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Decreased Liver Activity of Porphyrin–Metal Chelatase in Hepatic Porphyria caused by 3,5-Diethoxycarbonyl-1,4-dihydrocollidine

STUDIES IN RATS AND MICE

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1. A difference has been found between rats and mice in their sensitivity to the porphyrogenic effect of drugs. Mice are more sensitive than rats to 3,5-diethoxycarbonyl-1,4-dihydrocollidine, but less sensitive than rats to 2-allyl-2-isopropylacetamide. 2. Use has been made of this difference in sensitivity to ascertain the importance of the decrease of liver porphyrin–metal chelatase activity in porphyria caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine. Mice, which are more sensitive than rats to the stimulation of 5-aminolaevulinate caused by this drug, are also more sensitive with respect to the decrease of chelatase activity. 3. In both species, after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine, the ratio between chelatase activity and 5-aminolaevulinate activity is linear with respect to the reciprocal of the liver porphyrin concentration. This suggests that under these conditions the degree of porphyrin accumulation depends on the balance between rate of porphyrin formation and rate of porphyrin utilization. 4. Compound SKF 525-A (2-diethylaminoethyl 3,3-diphenylpropylacetate) when given before 3,5-diethoxycarbonyl-1,4-dihydrocollidine prevents the appearance of porphyria in the rat and also largely prevents the decrease of chelatase activity. In the mouse it is much less effective in preventing porphyria and it is almost completely inactive in protecting the chelatase from a decrease in activity. 5. Cycloheximide, when given before 3,5-diethoxycarbonyl-1,4-dihydrocollidine also inhibits the induction of 5-aminolaevulinate synthetase and the appearance of porphyria in the rat, but does not prevent the decrease of chelatase activity. These results suggest that two successive stages can be distinguished in the induction process: a first stage leading to inhibition of haem synthesis and a second stage requiring synthesis of protein in the liver and leading to stimulation of 5-aminolaevulinate synthetase.

Drugs like 2-allyl-2-isopropylacetamide and 3,5-diethoxycarbonyl-1,4-dihydrocollidine when administered to the rat stimulate markedly the activity of liver 5-aminolaevulinate synthetase and cause accumulation of porphyrins in the liver. A transient decline in the concentration of haem is observed in the microsomal fraction of the liver after a single dose of either drug (De Matteis, 1970; De Matteis & Gibbs, 1972), coincidental with the rise in activity of 5-aminolaevulinate synthetase. This suggests that these drugs may stimulate markedly the activity of the enzyme by lowering the concentration of haem in the liver and thereby decreasing the normal-feedback control. In the case of 2-allyl-2-isopropylacetamide the fall in liver haem concentration is due to increased destruction (De Matteis, 1971; Meyer & Marver, 1971; Levin *et al.*, 1972), whereas with 3,5-diethoxycarbonyl-1,4 dihydrocollidine inhibition of liver haem synthesis may also be involved, since a rapid decrease in the activity of liver porphyrin–metal chelatase (the enzyme which converts protoporphyrin into haem) follows the administration of this latter drug (De Matteis & Gibbs, 1972).

A difference has now been found between rats and mice in their sensitivity to the porphyrogenic activity of 3,5-diethoxycarbonyl-1,4-dihydrocollidine; use has been made of this species difference to ascertain the importance of the decreased activity of liver porphyrin–metal chelatase in relation to the induction of 5-aminolaevulinate synthetase and to the accumulation of porphyrins in the liver caused by this drug. Experiments have also been carried out with cycloheximide and compound SKF 525-A to find out more about the mechanism by which 3,5-diethoxycarbonyl-1,4-dihydrocollidine stimulates the activity of liver 5-aminolaevulinate synthetase.

Experimental

Methods

Treatment of animals. Male albino rats of the Porton (Wistar-derived) strain (160–180g) were kept

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in experimental cages designed to prevent coprophagy with trays below containing sawdust bedding. Male *LAC A* mice (derived from the CFW inbred strain) were 8–12 weeks of age at the time of the experiments; they were kept on Sterolit bedding (Mineral and Chemicals Corporation of America, Menlo Park, New Jersey, U.S.A.) for at least 3 weeks before and during the experiments. Both rats and mice were starved for 24h before treatment with drugs. The 3,5-diethoxycarbonyl-1,4-dihydrocollidine was either injected intraperitoneally or administered orally dissolved in arachis oil. 2-Allyl-2-isopropylacetamide was given by subcutaneous injection dissolved in 0.9% NaCl. Control animals received arachis oil or saline either orally or by injection, as appropriate. In other experiments rats and/or mice were given an intraperitoneal dose of either cycloheximide or compound SKF 525-A (2-diethylaminoethyl 3,3-diphenylpropylacetate) 45min before administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine.

Enzyme assays. 5-Aminolaevulinate synthetase was assayed by using total liver homogenate as the source of the enzyme (Abbritti & De Matteis, 1971–72). The amount of 5-aminolaevulinate formed was assayed as described by De Matteis (1971).

