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LIVER PORPHYRINOGEN CARBOXYLYASE IN HEXACHLOROBENZENE PORPHYRIC RATS. STUDIES WITH INTERMEDIATE PORPHYRINOGENS OF SERIES III AND WITH UROPORPHYRINOGEN I

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Abstract—1. The present work studies the action of hexachlorobenzene (HCB) on the decarboxylation of uroporphyrinogen (Urogen) I and III and also on the decarboxylation of intermediate porphyrinogens of series III under different conditions using liver of normal and porphyric rats as enzyme source.

2. The same enzyme is involved in the Urogen decarboxylation of both isomeric series I and III and catalyses the four steps in both cases. HCB affects all of them.

3. HCB blocks the four steps of Urogen III decarboxylation to the same degree, as a function of intoxication time.

4. HCB leads, in general, to an increase in the efficiency (K_m/V_{max}) of the porphyric system. These data can be interpreted as a reaction of the organism to overcome the enzymatic blockade.

INTRODUCTION

Uroporphyrinogen carboxylyase (EC 4.1.1.37) (PCL) catalyses the sequential removal of the four carboxyl groups of uroporphyrinogen (Urogen) to yield coproporphyrinogen (Coprogen) (Mauzerall and Granick, 1958). This decarboxylation occurs randomly with Urogen I and in a clockwise manner with the isomer III (Jackson *et al.*, 1976, 1977).

Studies of PCL kinetic properties, using Urogen III as substrate, have suggested that the decarboxylations might occur in two stages, the first, decarboxylation, being faster than the second one (Garcia *et al.*, 1973). It has been proposed that there are different sites for the entire decarboxylation reaction (Tomio *et al.*, 1970; de Verneuil *et al.*, 1980). The relative rates of decarboxylation of I and III isomers of Urogen have been investigated (Mauzerall and Granick, 1958; Aragonés *et al.*, 1972; de Verneuil *et al.*, 1980; Romeo and Levin, 1971; Kushner *et al.*, 1976 and others), but there is not yet agreement among the results obtained by these different groups.

It has been consistently demonstrated that hexachorobenzene (HCB) promotes a decrease in the PCL activity, this decrease being the primary cause of the porphyrinogenic action of this drug and other polyhalogenated aromatic hydrocarbons (Wainstok de Calmanovici *et al.*, 1984).

The present work explores the mode of action of HCB on the PCL and at the same time tries to shed light on the mechanism of the enzyme reaction. With this in mind, the effect of HCB on the enzymic decarboxylation of 8-, 7-, 6- and 5-COOH porphyrinogen of series III, as well as Urogen I was investigated using rat liver of both normal and porphyric animals.

Taking into account: (1) that the decrease of hepatic PCL is characteristic of human porphyria cutanea tarda (Kushner *et al.*, 1976; Elder *et al.*, 1978) and (2) that HCB porphyria is a good model studying this disease; the knowledge of how HCB acts on this enzyme would be useful to clarify the mechanism of the onset and exacerbation of this frequent type of human porphyria.

MATERIALS AND METHODS

Chemicals

HCB (commercial grade; composition: HCB, 95%; tetra- and penta-chlorobenzene, 5% (w/v) was generously given by Compañía Química S.A. Buenos Aires, Argentina. Uroporphyrin III was isolated from turacin, 7-COOH, 6-COOH and 5-COOH porphyrins III were extracted from the urine of HCB porphyric rats and uroporphyrin I was isolated from the urine of a human Günther erythropoietic porphyria (San Martín de Viale *et al.*, 1970). The corresponding porphyrinogens were obtained according to Mauzerall and Granick (1958).

Animals

Female Wistar rats (200 g) were housed individually in metabolic cages for collection of urine and fed Purina 3 diet (Cabeca S.C.A. Buenos Aires, Argentina) and water *ad libitum*. Porphyria was induced by daily administration of HCB. The drug was suspended in water (40 mg/ml) with the aid of Tween 20 (0.5 ml/100 ml of suspension) and adminis-

This work is dedicated to Dr Leloir on the occasion of his 80th birthday—6 September 1986.

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Abbreviations used: HCB—hexachlorobenzene; PCL—porphyrinogen carboxylyase; urogen—uroporphyrinogen; Coprogen—coproporphyrinogen.

tered by stomach tube (1 g/kg body wt). The state and course of the porphyria was followed investigating spectroscopically the presence of absorption bands in the visible part of the spectrum in the daily urine specimens. Once the animals acquired a severe porphyria (absorption bands in the green and red part in the spectrum) the chemical was given once every 2 or 3 days thus keeping the animals in a porphyric state with very few resulting deaths. The animals were used in this state of severe porphyria except for the intoxication time course experiments.

