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PORPHYRIN SYNTHESIS IN DRUG INDUCED HEPATIC PORPHYRIA*

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Abstract—Liver δ-aminolaevulate (ALA) synthetase and ALA dehydratase are induced to a greater extent in 3,5-diethoxy carbonyl-1,4-dihydrocollidine (DDC) injected mice as compared to the allyl isopropyl acetamide (AIA) injected rats. DDC treated mice do not show an increase in porphobilinogen (PBG) levels commensurate with the increase in ALA levels and the two enzyme activities, but accumulate enormous quantities of protoporphyrin in the liver. Normal mouse liver has an inherent greater capacity to convert PBG to porphyrins as compared to that of the rat. This together with the inhibition of iron incorporation into protoporphyrin *in vivo* at later stages of DDC administration can account for the large accumulation of protoporphyrin in these animals.

δ-AMINOLAEVULATE (ALA) synthetase, the first enzyme involved in haem biosynthesis, has been shown to be the limiting and regulatory enzyme of the pathway in animal liver.[1, 2] This enzyme is induced by drugs and hormones and is repressed by haem. It is held that the other enzymes of the pathway are present in non-limiting amounts.[3] While this may be true under normal conditions, additional regulatory sites are likely to be involved in different porphyrias where different intermediates accumulate in abnormal quantities.[3, 4] For example, acute intermittent porphyria is characterized by excessive excretion of the precursors ALA and porphobilinogen (PBG) as compared to the porphyrins themselves. In variegate porphyria urinary and faecal excretion of porphyrins is considerably increased.[5]

In the present investigation, the porphyrin synthesis in two types of drug induced porphyria has been investigated. These have been produced by allyl isopropyl acetamide (AIA) administration to rats and 3,5-diethoxy carbonyl-1-4-dihydrocollidine (DDC) administration to mice. The two resemble acute intermittent porphyria and variegate porphyria respectively.[5]

EXPERIMENTAL

Chemicals. AIA was generously provided by Hoffmann-LaRoche, Basle. DDC was synthesized by the method of Dematteis and Prior.[6] The final product was recrystallized twice from ethanol-water and melted at 128°. PBG was isolated from the urine of rabbits treated with lead. The method is based on the formation of an insoluble mercury salt and has been described in detail.[7] The crystalline PBG was found to be pure by paper and column chromatographic criteria. $^{59}FeCl_3$ was purchased from Bhabha Atomic Research Centre, Trombay.

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Treatment of animals. Male rats (100–110g) of the local Institute strain maintained on stock diet were given daily subcutaneous injections of AIA (500 mg/kg) in saline. The animals were killed every 24 hr by decapitation, 6 hr after the injection and the livers were removed after perfusion with saline. Male mice (25–28g) received a similar schedule of injections with DDC (400 mg/kg) in coconut oil. Control animals were treated with the vehicle only.

Estimation of liver and urinary ALA, PBG and porphyrin levels

For the estimation of ALA and PBG contents, portions of the liver homogenates were deproteinised with trichloracetic acid. The supernatant as well as suitably diluted urine samples were adjusted to pH 4·5–5·0 and after treatment with acetylacetone, PBG and ALA (converted to the pyrrole) were fractionated using Dowex-1 acetate columns as described by Marver *et al.*[8] The effluents containing PBG and ALA pyrrole were treated with equal volumes of modified Ehrlich reagent and the colour measured at 556 mμ.[9]

Porphyrins were estimated in portions of the liver homogenate by the method described by Sardesai, Waldman and Orten.[10]

^{59}Fe incorporation into liver haem. The animals were given intraperitoneal injections of $^{59}FeCl_3$ ($1{\cdot}3 \times 10^6$ counts/min), 30 min before killing. After killing the animals, haemin was isolated from perfused liver homogenates after adding 3 ml of carrier-blood.[11] Portions of the original homogenate were counted to measure total incorporation. The twice recrystallized haemin was solubilized and taken in ethyl acetate. The ethyl acetate layer was extracted with 3N HCl to remove any porphyrin contamination and then concentrated to dryness in vacuum. The residue was dissolved in alkaline pyridine and portions were used to measure radioactivity and haemin content.

