Stimulation of Liver 5-Aminolaevulinate Synthetase by Drugs and its Relevance to Drug-Induced Accumulation of Cytochrome *P*-450

STUDIES WITH PHENYLBUTAZONE AND 3,5-DIETHOXYCARBONYL-1,4-DIHYDROCOLLIDINE

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The relevance of the stimulation of 5-aminolaevulinate synthetase to the accumulation of cytochrome *P*-450 after administration of drugs was examined in rats treated with phenylbutazone and with 3,5-diethoxycarbonyl-1,4-dihydrocollidine. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine alone stimulated 5-aminolaevulinate synthetase without increasing the concentration of cytochrome *P*-450, whereas phenylbutazone alone increased the microsomal cytochrome *P*-450 without significantly affecting the activity of the enzyme. When the two drugs were given together both effects were found. It is concluded that if an increased amount of 5-aminolaevulinate and haem must be made to provide for the accumulation of cytochrome *P*-450, it need only be a small amount. It is also concluded from these findings that stimulation of the drug-metabolizing system on the one hand and marked enhancement of 5-aminolaevulinate synthetase activity and porphyria on the other are likely to result from different actions of the drugs. Evidence is presented suggesting that porphyrogenic drugs stimulate markedly the activity of 5-aminolaevulinate synthetase by lowering the concentration of haem in the liver, thereby decreasing the normal feedback control. With 3,5-diethoxycarbonyl-1,4-dihydrocollidine a rapid inhibition of mitochondrial ferrochelatase and of liver haem synthesis may be the primary mechanism involved.

Several chemically unrelated drugs cause hepatic porphyria in animals and stimulate the formation of porphyrins by liver cells cultured *in vitro* (De Matteis, 1967, 1971a).

There are a number of similarities between drug-induced porphyria and stimulation of drug-metabolizing enzymes. In both effects: (1) drugs act directly on liver cells and not through hormones; (2) several chemically unrelated drugs are equally effective; (3) lipid solubility is an important property of effective drugs; (4) the metabolic change can be prevented by inhibitors of protein synthesis and by glucose. An increase in the microsomal cytochrome *P*-450 accompanies the stimulation of drug-metabolizing enzymes and this may require increased haem synthesis and enhanced activity of 5-aminolaevulinate synthetase, the rate-limiting enzyme of the haem-biosynthetic pathway (Granick, 1966). Granick & Urata (1963) have found that the activity of this enzyme increases in the liver of animals made porphyric by drugs and Granick (1966) has suggested that the increase in microsomal cytochrome *P*-450 and stimulation of 5-aminolaevulinate synthetase observed after drugs may be two aspects of the same response of the liver cell to detoxify foreign compounds.

Experiments have now been carried out in rats after treatment with drugs to find out whether increased activity of the haem-biosynthetic pathway and porphyria on the one hand, and increase in microsomal cytochrome *P*-450 on the other, are closely related phenomena and can be regarded as resulting from a single action of the drugs. Some of the findings have been given in a preliminary form at the International Conference on the Porphyrins, Cape Town (De Matteis, 1971a).

Materials and Methods

Treatment of animals

Male albino rats of the Porton strain (150–180 g) were either killed at different times after a single dose of drugs or given two daily doses for 2 or 3 days and killed 17 h after the last dose. Finely powdered phenylbutazone (4-*n*-butyl-1,2-diphenyl-3,5-dioxo-pyrazolidine) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine were suspended in arachis oil and given by stomach tube. Control animals received the same volume of arachis oil. In the labelling experiment *in vivo* rats were starved for 24 h, then dosed with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (200 mg/kg)

or arachis oil. At 5 h later they were injected intravenously with $50\mu\text{Ci}$ of [^{59}Fe]ferric citrate/kg and killed by decapitation 10 min later.

Isolation of liver mitochondria and microsomal fraction

Mitochondria for 5-aminolaevulinate synthetase assays were obtained as described by De Matteis (1971b). Mitochondria for porphyrin-metal chelatase assay and for porphyrin determination were obtained in 0.25 M-sucrose as described by Jones & Jones (1969), washed once and resuspended in 0.25 M-sucrose. The microsomal fraction was obtained, washed and resuspended in 0.1 M-sodium phosphate buffer, pH 7.4, as described by Bond & De Matteis (1969).

Determination of microsomal cytochromes and protohaem

The microsomal cytochromes were determined as previously described (Bond & De Matteis, 1969) by using a Unicam SP.800 recording spectrophotometer. The total protohaem content of the washed microsomal fraction was determined as described by De Matteis (1971b).

Enzyme assays

5-Aminolaevulinate synthetase was assayed by using washed mitochondria as the source of the enzyme (De Matteis, 1971b). 5-Aminolaevulinate dehydratase (EC 4.2.1.24) was assayed as described by Gibson *et al.* (1955) with the difference that a sample of the deproteinized supernatant (0.5 ml) was mixed with 1 ml of water and 1.5 ml of Ehrlich reagent, 2 M with respect to HClO_4 (Mauzerall & Granick, 1956); the E_{555} was determined between 15 and 25 min after addition of Ehrlich reagent and a molar extinction coefficient of 61 000 (Mauzerall & Granick, 1956) used to calculate the amount of porphobilinogen present.

Porphyrin-metal chelatase was determined, with mesoporphyrin and Co^{2+} as substrates, by the spectrophotometric method developed by Jones & Jones (1969), except that the decrease in extinction of band IV of the porphyrin was followed by scanning the spectrum from 520 to 490 nm repeatedly, every 30–60 s, in a Unicam SP.800 spectrophotometer connected to a scale-expander. Mesoporphyrin (100 nmol) and mitochondria (5–8 mg of protein) in 2.75 ml of 200 mM-tris-HCl buffer, pH 8.2, containing 0.074% Tween 80, were incubated at 30°C in the presence of CoCl_2 (100 nmol).

