the decrease in the holoenzyme activity and the haem-saturation ratio) observed at 4h after administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine. The further early depletion of haem produced by combined administration of ethanol with the above porphyrogen was also prevented by compound SKF 525-A. The latter compound alone exerted no significant effects.

The effects of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (25mg/kg), ethanol (800mg/kg) or both

Table 5. *Prevention by compound SKF 525-A of the early depletion of liver haem by 3,5-diethoxycarbonyl-1,4-dihydrocollidine with or without combined ethanol administration*

Rats were starved for 24h before being killed and were given 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC; 50mg/kg), ethanol (800mg/kg) or both as described in Table 3. At 45min before the administration of DDC, some rats received an intraperitoneal injection of compound SKF 525-A (45mg/kg, dissolved in 0.9% NaCl). Control rats received the solvents. The values given are means±S.E.M. for each group of four rats.

Pretreatment ...	Kynurenine formed (μmol/h per g wet wt. of liver)			
	None		Compound SKF 525-A	
Treatment	Holoenzyme activity	Total enzyme activity	Holoenzyme activity	Total enzyme activity
Control	4.3±0.31	8.7±0.6	4.4±0.47	8.3±0.7
DDC	3.0±0.14	8.4±0.9	4.3±0.12	8.7±0.3
Ethanol	3.8±0.34	8.1±0.8	4.6±0.33	10.2±1.0
DDC+ ethanol	2.4±0.08	8.6±0.6	4.4±0.25	8.9±0.9

on tryptophan pyrrolase activity were also examined at 4h after administration to 24h-starved rats. The holoenzyme and total pyrrolase activities (in μmol of kynurenine formed/h per g wet wt. of liver±S.E.M. for each group of four rats) were as follows: control rats (4.8±0.5 and 10.6±1.7); ethanol-treated rats (4.6±0.4 and 10.5±0.8); porphyrogen-treated rats (4.2±0.1 and 9.6±1.3); porphyrogen plus ethanol-treated rats (4.7±0.4 and 10.0±0.5). These results therefore show that none of these single or combined treatments caused any decreases in the holoenzyme activity or the haem-saturation ratio.

Effects of lead acetate plus phenobarbitone on rat liver tryptophan pyrrolase activity and the haem-saturation ratio

As shown in Table 6, at 4h, lead acetate caused a 22% decrease ($P<0.05$) of the holoenzyme activity and a 34% decrease in the haem-saturation ratio in 24h-starved rats. Phenobarbitone decreased the holoenzyme activity by 25% ($P<0.025$), but this had little effect on the ratio because the total activity was also decreased (by 19%, though not significantly). Combined administration of the two compounds caused, at 4h, a further decrease in the holoenzyme activity (of 42–44%; $P<0.005$) and in the haem-saturation ratio below the values observed with either individual treatment. The 24h effects of the same treatments (Table 6) show that neither lead acetate nor phenobarbitone caused any significant changes when given alone, but that the two together increased

Table 6. *Early and late effects of lead acetate, phenobarbitone or both on rat liver tryptophan pyrrolase activity and the haem-saturation ratio*

Lead acetate (10mg/kg in 1ml of water) or phenobarbitone sodium (100mg/kg in 1ml of 0.9% NaCl) were each injected intraperitoneally either separately or together. Control rats received both solvents, whereas those treated with one compound also received an injection of the other solvent. The early effects were examined at 4h after injections in 24h-starved rats, whereas the late ones were studied at 24h after injections in 48h-starved animals. Values for the enzyme activity are means±S.E.M. for each group of four rats. Single ratio values are based on these means.

Treatment	Kynurenine formed (μmol/h per g wet wt. of liver)		Haem-saturation ratio
	Holoenzyme activity	Total enzyme activity	
Early effects			
Control	3.2±0.28	8.2±0.6	0.64
Lead acetate	2.5±0.14	8.5±0.7	0.42
Phenobarbitone	2.4±0.07	6.6±0.7	0.57
Lead acetate+ phenobarbitone	1.4±0.12	7.0±0.3	0.25
Late effects			
Control	2.6±0.15	7.5±0.9	0.53
Lead acetate	2.2±0.17	9.3±0.6	0.31
Phenobarbitone	2.4±0.25	5.9±0.4	0.68
Lead acetate+ phenobarbitone	3.7±0.26	5.8±0.1	1.76

the holoenzyme activity by 42% ($P<0.01$) above the control value and the ratio by over 3-fold.

Discussion

Importance of the early depletion of liver haem in the production of porphyria

Granick (1966) suggested that drugs enhance 5-aminolaevulinate synthase activity by interfering with the negative-feedback control of haem biosynthesis. There is considerable evidence (see the introduction) implicating the early depletion of the liver haem used by apo-(cytochrome *P*-450) and apo-(tryptophan pyrrolase) as one such interference. This concept in mammals does not apply to the chick-embryo (or chicken) liver system, because several drugs known not to cause an early depletion of liver haem in either system are capable of producing porphyria in the chick (or chicken) liver (Granick, 1966; Creighton & Marks, 1972) and this has been suggested (De Matteis, 1973, and references cited) to be due to the greater sensitivity of the latter system to drugs and also to the lability of its haem-biosynthetic pathway.

The early depletion of liver haem has been shown in

experiments with porphyrogens administered alone. The present results (Tables 1 and 3) suggest that a further early depletion of pyrrolase haem occurs under conditions of potentiation of experimental porphyria (see De Matteis, 1973; De Matteis & Gibbs, 1972, 1975). In addition, this further early depletion of haem is produced by almost all known exacerbators of human hepatic porphyrias, but not by drugs causing no exacerbation, and this distinction offers a possible laboratory test for screening potential therapeutic agents for possible exacerbation of the human disease. The results in Tables 2 and 4 suggest that, under conditions of potentiation of porphyria, haem utilization by apo-(tryptophan pyrrolase) is enhanced to a greater extent than with unpotentiated porphyria, a finding confirming the potentiation phenomenon. These results strongly suggest the importance of the early depletion of mammalian liver haem in the production of porphyria and its exacerbation by drugs and other compounds.

