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EFFECT OF DESFERRIOXAMINE ON THE DEVELOPMENT OF HEXACHLOROBENZENE-INDUCED PORPHYRIA*

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Abstract—The present work deals with the effect of desferrioxamine (DF) on hexachlorobenzene (HCB)-induced porphyria in female rats with the purpose of further investigation of the role of iron in the development of this porphyria. The results obtained show that the repeated injection of DF (three times a week: 100 mg/kg each i.m.) delayed and diminished remarkably the urinary excretion of precursors and porphyrins as well as the accumulation of the latter in liver promoted by HCB (1 g/kg daily given by stomach tube). This was probably due to attenuation by DF of the alterations produced by the fungicide in the two key enzymes: porphyrinogen carboxy-lyase (PCL) and δ-aminolaevulinate synthase (ALA-S). In fact, DF by reducing liver iron levels produced a smaller decrease of the target enzyme (PCL) and a concomitant smaller induction of ALA-S. DF alone did not modify any of these variables or the liver to body weight ratio. DF added at 10^{-2} and 10^{-3} M to the incubation media of ALA-S and PCL did not alter either of the enzymatic activities, whether in normal or HCB-porphyric preparations. The results obtained show that DF improved the biochemical picture during HCB porphyria. They suggest that iron plays an indirect role in the decrease of PCL enzyme, possibly at the HCB metabolization step. A common iron-involving mechanism for the production of porphyria by different chlorinated compounds is suggested.

Hepatic siderosis is a feature commonly accepted in human cutanea tarda porphyria [1]. On the other hand, it has been observed that phlebotomy leads to a remission of the disease [2] and that iron overload, in contrast, exacerbates the porphyric picture [3] or causes relapse in a phlebotomy-induced remission [4]. It has been also reported that chelation therapy with EDTA improves the clinical and biochemical picture of cutanea tarda porphyria caused by hexachlorobenzene (HCB‡) [5].

HCB promotes an experimental porphyria that resembles hepatic human cutanea tarda porphyria, so it is used as a model for its study [6, 7].

HCB markedly decreases porphyrinogen carboxy-lyase (PCL), during heme metabolism, with a concomitant increase of δ-aminolaevulinate synthase (ALA-S), the rate-limiting enzyme; this leads to a massive accumulation and excretion of higher carboxylated porphyrins [6–10].

Experimental porphyria induced by chlorinated hydrocarbons also seems to be associated with hepatic siderosis. It has been reported that hepatic iron content is increased by treatment of rats with HCB [11–13], polychlorinated biphenyls [14], or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [15].

In addition, it has been observed that iron overload accelerates and exacerbates the porphyria induced by HCB [8, 16], and that the PCL decrease, elicited by this drug treatment, is greater when the animals have been made previously siderotic [17]. Moreover, Smith *et al.* [18] reported that a strain of rats, markedly susceptible to the porphyrinogenic effects of HCB, has a hepatic non-haem iron content higher than that of a less susceptible strain.

On the other hand, it has been reported that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced porphyria does not develop in iron-deficient mice [19] and that the administration of desferrioxamine (DF) prevents hepatic porphyrin accumulation promoted by 2-allyl-2-isopropylacetamide (AIA) or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) treatment [20]. Although DF potentiates the induction of ALA-S promoted by AIA in chick embryo liver cells in culture [21, 22], it does not exert a synergistic effect on the induction of this enzyme by AIA or DDC in rat liver [20].

DF has high affinity for ferric ions, and it is used in clinical therapy as a specific iron chelator [23, 24]. As shown in humans, treatment with this drug increases serum ferritin and the concentration of iron in the liver [25], leading to an increase in urinary iron [26]. Marked biliary excretion of iron has been reported in rats treated with this chelator [27]. Administration of only this dose of this chelator to these animals reduces the hepatic ferritin iron content, the reduction being greater when a low iron diet is given in addition [28]. A marked decrease of liver ferritin iron was also achieved by repeated DF administration to rats [29].

* Dedicated to Dr Luis F. Leloir on the occasion of his 80th birthday—6 September 1986.