For assay of aniline hydroxylase and nitroanisole demethylase activities washed microsomal fractions (4.5–6 mg of protein) were incubated at 38°C with shaking (120 double oscillations/min) in an atmo-

sphere of O_2 . Each flask contained, in a total volume of 3 ml, the following components (final concentrations in parentheses): KCl (66.6 mM); sodium phosphate buffer (66.6 mM); MgCl_2 (14.0 mM); glucose 6-phosphate (4.5 mM); NADP (0.19 mM); glucose 6-phosphate dehydrogenase (0.5 unit/flask); as substrate, aniline (10 mM) or *o*-nitroanisole (3.33 mM). Incubation time was 10 min for aniline hydroxylase assay and 20 min for nitroanisole demethylase assay at a pH of 7.4 and 7.9 respectively. The *o*-nitrophenol and 4-aminophenol produced during the incubation were determined as described by Gilbert & Golberg (1965).

Haem synthesis in vitro

This was followed by measuring the incorporation of ^{59}Fe into haem by liver homogenates or by liver slices. Liver homogenates (10%, w/v) in 0.25 M-sucrose (4 ml) or 400 mg of liver slices were incubated in air at 38°C with shaking (100 cycles/min) in the presence of 500 μmol of sodium phosphate-potassium phosphate buffer, pH 7.4, or of Krebs-Ringer phosphate solution, pH 7.4 (Umbreit *et al.*, 1959) respectively, in a total volume of 5 ml. After a preliminary incubation of 2 min approx. 0.5 μCi of [^{59}Fe]ferric citrate was added and the incubation continued for a further 15 min. For liver slices the preliminary incubation was of 10 min and the incubation mixture contained also 30 nmol of unlabelled ferrous sulphate.

After addition of horse erythrocyte lysate (to provide carrier haem) haemin was crystallized by the method of Labbe & Nishida (1957). The dry crystals of haemin were dissolved in pyridine, the solution was filtered, a small sample taken for measurement of haem as the pyridine haemochrome and the remainder counted for radioactivity in a Packard Autogamma scintillation spectrometer. No change in specific radioactivity was observed after recrystallization of haemin from either experimental or control samples. A similar method was followed for the isolation of haemin from the liver of the rats injected with ^{59}Fe .

Analytical methods

Proteins were determined either by Aldridge's (1962) modification of the method of Robinson & Hogden (1940), or as described by Lowry *et al.* (1951) with crystalline bovine albumin as the standard. Isolated haem was determined as the pyridine haemochrome by the method of Rimington (1942). Porphyrins were extracted from total liver homogenates or from the mitochondrial fraction into 0.9 M- HClO_4 -ethanol (1:1, v/v) and measured fluorimetrically with protoporphyrin as the standard (Abbritti & De Matteis, 1971–72). 5-Aminolaevulinate was assayed in liver homogenates as described by De Matteis (1971b); the

chromogen remaining after extraction with ether was assumed to be entirely due to 5-aminolaevulinate.

Source of drugs and special chemicals

3,5-Diethoxycarbonyl-1,4-dihydrocollidine was obtained from Kodak Ltd., Kirkby, Liverpool, U.K.; it was recrystallized twice from aqueous ethanol. Phenylbutazone was a gift of Geigy Pharmaceuticals, Wythenshawe, Manchester 23, U.K., through the courtesy of Dr. P. D. Fowler. Protoporphyrin IX and 5-aminolaevulinate were obtained from Sigma (London) Chemical Co., London S.W.6, U.K., and mesoporphyrin IX was a gift of Dr. S. Schwartz, Department of Medicine, University of Minnesota, Minneapolis, Minn., U.S.A. [^{59}Fe]Ferric citrate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and had the specific radioactivity of 10mCi/mg of Fe (at the time of issue).

Calculation of results

Results are expressed as the arithmetic means $\pm$ S.E.M. and the means compared by Student's *t* test

(Fisher, 1934). To obtain the value of *P*, a degree of freedom $N_1 + N_2 - 2$ has been used throughout.

Results

In the present experiments two drugs were used: 3,5 - diethoxycarbonyl - 1,4 - dihydrocollidine and phenylbutazone. They were chosen because the former had been previously found to stimulate porphyrin production by liver cells cultured *in vitro* at the concentration of 1 μM , whereas phenylbutazone had been found to be inactive in that system up to the highest concentration employed, 0.2mM (Granick, 1966; De Matteis, 1971a), but it had been reported to stimulate the activity of drug-metabolizing enzymes in the liver of the rat (Conney *et al.*, 1960).

Concentration of cytochrome P-450 and 5-aminolaevulinate synthetase activity after treatment with drugs

When starved rats were given two daily doses of drugs for 2 days, 3,5-diethoxycarbonyl-1,4-dihydrocollidine was found to stimulate 5-aminolaevulinate

Table 1. *Effect of treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine or phenylbutazone or both on the size and protein content of the liver, on the concentration of protein and cytochromes of the microsomal fraction and on the 5-aminolaevulinate synthetase activity of the mitochondria*

All rats were starved throughout the whole experiment. Drugs were given by stomach tube twice a day for 2 days. Results are expressed as means $\pm$ S.E.M. with numbers of observations in parenthesis. **P* < 0.01; ***P* < 0.001, when compared with corresponding controls.