Porphyrin–metal chelatase was determined usually with mesoporphyrin and Co^{2+} as substrates, by a modification (De Matteis & Gibbs, 1972) of the spectrophotometric method developed by Jones & Jones (1969). Mitochondria for porphyrin–metal chelatase assay were obtained from a 10% (w/v) liver homogenate in 0.25M-sucrose. The supernatant of an 850*g* (10min) centrifugation was spun again at 17000*g* for 4min; the tightly packed mitochondrial pellet was washed once by resuspending it in a volume of 0.25M-sucrose equal to that of the original homogenate and was finally resuspended in sucrose.

Haem synthesis in vitro. This was followed by measuring the incorporation of ^{59}Fe into haem by liver homogenates. Rat or mouse liver homogenates (20%, w/v) in 0.25M-sucrose (1ml) were incubated at 38°C with shaking (100 cycles/min) in the presence of protoporphyrin (100nmol) and of sodium phosphate buffer (250μmol), pH7.4, in a total volume of 2.60ml. After a preliminary incubation of 2min, approx. 0.25μCi of [^{59}Fe]ferric citrate was added and the incubation continued for a further 15min. The incubation was carried out in air with the exception of the experiment described in Table 2 when it was performed anaerobically in Thunberg tubes under vacuum. After addition of horse erythrocyte lysate, haemin was crystallized by the method of Labbe & Nishida (1957), then the crystals were dissolved in pyridine and the solution counted for radioactivity in a Packard Autogamma scintillation spectrometer.

Analytical Methods. Proteins were determined by Aldridge's (1962) modification of the method of Robinson & Hogden (1940). Porphyrins were extracted from total liver homogenates into 0.9M-$HClO_4$–ethanol (1:1, v/v) and measured fluorimetrically with protoporphyrin as the standard (Abbritti & De Matteis, 1971–72). Isolated haemin was determined as the pyridine haemochrome by the method of Rimington (1942).

Calculation of results. Results are expressed as the arithmetic means ± S.E.M. As variances in experimental groups were often much greater than in controls, a non-parametric method (Wilcoxon ranking test; Snedecor & Cochran, 1967) was used to estimate *P*. To assess the level of significance of the coefficients of linear correlation a degree of freedom *N*-2 was used (Snedecor & Cochran, 1967).

Materials

3,5-Diethoxycarbonyl-1,4-dihydrocollidine was obtained from Kodak Ltd., Kirkby, Liverpool, U.K.; it was recrystallized four times from aq. ethanol. Cycloheximide, protoporphyrin IX and 5-aminolaevulinate were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. and mesoporphyrin IX from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Compound SKF 525A was a gift from Smith, Kline and French Laboratories Ltd., Welwyn Garden City, Herts., U.K. [^{59}Fe]Ferric citrate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. and had a specific radioactivity of 10mCi/mg of Fe (at the time of issue).

Results

Species differences in the liver response to the porphyrogenic activity of 2-allyl-2-isopropylacetamide and of 3,5-diethoxycarbonyl-1,4-dihydrocollidine

Rats and mice were starved for 24h, then given a single dose of either 2-allyl-2-isopropylacetamide subcutaneously or 3,5-diethoxycarbonyl-1,4-dihydrocollidine by intraperitoneal injection and killed 5h later. A difference was found between rats and mice in the degree of accumulation of porphyrins in the liver caused by either drug (Table 1): rats were more sensitive than mice to the porphyrogenic activity of 2-allyl-2-isopropylacetamide but less sensitive than mice to 3,5-diethoxycarbonyl-1,4-dihydrocollidine. A similar difference in sensitivity was observed between rats and mice when the latter drug was administered orally rather than by intraperitoneal injection.

Both drugs stimulated the activity of 5-aminolaevulinate synthetase in rats and mice (Table 1). In agreement with Hutton & Gross (1970), after administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine a statistically significant correlation ($P<0.01$) was noted between activity of the enzyme and concentration of porphyrins in both rats (Fig. 1*a*) and mice.

Table 1. *Effect of treating rats and mice with either 2-allyl-2-isopropylacetamide or 3,5-diethoxycarbonyl-1,4-dihydrocollidine on the activity of 5-aminolaevulinate synthetase and on the total porphyrin content of the liver*

Rats and mice were starved for 24h, then given either 2-allyl-2-isopropylacetamide subcutaneously or 3,5-diethoxycarbonyl-1,4-dihydrocollidine intraperitoneally and killed 5h later. Results given are means ±S.E.M. of the numbers of observations in parentheses. Probabilities of difference from corresponding controls are indicated thus: * $P \leqslant 0.5$; ** $P \leqslant 0.01$.