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‡ Abbreviations: HCB, hexachlorobenzene; DF, desferrioxamine; ALA, δ-aminolaevulinate; PBG, porphobilinogen; ALA-S, ALA-synthase; and PCL, porphyrinogen carboxy-lyase.

Several effects of DF on hemeprotein metabolism have been reported. The chelator inhibits ferrochelatase activity [20–22], stimulates microsomal heme oxygenase [20], and decreases cytochrome P-450 [20, 28]. It promotes, in some cases, accumulation of porphyrins [21, 22], but it does not induce ALA-S in either avian tissue culture [22] or rat liver [20, 28].

The purpose of the present work was to investigate the ability of DF, as a specific iron chelator, to prevent or diminish HCB-induced porphyria.

MATERIALS AND METHODS

Chemicals. HCB (commercial grade), with the following composition: HCB 95%, tetra- and pentachlorobenzene 5%, was a gift from Compañía Química S.A., Buenos Aires, Argentina. DF was a gift from CIBA-GEIGY Argentina S.A., División Farmacéutica, courtesy of Dr. Fortmüller. Uroporphyrin III was isolated from turacin [6]. Porphyrinogens were prepared with sodium amalgam according to Mauzerall and Granick [30]. Ion-exchange resins Dowex 1-X_8 and Dowex 50 W-X_8 (200–400 mesh) were A. G. grade from the Baker J. T. Chemical Co., Phillipsburg, NJ, U.S.A. Sephadex G-25 was purchased from Pharmacia, Uppsala, Sweden.

Animals. Female CHBBTHOM rats, weighing 160–180 g at the start of the experiment, were housed individually in metabolic cages for collection of 24-hr urine and were fed Purina 3 diet (Cabeca S.C.A., Argentina) and water *ad lib.* Animals were divided into four groups of twelve animals each: (1) normal, untreated control; (2) treated with DF three times a week by intramuscular injection (100 mg/kg body weight each); (3) treated with HCB (1 g/kg body weight) administered daily by stomach tube (the drug, 40 mg/ml, was suspended in water containing Tween 20, 0.5 ml/100 ml); and (4) treated with DF + HCB as in (2) and (3). DF was given at the start of HCB treatment and throughout the experiment. Animals were killed after 12–14 weeks of treatment. Livers were weighed and divided into two portions: (1) Homogenates for estimation of porphyrin content and PCL enzyme activity were made with 0.154 M KCl (1 g of liver was brought up to 5 ml with this buffer). They were centrifuged at 11,000 *g* for 20 min. Homogenate supernatant fraction from porphyric livers was filtered through a Sephadex G-25 column with 0.134 M potassium phosphate buffer, pH 7.0. The eluates with no, or trace, fluorescence were pooled and used as the enzyme preparation. (2) Livers were homogenized in a 0.9% (w/v) NaCl, 0.5 mM EDTA, 10 mM buffer Tris–HCl (pH 7.4) mixture (1 g of liver was brought up to 3 ml with this mixture) for determination of ALA-S activity. All procedures were carried out at 4°. Hepatic porphyrin content and enzyme activities were determined simultaneously in each animal on the same day, in duplicate.

Hepatic and urinary contents of porphyrins and precursors. Porphyrin content in liver was determined in 0.1 to 1-ml portions of whole homogenates as total free porphyrins in 5% (w/v) HCl as described by San Martín de Viale *et al.* [7].

Analyses of δ-aminolaevulinate (ALA), porphobilinogen (PBG) and porphyrins were done weekly on 24-hr urine specimens. They were determined in aliquots of 0.3 to 1 ml of urine. Two separate ion-exchange columns were used according to the method described by Wainstok de Calmanovici *et al.* [10].