Treatment and daily dose (ml/kg or mg/kg)

	Control (oil, 10ml)	3,5-Diethoxycarbonyl- 1,4-dihydrocollidine (200mg)	Phenylbutazone (150mg)	3,5-Diethoxycarbonyl- 1,4-dihydrocollidine (200mg)+phenylbutazone (150mg)
Liver wet wt. (g/100g body wt.)	3.58 $\pm$ 0.08 (15)	3.74 $\pm$ 0.1 (10)	4.38 $\pm$ 0.1 (12)**	4.86 $\pm$ 0.1 (10)**
Liver protein (mg/ total liver of 100g body wt.)	626.8 $\pm$ 24.2 (6)	675.9 $\pm$ 30.8 (6)	774.0 $\pm$ 16.1 (6)**	818.4 $\pm$ 16.2 (6)**
Microsomal protein (mg/g of wet liver)	19.1 $\pm$ 0.16 (10)	21.7 $\pm$ 0.6 (6)	25.0 $\pm$ 0.9 (6)**	24.0 $\pm$ 0.7 (6)*
Microsomal cyto- chrome P-450 (nmol/g of wet liver)	22.9 $\pm$ 2.3 (10)	24.5 $\pm$ 2.5 (6)	44.8 $\pm$ 5.8 (6)*	38.7 $\pm$ 3.0 (6)*
Microsomal cyto- chrome <i>b</i> ₅ (nmol/ g of wet liver)	16.1 $\pm$ 1.1 (10)	18.0 $\pm$ 1.3 (6)	19.4 $\pm$ 1.1 (6)	17.4 $\pm$ 0.5 (6)
5-Aminolaevulinate synthetase activity (nmol/min per 100mg of mito- chondrial protein)	0.89 $\pm$ 0.15 (4)	5.44 $\pm$ 1.23 (4)*	1.03 $\pm$ 0.28 (4)	5.30 $\pm$ 1.35 (4)*

synthetase without increasing the concentration of cytochrome *P*-450, whereas phenylbutazone increased the microsomal cytochrome without affecting the activity of the enzyme. When the two drugs were administered simultaneously both effects were observed. Phenylbutazone also increased the size of the liver and the protein content of the total liver as well as the protein content of the microsomal fraction (per g of wet liver; Table 1). Similar results were obtained for microsomal cytochrome *P*-450 content after 3 days of treatment, when an increased concentration of cytochrome *b*₅ was also observed after phenylbutazone administration.

The activity of two drug-metabolizing enzymes behaved as did cytochrome *P*-450 concentration (Table 2) and the porphyrin content of the total liver paralleled the activity of 5-aminolaevulinate synthetase (Table 3).

The mitochondrial fraction from the livers of rats given both drugs together exhibited a pink discoloura-

tion after 2–3 days of treatment. This was found to be associated with an accumulation of porphyrins in amounts greater than observed after administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine alone (Table 3). The finding of higher concentrations of liver porphyrins (Table 3) suggested that a greater degree of porphyria could be obtained by both drugs together than by 3,5-diethoxycarbonyl-1,4-dihydrocollidine alone.

This was confirmed in rats killed at different times after a single dose of drugs; here again phenylbutazone increased cytochrome *P*-450 without causing a significant stimulation of 5-aminolaevulinate synthetase (not even at doses fourfold those effective on cytochrome *P*-450), whereas 3,5-diethoxycarbonyl-1,4-dihydrocollidine stimulated the enzyme without increasing the concentration of the cytochrome; administration of both drugs resulted in both effects and in a greater stimulation of enzyme activity than was observed after 3,5-diethoxycarbonyl-1,4-di-

Table 2. *Effect of treatment of rats with 3,5-diethoxycarbonyl-1,4-dihydrocollidine or phenylbutazone or both for 2 days on the activity of o-nitroaniline demethylase and of aniline hydroxylase of the isolated microsomal fraction*

Rats were treated as indicated in the legend to Table 1. Results are the means $\pm$ S.E.M. of four observations and are expressed as nmol of product formed by 1 mg of protein during the whole of the incubation. **P* < 0.02; ***P* < 0.01, when compared with corresponding control.

Treatment	<i>o</i> -Nitroaniline demethylase (nmol of <i>o</i> -nitrophenol produced)	Aniline hydroxylase (nmol of 4-aminophenol produced)
Control (oil)	21.5 $\pm$ 2.5	6.7 $\pm$ 0.7
3,5-Diethoxycarbonyl-1,4-dihydrocollidine	19.3 $\pm$ 0.7	7.0 $\pm$ 0.2
Phenylbutazone	33.8 $\pm$ 2.6*	8.5 $\pm$ 0.5
3,5-Diethoxycarbonyl-1,4-dihydrocollidine + phenylbutazone	35.8 $\pm$ 1.6**	9.4 $\pm$ 0.6

Table 3. *Concentrations of porphyrins in the total liver homogenate and in its mitochondrial fraction after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine or phenylbutazone or both for 2 days*

Rats were treated as indicated in the legend to Table 1. Results given are the means $\pm$ S.E.M. of four observations. **P* < 0.01; ***P* < 0.001, when compared with control. ****P* < 0.05, when compared with corresponding value obtained with 3,5-diethoxycarbonyl-1,4-dihydrocollidine alone.