Conversion of PBG to porphyrins. For this, the method described by Schwartz and Watson[12] was used. Liver homogenates were prepared using 3 ml of 0·1 M phosphate buffer (pH 7·4) per g of the tissue. Six ml of the homogenate was incubated with 200 μg of PBG for 6 hr at 37° without shaking. The reaction was stopped by adding 4 vol. of ethylacetate–acetic acid mixture (4:1, v/v) and the total porphyrins formed was estimated as described earlier.

Assay of ALA synthetase and ALA dehydratase. ALA synthetase was assayed in liver homogenates by the procedure described by Marver *et al.*[13] The liver was homogenised in 3 vol. of 0·9% sodium chloride containing 0·5 mM EDTA and 10 mM tris, pH 7·4. The incubation mixture contained 0·5 ml of homogenate, 200 μmoles of glycine, 20 μmoles of EDTA, 150 μmoles of tris–HCl buffer at a final pH of 7·2 in a total volume of 2 ml. The incubation was carried out at 37° for 60 min. The reaction was terminated by the addition of 0·5 ml of 25% trichloracetic acid. ALA was estimated in the trichloracetic acid supernatant. In the case of mice 0·4 μmole of pyridoxal phosphate was included in the incubation mixture.

ALA dehydratase in liver homogenates was assayed by the method of Gibson, Neuberger and Scott.[14] The homogenate in 0·05 M potassium phosphate buffer (pH 6·8) was preincubated with glutathione (10 μmoles) for 30 min at 37°. The reaction was started with the addition of ALA hydrochloride (6 μmoles). The total volume of the reaction mixture was 2 ml. After incubation for 60 min the reaction was stopped with 1 ml of 12·5% trichloracetic acid containing 0·02 M $HgCl_2$. After centrifugation, a portion of the supernatant was used to estimate PBG content.[9]

The two enzyme activities were found to give a linear time-course curve for at least 2 hr under the conditions of assay.

RESULTS AND DISCUSSION

Figure 1 gives the ALA, PBG and protoporphyrin levels in livers of rats and mice given AIA and DDC respectively. In DDC administered mice despite the accumulation of high concentrations of ALA in the liver during the initial stages of drug administration, PBG levels are not proportionately high. In AIA treated rats, the

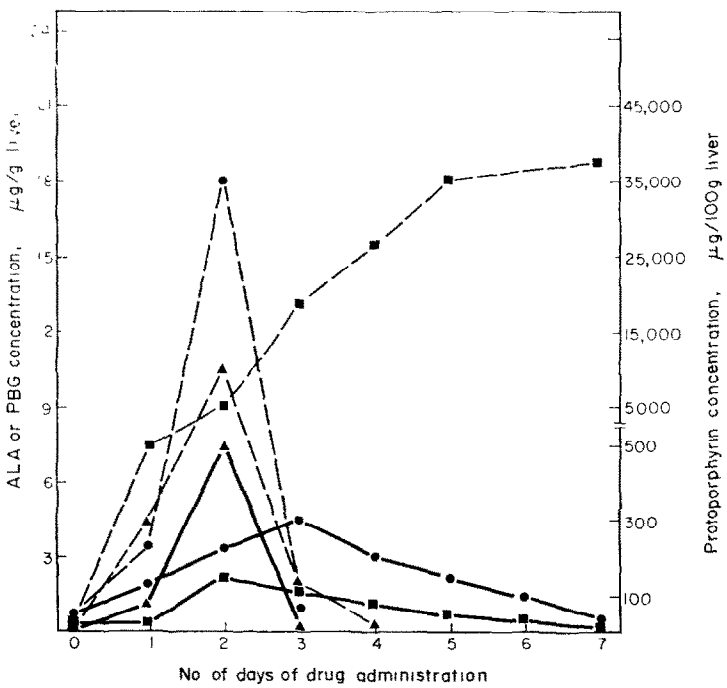

FIG. 1. Effect of AIA and DDC administration on liver ALA, PBG and protoporphyrin levels of rats and mice. The experimental details are given in text. Each point represents the average of two independent determinations and livers from five animals were pooled in each determination. The solid and dashed lines represent the values obtained with AIA treated rats and DDC treated mice respectively. ●—ALA; ▲—PBG; ■—Protoporphyrin.