Enzyme preparation

Animals were killed by decapitation. After bleeding livers were removed, immediately cooled on ice, washed with 0.9% (w/v) NaCl and weighed. They were homogenized in 5 vol. (v/w) of 0.154 M KCl. Homogenates were centrifuged at 11,000 ***g*** at 0–2°C for 20 min and the supernatant thus obtained was used as enzyme preparation in the case of normal rats. Homogenate supernatants from porphyric rats were then filtered through Sephadex G-25 column to remove the endogenous porphyrins as detailed Ríos de Molina *et al.* (1980) and the eluates used as enzyme source.

Determination of PCL activity under standard conditions

Incubations were carried out in a mixture containing in a final volume of 3 ml, 0.067 M potassium phosphate buffer, pH 7.0, 1 mM reduced glutathione, 0.1 mM EDTA, 2.5–2.9 μM Urogen III and postmitochondrial supernatant from normal rats or Sephadex-G-25 eluates of HCB-treated rats as enzyme source. Incubations were carried out under vaccum in Thunberg tubes at 37°C for 30 min in the dark with mechanical shaking. Enzyme activity was determined by estimating the relative amount of each porphyrin with different carboxyl number, present at the end of the incubation, by means of chromatographic and spectrophotometric techniques (Tomio *et al.*, 1980). Results are expressed as substrate disappearance (the sum of formed products) or as end-product formation (Coprogen).

Protein determination

Proteins were determined by the method of Lowry *et al.* (1951).

RESULTS

Effect of HCB on PCL, using the standard system to measure activity

The data shown in Table 1 were obtained through using the standard incubation conditions for measuring activity; i.e. a concentration of 2.8 μM of Urogen III as substrate was employed.

It is observed that the amount of each intermediate decreases as the reaction proceeds. Thus, while there is a 69 and 23% conversion of Urogen into 7–COOH porphyrinogen when normal or porphyric preparations are used respectively, only 38% (normal) and as little as 4% (porphyric) reached the level of the 5–COOH porphyrinogen intermediate.

When we analyze the percentages of the different porphyrinogens at the end of the incubation it is evident that (i) with either normal or porphyric liver there is accumulation of the first decarboxylation product, the 7–COOH porphyrinogen; in relation to the amounts of the 6-COOH and 5-COOH intermediates, such accumulation is even greater with the enzyme coming from the porphyric rats; (ii) in any case, the percentages of 6– and 5–COOH porphyrinogens are low, and it was consistently found that these values were the same for both intermediates when using the normal PCL, while the ratio of 6– to

Table 1. Porphyrinogen carboxylyase activities in liver of normal and HCB-porphyric rats under the standard conditions

Enzyme	Porphyrinogens (%)					Enzyme activities*					
						Urogen decarboxylation		7–COOH porphyrinogen decarboxylation		6–COOH porphyrinogen decarboxylation		Coprogen formation
	Uro	7–COOH	6–COOH	5–COOH	Copro	U/mg protein	U	U/mg protein	U	U/mg protein	U	U/mg protein
Normal	31	27	4	4	34	0.49	4.60	0.38	3.12	0.26	2.88	0.24
Porphyric	77	17	2	1	3	0.12 (76)	0.48	0.03 (90)	0.32	0.02 (92)	0.24	0.015 (94)

Porphyrinogen carboxylyase activities were measured as described in the Experimental section. The postmitochondrial supernatant of normal rats or Sephadex G-25 eluates of HCB-treated rats were used as enzyme source. The amount of protein incubated ranged from 12 to 16 mg. Relative specific activities are expressed as percentage decrease compared with normal rats, in parentheses. Each value represents the average of two experiments in which two livers for each group were assayed separately.