Treatment and daily dose (ml or mg/kg)	Porphyrins in total homogenate (nmol/g of wet liver)	Porphyrins in mitochondrial fraction (nmol/100mg of protein)
Control (oil, 10ml)	0.87 $\pm$ 0.14	Not measurable
Phenylbutazone (150mg)	1.05 $\pm$ 0.19	Not measurable
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (200mg)	22.5 $\pm$ 3.0 **	3.2 $\pm$ 0.5
Phenylbutazone (150mg) + 3,5-diethoxycarbonyl- 1,4-dihydrocollidine (200mg)	50.4 $\pm$ 11.2 *	31.2 $\pm$ 10.6***

Table 4. *Effect of a single dose of drugs on the 5-aminolaevulinate synthetase activity of the mitochondria and on the concentration of microsomal cytochrome P-450 of the liver of the rat*

All rats were starved for 24h, then given an oral dose of drugs and their starvation was continued. Results are given as means $\pm$ S.E.M. of the numbers of observations in parenthesis. * $P < 0.01$; ** $P < 0.001$, when compared with corresponding controls.

Treatment and dose (ml/kg or mg/kg)	5-Aminolaevulinate synthetase activity (nmol/min per 100mg of mitochondrial protein)		Cytochrome P-450 at 24h	
	At 5h	At 17h	(nmol/g of wet liver)	(nmol/mg of microsomal protein)
Oil (10ml)	2.18 $\pm$ 0.30 (8)	2.88 $\pm$ 0.66 (4)	23.0 $\pm$ 1.5 (6)	1.22 $\pm$ 0.09 (6)
3,5-Diethoxycarbonyl-1,4- dihydrocollidine (200mg)	8.37 $\pm$ 1.87 (10)*	9.10 $\pm$ 1.07 (4)*	24.0 $\pm$ 1.4 (5)	1.25 $\pm$ 0.06 (5)
(400mg)	11.60 $\pm$ 2.49 (4)*	—	28.8 $\pm$ 2.25 (7)	1.35 $\pm$ 0.09 (7)
Phenylbutazone (75mg)	—	—	36.9 $\pm$ 3.38 (8)*	1.62 $\pm$ 0.15 (8)
(150mg)	2.44 $\pm$ 0.37 (6)	3.45 $\pm$ 1.05 (6)	46.4 $\pm$ 1.44 (4)**	1.96 $\pm$ 0.21 (4)*
(300mg)	2.48 $\pm$ 0.40 (5)	4.41 $\pm$ 2.22 (4)	47.0 $\pm$ 3.3 (5)**	1.96 $\pm$ 0.11 (5)**
3,5-Diethoxycarbonyl-1,4- dihydrocollidine (200mg) + phenyl- butazone (150mg)	11.04 $\pm$ 2.53 (6)*	15.77 $\pm$ 2.77 (4)*	42.6 $\pm$ 2.5 (5)**	2.03 $\pm$ 0.07 (5)**

hydrocollidine alone (Table 4 and Fig. 1). A rise in enzyme activity was already evident 2.5h after dosing with the latter drug, whereas most of the cytochrome P-450 increase caused by phenylbutazone took place between 13 and 24h, at the time when the 5-aminolaevulinate synthetase activity was returning to normal values. A transient decline in cytochrome P-450 concentration was observed at 5h after administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine either alone or together with phenylbutazone, coincidental with the rise in activity of 5-aminolaevulinate synthetase (Fig. 1). The changes in concentration of cytochrome P-450 were statistically significant as compared with appropriate controls, also when expressed as nmol/mg of microsomal protein.

The total haem content of the washed microsomal fraction was also measured and compared with the concentration of microsomal cytochromes. The changes in microsomal P-450 content observed after administration of drugs were reflected in similar changes in the total haem content and this applied to both the transient fall observed at 5h and the late increase observed after phenylbutazone treatment.

Effect of drug administration on the 5-aminolaevulinate concentration and 5-aminolaevulinate dehydratase activity of the liver

The 5-aminolaevulinate dehydratase activity was measured 5 and 13h after a single dose of drugs (Table 5). The only significant change was observed

at 13h in rats given 3,5-diethoxycarbonyl-1,4-dihydrocollidine and phenylbutazone together, which showed an activity nearly twice the control value. Table 5 shows that this treatment also markedly raised the concentration of 5-aminolaevulinate in the liver; this finding is noteworthy in view of the correlation between liver concentrations of 5-aminolaevulinate and liver activity of 5-aminolaevulinate dehydratase, which has been reported after administration of 5-aminolaevulinate to the mouse (Onisawa & Labbe, 1962).

*Effect of drug administration on the incorporation of ^{59}Fe into liver haem *in vitro* and *in vivo**

The incorporation of ^{59}Fe into haem *in vitro* was studied by using liver preparations from rats given a dose of drugs 5 or 17h before killing. Liver homogenates were usually employed; in one experiment liver slices were used.

At both 5 and 17h after a single dose of drugs it was found that 3,5-diethoxycarbonyl-1,4-dihydrocollidine given either on its own or together with phenylbutazone greatly stimulated the incorporation of ^{59}Fe into haem by liver slices, whereas phenylbutazone did so only slightly (Table 6). As observed for 5-aminolaevulinate synthetase and for 5-aminolaevulinate dehydratase the effect of the two drugs given together was greater than the effect of 3,5-diethoxycarbonyl-1,4-dihydrocollidine alone.