Enzyme activities. ALA-S was assayed in whole homogenates by the method of Marver *et al.* [31]. PCL activities in 11,000 *g* supernatant fraction from normal and DF livers or in eluates of Sephadex G-25 from porphyric livers were determined according to Wainstok de Calmanovici *et al.* [10], but the porphyrins formed were separated and quantified by high performance liquid chromatography. The system employed was a Waters Associates liquid chromatograph with a model 440 Absorbance detector used at 404 nm and a model 6000 A solvent delivery system with a UGK injector. Separations were performed using a μPorasil column (30 cm × 4 cm) and a solvent system of *n*-heptane–methyl acetate (6 : 4), at a flow rate of 1.5 ml/min and 700 psi as described Gray *et al.* [32]. Porphyrins as methyl esters were quantified using calibration curves prepared for uroporphyrin, hepta-carboxyporphyrin, hexa-carboxyporphyrin, penta-carboxyporphyrin and coproporphyrin, all as methyl esters. A linear relationship between the area under the peak and the amount of porphyrins expressed as nmoles was found in the range of 0.025 to 0.1 nmoles of porphyrins. Proportionality coefficients (area/nmole) were: 24 and 12.2 (uroporphyrin methyl ester), 19 and 9.9 (hepta-carboxyporphyrin methyl ester), 17 and 8.7 (hexa-carboxyporphyrin methyl ester), 26 and 12.2 (penta-carboxyporphyrin methyl ester), and 23 and 10.5 (coproporphyrin methyl ester), using 0.05 and 0.1 absorbance units full scale respectively. The first stage of PCL activity, i.e. uroporphyrinogen decarboxylation is expressed as hepta- + hexa- + penta- + tetra-carboxyporphyrins formed per 30 min per mg of protein. The second stage of PCL activity, i.e. coproporphyrinogen formation, is expressed as coproporphyrin formed per 30 min per mg of protein [33].

Protein determination. Proteins were determined by the method of Lowry *et al.* [34] with bovine serum albumin as standard.

Statistical treatment of results. The non-parametric test of Kruskal Wallis [35] for differences of location in ranked data grouped by a single classification was used, since there was not homoscedasticity, and a normal distribution could not be assured.

RESULTS

Urinary excretion of precursors and porphyrins. The contents of ALA, PBG and porphyrins were determined weekly in 24-hr urine specimens from four lots of animals (normal, DF, HCB, and HCB + DF) in order to detect the relative times of onset of the porphyria. The results obtained are shown in Fig. 1. As can be seen, DF did not modify the normal excretion of ALA (23 μg/24 hr), PBG (8.6 μg/24 hr) and total porphyrins (2.2 μg/24 hr). These normal excretions ranged between (13.1–32.0), (2.3–9.0) and (1.0–2.5) μg/24 hr respectively.

