Biochem. J. (1977) **168**, 393–400
Printed in Great Britain

Porphyrins and Porphyrinogen Carboxy-lyase in Hexachlorobenzene-Induced Porphyria

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(*Received* 4 *July* 1977)

1. Qualitative and quantitative studies of the porphyrins and the porphyrinogen carboxy-lyase of the liver, spleen, kidney, Harderian gland and erythrocytes from normal rats and from those with hexachlorobenzene-induced porphyria were carried out. 2. Hexachlorobenzene has no effect on erythrocyte porphyrin content, but produces a decrease in that of Harderian gland and an increase in the porphyrin content of the kidney and spleen, and a marked increase in the liver (1 μmol/g of tissue). Octacarboxylic (isomer III) and heptacarboxylic porphyrins accumulated in kidney, spleen and liver, the former porphyrin being predominant. 3. Hexachlorobenzene has no effect on the activity of porphyrinogen carboxy-lyase in erythrocytes; there is a slight decrease in enzyme activity in the Harderian gland, and a marked decrease in the liver and kidney enzyme activities. In the liver the removal of each carboxyl group from uroporphyrinogen III appears to be affected by this treatment. 4. The liver is the principal site of action of hexachlorobenzene, with the kidney next in decreasing order of effect, and erythropoietic tissue is unaffected. The marked decrease in porphyrinogen carboxy-lyase activities observed in liver and kidney could explain the high accumulation of octacarboxylic and heptacarboxylic porphyrins found in these tissues. 5. The results are discussed in relation to changes promoted by hexachlorobenzene in other enzymes of the haem pathway.

Porphyrinogen carboxy-lyase (uroporphyrinogen decarboxylase, EC 4.1.1.37) catalyses the stepwise removal of four carboxyl groups from uroporphyrinogen (eight carboxyl groups) to coproporphyrinogen (four carboxyl groups). The enzyme has been partially purified and some of its properties studied in material from bacterial (Hoare & Heath, 1959), avian (Tomio *et al.*, 1970) and mammalian sources (Mauzerall & Granick, 1958; Romeo & Levin, 1971; Aragonés *et al.*, 1972). Taking into account the relatively high accumulation of phyriaporphyrinogen (heptacarboxylic porphyrinogen) observed both *in vivo* and *in vitro* and the presence of small amounts of hexacarboxylic and pentacarboxylic porphyrinogens that were constant during the incubation, two metabolic stages were suggested in the mechanism of uroporphyrinogen decarboxylation: octacarboxylic → heptacarboxylic → tetracarboxylic porphyrinogen (Tomio *et al.*, 1970). The first stage is a fast decarboxylation reaction, as suggested by the different rates ($v_1 > v_2$) and 'activation energies' ($E_1 < E_2$) for the two stages, indicating an easier removal of the first carboxyl group from uroporphyrinogen (Tomio *et al.*, 1970; García *et al.*, 1973). The second stage is more susceptible than the first to a number of chemical and physical agents (San Martín de Viale *et al.*, 1969).

Hexachlorobenzene produces a type of porphyria in humans (Schmid, 1960; Cetingil & Ozen, 1960; Cam & Nigogosyan, 1963) and in several animal species (Ockner & Schmid, 1961; De Matteis *et al.*, 1961; San Martín de Viale *et al.*, 1970; Vos *et al.*, 1971; Strik, 1973*a*,*b*) that resembles the hepatic human cutanea tarda porphyria (Cam & Nigogosyan, 1963; San Martín de Viale *et al.*, 1970), in which the chemical feature is the increased urinary excretion of porphyrins with four to eight carboxyl groups (Nacht *et al.*, 1970).

Experimental porphyria in rats is characterized by an increased excretion of porphyrins, essentially isomer III (San Martín de Viale *et al.*, 1970), and by a massive accumulation in liver of uroporphyrin III and heptacarboxylic porphyrin (phyriaporphyrin) (San Martín de Viale *et al.*, 1970; Taljaard *et al.*, 1972).

The high accumulation and excretion of uroporphyrin observed both in hexachlorobenzene-porphyric rats and in the human cutanea tarda porphyria (Dowdle *et al.*, 1970) suggests a deficiency in the decarboxylation process of uroporphyrinogen catalysed by porphyrinogen carboxy-lyase. Tal-

jaard *et al.* (1972) found no enzyme activity in the livers of rats receiving iron before hexachlorobenzene feeding, but they could not demonstrate a decrease in the porphyrinogen carboxy-lyase in porphyric rats treated only with hexachlorobenzene.

The purposes of the present investigation were: (1) to perform a qualitative and quantitative study of the porphyrins present in liver, spleen, kidney, Harderian gland and erythrocytes of normal and hexachlorobenzene-porphyric rats to determine if the metabolic disturbances are limited to the liver or affect other organs, particularly the erythropoietic tissue; (2) to study the porphyrinogen carboxy-lyase activities in these tissues to determine if the enzyme is actually affected by hexachlorobenzene, and, if so, in which decarboxylation step the effect is located.