	Rat		Mouse	
Treatment and dose (ml/kg or mg/kg)	5-Aminolaevulinate synthetase (nmol/min per g of wet liver)	Total porphyrins (nmol/g of wet liver)	5-Aminolaevulinate synthetase (nmol/min per g of wet liver)	Total porphyrins (nmol per g of wet liver)
Saline (20ml)	2.14±0.5 (6)	0.141±0.03 (8)	3.52±1.02 (4)	0.065±0.01 (5)
2-Allyl-2-isopropylacetamide (50mg)	5.94±1.4 * (4)	2.83 ±1.1* (6)	9.38±1.84 (4)	0.150±0.04 (5)
2-Allyl-2-isopropylacetamide (200mg)	5.07±0.7** (8)	8.08 ±0.9** (10)	7.99±2.34 (4)	0.546±0.126** (6)
2-Allyl-2-isopropylacetamide (400mg)	9.88±1.9** (4)	6.74 ±1.25** (6)	5.87±1.49 (4)	0.277±0.051** (4)
Oil (10ml)	1.44±0.08 (3)	0.130±0.02 (10)	3.84±0.77 (6)	0.080±0.014 (10)
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (5mg)	1.10±0.14 (7)	0.137±0.02 (9)	4.66±0.67 (6)	1.77±0.55** (10)
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (10mg)	1.55±0.21 (3)	0.159±0.02 (10)	8.21±1.24* (5)	4.54 ±1.03** (8)
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (25mg)	3.62±1.07 (4)	0.66 ±0.26** (4)	—	—
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (50mg)	6.68±0.78 (3)	3.82 ±0.77** (11)	13.79±2.96 (3)	11.95 ±1.1** (6)
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (100mg)	10.31±1.08** (7)	10.68 ±1.30** (12)	17.2 ±4.9 (3)	15.1 ±1.1** (6)

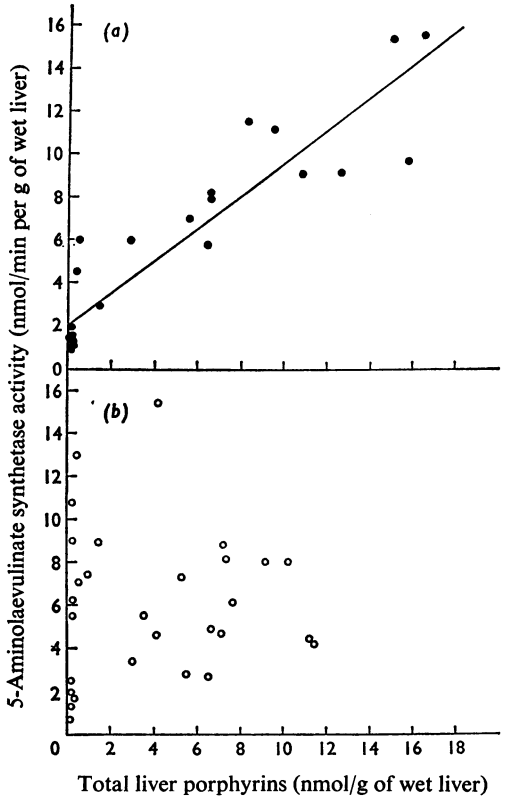

Fig. 1. *Relationship between activity of liver 5-aminolaevulinate synthetase and liver concentration of total porphyrins in rats made porphyric by treatment with either 3,5-diethoxycarbonyl-1,4-dihydrocollidine (a) or 2-allyl-2-isopropylacetamide (b)*

Rats were treated with different doses of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (●) or of 2-allyl-2-isopropylacetamide (○) as indicated in the legend to Table 1. Symbols refer to enzyme activity and porphyrin concentration observed in the individual animals.

(The coefficient of linear correlation was 0.92 in rats and 0.68 in mice with a degree of freedom of 25 and 20 respectively.) However, no correlation was found between activity of 5-aminolaevulinate synthetase and concentration of porphyrins in the liver of rats treated with 2-allyl-2-isopropylacetamide (Fig. 1*b*); also, this latter drug caused a far greater accumulation of porphyrins in rat liver than in mouse liver, even though similar amounts of enzyme activity were found in both species (Table 1). This lack of correlation suggests that either the activity of 5-aminolaevulinate synthetase as measured *in vitro* in our experiments does not always accurately reflect the

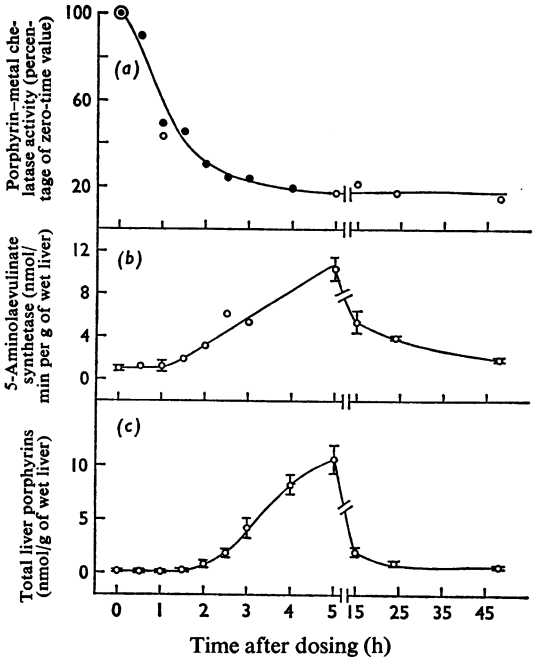

Fig. 2. *Effect of a single dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine on (a) the porphyrin–metal chelatase activity, (b) the 5-aminolaevulinate synthetase activity and (c) the total porphyrin concentration of rat liver*