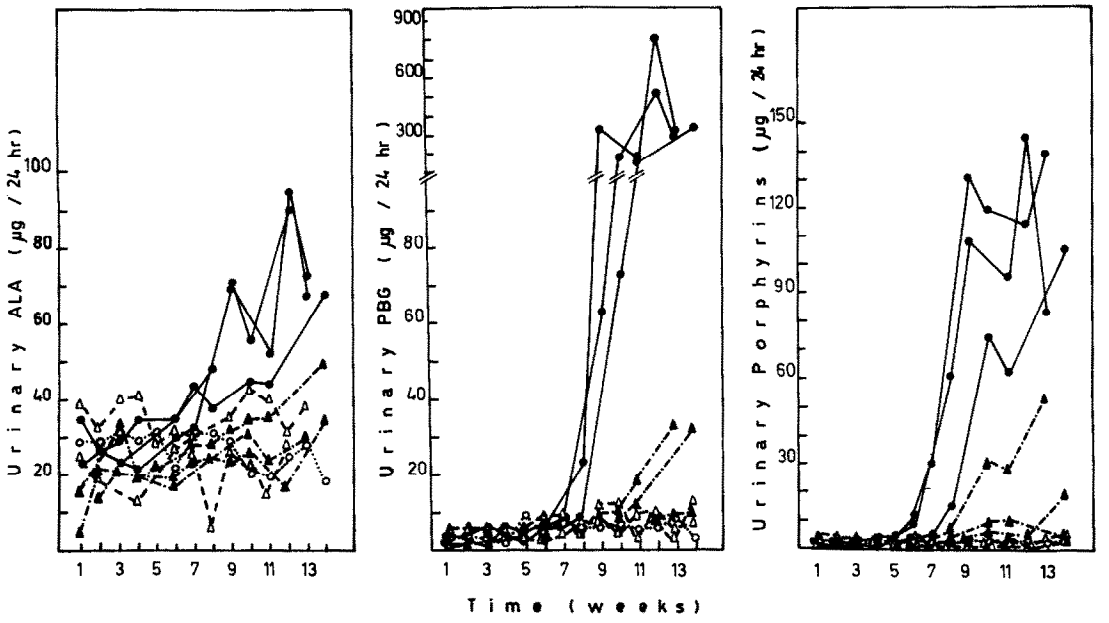

Fig. 1. Time courses of urinary excretion of ALA, PBG, and total porphyrins. Female rats were treated daily with HCB (1 g/kg by gastric intubation) for 14 weeks and were injected simultaneously three times a week with DF (100 mg/kg). ALA, PBG and porphyrins were estimated as described in Materials and Methods. Each point represents urinary excretion of individual and representative rats of each group for animals treated with HCB (●), HCB + DF (▲), DF (△) and the mean value from five rats for normals (○).

In rats treated with HCB alone, the levels of urinary ALA and PBG increased with respect to the normals at week 8, reaching values of 90 μg/24 hr and 800 μg/24 hr, respectively, at week 12. Porphyrin levels in the urine were augmented 2 weeks earlier, attaining values of 140 μg/24 hr at week 12. Animals receiving the iron chelator together with the porphyrinogenic drug showed normal or slightly increased excretions of ALA and PBG by 14 weeks of treatment (Fig. 1). In this lot of animals, the porphyrins started to rise at week 10, and only in one case did they reach a value of 50 μg/24 hr, whereas levels above 10 or 20 μg/24 hr were not reached in the remaining cases, i.e. the excretion of porphyrins in the HCB + DF group started to increase 4 weeks later and kept the values markedly lower than those with HCB alone.

Liver to body weight ratio and hepatic porphyrin accumulation. The liver to body weight ratios were the same in the normal and DF-treated groups, the values being 0.040 and 0.043 respectively. This ratio increased to about 0.054 in the animals either receiving HCB alone or in combination with the chelator.

With respect to the accumulation of porphyrins in liver (Fig. 2) significant differences were not found between normal and DF-treated groups. The administration of fungicide produced a noticeable increase (56-fold) in the porphyrin content of liver. This high accumulation promoted by the porphyrinogenic drug was decreased significantly ($P < 0.01$) to levels approaching the normal values when DF was given in addition to HCB.

Enzymatic activity of ALA-S. As can be seen in

Table 1. Addition of DF to the incubation medium of ALA-S and PCL

Enzyme preparation	Additive	Final conc (mM)	ALA-S (nmoles ALA/g liver/hr)	PCL: First stage porphyrins of 7- + 6- + 5- + 4-COOH (Specific activity, nmoles porphyrins/mg protein/30 min)	PCL: Second stage (porphyrin of 4-COOH) (Specific activity, nmoles porphyrins/mg protein/30 min)
Normal	—		11.8	0.626	0.084
	DF	1	9.1	0.634	0.078
		10	11.3	0.621	0.077
Porphyric	—		46.2	0.251	0.002
	DF	1	48.4	0.256	0.002
		10	42.6	0.262	0.002

Assay mixtures contained the components detailed in Figs. 3 and 4. DF was added in aqueous solution in the above indicated concentrations. Each value represents the average of two experiments.