Experimental

Materials

Hexachlorobenzene (commercial grade) was a generous gift from Compañía Química S.A., Buenos Aires, Argentina, and had the following composition: hexachlorobenzene, 95%; tetra- and penta-chlorobenzene, 5%.

Porphyrins and porphyrinogens. Uroporphyrin III was isolated from turacin and phyriaporphyrin III from the urine of hexachlorobenzene-porphyric rats as previously described (San Martín de Viale *et al.*, 1970). Porphyrinogens were prepared with sodium amalgam as described by Mauzerall & Granick (1958).

Methods

Animals. Porphyria was induced in female Wistar rats (200g) by daily administration of hexachlorobenzene. The drug, suspended in water (40mg/ml) with the aid of Tween 20 (0.5ml/100ml of suspension), was given by stomach tube at a dose of 1g/kg body wt. Since a severe porphyria was established in the rats, the drug was given once every 2–3 days, thus keeping the animals in a porphyric state with very few resulting deaths. Animals with severe porphyria after 3–4 months of drug treatment were used in the present work. The state and course of the porphyria was followed as previously described (San Martín de Viale *et al.*, 1970).

Preparation of the tissues. Animals of both groups (normal and hexachlorobenzene-porphyric) were used in each experiment. The animals, previously anaesthetized with diethyl ether, were bled and their organs subsequently removed. The organs of two to three animals per group were pooled for each determination.

(*a*) Preparations from blood. Blood was removed from the abdominal aorta with a 12-gauge needle and collected in a tube containing heparin (0.2mg/ml). Washed erythrocytes obtained as described previously (San Martín de Viale & Grinstein, 1968) were suspended in 1vol. of water; the haemolysate was obtained by twice freezing and thawing the preparation, and the haemolysate supernatant by centrifuging the haemolysate at 35000g at 0–2°C for 60min.

(*b*) Preparations from liver, spleen, kidney and Harderian gland. The different organs removed immediately after bleeding were cooled on ice, pooled, weighed and washed with 0.9% (w/v) NaCl. The livers, spleen and kidneys were homogenized in 5vol. (v/w) of 0.154M-KCl. Frozen Harderian gland was ground with metal-free sand in a mortar (Tomio & Grinstein, 1968) and homogenized in 10vol. (v/w) of 0.134M-potassium phosphate buffer, pH7.0. Homogenates were centrifuged at 11000g at 0–2°C for 20min and the supernatant thus obtained was used as enzyme preparation. Homogenate supernatants for porphyric livers and kidneys were then filtered through a Sephadex G-25 column (2.4cm × 30cm), equilibrated and eluted with 0.134M-potassium phosphate buffer, pH7.0. The eluates with no or little fluorescence were pooled and used as enzyme preparations for these two porphyric organs.

Porphyrin content. Porphyrin content in liver, spleen, kidney and erythrocytes was determined as free porphyrins in 5% (w/v) HCl after precipitation of proteins and quantitative extraction of the porphyrins performed in two different ways: (*a*) for haemolysates, by the addition of 5vol. of ethyl acetate/acetic acid (3:1, v/v) and later transfer of porphyrins from ethyl acetate to 5% HCl as detailed elsewhere (San Martín de Viale & Grinstein, 1968); (*b*) for homogenates, by the addition of conc. HCl to give a final concn. of 5% as described previously (Tomio *et al.*, 1970). Porphyrin content in Harderian gland was determined as methyl esters in chloroform. For this the glands were ground in a mortar with methanol/H_2SO_4 (19:1, v/v); the centrifuged supernatant was separated and the protein residue was repeatedly extracted until red fluorescence could no longer be detected. The pooled extracts were kept in the dark for 14h and the esterified porphyrins were then transferred to chloroform (Falk, 1964).

Total porphyrin content in the tissues was determined spectrophotometrically by using the correction formula quoted by Rimington & Sveinsson (1950) and the extinction coefficient calculated as described previously (Tomio *et al.*, 1970).

When the porphyrin content was small, porphyrins were measured fluorimetrically with an Aminco–Bowman spectrophotofluorimeter, by using the graphical correction detailed elsewhere (Martínez & Mills, 1971) and using calibration curves for uroporphyrin, coproporphyrin and protoporphyrin in 5% HCl.

Total free porphyrins were evaporated to dryness in a desiccator under vacuum and then esterified and transferred to chloroform by the usual techniques (Falk, 1964). Porphyrins were separated by paper chromatography (Falk & Benson, 1953) and the percentage of each was spectrophotometrically measured as previously described (Tomio *et al.*, 1970).