Rats were starved for 24h, then given an intraperitoneal dose of drug (100mg/kg) and their starvation was continued. Values given for 5-aminolaevulinate synthetase and for porphyrin concentration are the means±S.E.M. of at least four observations or the results obtained with the pooled liver homogenates from two animals. The chelatase activity is expressed as percentage of the zero-time values of either the Co^{2+} mesoporphyrin chelatase of isolated mitochondria (○; each point is the mean of four observations) or of the incorporation of ^{59}Fe into protoporphyrin by liver homogenates (●; each point is the mean of two observations). The zero-time value of Co^{2+} mesoporphyrin chelatase was 1.55±0.1nmol of metalloporphyrin formed/min per mg of mitochondrial protein. Note that the time scale of the left hand side of the figure has been expanded.

rate of 5-aminolaevulinate formation *in vivo*, or that some other factor apart from 5-aminolaevulinate synthetase activity is of importance for the accumulation of porphyrins in the liver of rats treated with 2-allyl-2-isopropylacetamide (see also, Abbritti & De Matteis, 1971–72).

In the experiments described below use was made

of the difference between rats and mice in their response to 3,5-diethoxycarbonyl-1,4-dihydrocollidine to study the mechanism involved in the induction of 5-aminolaevulinate synthetase and of porphyria by this drug.

Decreased liver porphyrin–metal chelatase activity in porphyria caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine

When a single dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine was given intraperitoneally to the rat there was a rapid decrease in liver porphyrin–metal chelatase before any increase in either activity of 5-aminolaevulinate synthetase or in liver porphyrin concentration (Fig. 2; see also De Matteis & Gibbs, 1972). The degree of decrease in chelatase activity was essentially similar whether the activity of the enzyme was measured spectrophotometrically with Co^{2+} and mesoporphyrin as substrates or isotopically with ^{59}Fe and protoporphyrin. An increase in liver porphyrin concentration and in 5-aminolaevulinate synthetase activity was first seen when the activity of the chelatase was decreased by about 70%. In the second part of the experiment both the activity of 5-aminolaevulinate synthetase and the concentration

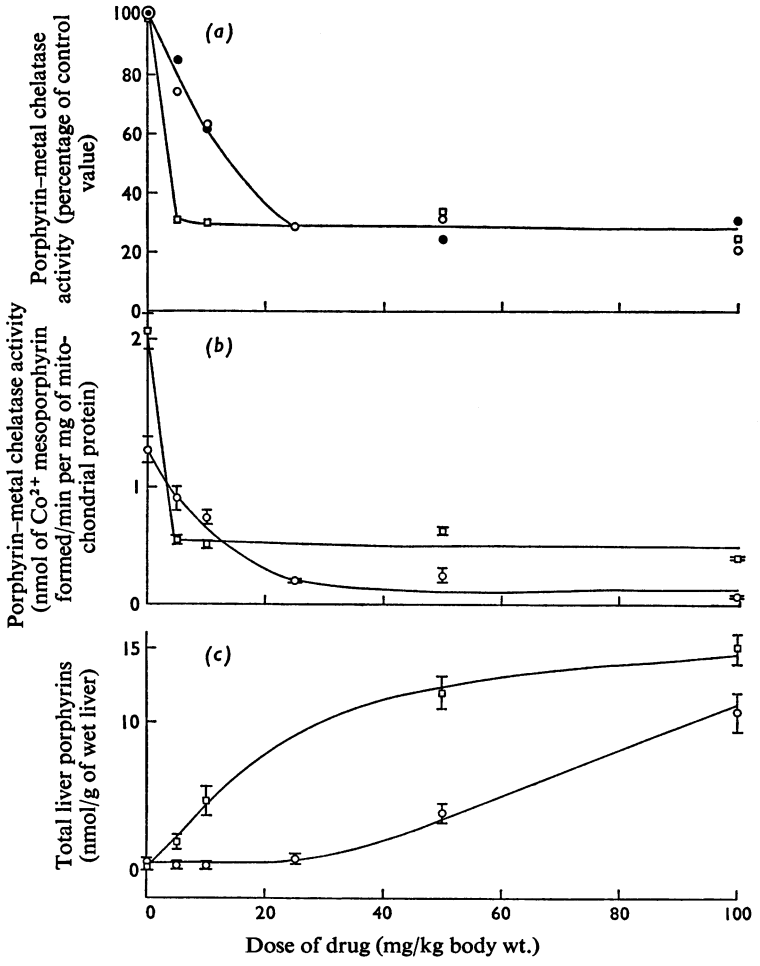

Fig. 3. *Effect of treating rats or mice with 3,5-diethoxycarbonyl-1,4-dihydrocollidine on (c) the total porphyrin content of the liver and (a) and (b) the porphyrin–metal chelatase activity of isolated liver mitochondria*

Rats (○) and mice (□) were treated as indicated in the legend to Table 1. In the case of rats the chelatase activity was also measured by the incorporation of ^{59}Fe into protoporphyrin by liver homogenates and the activity expressed as percentage of the control value (●; each point the mean of two observations).

of porphyrins showed a gradual decrease, but the chelatase activity remained very low (Fig. 2).

