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Studies on the Mechanism of Experimental Porphyria Produced by 3,5-Diethoxycarbonyl-1,4-dihydrocollidine

ROLE OF A PORPHYRIN-LIKE INHIBITOR OF PROTOHAEM FERRO-LYASE

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A green porphyrin-like pigment with inhibitory properties towards protohaem ferro-lyase activity was isolated from the livers of mice and rats after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine. Mice, which are more sensitive to the porphyrogenic properties of the drug, produce more inhibitor. The non-porphyrogenic analogue 3,5-diethoxycarbonylcollidine does not cause accumulation of the pigment in the liver. The inhibitory substance is present in control liver at low but measurable concentrations.

The porphyrogenic agent 3,5-diethoxycarbonyl-1,4-dihydrocollidine produces a marked inhibition of protohaem ferro-lyase activity (EC 4.99.1.1) *in vivo* that probably accounts for the large accumulation of protoporphyrin seen in the experimental hepatic porphyria caused by this drug (Onisawa & Labbe, 1963). The inhibition of protohaem ferro-lyase after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine has been confirmed in other laboratories (Tephly *et al.*, 1971; De Matteis *et al.*, 1973); the mouse is more sensitive than the rat, and the effect is rapid in both species. Although Abbritti & De Matteis (1973) showed that 3,5-diethoxycarbonyl-1,4-dihydrocollidine promotes an increased breakdown of hepatic haem, studies on the inhibition of haem biosynthesis have been more numerous. We now report that administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine leads to the accumulation of a green porphyrin-like pigment that has strong inhibitory activity towards protohaem ferro-lyase. Labelling studies suggest that the pigment may arise from haem, and the mouse, which is markedly more sensitive than the rat to the porphyrogenic effect of 3,5-diethoxycarbonyl-1,4-dihydrocollidine, also produces much more pigment than does the rat. In addition, an analogue that does not cause inhibition of the enzyme *in vivo* (De Matteis & Gibbs, 1975) also fails to promote accumulation of the inhibitory pigment in the liver.

Materials and Methods

Animals

Male 23–28g mice of the LACA strain and male 200g Porton (Wistar-derived) rats were allowed food and water *ad libitum*. 5-Amino[4-^{14}C]laevulinate (10μCi; 0.17μmol/kg) was injected intraperitoneally. Mice were killed by cervical dislocation and rats were decapitated; their livers were quickly removed and homogenized in ice-cold 0.25M-sucrose. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine and 3,5-diethoxycarbonylcollidine were prepared and purified by the methods given by De Matteis & Gibbs (1975) and administered intraperitoneally dissolved in arachis oil.

Enzyme assays and analytical procedures

Protohaem ferro-lyase activity was determined by the method of Jones & Jones (1969) by using a Perkin–Elmer model 356 dual-wavelength recording spectrophotometer and employing Co^{2+} and mesoporphyrin as substrates. Mitochondria were prepared as described by De Matteis *et al.* (1973) and suspended in sucrose at a concentration of 20mg of protein/ml. This preparation was stable for about 4 days when kept at 4°C. Scintillation counting of radioactivity was performed by using a Philips PW 4510 analyser, with correction for quench by an external-standard channels-ratio programme. Protein was determined by the method of Aldridge (1962).

Extraction and purification of the inhibitor

Liver homogenates (50%, w/v, in 0.25M-sucrose, equivalent to 6g wet wt. of liver) were extracted twice with an equal volume of acetone/ether (4:1, v/v). The pellet obtained by centrifugation was suspended in 12ml of acetone, and 0.2ml of conc. HCl was added. The acetone/HCl extraction was repeated, and the combined supernatants were evaporated to dryness under a stream of O_2-free N_2 and dissolved in 1ml of acetone for Sephadex LH-20

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chromatography. This was carried out in freshly prepared 20 cm × 1.0 cm columns of Sephadex LH-20, and acetone was used to pack the gel and to elute the fractions.

Studies on protohaem ferro-lyase inhibition

Fractions were evaporated to dryness under O_2-free N_2 and immediately dissolved in dimethyl sulphoxide, and 0.05 ml portions of various dilutions were preincubated with 0.4 ml of mitochondrial suspension and 0.2 ml of 0.5 M-Tris/HCl buffer, pH 8.2, at 37°C for 15 min, a time found to be necessary to obtain maximal inhibition; 0.4 ml samples of these preincubation mixtures were then removed and protohaem ferro-lyase activity was determined (Jones & Jones, 1969) in a final volume of 2.75 ml. Under these conditions dimethyl sulphoxide alone did not inhibit. In preliminary studies it was shown that inhibition of enzyme activity, once established, was not reversible by dialysis overnight against 400 vol. of 0.1 M-Tris/HCl buffer, pH 8.2. Thus, by assuming irreversible inhibition of the enzyme and by adjusting the starting enzyme activity to approx. 12.0 nmol of porphyrin utilized/min per 0.65 ml of preincubation mixture, it was possible to define a unit of inhibitor as that amount which produced a 50% inhibition of protohaem ferro-lyase activity under these standard conditions.