Determination of porphyrinogen carboxy-lyase activity. Porphyrinogen carboxy-lyase activities in normal and porphyric tissues were determined as described by Tomio *et al.* (1970). Incubation mixtures contained, in a final volume of 4 ml, 0.067 M-potassium phosphate buffer, pH 7.0, 1 mM-reduced glutathione, 0.1 mM-EDTA, 2 μM-porphyrinogens and enzyme preparations as indicated in the legends of the Tables. The assays were carried anaerobically in Thunberg tubes at 37°C for 30 min in the dark with mechanical shaking. The incubations were stopped with 5 vol. of ethyl acetate/acetic acid (3:1, v/v) for the haemolysate supernatant and with conc. HCl (final concn. 5%) for the other tissue preparations and then processed as described by Tomio *et al.* (1970).

Proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

When it was not possible to separate the endogenous porphyrins from enzyme preparations in porphyric tissue, appropriate corrections were performed in the determination of porphyrinogen carboxy-lyase activities. Thus, the endogenous amount of each porphyrin was subtracted from the respective porphyrins present in the assays with uroporphyrinogen III or phyriaporphyrinogen III after incubation.

Statistical treatment of results. Results are expressed as the arithmetic means±S.E.M. and the means compared by Student's t test. To obtain the value of P, a degree of freedom n_1+n_2-2 was used throughout.

Results

Effect of hexachlorobenzene on the porphyrin content of several rat tissues

The results (Table 1) show that hexachlorobenzene promotes an increase in the porphyrin content of spleen, kidney and liver, and that the concentration in the liver increased to 1 μmol/g of tissue. The mean values for liver, kidney and spleen were respectively 3900, 300 and 120 times greater in porphyric than in normal animals. Hexachlorobenzene had no apparent effect on rat erythrocytes, since the same small amount of porphyrins was found in both porphyric and normal rats. On the other hand, hexachlorobenzene produced a decrease of about 30% in the high content of porphyrins normally present in the Harderian gland.

Table 1. *Effect of hexachlorobenzene on porphyrin accumulation in several rat tissues*

Porphyrin content in liver, spleen, kidney and erythrocytes of normal and porphyric animals was determined in 5% HCl from the homogenates or haemolysates respectively. Content in Harderian gland was determined in chloroform. Details of the isolation procedure are given in the Experimental section. The relative quantities of each porphyrin were determined by the chromatographic and spectrophotometric methods described in the Experimental section. Results are expressed as means±S.E.M. of the numbers of experiments indicated in parentheses. The tissues of two animals were pooled in each experiment. Probabilities of differences from corresponding controls are indicated thus: **, $P<0.2$; *, $P<0.001$.

		Relative quantities of porphyrins (%)					
Tissue	Homogenate or erythrocyte haemolysate (nmol of total porphyrins/g of tissue or /100 ml of erythrocytes)	Octa-carboxylic	Hepta-carboxylic	Hexa-carboxylic	Penta-carboxylic	Tetra-carboxylic	Di-carboxylic
Liver							
Normal	0.30 ±0.02 (4)	—	—	—	—	Traces	Traces
Hexachlorobenzene	1177 ±47 (8)*	72±1	28±1	—	—	—	—
Spleen							
Normal	0.094±0.011 (5)	Traces	Traces	Traces	Traces	Traces	Traces
Hexachlorobenzene	11.1 ±0.5 (5)*	74±4	26±4	—	—	—	—
Kidney							
Normal	0.36 ±0.07 (4)	Traces	Traces	—	—	Traces	Traces
Hexachlorobenzene	109 ±19 (7)*	86±2	14±2	—	—	—	—
Harderian gland							
Normal	612 ±92 (4)	5±1	3±1	6±1		6±1	80±3
Hexachlorobenzene	425 ±89 (4)**	10±1	4±1	6±1		6±2	74±2
Erythrocytes							
Normal	26 ±4 (3)	—	—	—	—	Traces	Traces
Hexachlorobenzene	29 ±4 (3)	—	—	—	—	Traces	Traces

Studies on the nature of porphyrins showed that coproporphyrin (tetracarboxylic) and protoporphyrin (dicarboxylic) were always present in small amounts in normal liver, spleen and kidney. Traces of uroporphyrin and phyriaporphyrin were also detected in normal kidney. In contrast, in all these organs hexachlorobenzene produced an accumulation of porphyrins consisting only of uroporphyrin (octacarboxylic) and phyriaporphyrin (heptacarboxylic). Coproporphyrin and protoporphyrin were not detected. On a molar basis, the ratio of uroporphyrin to phyriaporphyrin was 72:28 for liver, 74:26 for spleen and 86:14 for kidney. All the intermediates between uroporphyrin and protoporphyrin accumulated in both normal and porphyric Harderian glands. Moreover, there were no differences in the type of porphyrins present in erythrocytes as a result of hexachlorobenzene treatment, i.e. only traces of coproporphyrin and protoporphyrin were detected in normal as well as in porphyric erythrocytes.