In a further experiment the effect of administering increasing doses of 3,5-diethoxycarbonyl-1,4-dihydrocollidine on the activity of the chelatase was studied in both rats and mice and related to the stimulation of 5-aminolaevulinate synthetase (Table 1) and to the accumulation of porphyrins in the liver (Fig. 3). Mice, which are more sensitive than rats to the effect of 3,5-diethoxycarbonyl-1,4-dihydrocollidine on the activity of 5-aminolaevulinate synthetase and liver porphyrin content, are also more sensitive with respect to the decrease in chelatase activity: in both species an approximately 70% decrease in chelatase activity was accompanied by an increase in liver porphyrin content and this was achieved by 5mg/kg body wt. in mice and by 25mg/kg in rats. Table 1 and Fig. 3 also show that in both rats and mice the administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine in doses larger than the minimum porphyrogenic doses mentioned above was followed by a further increase in 5-aminolaevulinate synthetase activity and in porphyrin concentration without a further decrease in chelatase activity.

Mouse liver mitochondria are more active (per mg of protein) than rat liver mitochondria in catalysing the incorporation of Co^{2+} into mesoporphyrin *in vitro* (Fig. 3). When the incorporation *in vitro* of ^{59}Fe into protoporphyrin by liver homogenates was used to measure the activity of the chelatase, it was, however, found that mouse liver was less active than rat liver. Nevertheless the decrease in activity caused by administering 3,5-diethoxycarbonyl-1,4-dihydrocollidine, in a dose (10mg/kg) which is porphyrogenic in the mouse but not in the rat, was again appreciably greater in the mouse than in the rat (Table 2). Therefore with two different methods of assay of the chelatase, the decrease in enzyme activity caused by treatment with the drug was found to be more marked in the mouse, the species that is also more sensitive with respect to the induction of 5-aminolaevulinate synthetase and to the accumulation of porphyrins.

Effect of prior treatment with compound SKF 525-A or with cycloheximide on the induction of porphyria by 3,5-diethoxycarbonyl-1,4-dihydrocollidine

Compound SKF 525-A, when administered to rats before 3,5-diethoxycarbonyl-1,4-dihydrocollidine completely prevented the induction of 5-aminolaevulinate synthetase and the accumulation of porphyrins caused by the latter drug and also almost completely prevented the decrease in chelatase activity. In contrast prior treatment with cycloheximide inhibited the induction of 5-aminolaevulinate synthetase and the appearance of porphyria without preventing the decrease in activity of the chelatase (Table 3). A possible interpretation for the different effect of the two inhibitors is discussed below.

In the mouse compound SKF 525-A only partially inhibited the porphyria caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine and was almost completely inactive in protecting the liver chelatase from a decrease in activity (Table 4). Therefore compound SKF 525-A could only prevent porphyria in the species (the rat) where the loss of chelatase activity was also largely prevented.

Discussion

Species differences in the induction of hepatic porphyria by drugs

Gross & Hutton (1971) described genetic differences between several inbred strains of mice in the

Table 2. *Effect of treating rats and mice with 3,5-diethoxycarbonyl-1,4-dihydrocollidine 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 200mg of wet liver) were incubated for 15min in the presence of [^{59}Fe]ferric citrate (100000c.p.m.) under the conditions described in the Experimental section. At the end of the incubation 10mg of carrier haem was added.

	Incorporation of ^{59}Fe into haem (c.p.m./mg of haem)	
Treatment and dose (ml/kg or mg/kg)	Rat	Mouse
Control (oil, 10ml)	2470	1770
	2500	1950
	Average 2490	Average 1860
3,5-Diethoxycarbonyl-1,4-dihydrocollidine (10mg)	2280	670
	1690	870
	Average 1990	Average 770
Decrease caused by the drug (%)	20	59

Table 3. *Effect of pretreatment with compound SKF 525-A or with cycloheximide on the increase in 5-aminolaevulinate synthetase activity and total porphyrin content and on the decrease of porphyrin–metal chelatase activity in rat liver caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine*

Rats were starved for 24h, then injected with arachis oil (10ml/kg body wt.) or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (100mg/kg body wt.) intraperitoneally and killed 4h later. Compounds SKF 525-A, cycloheximide or saline alone were given 45min before either oil or 3,5-diethoxycarbonyl-1,4-dihydrocollidine as described in the Experimental section. The results are expressed as means ±S.E.M. of the numbers of observations in parentheses. Probabilities of differences from animals receiving the same treatment after pretreatment with saline alone are indicated thus: * $P<0.05$; ** $P<0.01$. Probabilities of difference from animals receiving oil alone after the same pretreatment are indicated thus: † $P<0.05$; †† $P<0.01$.