*Enzyme activities are expressed as nmol of porphyrins/30 min (U) or as U/mg protein, as indicated

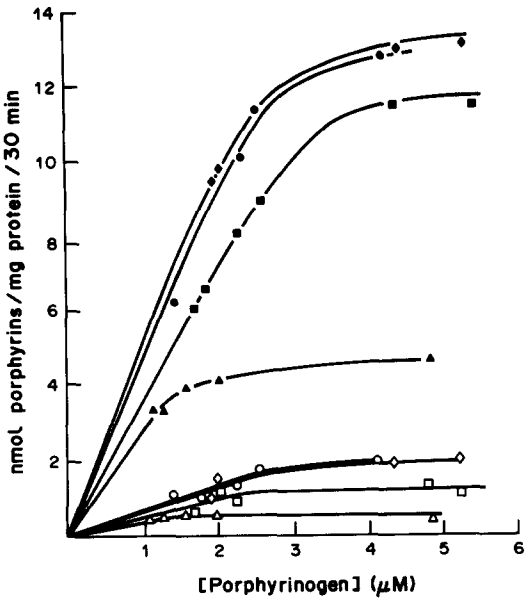

Fig. 1. Effect of substrate concentration on the decarboxylation of Urogen (■, □), 7–COOH porphyrinogen (▲, △), 6–COOH porphyrinogen (◆, ◇) and 5–COOH porphyrinogen (●, ○) by normal (solid symbols) and porphyric rat (open symbols) liver enzyme. Enzyme activities were measured as described in the Experimental section and were expressed as the sum of Coprogen and intermediate porphyrinogens formed from the substrates/ml of protein per 30 min. Each point is the average of assays performed by duplicate. Experiments were conducted 3–5 times to confirm results.

5–COOH was always 2 in porphyric samples; (iii) as far as the final product of the decarboxylation reaction, Coprogen, the amount formed by the HCB preparation is very low and, correspondingly, the percentage of remaining substrate is much greater; (iv) HCB seems to affect all four steps involved in the Urogen to Coprogen decarboxylation.

Saturation curves of PCL by its initial and intermediate substrates

To elucidate if the effect of HCB was more pronounced in the last steps of decarboxylation process than in the first ones or if this was an apparent phenomenon due to a lower enzyme saturation with the corresponding substrate, experiments were carried out using the different substrates at varying saturating concentrations. Thus, the activity of PCL as a function of the concentration of substrate of isomeric III series with different number of carboxyl groups was analyzed. The results obtained were plotted in Fig. 1. As can be seen, the saturation of PCL from normal liver by 5–COOH and 6–COOH was reached at a concentration between 4–5 μM, by Urogen between 3.5–4 μM, while with 7–COOH porphyrinogen the corresponding plateau was reached at 1.5–2 μM. The data from porphyric animals are less accurate due to the low activity of the enzyme and the relatively great fluctuation of results when higher porphyrinogen concentrations were used. Nevertheless, the plots seem to indicate that, as found for normal preparations, the concentration of 7–COOH porphyrinogens necessary to saturate the enzyme, is much lower than that corresponding to the other porphyrinogens when used as substrates. In addition, it can be observed that the relative order of the curves corresponding to the different substrates was the same for normal and porphyric systems. Consequently the following ratio for the rate of decarboxylation: 7-COOH < Uro < 5-COOH ≃ 6-COOH porphyrinogen can be established for both the normal and HCB liver enzyme.

From the (S) vs $(S)/v$ plots the values of K_m and V_{max} for the different substrates were calculated and the results obtained are shown in Table 2. It can be seen that K_m values for porphyric preparations were always lower than those for normal livers. This difference was more noticeable at the 5–COOH porphyrinogen level.

The porphyric V_{max} values were also lower than normal ranging between 8 and 13% of the latter.

Linear correlation coefficients were close to 1 with normal preparations while they shifted from nearly 1 to 0.8 in the intoxicated livers.

Evaluation of the effect of HCB on the different decarboxylation steps, under saturating conditions for each of its substrates

Table 3 shows the values for the activity of PCL when the different porphyrinogens of series III were used at saturating conditions. The percentages of inhibition of activity in porphyric animals with respect to normals in each decarboxylating step from Urogen to Coprogen are also indicated. It was found that in the case of the enzyme from intoxicated animals for all the porphyrinogens tested, the rate of removal of the first carboxyl, decreases from 82 to 85%; while that of Coprogen formation was always reduced over 90% at the same time it was lower as the intermediate steps involved increase. Rate of Coprogen formation from: Urogen ≤ 7–COOH < 6–COOH < 5–COOH porphyrinogen.