In the experiments described in Table 6 the rate of haem synthesis *in vitro* was studied by measuring the

Fig. 1. Effect of a single dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine or phenylbutazone or both on the 5-aminolaevulinate synthetase activity of the mitochondria and on the concentration of microsomal cytochrome P-450 of rat liver

Rats were starved for 24h, then given an oral dose of drugs and their starvation was continued. Doses (in ml/kg or mg/kg) were as follows: control, 10ml of oil (○); 3,5-diethoxycarbonyl-1,4-dihydrocollidine, 200mg (●); phenylbutazone, 150mg (□); 3,5-diethoxycarbonyl-1,4-dihydrocollidine (200mg) and phenylbutazone (150mg) together (■). Results given are the means $\pm$ S.E.M. of four observations, unless indicated otherwise (in parentheses).

incorporation of ^{59}Fe into the protoporphyrin present as such in the liver or generated endogenously during the incubation. Under these conditions the increased incorporation of ^{59}Fe caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine could be due either to a stimulation of the activity of the chelatase (i.e. the enzyme that chelates iron into protoporphyrin to produce haem) or to an increased production of the protoporphyrin substrate. To distinguish between these two possibilities further experiments were carried out on liver homogenates from animals given a single dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine in oil or oil alone 5h before killing.

When the incorporation of ^{59}Fe into haem was studied in the presence of increasing concentrations of exogenous 5-aminolaevulinate or protoporphyrin, it was found that porphyric and control homogenates behaved in quite a different fashion (Fig. 2 and Table

7). With homogenates from porphyric animals the addition of either 5-aminolaevulinate or protoporphyrin did not produce great changes in the incorporation rate of ^{59}Fe into haem. The incorporation rate of control homogenates was, on the contrary, very sensitive to the addition of either substrate: a three-fold stimulation was observed with 5-aminolaevulinate and an even greater stimulation was obtained after addition of protoporphyrin, when incorporation far greater than the maximum observed with porphyric livers was obtained. Therefore the effect of 3,5-diethoxycarbonyl-1,4-dihydrocollidine treatment on the incorporation of ^{59}Fe into haem *in vitro* was found to depend on whether exogenous protoporphyrin was added to the liver homogenate or not (Table 8). In the absence of exogenous protoporphyrin, 3,5-diethoxycarbonyl-1,4-dihydrocollidine treatment stimulated the incorporation of ^{59}Fe into haem above the value of the corresponding controls, probably on account of the increased amount of protoporphyrin already present in the liver or generated during the incubation because of the porphyric state; in the presence of saturating amounts of protoporphyrin added from the outside (i.e. under conditions where the incorporation rate is mostly, if not entirely, dependent on the activity of the chelatase) treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine inhibited the incorporation of ^{59}Fe into haem.

This latter finding suggested that the activity of the chelatase enzyme was inhibited in porphyria induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine. Measurement of the chelatase activity by another method (Jones & Jones, 1969), in mitochondria isolated 1 or 5h after a single dose of drugs, showed that this was indeed the case (Table 9); a significant inhibition was observed as early as 1h after dosing with 3,5-diethoxycarbonyl-1,4-dihydrocollidine and by 5h the degree of inhibition (67%) was virtually identical with that found by the ^{59}Fe method (68%). Tables 7 and 8 also show that phenylbutazone did not inhibit chelatase activity and that when the two drugs were given together the inhibition observed (58% with either method) was slightly less than with 3,5-diethoxycarbonyl-1,4-dihydrocollidine alone.

Since the chelatase is located inside the inner membrane of the mitochondrion (Jones & Jones, 1969) the possibility was considered that 3,5-diethoxycarbonyl-1,4-dihydrocollidine might inhibit the access of the porphyrin substrate to the enzymic site, giving rise to an apparent decrease in chelatase activity. This appears to be unlikely, however, as this effect of the drug could not be abolished by either exposing the mitochondria to ultrasound treatment or by doubling the concentration of detergent in the spectrophotometric assay of the chelatase.

When the incorporation of ^{59}Fe into liver haem was measured *in vivo* after treatment with 3,5-diethoxy-

Table 5. *Effect of a single dose of drugs on the concentration of 5-aminolaevulinate and on the activity of the 5-aminolaevulinate dehydratase of the liver*

All rats were starved for 24h, then given an oral dose of drugs and their starvation was continued. Results are given as means $\pm$ S.E.M. of the numbers of observations in parentheses. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, when compared with corresponding controls.

Treatment and dose (ml/kg or mg/kg)	Liver 5-aminolaevulinate dehydratase (μ mol of porphobilinogen formed/h per g of liver)		Liver 5-aminolaevulinate concentrations (nmol/g of wet liver)	
	At 5h	At 13h	At 5h	At 17h
Oil (10ml)	1.8 $\pm$ 0.07 (4)	1.5 $\pm$ 0.2 (6)	47.7 $\pm$ 6.4	35.1 $\pm$ 4.3
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (200mg)	1.9 $\pm$ 0.07 (4)	1.7 $\pm$ 0.2 (6)	55.4 $\pm$ 3.0	50.9 $\pm$ 7.7
Phenylbutazone (150mg)	1.6 $\pm$ 0.07 (4)	1.5 $\pm$ 0.2 (6)	37.5 $\pm$ 5.6	35.4 $\pm$ 4.6
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (200mg) + phenylbutazone (150mg)	1.8 $\pm$ 0.13 (4)	2.9 $\pm$ 0.3 (6)**	88.8 $\pm$ 13.5*	213.8 $\pm$ 25.8***

Table 6. *Effect of a single dose of drugs on the incorporation of ^{59}Fe into haem by liver slices at 5h and 17h after dosing*

The animals were starved for 24h, then dosed and killed 5 or 17h later. Liver slices (400mg) were incubated for 15 min in the presence of 0.6 nmol of [^{59}Fe]ferric citrate (500000 c.p.m.) and of 30 nmol of unlabelled ferrous sulphate, with shaking. At the end of the incubation 13 mg of carrier haem was added.