Pretreatment and dose (ml/kg or mg/kg)	Treatment and dose (ml/kg or mg/kg)	5-Aminolaevulinate synthetase activity (nmol/min per g of wet liver)	Total porphyrins (nmol/g of wet liver)	Porphyrin–metal chelatase activity (nmol of Co^{2+} mesoporphyrin formed/min per mg of mitochondrial protein)
Saline (5ml)	Oil (10ml)	2.41±0.19 (4)	0.06±0.02 (8)	1.084±0.08 (4)
Saline (5ml)	3,5-Diethoxycarbonyl-1,4-dihydrocollidine (100mg)	11.95±0.5† (4)	8.14±0.77†† (10)	0.453±0.04†† (6)
SKF 525-A (40mg)	Oil (10ml)	1.73±0.17 (4)	0.14±0.03* (8)	0.960±0.04 (4)
SKF 525-A (40mg)	3,5-Diethoxycarbonyl-1,4-dihydrocollidine (100mg)	1.83±0.3 * (4)	0.17±0.04** (9)	0.920±0.10** (5)
Cycloheximide (40mg)	Oil (10ml)	0.74±0.32* (4)	<0.06 (4)	1.060±0.04 (4)
Cycloheximide (40mg)	3,5-Diethoxycarbonyl-1,4-dihydrocollidine (100mg)	0.24±0.10* (4)	<0.06** (4)	0.290±0.03*† (4)

Table 4. *Effect of pretreatment with compound SKF 525-A on the increase in total porphyrin content and on the decrease of porphyrin–metal chelatase activity in mouse liver caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine*

Mice were starved for 24h, then injected with arachis oil (10mg/kg body wt.) or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (100mg/kg body wt.) intraperitoneally and killed 4h later. Compound SKF 525-A or saline alone were given 45min before either oil or 3,5-diethoxycarbonyl-1,4-dihydrocollidine as described in the Experimental section. The results are expressed as means ±S.E.M. of the numbers of observations in parentheses. Probabilities of difference from animals receiving oil alone after the same pretreatment are indicated thus * $P<0.05$.

Pretreatment and dose (ml/kg or mg/kg)	Treatment and dose (ml/kg or mg/kg)	Total porphyrins (nmol/g of wet liver)	Porphyrin–metal chelatase activity (nmol of Co^{2+} mesoporphyrin formed/min per mg of mitochondrial protein)
Saline (5ml)	Oil (10ml)	<0.06 (4)	1.807±0.10 (4)
Saline (5ml)	3,5-Diethoxycarbonyl-1,4-dihydrocollidine (100mg)	6.98±2.25* (4)	0.503±0.08* (4)
SKF 525-A (40mg)	Oil (10ml)	<0.06 (4)	1.834±0.11 (4)
SKF 525-A (40mg)	3,5-Diethoxycarbonyl-1,4-dihydrocollidine (100mg)	3.65±1.19* (4)	0.63 ±0.1 * (4)

induction of liver 5-aminolaevulinate synthetase by drugs; in their experiments strains that were more sensitive to 3,5-diethoxycarbonyl-1,4-dihydrocollidine were also more sensitive to 2-allyl-2-isopropylacetamide, suggesting that the genetic variations applied to some step involved in the induction of 5-aminolaevulinate synthetase by both drugs. In the present experiments, on the contrary, mice were more sensitive than rats to 3,5-diethoxycarbonyl-1,4-dihydrocolidine but not to 2-allyl-2-isopropylacetamide. Therefore something which applies specifically to each drug is likely to be involved in this difference in response between rats and mice.

The reason why mice are more sensitive than rats to the effects of 3,5-diethoxycarbonyl-1,4-dihydrocollidine is not known. It could depend on a difference in sensitivity of the target in the liver (like the mitochondrial chelatase; see below) or on the way in which the two species distribute and metabolize the drug. Racz & Marks (1972) have suggested on the basis of results obtained in the chick embryo that it is the unchanged drug which is porphyrogenic and that its metabolism results in loss of porphyrogenic activity. If this also applied to the induction of porphyria in the liver of rodents, a greater rate of metabolism of 3,5-diethoxycarbonyl-1,4-dihydrocollidine by the rat (as compared with the mouse) might explain the difference in sensitivity between the two species. Mice are also more sensitive than rats to the porphyrogenic activity of griseofulvin (Weston Hurst & Paget, 1963; F. De Matteis, unpublished work). The metabolism of griseofulvin has been studied in both species, but there is no major quantitative difference between rats and mice in the rate at which they metabolize griseofulvin (Synchowicz & Wong, 1966; Lin *et al.*, 1972).

The following findings could be interpreted to indicate that a metabolite of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (rather than the parent compound) is involved in the decrease in chelatase activity and in the induction of porphyria in rodents: (1) when added *in vitro* to partially solubilized rat liver mitochondria under the conditions employed for the assay of the incorporation of Co^{2+} into mesoporphyrin, 3,5-diethoxycarbonyl-1,4-dihydrocollidine at the concentration of 800μg/ml failed to inhibit the chelatase. Similar observations have been reported by Onisawa & Labbe (1963) and by Tephly *et al.* (1971). (2) In newborn rats, which have low activity of drug-metabolizing enzymes in the liver (Conney, 1967), the drug causes only a small decrease in chelatase activity and only marginally raises the concentration of porphyrins in the liver without increasing the activity of liver 5-aminolaevulinate synthetase (F. De Matteis, unpublished work). (3) Compound SKF 525-A, an inhibitor of drug-metabolizing enzymes, prevents the decrease in chelatase activity and the induction of 5-aminolaevulinate caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine in rat liver. All this evidence is only indirect, however. Also compound SKF 525-A failed to prevent the decrease in chelatase activity and the induction of porphyria in mouse liver. Therefore the significance of the metabolism of 3,5-diethoxycarbonyl-1,4-dihydrocollidine in relation to its porphyrogenic activity cannot yet be conclusively assessed.