As for the percentage of intermediates at the end of incubation, looking first at the behavior of the normal liver enzyme, when Urogen III was used as

Table 2. Estimates of apparent K_m and V_{max} of porphyrinogen carboxylyase from liver of normal and porphyric rats

	K_m (μM)		V_{max} (nmol porphyrins/mg protein per 30 min)		Linear correlation coefficient		Efficiency K_m/V_{max}	
Substrate	Normal	Porphyric	Normal	Porphyric	Normal	Porphyric	Normal	Porphyric
Urogen	3.24	1.06	18.7	1.4 (7.5)	0.96	0.89	0.17	0.76
7–COOH porphyrinogen	0.72	0.29	5.3	0.6 (11)	0.99	0.91	0.13	0.48
6–COOH porphyrinogen	1.43	1.07	16.5	2.2 (13)	0.99	0.80	0.09	0.49
5–COOH porphyrinogen	2.68	0.11	20.4	1.8 (9)	0.92	0.86	0.13	0.06

Porphyrinogen carboxylyase activities were measured as described in Fig. 1.

Table 3. Porphyrinogen carboxylyase activities in liver of normal and HCB-porphyric rats under saturating conditions

Substrate (μM)	Enzyme	Porphyrinogens (%)					Enzyme activities (nmol porphyrins/mg protein per 30 min)			
		Uro	7–COOH	6–COOH	5–COOH	Copro	Urogen decarboxylation	7–COOH porphyrinogen decarboxylation	6–COOH porphyrinogen decarboxylation	Coprogen formation
Urogen (3.4)	Normal	40.5	30	4.6	4.6	20.3	0.81	0.40	0.34	0.27
	Porphyric	80.8	14	2.8	1.1	1.3	0.15 (82)	0.040 (90)	0.019 (94)	0.011 (96)
7–COOH porphyrinogen (1.8)	Normal		63	9	8	21		0.37	0.28	0.21
	Porphyric		89	5.6	3	2.4		0.058 (84)	0.026 (91)	0.011 (95)
6–COOH porphyrinogen (4.8)	Normal			51	19	30			1.31	0.80
	Porphyric			86.5	8	5.5			0.20 (85)	0.083 (90)
5–COOH porphyrinogen (4.5)	Normal				53	47				1.20
	Porphyric				87	13				0.20 (84)

Porphyrinogen carboxylyase activities were measured as described in Fig. 1. The substrates employed and their corresponding concentrations were as indicated. Percentage decreases compared with normal rats are indicated in parentheses.

Table 4. Enzymatic activities of control and HCB treated rats by using Urogen III or I as substrates at 1.7 μM

Enzyme	Isomeric series	Porphyrinogens (%)					Enzyme activities (nmol porphyrins/mg protein per 30 min)					
		Uro	7–COOH	6–COOH	5–COOH	4–COOH	Urogen decarboxylation	$\frac{U_I}{U_{III}} \times 100$	$\frac{p}{N} \times 100$	Coprogen formation	$\frac{U_I}{U_{III}} \times 100$	$\frac{P}{N} \times 100$
Normal	III	28	23	5.5	5.5	38	0.612			0.327		
	I	89	0.8	0.3	4.4	5.5	0.085	(14)		0.044	(14)	
Porphyric	III	84	11	2.3	1.2	1.5	0.085		(14.0)	0.009		(2.8)
	I	98	0.3	0.6	0.8	0.3	0.003	(3.5)	(3.5)	<0.001	(11)	(2.3)

U_I/U_{III}: ratio between the enzyme activities using Urogen I and Urogen III as substrate. P/N: ratio between the enzyme activities using the enzymes coming from porphyric and normal rats.

substrate, once again great accumulation of the 7–COOH porphyrinogen and lower and equal amount of the 6– and 5–COOH intermediates were observed. A similar pattern emerged when the substrate was the 7–COOH porphyrinogen. However, starting from the 6–COOH porphyrinogen there was only about 50% conversion to less carboxylated porphyrinogens and from these the percentage of the 5–COOH intermediate was 2.5–4 times that estimated when Urogen or 7–COOH porphyrinogen were the substrates.

On the other hand, when the data corresponding to the porphyric preparation were analyzed, it was observed that using either Urogen or 7–COOH porphyrinogen as substrates; the amount of 6– and 5–COOH intermediates was lower but not equal, as with normal liver, and as already noted, percentage of the former was about twice that of the latter.

It is interesting to point out that for both the normal and porphyric enzyme the same percentage of remaining substrate was obtained when either 5– or 6–COOH porphyrinogens were used as substrates.

Comparative effect of HCB on the decarboxylation of series I and III

When the activity of PCL from both normal and porphyric livers was comparatively studied using Urogen I and III as substrate (Table 4) data already obtained with the latter substrate were reproduced; on the other hand the former substrate produced a noticeable accumulation of 5–COOH porphyrinogen in amounts nearly equal to those of the final product, in the normal system. The enzyme from intoxicated animals formed instead, almost the same and extremely low quantities of all intermediates products from 7– to 4–COOH; however although these absolute values were hardly reproducible, due to the low activity of the enzyme, we can nevertheless assure that they were always below 1%.