maximal level of PBG reached is higher than the maximal level of ALA recorded in the liver of these animals. In addition, there is an enormous increase in protoporphyrin levels of DDC treated mouse liver which has been observed by other workers as well.[6, 15, 16] The liver pattern of ALA and PBG levels is also reflected in the urinary excretion picture (Table 1). The urinary ALA/PBG ratio is in favour of ALA in the normal rat and mouse. In rats receiving AIA the ratio shifts in favour of PBG as has been observed by Dematteis and Prior,[6] but in mice receiving DDC the ratio is in favour of ALA at the time of excretion of peak levels of metabolites.

Figure 2 indicates that ALA synthetase as well as ALA dehydratase are induced to higher levels in DDC treated mice as compared to AIA treated rats. However, as

TABLE 1. LEVELS OF ALA AND PBG IN THE URINE OF DRUG TREATED ANIMALS

Number and days of drug administration	Rat (AIA)			Mice (DDC)		
	ALA (mg/l.)	PBG (mg/l.)	ALA/ PBG	ALA (mg/l.)	PBG (mg/l.)	ALA/ PBG
0	2·1	1·1	1·91	6·2	4·3	1·44
1 (0–24 hr)	3·3	2·6	1·27	41·2	5·7	7·23
2 (24 hr–48 hr)	7·5	25·6	0·29	15·0	18·0	0·83
3 (48 hr–72 hr)	5·0	10·1	0·50	3·7	6·8	0·54
4 (72 hr–96 hr)	3·0	3·7	0·81	3·7	5·5	0·67

Urine was collected for a 24 hr period. Each value represents the average of two independent determinations and each determination was made from pooled urine samples from at least eight animals.

indicated in Fig. 1 and Table 1 levels of PBG commensurate with the high levels of ALA synthetase and ALA dehydratase could not be detected in DDC treated mice. One possible reason for this situation is the inherent greater efficiency of mouse liver homogenates to convert PBG to porphyrins (Table 2). This efficiency decreases with successive DDC administration possibly due to a feed-back inhibitory effect of the accumulated porphyrins. Thus, the initial increase in porphyrin levels in DDC

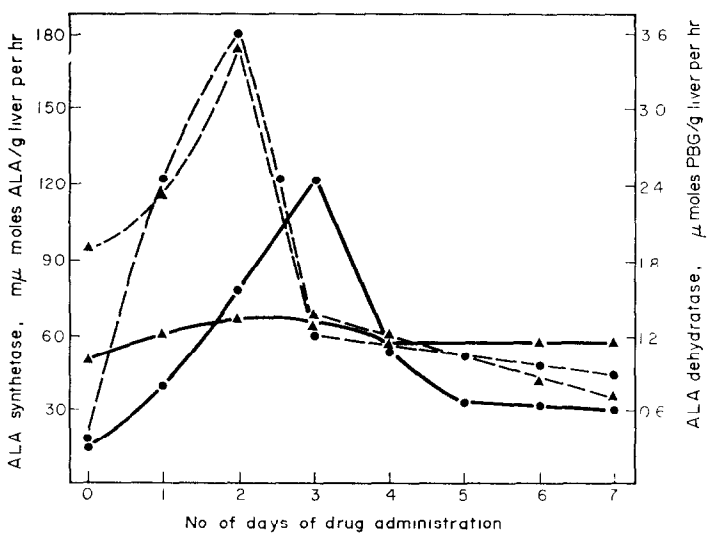

FIG. 2. Effect of AIA and DDC administration on liver ALA synthetase and ALA dehydratase of rats and mice. The experimental details are given in text. Each point represents the average of two independent determinations and livers from five animals were pooled in each determination. The solid and dashed lines represent the values obtained with AIA treated rats and DDC treated mice respectively. ●—ALA Synthetase; ▲—ALA Dehydratase.