Decrease in the liver activity of porphyrin–metal chelatase and accumulation of porphyrins in the liver

Onisawa & Labbe (1963) first described an inhibition of the synthesis of haem in the liver of mice treated for several days with 3,5-diethoxycarbonyl-1,4-dihydrocollidine. They suggested that inhibition of the conversion of protoporphyrin into haem might explain the very marked accumulation of protoporphyrin which is a characteristic of this type of hepatic porphyria. This interpretation is supported by the findings obtained in this present work. First decreased chelatase activity precedes the accumulation of liver porphyrins, which is compatible with the existence of a causal relationship between these two effects of the drug. Secondly there exists a good correlation in both rats and mice between the doses of drug required to decrease chelatase activity and the doses required to increase the concentration of porphyrins in the liver. Finally compound SKF 525-A prevented porphyria only in the species (the rat) where the decrease in chelatase activity was also largely prevented. Also in favour of this concept that the loss of chelatase activity is important for the accumulation of porphyrins in the liver is the finding (De Matteis, 1973 and unpublished work) of a good correlation between inhibition of the chelatase and concentration of liver porphyrins after administration of compounds chemically related to griseofulvin, another drug that causes a marked accumulation of protoporphyrin in the liver (De Matteis & Rimington, 1963).

The results obtained with cycloheximide and 3,5-diethoxycarbonyl-1,4-dihydrocollidine given together (Table 3) indicate that a marked decrease in chelatase activity is not sufficient on its own to cause accumulation of porphyrins in the liver. An increase in 5-aminolaevulinate synthetase activity is also probably not sufficient on its own (Creighton *et al.*, 1971; Abbritti & De Matteis, 1971–72): for example, in Table 1 there is a marked difference in the liver porphyrin content between mice treated with 10mg of 3,5-diethoxycarbonyl-1,4-dihydrocollidine/kg and those treated with 50mg of 2-allyl-2-isopropylacetamide/kg, a drug that does not inhibit the chelatase activity (Onisawa & Labbe, 1963), even though the 5-aminolaevulinate synthetase activities are quite similar in the two groups. The important factor in determining the accumulation of porphyrins in the liver is probably the ratio between rate of porphyrin

utilization and rate of 5-aminolaevulinate and porphyrin formation.

This interpretation is supported by the finding of a statistically-significant linear correlation between the ratios of the activities of chelatase and 5-aminolaevulinate synthetase on the one hand and the reciprocal of the liver porphyrin concentration on the other (see Fig. 4, where the logs have been plotted to bring the range of experimental results to a convenient scale). When the mean activities of the Co^{2+} mesoporphyrin chelatase are expressed as 5-aminolaevulinate equivalents metabolized/min by 1 g of wet liver and are then divided by the corresponding activities of 5-aminolaevulinate synthetase, it is found that the ratio of the two enzymic activities is directly related to the reciprocal of the liver porphyrin concentration. This relationship indicates that over the large range of enzyme activities and porphyrin concentrations observed in these experiments the amount of liver porphyrins is directly proportional to the synthetase activity and inversely proportional to the activity of the chelatase. The existence of this relationship is compatible with the concept that 5-aminolaevulinate synthetase is the rate-limiting enzyme in porphyrin biosynthesis and also with the finding that protoporphyrin is the main porphyrin that accumulates in the liver after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (Onisawa & Labbe, 1963), since this porphyrin is the direct substrate for the chelatase enzyme. The results observed in rats given cycloheximide and 3,5-diethoxycarbonyl-1,4-dihydrocollidine together follow the same general relationship.

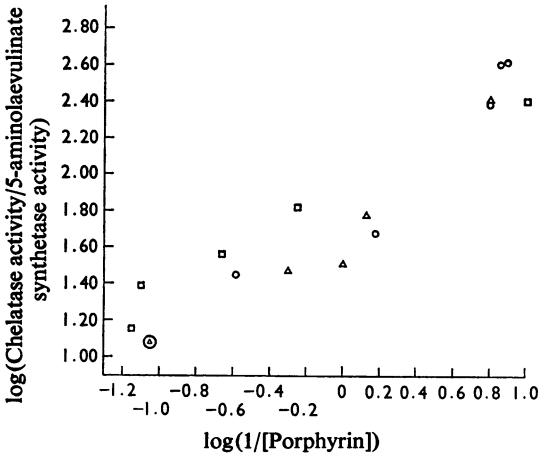

Fig. 4. *Relationship between the ratio of the activities of chelatase and 5-aminolaevulinate synthetase and the reciprocal of liver porphyrin concentration*

The Co^{2+} mesoporphyrin chelatase activities were corrected to nmol of 5-aminolaevulinate equivalents metabolized/min by 1 g of wet liver by multiplying the average values given in Figs. 2 and 3 first by 8 (8 mol of 5-aminolaevulinate are equivalent to 1 mol of porphyrin) then by 60 (mg of mitochondrial protein/g of liver of a starved rat). The corrected chelatase activity was then divided by the corresponding activity of 5-aminolaevulinate synthetase (Table 1) and the ratio related to the reciprocal of the liver porphyrin concentration. The following coefficients of linear correlation were obtained: 0.99 in rats killed at different times after a single dose of drug (△); 0.99 and 0.97 respectively in mice (□) and rats (○) killed 5 h after either oil or increasing doses of the drug. The value of P was <0.01 in all cases.