Treatment and dose (ml/kg or mg/kg)	Incorporation of ^{59}Fe into haem (c.p.m./mg of haem)	
	At 5h	At 17h
Control (oil, 10ml)		
rat 1	23.5	38.6
rat 2	24.3	39.3
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (200mg)		
rat 1	122.1	97.5
rat 2	111.1	370.0
Phenylbutazone (150mg)		
rat 1	55.9	60.0
rat 2	28.8	57.8
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (200mg) + phenylbutazone (150mg)		
rat 1	299.3	368.0
rat 2	121.6	448.0

carbonyl-1,4-dihydrocollidine, it was found not to be increased above control values as observed *in vitro* with either liver slices or homogenates incubated without exogenous substrates, but possibly to be slightly decreased (Table 10). A similar difference

between incorporation of ^{59}Fe into liver haem *in vivo* and *in vitro* has been observed by Abbritti *et al.* (1972) in mice treated with this drug. The reason for this discrepancy between results *in vivo* and *in vitro* is not known.

Fig. 2. Incorporation of ^{59}Fe into haem *in vitro* by liver homogenates from rats treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine and by liver homogenates from control rats

Effect of adding increasing amounts of 5-aminolaevulinate (○, control; □, 3,5-diethoxycarbonyl-1,4-dihydrocollidine) or protoporphyrin (●, control; ■, 3,5-diethoxycarbonyl-1,4-dihydrocollidine). The animals (three rats in each of the four experiments) were starved for 24 h, then given the drug (200 mg/kg) or oil (10 ml/kg) orally, killed 5 h later and their liver homogenates pooled. The incubation conditions were as indicated in the legend to Table 7.

Discussion

Synthesis of liver haem and control of cytochrome P-450 formation

Several drugs such as phenobarbitone and benzpyrene when administered to the rat raise the concentration of cytochrome P-450 in the liver microsomal fraction (Conney, 1967). Marver (1969) and Baron & Tephly (1969a, 1970) have reported that a severalfold increase in both activity of 5-aminolaevulinate synthetase and rate of liver haem synthesis precedes the increase in cytochrome P-450 after either phenobarbitone or benzpyrene treatment. Baron & Tephly (1969b) have also reported that the accumulation of cytochrome P-450 caused by phenobarbitone in rat liver can be prevented by administration of large doses of 3-amino-1,2,4-triazole, an inhibitor of hepatic haem synthesis. These findings have all been interpreted to indicate that an increased supply of haem is the primary event leading to the accumulation of the microsomal haemoprotein (Baron & Tephly, 1970). The role of haem in this proposed mechanism would be similar to that claimed for haem in controlling and stimulating the formation of haemoglobin by reticulocytes (London *et al.*, 1964) and embryonic erythroid cells (Wilt, 1965; Levere & Granick, 1967; Wainwright & Wainwright, 1966).

There are several reasons why this interpretation should be viewed with caution. First, the results obtained by Baron & Tephly (1969b) with 3-amino-1,2,4-triazole may merely indicate that haem, as an essential constituent of cytochrome P-450, is required

Table 7. Effect of the addition of glycine, 5-aminolaevulinate or protoporphyrin on the incorporation of ^{59}Fe into haem by liver homogenates

The animals (three rats in each group) were starved for 24 h, then given 3,5-diethoxycarbonyl-1,4-dihydrocollidine (200 mg/kg) or oil (10 ml/kg) orally, killed 5 h later and their liver homogenates pooled. Liver homogenates (equivalent to 400 mg of wet liver) were incubated for 15 min in the presence of [^{59}Fe]ferric citrate (100000 c.p.m.) with shaking (100 cycles/min). Each homogenate was incubated both with and without exogenous substrates. At the end of the incubation 20 mg of carrier haem was added.

Addition	Incorporation of ^{59}Fe into haem (c.p.m./mg of haem)	
	Control (oil)	With 3,5-diethoxycarbonyl-1,4-dihydrocollidine
None	56	164
Glycine (400 μmol)	62	177
5-Aminolaevulinate (300 nmol)	177	157
Protoporphyrin (300 nmol)	1026	278
Protoporphyrin and 5-aminolaevulinate (300 nmol each)	1020	280

Table 8. *Effect of a single dose of drugs on the incorporation of ^{59}Fe into haem by liver homogenates*

The animals were starved for 24h, then dosed and killed 5h later. Liver homogenates (equivalent to 400mg of wet liver) were incubated for 15min in the presence of [^{59}Fe]ferric citrate (100000c.p.m.) with shaking (100 cycles/min). Each homogenate was incubated in the presence of either glycine (400 μmol) or protoporphyrin (300nmol). At the end of the incubation 20mg of carrier haem was added. Results are presented as means $\pm$ S.E.M. of four observations. * $P < 0.01$; ** $P < 0.001$, when compared with corresponding controls.

Treatment and dose (ml/kg or mg/kg)	Incorporation of ^{59}Fe into haem		
	With glycine (A) (c.p.m./mg of haem)	With protoporphyrin (B) (c.p.m./mg of haem)	Stimulation due to protoporphyrin (B/A)
Control (oil, 10ml)	78 $\pm$ 9.8	534 $\pm$ 39	6.93 $\pm$ 0.64
Phenylbutazone (150mg)	69 $\pm$ 6.0	435 $\pm$ 26	6.41 $\pm$ 0.50
3,5-Diethoxycarbonyl-1,4-dihydro- collidine (200mg)	149 $\pm$ 24.6*	172 $\pm$ 17.7**	1.21 $\pm$ 0.12**
3,5-Diethoxycarbonyl-1,4-dihydro- collidine (200mg) + phenyl- butazone (150mg)	261 $\pm$ 28.8**	220 $\pm$ 26.6**	0.84 $\pm$ 0.01**

for the formation of the haemoprotein and that, under conditions in which the synthesis of haem is inhibited, haem supply may become rate-limiting. A similar interpretation has been proposed by Wilt (1968) for the stimulation by haem of haemoglobin synthesis *in vitro*. Secondly, the results obtained in the present work with phenylbutazone indicate that a considerable accumulation of cytochrome P-450 can be observed without great changes in either 5-aminolaevulinate synthetase activity or rate of haem synthesis *in vitro*. Finally, it has been found (De Matteis, 1971a) that the administration of 5-aminolaevulinate or haem to either normal or phenylbutazone-treated rats did not increase the cytochrome P-450 above the concentrations observed in the respective controls (which had not been given haem or its precursor); this also suggests that an increased supply of 5-aminolaevulinate and haem is probably not sufficient in itself to stimulate the formation of the cytochrome.