Similar conclusions can be drawn from a different potentiation system involving combined administration of lead acetate and phenobarbitone (see Maxwell & Meyer, 1976). This system is also associated with a further early depletion of pyrrolase haem and a later increase in haem utilization by the apoenzyme (Table 6). These results also suggest that the early depletion of haem may be a more general phenomenon in experimental porphyria.

The mechanism(s) by which clinical and experimental exacerbators of porphyria cause further early depletion of pyrrolase haem is not understood at present. Since these compounds alone do not exert such effects, it may be concluded that they do not act directly by inhibiting haem synthesis or increasing haem degradation. Available evidence supports this conclusion (see De Matteis, 1975; Satyanarayana Rao *et al.*, 1972). Other possibilities are: (1) enhancement of metabolism of the porphyrogen; (2) enhancement of the action of the porphyrogen metabolite; (3) removal of pyrrolase haem by an unknown mechanism(s), e.g., enhancement of haem oxygenase activity which, in the presence of the original depletion of haem, may lead to the observed effects. The results in Table 5 suggest that compound SKF 525-A, an inhibitor of drug-metabolizing enzymes (Foutes & Brodie, 1955) that has been shown (Badawy & Evans, 1973) to prevent the early depletion of pyrrolase haem by porphyrogens, also prevents the further early depletion caused by joint ethanol administration. This also suggests that the ethanol effect requires a metabolized, not an intact, porphyrogen molecule. The inability of ethanol to render an inactive dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (25mg/kg, see Fig. 1) capable of depleting pyrrolase haem (see the text) suggests that ethanol does not enhance the metabolism of this porphyrogen. A detailed examination of the other possibilities is required.

Relationship between tryptophan pyrrolase, the regulatory free haem and porphyria

The present results and those previously reported (Badawy & Evans, 1973, 1975; Badawy, 1977*a*) strongly suggest that rat liver tryptophan pyrrolase utilizes the free haem pool involved in the regulation of haem biosynthesis, because changes in the haem saturation of the apoenzyme occur under several conditions leading to altered haem synthesis and degradation. These findings confer on tryptophan pyrrolase a unique position among liver haem proteins in relation to haem utilization and possibly also regulation.

The precise concentration, location and mechanism(s) of action of the regulatory free haem pool are not clearly understood at present. Current theories on the latter aspect suggest an inhibition of transport of newly synthesized 5-aminolaevulinate synthase from cytosol to mitochondria (in rat liver) (Hayashi *et al.*, 1972) or repression of synthase synthesis (Granick *et al.*, 1975). Whichever mechanism(s) is involved, the regulatory free haem pool must primarily be present in the cytosol, because it is the only access for mitochondria-derived haem to the site(s) of synthase synthesis and/or repression. The importance of the cytosol is further suggested by the finding *in vitro* (Israels *et al.*, 1975; Yoda & Israels, 1972) that cytosolic proteins, with relatively low and non-specific affinity sites for haem, are required to contact the mitochondria to facilitate the exit of haem into the cytosol.

There is no direct information on the concentration of cytosolic haem or that of its regulatory fraction. It may, however, be useful to consider the indirect evidence. Thus, on the basis of repression studies with chick embryo liver, Granick *et al.* (1975) suggested that a steady-state concentration of free or readily exchangeable haem poised at a dissociation constant [of a postulated haem-apoprotein (co-repressor) complex] of 0.1 μM would be most suitable for the regulation of synthase synthesis. This concentration represents low-affinity sites for haem, in contrast with higher ones exhibited by catalase and cytochrome *P*-450. This suggests that cytosolic haem-binding proteins (with low-affinity sites) are involved in haem regulation. The low-affinity property would seem particularly important in this respect, since it allows more rapid reversible changes in haem binding to occur in response to those in haem metabolism, and tryptophan pyrrolase seems well suited for this function. Ligandin (see Ketterer *et al.*, 1976) has a dissociation constant for haem similar to that suggested by Granick *et al.* (1975) for the haem–apoprotein complex, but it remains to be seen whether the utilization of haem by ligandin is as versatile as that by pyrrolase.

The finding (Fig. 2) that 1 μM-haematin fully saturates apo-(tryptophan pyrrolase) in liver homogenates

in vitro and the suggestion (Feigelson & Greengard, 1961) that only one-tenth of the above concentration is required by cytosolic preparations suggest that pyrrolase uses very little haem. It may be calculated from Fig. 2 that each 14% difference in pyrrolase saturation with haem represents a 0.2 μM-haematin concentration change in homogenates, or 0.02 μM in the cytosol. By using this latter value, it may be possible to calculate the concentration of cytosolic haem. The largest depletion of pyrrolase haem, by phenylbutazone plus 3,5-diethoxycarbonyl-1,4-dihydrocollidine (Table 3), represents a 65% decrease in the holoenzyme activity, or a loss of haem of 0.093 μM by the cytosol. This is similar to that (0.1 μM) suggested by Granick *et al.* (1975) for free (or readily exchangeable) haem. It may therefore be suggested that the regulatory free haem concentration in the cytosol is similar to, or lower than, the above value. Although indirect, these calculations and the response of pyrrolase to changes in haem metabolism suggest that this enzyme may play an important role in haem regulation. Further work is required to examine this possibility.

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