When either the first step (Urogen decarboxylation) or the last (Coprogen formation) was considered to evaluate PCL activity, it was found that normal livers exhibit only 14% of activity towards Urogen I as compared to the III isomer. This relation was even lower (3.5%) with the porphyric system. The relative activity, porphyric respect to normal (P/N), was also less with the series I (3.5%) than with the series III (14%).

Kinetic of enzyme activity decrease after HCB poisoning

The activity of PCL was followed along the course of HCB intoxication, using either the standard conditions of Urogen concentration (open symbols) or substrate saturating concentrations of the enzyme (solid symbols) (Fig. 2). It was found that inhibition was always lower in the latter situation.

The percentage of inhibition for the different steps of Urogen decarboxylation measured under substrate saturation conditions of the enzyme with a preparation obtained from animals at an intermediate stage of the porphyria onset was also estimated. It was found that the percentage of inhibition for each of the four steps involved fluctuated about a 30% (data not shown).

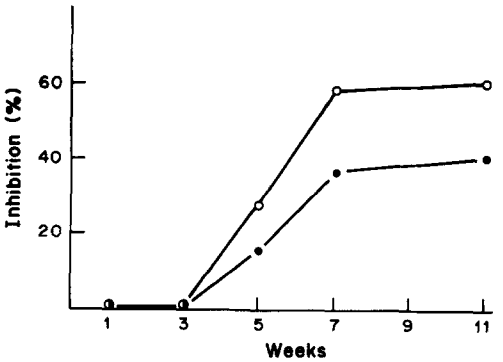

Fig. 2. Effect of using saturating (●) (7.0 μM) and not saturating (○) (2.3 μM) concentrations of Urogen III on the degree of inhibition of PCL activity, along the time of drug administration. Percentage decreases were calculated with respect to normal values and measuring the activity as Urogen disappearance. Each value represents the average of two experiments in which two livers for each group were assayed separately.

DISCUSSION

Previous studies from this laboratory have shown that the progress of the HCB intoxication is followed by a gradual decrease of PCL activity (Wainstok de Calmanovici *et al.*, 1984). When this effect was carefully studied at the level of each of the four steps converting Urogen into Coprogen (Table 1), apparently the decrease was becoming greater as the reaction proceeded from left to right; the possibility of a major action at the level of 6–COOH porphyrinogen decarboxylation was also suggested due to the accumulation of greater amounts of this intermediate in relation to the 5–COOH metabolite. This sort of differential inhibition by HCB could have been ascribed to unsaturating levels of the different substrate for the enzyme. This hypothesis was ruled out when the activity was determined employing saturating concentrations of all the intermediate porphyrinogens, under these circumstances it was clearly demonstrated that HCB would affect the four steps of Urogen III decarboxylation to the same degree.

Urogen → 7–COOH → 6–COOH
→ 5–COOH → Coproporphyrinogen

The observation that the same decrease was obtained for all steps (about 84%) would be in accordance with the hypothesis that a single protein catalyses the four steps of Urogen III decarboxylation (Cornford *et al.*, 1964; Tomio *et al.*, 1970; Smith and Francis, 1979; de Verneuil *et al*, 1978; Straka and Kushner, 1980).

In this respect, de Verneuil *et al.* (1983) got to the same conclusions with the erythrocytes enzyme from subjects with the genetic defect of porphyria cutanea tarda, thus about 50% decrease was obtained using either of the four substrates corresponding to the series III.

When the action of HCB was studied in a liver preparation coming from animals half way to complete intoxication, under saturating substrate, it was confirmed that the percentages of decrease were of the same order in all steps (about 30 ± 3%). These

findings would reinforce the fact that HCB affects in a progressive and similar way all the four steps involved in the whole decarboxylation of Urogen.

The plots of activity vs substrate concentration showed that the lower values of V_{max} and apparent K_m corresponded to the 7–COOH porphyrinogen, for both normal and porphyric livers. This was corroborated when the V_{max} and apparent K_m values were calculated after plotting (S) vs $(S)/v$.

The inhibitory effects of the intermediate substrates, up to the concentrations assayed, were not apparently too significant, since when the remaining substrates were plotted as a function of the initial substrates, a straight line was obtained (figure not shown).