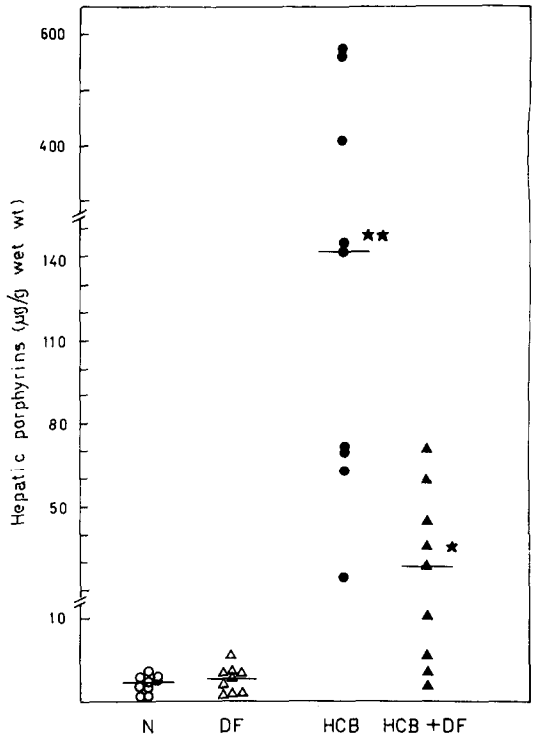

Fig. 2. Effect of DF treatment on hepatic porphyrin accumulation caused by HCB. Treatment conditions were as described in the legend of Fig. 1. Rats were killed at 12–14 weeks of treatment, the livers were removed, and the porphyrins were isolated. Total free porphyrin contents were measured in 5% (w/v) HCl as indicated in Materials and Methods. Each point represents the value corresponding to an individual animal of each group for animals treated with HCB (●), HCB + DF (▲), DF (△) and for the normals (○). For each group the median is represented by (—). Key: * significantly different from HCB group ($P < 0.01$); and ** significantly different from normal group ($P < 0.005$).

Fig. 3, the iron chelator reduced nearly to normal the activity of the rate-limiting enzyme ALA-S, increased 3-fold by the action of the fungicide. This decrease elicited by DF in the combined treatment was statistically significant ($P < 0.005$). It was also observed that DF alone did not alter significantly the activity of this enzyme.

Enzyme activity of PCL. Like the other variables studied, PCL in both stages of decarboxylation was not affected significantly by treatment with DF alone (Fig. 4). On the contrary, the first stage as well as the second stage of this enzyme were strongly decreased by the action of HCB, the decrease being 55 and 98% respectively. In the presence of iron chelator, this striking diminution of PCL promoted by the halogenated compound was partially reversed. The decrease observed in the DF + HCB group was 22 and 60% for the first and second stages, respectively, this decrease being significantly lower ($P < 0.025$) than those observed when the porphyrinogenic drug was given alone.

Effect in vitro *of DF on the enzyme activities.* Table 1 shows the results obtained when DF was added to the incubation medium of ALA-S and PCL at concentrations of 10^{-2} and 10^{-3} M. As can be seen, the chelator did not modify either of the enzyme activities studied in normal or in porphyric preparations.

DISCUSSION

Kinetics studies with HCB have demonstrated that PCL is the target enzyme in the action of HCB on the haem pathway [10]. Its strong decrease may be due to the presence of a thermostable, or partially thermostable, inhibitor present in HCB porphyric preparations [36]. Such diminution seems not to be ascribable to a decreased synthesis of PCL [37].

The possibility arises that this inhibitor of PCL was the iron augmented in porphyric liver [11–13]. Several observations, however, suggest that iron is not a direct inhibitor of the enzyme but rather exerts its action on PCL in an indirect way. In fact: (a) the concentrations at which iron inhibits PCL are higher, than those corresponding to a porphyric liver, in a PCL incubation medium [13], (b) the very low PCL activity of an HCB porphyric liver preparation is not