Results

Onisawa & Labbe (1963) reported that hepatic cytosolic fractions obtained from mice receiving 200 mg of 3,5-diethoxycarbonyl-1,4-dihydrocollidine/kg body wt. per day for 7 days and killed 24 h after the last injection slightly inhibited protohaem ferro-lyase activity of rat liver mitochondria. We have found that a marked inhibition of the enzyme was produced by hepatic cytosol fractions obtained from either rats or mice 1 h after a single dose of the drug. An inhibitor could be extracted from the liver homogenate of these animals by acetone/HCl and further purified by Sephadex LH-20 chromatography, where it was eluted immediately after haem and before authentic protoporphyrin. All fractions with inhibitory activity displayed a green colour in visible light, and, when dried on a silica-gel chromatographic plate, a scarlet-red fluorescence was noted under u.v. light.

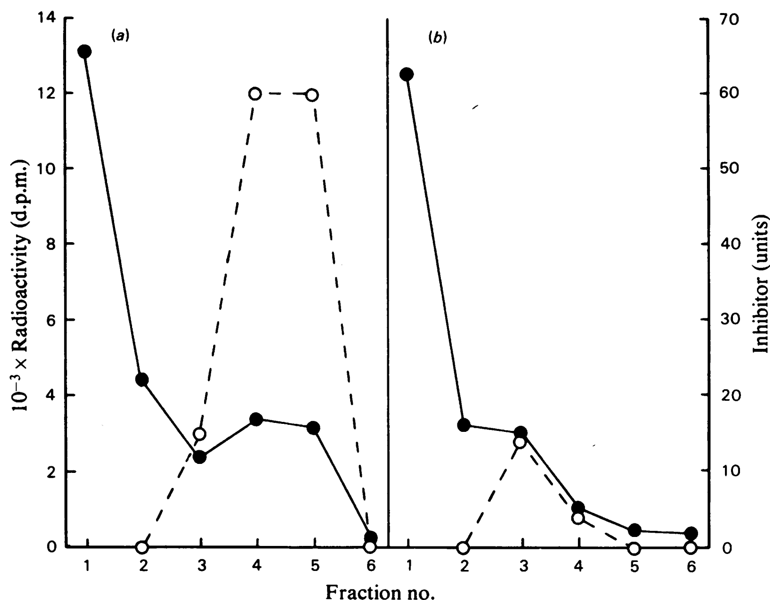

Fig. 1. *Distribution of inhibitory activity towards protohaem ferro-lyase and radioactivity in fractions eluted from Sephadex LH-20*

Mice (*a*) and rats (*b*) were injected with 5-amino[4-^{14}C]laevulinic acid (10 μCi/kg, intraperitoneally); 2 h later they were given 3,5-diethoxycarbonyl-1,4-dihydrocollidine (25 mg/kg) and killed after a further 1 h. Liver acid/acetone extracts were prepared and chromatographed on Sephadex LH-20 as described in the Materials and Methods section. After elution of the first 22 ml, which contained the bulk of the haem, six 3 ml fractions were collected to recover quantitatively the inhibitor. A 0.3 ml sample of each fraction was taken for counting of radioactivity (●) and 1 ml for the determination of inhibitor activity (○). Results presented were obtained from the pooled livers of eight mice and two rats, and were confirmed at least once.

Since certain green pigments have been shown to be degradation products of haem (De Matteis, 1971; Unseld & De Matteis, 1976; McDonagh *et al.*, 1976), mice and rats were injected with a tracer dose of 5-amino[4-^{14}C]laevulinate in order to label hepatic haem. Then 2h later, at a time when precursor pools are no longer labelled and further incorporation of label into haem is minimal, 3,5-diethoxycarbonyl-1,4-dihydrocollidine was injected and the animals were killed after a further 1h. Results of a typical experiment are shown in Fig. 1, where the amount of inhibitor obtained from mouse (Fig. 1*a*) and rat (Fig. 1*b*) liver is related to the amount of radioactivity present. About 8 times more inhibitor is found in the mouse liver fractions than in those from rat (see also Table 1), The amount of radioactivity associated with inhibitor fractions was also greater with mouse liver, but a precise quantitative comparison cannot be made, as haem most probably contributed to the radioactivity of these fractions.

All fractions obtained from the experiments reported in Fig. 1 were analysed spectrally, and the spectra of the most inhibitory fractions of either mouse or rat liver are shown in Fig. 2(*a*). The green fractions with inhibitory activity exhibited a Soret absorption peak at 411nm quite distinct from the

Table 1. *Units of inhibitor of protohaem ferro-lyase recovered from the livers of mice given oil,* 3,5-*diethoxycarbonylcollidine or* 3,5-*diethoxycarbonyl*-1,4-*dihydrocollidine and rats treated with the latter drug*

Mice and rats were injected intraperitoneally with oil alone, 3,5-diethoxycarbonyl-1,4-dihydrocollidine or 3,5-diethoxycarbonylcollidine in oil and killed 1h later. The inhibitor was extracted, chromatographed and assayed as described in the legend to Fig. 1. The values presented are those of a typical experiment, which was confirmed at least once, and represent the total inhibitory activity recovered from the pooled livers of four to 20 mice and of two rats. The unit of inhibitor is defined in the text.