The V_{max} values for the normal enzyme can be arranged as follows:

5–COOH $\geqslant$ Urogen > 6–COOH $\gg$ 7–COOH porphyrinogens

This order would suggest that, starting from the second step, decarboxylation of less carboxylated substrates occurs at higher speed. These results are in agreement with those of Straka and Kushner (1983) and Smith and Francis (1981).

In a similar fashion the K_m values can be ordered in the following way:

Urogen $\geqslant$ 5–COOH > 6–COOH > 7–COOH porphyrinogen

Indicating that with the exception of the first substrate, the existence of certain relation between the obtained values and the number of carboxyls was again verified. In addition, it can be deducted that the smaller the carboxyl number the smaller its affinity. The same relation was found by Smith and Francis (1981).

However, the data obtained for the efficiencies expressed as K_m/V_{max} here presented differs in part from those reported by these authors. In fact, in the present work it was found that the efficiency for the first two substrates (Urogen: 0.17, 7–COOH porphyrinogen: 0.14) is quite similar to the 5–COOH porphyrinogen (0.13), and it was 1.5–2-fold greater than that of the 6-COOH porphyrinogen (0.09). On the other hand, Smith and Francis (1981) had reported similar efficiencies for Urogen and 7–COOH porphyrinogen, but these values were about half of those corresponding to 6–COOH and 5–COOH porphyrinogens.

The higher efficiency found here for the first decarboxylation of Urogen would fit with the assumption that the first reaction (first step) is the one the cell needs to be performed at higher speed, because it is the point of arrival of the initial substrate the concentration of which would directly affect the enzyme activity. It is postulated that the rate of the other decarboxylating steps could be controlled by the enzyme itself. The second substrate would also affect its own decarboxylation in a way dependent on the speed of the first step, this being high. From then onwards, there would not be sharp changes in substrate concentration since this would be limited by the slowness of the second step.

When these results were compared with those obtained from porphyric animals it was evidence that all the V_{max} were reduced to about one-tenth of the normal values. Regarding the K_m values for each substrate they were also diminished but not in such a magnitude, thus leading in general to an increase in the efficiency of the porphyric system.

These data can be interpreted as a reaction of the organism to overcome the enzymatic blockade and would be partly in agreement with those of Straka and Kushner (1983) who found that at high substrate concentrations, condition quite similar to the accumulation of porphyrinogens produced by HCB in the liver of porphyric animals, the enzyme would respond by increasing the efficiency with respect to the 5–COOH and 6–COOH porphyrinogens.

In plots of v vs (S) for Urogen and 7–COOH porphyrinogen III, using a wide and more detailed range of concentrations (data not shown) what was initially considered as a shoulder, it was in fact a definite plateau. Quite similar observations were reported by Straka and Kushner (1983) using 5– and 6–COOH porphyrinogens. When the graphics presented by other groups (Smith and Francis, 1979, 1981; Cantoni *et al.*, 1984) were carefully analyzed the existence of such shoulders was also visualized. These results would be in accordance with the suggestion of one enzyme with different active centers for the substrates with different carboxyl numbers, with independent K_m and V_{max} values. This would also be in agreement with our first proposal of at least two active centers for the enzyme (Tomio *et al.*, 1970; García *et al.*, 1973) and the recent hypothesis of four different sites (de Verneuil *et al.*, 1980). However this point must be further investigated.

The analysis of the results using Urogen I as substrate showed that the relative activities respect to the assay with Urogen III coincide at least for the first and last step. These data would suggest that a single enzyme catalyzes the decarboxylation of Urogen I and its intermediates to give Coprogen I, just as it happens with the series III and, in addition, that the same enzyme would be involved in the decarboxylation of both isomeric series I and III. On the other hand, we confirmed that the Urogen I decarboxylation rate is much lower than that of isomer III in agreement with other authors (Mauzerall and Granick, 1958; Cornford, 1964; Tomio *et al*, 1970; Aragonés *et al.*, 1972; Smith and Francis, 1973, 1981; de Verneuil *et al.*, 1980) but at variance with the results of Romeo and Levin (1970) and Kushner *et al.* (1975).

The decrease promoted by HCB on the enzyme using Urogen I as substrate was apparently more noticeable than in the case of series III. This would indicate that HCB greatly affects decarboxylation of series I. But this can not be assured considering the low sensibility of the method in the case of very low values of activity such as those corresponding to porphyric preparation assayed with Urogen I. That would be the reason why the same decrease was not obtained at the first and fourth step level when the activities of the enzyme with isomer I were compared with those of isomer III.