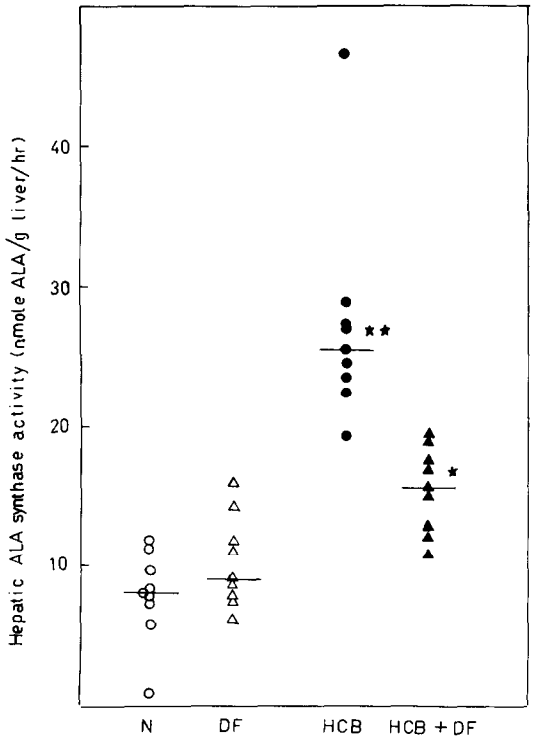

Fig. 3. Effect of the chelator on the increased activity of ALA-S caused by HCB. Treatment conditions are described in Fig. 1. Rats were killed at 12–14 weeks of treatment, livers were immediately removed and homogenized, and ALA-S activity was measured as described in Materials and Methods. Each point represents the value corresponding to an individual animal of each group for animals treated with HCB (●), HCB + DF (▲), DF (△) and for the normals (○). For each group the median is represented by (—). Key: * significantly different from HCB group ($P < 0.005$), and ** significantly different from normal group ($P < 0.005$).

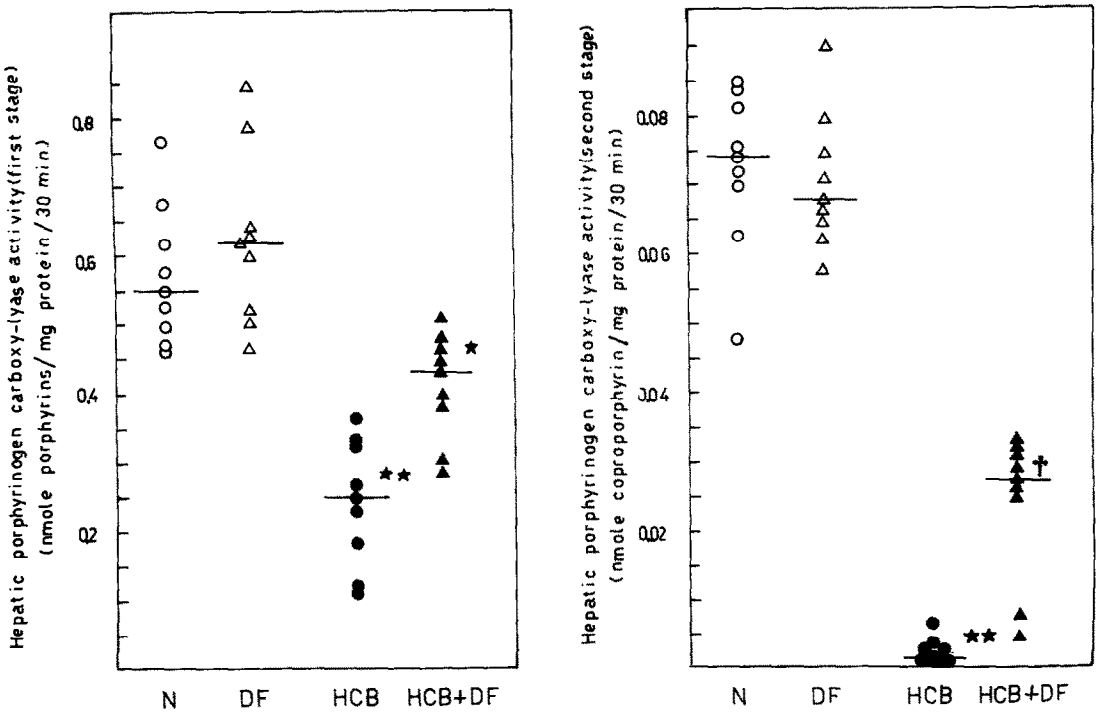

Fig. 4. Effect of DF on the decrease promoted by HCB on PCL. Treatment conditions were as described in the legend of Fig. 1. Rats were killed at 12–14 weeks of treatment, livers were immediately removed and homogenized, and PCL activity was measured as described in Materials and Methods. Each point represents the value corresponding to an individual animal of each group for animals treated with HCB (●), HCB + DF (▲), DF (△) and for the normals (○). For each group the median is represented by (—). Key: (*) significantly different from HCB group ($P < 0.025$), (†) significantly different from HCB group ($P < 0.005$), and (**) significantly different from normal group ($P < 0.005$).

increased by the addition of chelating agents ([13] and Table 1), (c) iron seems not to act as a repressor of PCL [16, 17, 38], and (d) the decrease of PCL produced by HCB treatment can be modified by the hepatic iron levels [16, 17].