Species	Treatment (and dose)	Liver inhibitor content (units/10g of liver)
Mouse	Arachis oil (10ml/kg)	2.5
Mouse	3,5-Diethoxycarbonylcollidine (100mg/kg)	6.6
Mouse	3,5-Diethoxycarbonyl-1,4-dihydrocollidine (25mg/kg)	225.0
Rat	3,5-Diethoxycarbonyl-1,4-dihydrocollidine (25mg/kg)	30.0

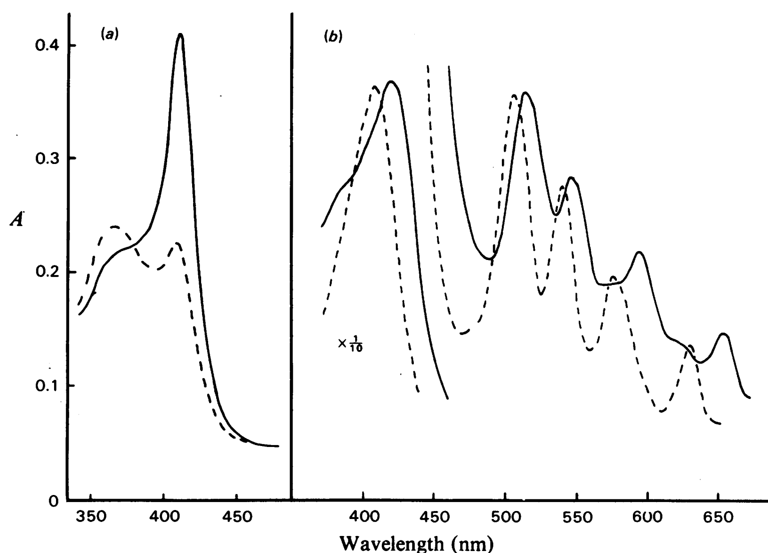

Fig. 2. *Absorption spectra of inhibitory green pigments from rat and mouse liver: comparison with the spectrum of protoporphyrin*

The spectra (Soret region) of the most inhibitory fractions obtained from either rat (----) or mouse (——) liver in the experiment of Fig. 1 (and determined as eluted from the column) are shown in (*a*). In (*b*) the complete visible-absorption spectrum of haem-free green pigment (——) obtained from the liver of treated mice is compared with that of authentic protoporphyrin (----) (both pigments dissolved in pyridine). Note that in (*b*) the scale of the ordinates has been decreased 10-fold for the Soret region.

haem peak that was observed at 380nm in the same solvent. With the rat fraction the spectrum was complicated by the presence of both peaks; nevertheless the amount of material absorbing at 411nm was clearly less than that seen in the mouse fraction. A good correlation between inhibitory activity, radioactivity from 5-amino[4-^{14}C]laevulinate and absorption at 411nm was also found during comparison of different fractions from the same column.

A purified preparation of green pigment, when evaporated to dryness and dissolved in pyridine, showed an aetio-type spectrum with all absorption maxima shifted towards the red as compared with those of authentic protoporphyrin (Fig. 2*b*).

The total amount of inhibitor obtained from the liver of the experiment of Fig. 1 is compared in Table 1 with the amount similarly obtained from the liver of control mice or of mice given the analogue 3,5-diethoxycarbonylcollidine, previously shown not to cause inhibition of protohaem ferro-lyase *in vivo* (De Matteis & Gibbs, 1975). Control livers were found to contain a similar green inhibitory pigment at low but measurable concentrations, and 3,5-diethoxycarbonylcollidine failed to increase significantly the amount of inhibitor over that of the controls; in these two groups the Soret absorption again correlated well with the inhibitory activity.

The inhibitory green pigment found after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine could be further separated from haem and purified by t.l.c on silica-gel plates by using a developing system of chloroform/methanol/acetic acid (40:10:3, by vol). The green band (R_F 0.60) displayed scarlet fluorescence under u.v., and after elution with methanol was shown to inhibit protohaem ferro-lyase activity.

Discussion

This work has provided conclusive evidence for the accumulation of an inhibitor of protohaem ferro-lyase in the liver of animals treated with the porphyrogenic agent 3,5-diethoxycarbonyl-1,4-dihydrocollidine. The following lines of evidence suggest that the inhibitor is a porphyrin-like pigment: (1) it is extracted under conditions that lead to porphyrin isolation; (2) its spectrum is porphyrin-like; (3) it fluoresces red under u.v. light; (4) it is labelled by 5-amino[4-^{14}C]laevulinate, a precursor of haem.

The finding of a porphyrin-like green pigment with inhibitory properties towards protohaem ferro-lyase in the liver of control mice is of considerable interest. If this control pigment and that accumulating after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine prove to be identical, then the possibility should be considered that the drug acts by accelerating a normally occurring process, perhaps a pathway of haem degradation. The significance of such a substance in the human porphyrias is not known as yet, but a possible role of similar endogenously produced inhibitors in the production of the partial blocks in haem biosynthesis should be investigated.

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