When the first step of Urogen III decarboxylation was analyzed as function of intoxication time, it was confirmed that the decrease elicited by the drug on

the enzyme was made evident more noticeably using the standard system than under substrate saturating conditions. At high substrate concentrations and low protein concentration, only the 7–COOH porphyrinogen was obtained while all the intermediate products appeared under the standard conditions, thus explaining the reason of its higher sensibility. That is why this last system would be the more appropriate to comparatively analyze the behavior of normal and porphyric preparations. In addition, Smith and Francis (1981) reported that the activity estimated at 1 μM was considered to be a better indication of real relative rates owing to substrate/product inhibition, at this concentration we did not find inhibition either.

These results also showed that a short time passes before a decrease in PCL activity could be detected and then this decrease turns more noticeably as a function of intoxication time until reaching a plateau. Smith and Francis (1981) consider that it is the production of the enzyme that is inhibited but that this does not become immediately apparent due to the slow turnover of the enzyme. Our previous experience (Ríos de Molina *et al.*, 1980; Wainstok de Calmanovici *et al.*, 1984) and the data reported by Elder and Sheppard (1982) would suggest that in fact a defective enzyme and not a lower amount of it would be synthesized. However, the explanation of the delayed appearance of the effect could be equally based on the slow turnover of the enzyme and thinking besides in the existence of an inhibitor which could bind the enzyme during the process of its synthesis (Wainstok de Calmanovici *et al.*, 1984; Billi *et al.*, 1986).

SUMMARY

(1) The present work explores the mode of action of the hexachlorobenzene (HCB) on the decarboxylation of porphyrinogens of both isomeric series I and III at the time it tries to shed light on the mechanism of porphyrinogen carboxylyase action. With this purpose porphyrinogens with different number of carboxyl groups and enzyme liver preparation from normal and HCB porphyric rats were used under different conditions.

(2) The present results would suggest that a single enzyme catalyzes the decarboxylation of uroporphyrinogen (Urogen) III and its intermediates to give coproporphyrinogen III, just as it happens with the series I. The same enzyme would be involved in the decarboxylation of both isomeric series I and III.

(3) 7–COOH→6–COOH porphyrinogen is the rate limiting step in the series III decarboxylation both in normal and porphyric conditions. It was clearly demonstrated that HCB affects the four steps of Urogen III decarboxylation to the same degree. The drug blocks in a aprogressive and similar way all the four steps involved as a function of intoxication time. Series I decarboxylations were also decreased by HCB action.

(4) The lower values of V_{max} and apparent K_m corresponded to the 7–COOH porphyrinogen III for both normal and porphyric livers.

(5) With the exception of the first substrate, for all the others it can be deducted (normal enzyme, isomer III substrates) that the smaller the carboxyl number the greater its K_m and the smaller its V_{max}.

(6) HCB leads in general to an increase in the efficiency (K_m/V_{max}) of the porphyric system. These data can be interpreted as a reaction of the organism to overcome the enzymatic blockade.

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REFERENCES

Aragones A. Garcia R. C., San Martin de Viale L. C., Tomio J. M. and Grinstein M. (1972) Estudios de la transformación de uroporfirinógenos en coproporfirinógenos por decarboxilasas de eritrocitos de pollo e hígado de rata. *An. Asoc. quím. argent.* **60,** 239–248.

Billi S. C., Koss G. and San Martin de Viale L. C. (1986) Screening for the ability of hexachlorobenzene metabolites to decrease rat liver porphyrinogen carboxylyase. *Res. Commun. Chem. Path. Pharmac.* **51,** 325–336.

Cantoni L., Dal Fiume D. and Ruggieri R. (1984) Decarboxylation of uroporphyrinogen I and III in 2,3,7,8-tetrachlorodibenzo-p-dioxin induced porphyria in mice. *Int. J. Biochem.* **16,** 561–565.

Cornford P. (1964) Transformation of porphobilinogen into porphyrins by preparations from human erythrocytes. *Biochem. J.* **91,** 64–73.

Elder G. H., Evans J. O. and Matlin S. A. (1976) The effect of the porphyrinogenic compound hexachlorobenzene on the activity of hepatic uroporphyrinogen decarboxylase in the rat. *Clin. Sci. Mol. Med.* **51,** 71–80.

Elder G. H., Path M. R. C., Lee G. B. and Tovey J. A. (1978) Decreased activity of hepatic uroporphyrinogen decarboxylase in sporadic porphyria cutanea tarda. *New Engl. J. Med.* **299,** 274–278.