Although several principal metabolites of HCB have been shown not to induce porphyria [39], indirect evidence from phenobarbitone pretreatment studies suggests that the porphyrinogenic action of HCB may be mediated through an epoxide, radical, or other short-half-life reactive metabolite [10]. Iron might be required at some HCB metabolism step to improve the formation of such metabolites [21], which in turn would promote the striking diminution of PCL. Agus rats, having a hepatic iron content higher than the Wistar strain and being more susceptible to the porphyrinogenic action of HCB [18], do have a higher liver concentration of its metabolites [40]. On the other hand, it was reported that although iron overload increased vulnerability to porphyria in certain mice strains this was not correlated with inducibility of aryl hydrocarbon hydroxylase [16].

The smaller induction of ALA-S by HCB in the presence of DF is in agreement with Liem *et al.* [20] who reported that DF does not have a synergistic effect on induction of ALA-S by AIA and that this chelator decreases the induction of this enzyme promoted by DDC in rat liver. This lack of synergism by DF on the induction of ALA-S by these porphyrinogenic drugs differs from that reported by Sinclair and Granick [22] in chick embryo liver cells in culture where DF increases the induction of ALA-S by AIA. The different effects produced by DF can be ascribed to the different experimental conditions. The present observations, that DF alone did not alter ALA-S or the other variables studied and that it does not have a synergistic effect on the induction of ALA-S by HCB, would indicate that the blockade of ferrochelatase produced by DF may not have affected the haem regulatory pool as to induce ALA-S. Nevertheless, an additional and direct role of iron in the induction of ALA-S cannot be discarded and would be supported by reports on the *in vivo* effects of iron administration [41, 42] and iron deficiency [28] on the activity of this enzyme. On the other hand, a direct effect of the metal on the activity of this enzyme seems not to contribute to the enhanced ALA-S activity in the HCB porphyric liver since the presence of chelator was not able to decrease it *in vitro* (Table 1).

The results of the present work show that the repeated administration of DF delays and diminishes remarkably the excretion of precursors and porphyrins as well as the accumulation of these in liver. This is a consequence of the attenuating effect that DF exerts on the alterations produced by HCB in the two key enzymes: PCL and ALA-S. Taking into account the above considerations, DF by reducing liver iron levels would lead to lower HCB metabolism, thus producing a smaller decrease of the target

enzyme and, consequently, a concomitant smaller induction of ALA-S. This latter enzyme induction can also contribute, in a direct way, to the lower iron content elicited by the chelating agent. All this would lead to a smaller accumulation and excretion of precursors and highly carboxylated porphyrins.

The results of the present work are consistent with those of Blekkenhorst *et al.* [43] and show that the reduction of iron levels produced by DF decrease more markedly the porphyrinogenic effect of HCB than did the bleeding.

The smaller inhibition of PCL observed in the present work as a result of DF is in line with the results of Sweeney *et al.* [19] who observed that bleeding and a subsequent iron-deficient diet prevent the decrease of PCL produced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and with the observation of Sinclair and Granick [21] that, in the presence of DF, lindane and polychlorinated biphenyls produce accumulation of protoporphyrin instead of uroporphyrin. Recently, Sinclair *et al.* [44] reported that chelator reverses the PCL decrease promoted by halogenated biphenyls by inhibiting synthesis of cytochrome P-448.

This would suggest that, perhaps, these chlorinated compounds produce porphyria by a common mechanism, since they share the ability to decrease PCL *in vivo* and to induce a porphyria in which iron plays a synergistic role.

In this respect two possibilities can be considered: (1) the PCL inhibitor is the same for all of them, i.e. OH· or CL· since iron is known to be a catalyst of free radical formation [45, 46]; and (2) the PCL inhibitor is unique and characteristic for each chlorinated hydrocarbon but shares the common feature of being a metabolic product of the drug, and this process is enhanced by the iron. Other studies may be undertaken to check these hypotheses.

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