In the liver of control animals the Co^{2+} mesoporphyrin chelatase is about 400-fold in excess of the synthetase and when porphyrins accumulate the chelatase is still in excess though by a factor of 60 or less. In the rats given cycloheximide and 3,5-diethoxycarbonyl-1,4-dihydrocollidine together there is no increase in porphyrin content in the liver and, since both the chelatase and the synthetase are inhibited, the ratio of the two enzyme activities is still very high (above 400).

When porphyrins started to show a clear increase in the liver a ratio of the chelatase activity to the 5-aminolaevulinate synthetase activity equal or less than 1 might have been expected, but this was not found. Instead the Co^{2+} mesoporphyrin chelatase was still well in excess of the synthetase (by a factor of 60). Most of the excess of chelatase activity is probably due to the use of Co^{2+} and mesoporphyrin as substrates in the enzyme assay, since these are better utilized by the mitochondrial chelatase than are the physiological substrates Fe^{2+} and protoporphyrin. For example, when the incorporation of Fe^{2+} into protoporphyrin was assayed spectrophotometrically with ultrasonically treated mitochondria from the liver of rats starved for 24 h and in the presence of succinate to promote anaerobiosis (Jones & Jones, 1969), the rate of metalloporphyrin formation in nmol/min per mg of protein was 0.141 ± 0.004 (5). This rate is nine times slower than that obtained with Co^{2+} and mesoporphyrin. Therefore when porphyrins start accumulating in the liver the Fe^{2+} protoporphyrin chelatase is still likely to be in excess of the synthetase though only by a factor of about 7. It is not known whether this excess of ferrochelatase reflects the situation *in vivo*.

Decreased liver chelatase activity and induction of liver 5-aminolaevulinate synthetase

Haem exerts a negative-feedback control on 5-aminolaevulinate synthetase (reviewed by Granick &

Sassa, 1971). The possibility has therefore been considered (De Matteis & Gibbs, 1972) that inhibition of liver haem synthesis (and the consequent interference with the negative-feedback control of haem) may be at least part of the mechanism by which 3,5-diethoxycarbonyl-1,4-dihydrocollidine stimulates markedly the activity of 5-aminolaevulinate synthetase. This interpretation is supported by the finding in the present work [and by the similar finding obtained with griseofulvin and its analogues (F. De Matteis, unpublished work)] that under conditions where the decrease in chelatase activity was more pronounced there was not only a more marked accumulation of liver porphyrins, but also a greater induction of 5-aminolaevulinate synthetase activity. This interpretation is also supported by the findings in avian hepatocytes cultured *in vitro* (Strand *et al.*, 1972) that inhibition of the synthesis of haem by other agents also results in increased activity of the synthetase.

The stimulation of 5-aminolaevulinate synthetase caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine could be prevented by administering to the rat either compound SKF 525-A or cycloheximide before the drug. An important difference was that only compound SKF 525-A was also capable of preventing the decrease in the chelatase activity caused by the drug. These results suggest that if a decrease in chelatase activity is involved in the induction of 5-aminolaevulinate synthetase by 3,5-diethoxycarbonyl-1,4-dihydrocollidine it is not the only factor involved. Perhaps there are two successive stages in the induction process (cf. De Matteis, 1973): a first stage leading to inhibition of haem synthesis and a second stage requiring synthesis of protein in the liver and leading to stimulation of 5-aminolaevulinate synthetase.

Both in rats and mice killed 5h after administration of increasing doses of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (Fig. 3) and in rats killed at different times after a standard dose of drug (Fig. 2) changes were found in the activity of 5-aminolaevulinate synthetase which were not accompanied by corresponding changes in the activity of the chelatase. This also suggests that some other mechanism, apart from the decrease in chelatase activity may be involved in the induction of the synthetase by the drug. Recent studies on drug interaction in experimental hepatic porphyria (De Matteis & Gibbs, 1972; De Matteis, 1972 and unpublished work) have suggested the existence of two different mechanisms leading to stimulation of 5-aminolaevulinate synthetase by drugs. One of these (so far documented only for 3,5-diethoxycarbonyl-1,4-dihydrocollidine) probably involves inhibition of the chelatase; once the chelatase is inhibited to a sufficient degree by 3,5-diethoxycarbonyl-1,4-dihydrocollidine the response of 5-aminolaevulinate synthetase can be increased by administering one of many lipid-soluble drugs (which are relatively ineffective on their own). The exact mechanism underlying this potentiation effect is not yet known. It is possible that 3,5-diethoxycarbonyl-1,4-dihydrocollidine may have two actions: a specific one related to the effect on the chelatase and an aspecific 'potentiation' action common to several lipid-soluble drugs.

The ability of 3,5-diethoxycarbonyl-1,4-dihydrocollidine to increase the rate of liver haem degradation (G. Abbritti & F. De Matteis, unpublished work) is an alternative or additional mechanism by which the drug might potentiate its main effect on 5-aminolaevulinate synthetase.

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