Garcia R. C., San Martin de Viale L. C., Tomio J. M. and Grinstein M. (1973) Porphyrin biosynthesis—X. Porphyrinogen carboxylyase from avian erthyrocytes. Further properties. *Biochim. biophys. Acta* **309,** 203–210.

Jackson A. H., Rao K. R. N., Supphayen D. M. and Smith S. G. (1977) Intermediates between Uroporphyrinogen-1 and Coproporphyrinogen-1. *J. Chem. Soc. Chem. Commun.* **20,** 696–698.

Jackson A. H., Sancovich H. A., Ferramola A. M., Evans N., Games D. E., Matlin S. A., Elder G. H. and Smith S. G. (1976) Macrocyclic intermediates in the biosynthesis of porphyrins. *Philos. Trans. R. Soc. Lond. Ser. B* **273,** 191–206.

Kushner J. P., Barbuto A. J. and Lee G. R. (1976) An inherited enzymatic defect in porphyria cutanea tarda. Decreased uroporphyrinogen decarboxylase activity. *J. clin. Invest.* **58,** 1089–1097.

Lowry O. H., Rosenbrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

Mauzerall D. and Granick S. (1958) Porphyrin biosynthesis in erthrocytes—III. Uroporphyrinogen and its decarboxylase. *J. biol. Chem.* **232,** 1141–1162.

Rios de Molina M. del C., Wainstok de Calmanovici R. and San Martin de Viale L. C. (1980) Investigations on the presence of porphyrinogen carboxylase inhibitor in the liver of rats intoxicated with hexachlorobenzene. *Int. J. Biochem.* **12,** 1027–1032.

Rios de Molina M. del C., Wainstok de Calmanovici R., Tomio J. M. and San Martin de Viale L. C. (1975)

Porfiria inducida en ratas por hexaclorobenceno. *An. Asoc. quím. argent.* **63,** 313–332.

Romeo G. and Levin E. (1971) Uroporphyrinogen decarboxylase from mouse spleen. *Biochim. biophys. Acta* **230,** 330–341.

San Martin de Viale L. C., Rios de Molina M. del C., Wainstok de Calmanovici R. and Tomio J. M. (1975) Experimental porphyria induced in rats by hexachlorobenzene. Porphyrins in human diseases. *Ist International Porphyrin Meeting on Porphyrins in Human Diseases* (Edited by Doss M.), 225–232. Kargel, Basel.

San Martin de Viale L. C., Rios de Molina M. del C., Wainstok de Calmanovici R. and Tomio J. M. (1977) Porphyrins and porphyrinogen carboxylyase in hexachlorobenzene-induced porphyria. *Biochem. J.* **168,** 393–400.

San Martin de Viale L. C., Viale A. A., Nacht S. and Grinstein M. (1970) Experimental porphyria induced in rats by hexachlorobenzene. A study of the porphyrins excretred by urine. *Clin. chim. Acta* **28,** 13–23.

Smith A. G. and Francis J. E. (1981) Investigations of rat liver uroporphyrinogen decarboxylase. Comparisons of porphyrinogens I and III as substrates and the inhibition by porphyrins. *Biochem. J.* **195,** 241–250.

Straka J. G. and Kushner J. P. (1983) Purification and characterization of bovine hepatic uroporphyrinogen decarboxylase. *Biochemistry* **22,** 4664–4672.

Tomio J. M., Garcia R. C., San Martin de Viale L. C. and Grinstein M. (1970) Porphyrin biosynthesis. Porphyrinogen carboxylyase from avian erythrocytes. Purification and properties. *Biochim. biophys. Acta* **198,** 353–363.

Verneuil H. de, Aitken G. and Normann Y. (1978) Familial and sporadic porphyria cutanea. *Hum. Genet.* **44,** 145–151.

Verneuil H. de, Grandchamp B. and Normann Y. (1980) Some kinetic properties of human red cell uroporphyrinogen decarboxylase. *Biochim. biophys. Acta* **611,** 174–186.

Verneuil H. de, Sassa S. and Kappas A. (1983) Purification and properties of uroporphyrinogen decarboxylase from human erythrocytes. A single enzyme catalyzing the four sequential decarboxylations of uroporphyrinogens I and III. *J. biol. Chem.* **258,** 2454–2460.

Wainstok de Calmanovici R. W., Rios de Molina M. del C., Taira de Yamasato M. C., Tomio J. M. and San Martin de Viale L. C. (1984) Mechanism of hexachlorobenzene-induced porphyria in rats. Effect of phenobarbitone pretreatment. *Biochem. J.* **218,** 753–763.