Mechanism(s) of stimulation of 5-aminolaevulinate synthetase by drugs

A distinction must be made between the large increase in 5-aminolaevulinate synthetase activity observed in the drug-induced hepatic porphyrias of the experimental animal and the small stimulation of the enzyme frequently observed after drugs without a significant accumulation of porphyrins in the liver. The latter is of common occurrence after administration of many drugs that increase the size of the liver and the concentration of cytochrome P-450 in the liver microsomal fraction (Wada *et al.*, 1968; Abbritti & De Matteis, 1971-72). For example, in the present work phenylbutazone marginally increased

the activity of the enzyme and slightly stimulated the incorporation of ^{59}Fe into haem by liver slices, a finding which also suggested a small stimulation of the production of haem precursors. It is possible that the small effect exercised by so many drugs on 5-aminolaevulinate synthetase activity is in some way related to their property of increasing the size of the liver and the activity of many liver enzymes (De Matteis, 1971a). Or it is possible that by increasing the amount of the cytochrome P-450 apoprotein these drugs may increase haem utilization and therefore also, to a small extent, the rate of haem and 5-aminolaevulinate production.

A mechanism of this type, involving either a general alteration of liver protein metabolism or a concerted increase in the production of the haem and protein moieties of cytochrome P-450, is not sufficient, however, to explain why with some drugs the increase in 5-aminolaevulinate synthetase becomes very marked and is accompanied by the appearance of porphyria. The experiments conducted here with 3,5-diethoxycarbonyl-1,4-dihydrocollidine and phenylbutazone clearly indicate that there are some drugs that are powerful inducers of 5-aminolaevulinate synthetase or of cytochrome P-450 but not of both; this finding, and the observation that when the two drugs were given together both effects were observed, strongly suggests that marked stimulation of 5-aminolaevulinate synthetase and porphyria on the one hand and increase in cytochrome P-450 and drug-metabolizing enzymes on the other result from two different actions. The same is probably true for the increase in liver size and protein content, which can also be dissociated from the appearance of porphyria (see also De Matteis, 1966). Therefore, for

Table 9. *Effect of a single dose of drugs on the chelatase activity of isolated mitochondria*

The animals were starved for 24h, then dosed and killed 1 or 5 h later. Results given are means $\pm$ S.E.M. of the number of observations in parenthesis. * $P < 0.05$, when compared with '0 time' value; ** $P < 0.01$; *** $P < 0.001$, when compared with control value.

Treatment and dose (ml or mg/kg)	Time of killing (h after dosing)	Porphyrin-metal chelatase activity (nmol of Co^{2+} mesoporphyrin formed/min per mg of protein)
None (0 time)	—	1.05 ± 0.10 (3)
Control (oil, 10ml)	5	1.00 ± 0.06 (5)
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (200mg)	1	0.78 ± 0.03 (4)*
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (200mg)	5	0.33 ± 0.02 (8)***
Phenylbutazone (150mg)	5	1.07 ± 0.11 (4)
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (200mg) + phenylbutazone (150mg)	5	0.42 ± 0.13 (4)**

Table 10. *Effect of a single oral dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine on the incorporation of ^{59}Fe into liver haem in the rat in vivo*

Rats were starved for 24h, then given 3,5-diethoxycarbonyl-1,4-dihydrocollidine (200mg/kg) or oil (10ml/kg) orally. At 5h later they were injected intravenously with $50\mu\text{Ci}$ (10.3×10^6 c.p.m.) of [^{59}Fe]ferric citrate/kg and killed 10min later.

Treatment	Radioactivity (c.p.m.) recovered		Radioactivity present in haem (% of total liver radioactivity)
	In total liver	In total haem of the whole liver	
Control (oil)	124000 ± 14000 (4)	2500 ± 300 (4)	2.0 ± 0.3 (4)
3,5-Diethoxycarbonyl-1,4-dihydrocollidine	108000 ± 13000 (3)	1800 ± 500 (3)	1.6 ± 0.2 (3)

porphyrinogenic compounds such as 3,5-diethoxycarbonyl-1,4-dihydrocollidine, some additional (or alternative) mechanism is likely to be involved in the stimulation of 5-aminolaevulinic synthetase, a mechanism that concerns more specifically the regulation of haem biosynthesis.