TABLE 2. CONVERSION OF PBG TO PORPHYRINS BY LIVER HOMOGENATES OF DRUG TREATED ANIMALS

Number and days of drug administration	Rat (AIA)	Mice (DDC)
	Total porphyrins (μg/g liver)	Total porphyrins (μg/g liver)
0	14·4 ± 2·1	26·1 ± 2·7
2	14·8 ± 1·3	22·0 ± 1·3
3	14·8 ± 2·0	15·5 ± 2·1

The amount of PBG present in the incubation mixture was adjusted to 200 μg. Incubation period was 6 hr. The values represent mean ± S.E. obtained from four independent determinations.

treated mice can be explained as due to high levels of ALA synthetase and ALA dehydratase registered as well as a greater conversion of PBG to porphyrins. The importance of the animal species is emphasized by the fact that DDC fails to elicit a porphyric response in rats of the magnitude observed in mice (Table 3). Similar results have been obtained by Dematteis and Prior.[6]

TABLE 3. COMPARISON OF THE EFFECTS OF DDC IN RATS AND MICE

Number and days of DDC administration	Rat		Mice	
	ALA synthetase (mμmoles ALA/g liver/hr)	Protoporphyrin (μg/100 g liver)	ALA synthetase (mμmoles ALA/g liver/hr)	Protoporphyrin (μg/100 g liver)
0	15·5	12·5	18·9	13·1
2	60·5	51·3	160·9	3600
6	25·6	885	45·2	26,500

Onisawa and Labbe[15] have reported of an inhibition of ferrochelatase in mice treated with DDC for 7 days and attributed this as being responsible for porphyrin accumulation. Such an effect was not seen in AIA treated rats. The results presented in Table 4 indicate that ^{59}Fe incorporation *in vivo* into liver haem is not significantly inhibited in the first 3 days of DDC administration. Subsequently however, there is inhibition of ^{59}Fe uptake into the liver as well as that of the fraction incorporated into liver haem. Onisawa and Labbe[15] have also reported of the inhibition of total ^{59}Fe incorporation into the liver of DDC treated mice. Wada *et al.*[16] have reported of an inhibition of (2-^{14}C) glycine into liver haem in mice treated with DDC for 24 hr. The results of Wada *et al.*[16] can also be explained as due to the dilution of the label by the increased endogenous levels of protoporphyrin in DDC treated mice and actual inhibition of iron incorporation into protoporphyrin may be brought about later (Table 4). It is known that an excess of protoporphyrin inhibits the enzyme ferrochelatase[17] and an inhibitory level of protoporphyrin may be reached only with successive doses of DDC.

TABLE 4. ^{59}Fe INCORPORATION INTO HAEM *in vivo* IN DDC TREATED MICE

Number and days of DDC administration	Total incorporation (counts/min/g liver)	Incorporation into haem	
		(counts/min/mg haemin)	(% Total)
0	63,900	15·8	0·025
1	53,700	11·4	0·021
3	54,000	10·5	0·019
5	45,800	7·4	0·016
8	25,800	2·6	0·012

$^{59}FeCl_3$ ($1·3 \times 10^6$ counts/min) was injected intraperitoneally to mice 30 min before killing. The values represent the average of two independent determinations and livers from three animals were pooled in each determination.

Another point of interest is the striking decrease in the porphyric response of the animals on continuous drug administration (Fig. 1). This can be explained in terms of the response of ALA synthetase and ALA dehydratase which after reaching a peak in the first 2–3 days of drug administration, do not respond to the subsequent doses of the drug (Fig. 2). This has been shown to be the characteristic of drug induced effects in general.[18] However, the ALA synthetase levels are maintained at two to three times the normal in animals receiving successive doses of AIA and DDC. This together with the block at the level of iron incorporation into protoporphyrin, manifested as a result of the administration of successive doses of DDC, can explain the maintenance of high levels of liver protoporphyrin in DDC treated mice. Another feature of DDC treated mice is the fall in ALA dehydratase level to about 40 per cent the controls on continuous drug administration (Fig. 2). Onisawa and Labbe[14] detected only about 20 per cent inhibition of ALA dehydratase by protoporphyrin at 10^{-4} M *in vitro*. Recently[19] coproporphyrinogen III has been found to inhibit this enzyme at a lower concentration to a greater extent and this may be an additional contributory factor for the decrease in ALA dehydratase level in mice receiving successive doses of DDC.

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