Inhibition of liver haem synthesis in porphyria caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine

A transient decline in microsomal cytochrome P-450 content and haem has been observed in this work after a single dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine to the rat, coincidental with the rise in activity of 5-aminolaevulinic synthetase. Similar findings have been reported after administration to the rat of another porphyrinogenic compound, 2-allyl-2-isopropylacetamide (De Matteis, 1970; Meyer & Marver, 1971) and of certain porphyrinogenic drugs chemically related to 2-allyl-2-isopropylacetamide (Abbritti & De Matteis, 1971–72). A loss of microsomal cytochrome P-450 and haem has also

been observed in rabbits and mice after administration of either 3,5-diethoxycarbonyl-1,4-dihydrocollidine or 2-allyl-2-isopropylacetamide and also after feeding with another porphyria-inducing compound, griseofulvin (Wada *et al.*, 1968; Waterfield *et al.*, 1969). In addition, 3,5-diethoxycarbonyl-1,4-dihydrocollidine and the other porphyrinogenic drugs mentioned above have all been reported to lower the activity of liver catalase (De Matteis, 1967; Abbritti & De Matteis, 1971–72), a finding which may indicate that they decrease the concentration of more than one pool of haem in the liver. Since haem is known to exercise a feedback control on the activity of the biosynthetic pathway of porphyrins at the level of 5-aminolaevulinic synthetase, these observations suggest that these drugs might stimulate markedly the activity of the enzyme and induce porphyria by lowering the concentration of haem in the liver.

There are two main mechanisms by which a drug might decrease the concentration of liver haem: (a) by promoting destruction of haem or (b) by inhibiting its synthesis. Destruction of liver haem is involved in

the loss of cytochrome *P*-450 caused by 2-allyl-2-isopropylacetamide (De Matteis, 1971*b*; Meyer & Marver, 1971) and related drugs (Abbritti & De Matteis, 1971–72). For 3,5-diethoxycarbonyl-1,4-dihydrocollidine previous findings and the results of the present work suggest that the drug inhibits the synthesis of liver haem. Onisawa & Labbe (1963) have reported an inhibition of the synthesis of haem in the liver of mice treated for several days with this drug and Tephly *et al.* (1971) have described an inhibition of the ferrochelatase in the liver of rats 24 h after a single dose. In our experiments, the activity of the mitochondrial chelatase was found to be inhibited as early as 1 h after administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine, when the 5-aminolaevulinate synthetase activity was still normal or just starting to rise; so it is possible that inhibition of the conversion of protoporphyrin into haem may not only explain the very marked accumulation of protoporphyrin in the liver, which is characteristic of this type of hepatic porphyria (Onisawa & Labbe, 1963), but also represent the primary lesion responsible for the stimulation of 5-aminolaevulinate synthetase and the overproduction of porphyrins.

The experiments on the incorporation of ^{59}Fe into haem *in vitro* (Fig. 2 and Table 7) require further comment. From the results obtained with control homogenates it can be concluded that the rate of haem synthesis in normal liver is primarily controlled by the supply of 5-aminolaevulinate and protoporphyrin and not by the activity of the chelatase, which is in large excess; this conclusion is in agreement with the concept that 5-aminolaevulinate synthetase is the rate-limiting step in the pathway of haem biosynthesis (Granick, 1966). The finding that protoporphyrin was much more effective than 5-aminolaevulinate in stimulating the incorporation of ^{59}Fe into haem by liver homogenates from control animals suggests, on the other hand, that under conditions where the supply of 5-aminolaevulinate becomes excessive one or more of the enzymes of the pathway concerned with its further metabolism to protoporphyrin may become rate-limiting. The results obtained with porphyric homogenates are best explained by the rapid inhibition of the chelatase and by the accumulation of protoporphyrin in the liver, which are observed after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine; under these conditions the supply of protoporphyrin is not limiting and the rate of haem synthesis appears to be determined primarily by the activity of the chelatase.

Potentiation by phenylbutazone of porphyria caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine

The reason why administration of phenylbutazone and 3,5-diethoxycarbonyl-1,4-dihydrocollidine to-

gether resulted in a greater stimulation of 5-aminolaevulinate synthetase and in a more marked degree of porphyria than observed with 3,5-diethoxycarbonyl-1,4-dihydrocollidine alone is not known. It will be noted that although the effects of the two drugs given together on liver size (and total protein content) were roughly equal to the sum of the effects found when either drug was administered individually, this was not the case for the stimulation of 5-aminolaevulinate synthetase and 5-aminolaevulinate dehydratase and for the accumulation in the liver of 5-aminolaevulinate and porphyrins, where a considerably greater effect was noted. This observation, and the finding that when the two drugs were given together the inhibition of the chelatase and the decrease in cytochrome *P*-450 were not greater than when 3,5-diethoxycarbonyl-1,4-dihydrocollidine was given on its own, suggest that stimulation of 5-aminolaevulinate synthetase by the latter drug and potentiation of this effect by phenylbutazone may be through different mechanisms. Perhaps 3,5-diethoxycarbonyl-1,4-dihydrocollidine acts by inhibiting the synthesis of liver haem and by lowering the concentration of haem available for control of 5-aminolaevulinate synthetase, whereas phenylbutazone, by causing an accumulation of cytochrome *P*-450 apoprotein, stimulates haem utilization and thereby decreases the concentration of this 'regulatory' haem still further. Alternatively, the potentiation by phenylbutazone of porphyria caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine may be explained by the marked effect on liver protein metabolism exhibited by the former drug. If the stimulation of 5-aminolaevulinate synthetase by 3,5-diethoxycarbonyl-1,4-dihydrocollidine involves accumulation of enzyme protein (Granick, 1966) (rather than, or as well as, activation of an already existing enzyme), then it is possible that this response might be further increased under conditions in which the overall rate of liver protein accumulation is also increased.

Like phenylbutazone, thiopentone [5-ethyl-5-(1-methylbutyl)thiobarbituric acid] and phenobarbitone (5-ethyl-5-phenylbarbituric acid) can also potentiate the porphyria caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (F. De Matteis, unpublished work). These findings may be of relevance to the exacerbation of human genetic porphyria caused by barbiturates and other drugs.

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