Porphyrinogen carboxy-lyase activities: stepwise decarboxylation of uroporphyrinogen III and phyriaporphyrinogen III

The large amounts of porphyrins accumulated in liver, spleen and kidney of hexachlorobenzene-porphyric rats were present in the homogenates not as porphyrinogens but as porphyrins, since the percentages of uroporphyrin and phyriaporphyrin were the same for the control assays with or without incubation at 37°C for 30min. Large amounts of endogenous uroporphyrin and phyriaporphyrin hinder the determination of porphyrinogen carboxy-lyase activity for the following reasons. (*a*) They decrease the sensitivity of tetra-, penta- and hexacarboxylic porphyrin determination that was by a method involving comparison of amounts of these compounds; (*b*) they can produce autoxidation of their respective porphyrinogens used as substrates; (*c*) they could inhibit the enzyme.

To separate the porphyrins from the enzyme, homogenates of porphyric liver, spleen, kidney and Harderian glands were passed through Sephadex G-25 columns. As shown in Table 2, by this procedure it was possible to achieve a good separation (99%) of endogenous porphyrins from liver homogenates, and a partial but quite good separation (85%) from kidney homogenates. The number of the first tubes eluted from the column that contained protein without fluorescence was greater for liver. The following fractions contained some fluorescence accompanying the protein. These are responsible for the small amount of remaining porphyrins in the pool (Table 2) used as enzyme source. This amount is very small compared with that of porphyrinogens used as substrate in the determination of porphyrinogen carboxy-lyase activities. Studies on the nature of porphyrins before and after gel filtration showed that uroporphyrin is retained to a greater extent than phyriaporphyrin by the dextran, since the percentage of phyriaporphyrin was greater in the eluates of the column (Table 2). Hexa-, penta- and tetra-carboxylic porphyrins were not detected. Attempts to separate the porphyrins present in Harderian-gland homogenates and in spleen were unsuccessful despite varying the size of the column as well as the ionic strength and/or the nature of the homogenizing mixture (0.154M-KCl, 0.2M-KCl, 0.1M-Tris/HCl, pH 7.0, were all tried). The porphyrinogen carboxy-lyase activity of homogenate supernatant from normal rats was determined before and after this gel filtration to test whether the treatment altered the enzyme activity. The results showed that gel filtration did not alter any of the specific activities of the different decarboxylation steps in normal liver, thus indicating that no cofactor was removed. The total activity was the same before and after this procedure.

Table 2. *Gel filtration of endogenous porphyrins from hexachlorobenzene-porphyric livers and kidneys*

Assay mixtures contained 0.067M-potassium buffer, pH 7.0, 1 mM-reduced glutathione, 0.1 mM-EDTA and homogenate supernatant (11000*g*) from hexachlorobenzene-porphyric livers or kidneys or this supernatant filtered through a Sephadex G-25 column (2.4×30cm) previously equilibrated and developed with 0.134M-potassium phosphate buffer, pH 7.0. Assay methods were as indicated in Table 1. Results are expressed as means±S.E.M. of four experiments. The tissues of two animals were pooled in each experiment. Probabilities of differences from corresponding controls are indicated thus: *, $P < 0.001$.

Enzyme preparation (homogenate supernatant)	Porphyrin content (nmol of total porphyrins/mg of protein)	Porphyrin eliminated (%)	Nature of remaining porphyrins (%)	
			Octacarboxylic	Heptacarboxylic
Liver				
Hexachlorobenzene	4.72 ±0.27	—	73±1	27±1
Hexachlorobenzene (Sephadex G-25 treatment)	0.066±0.011	98.6*	64±1	36±1
Kidney				
Hexachlorobenzene	0.459±0.006	—	83±2	17±2
Hexachlorobenzene (Sephadex G-25 treatment)	0.070±0.002	84.8*	63±3	37±3

Table 3. *Porphyrinogen carboxy-lyase activities in liver and kidney of normal and hexachlorobenzene-porphyric rats with uroporphyrinogen III as substrate*

Incubation mixtures contained 0.067 M-potassium phosphate buffer, pH 7.0, 1 mM-reduced glutathione, 0.1 mM-EDTA, 2 μM-uroporphyrinogen III and, as enzyme preparation, homogenate supernatant for normal liver (9 mg of protein) and normal kidney (25 mg of protein) or homogenate supernatant after treatment on a Sephadex G-25 column for porphyric liver (7.5 mg of protein) (*a*), (15 mg of protein) (*b*), or porphyric kidney (17 mg of protein) in a final volume of 4 ml. Assay mixtures were incubated at 37°C for 30 min anaerobically in the dark. Other details were as indicated in Table 2. Enzyme activities (nmol of porphyrins/30 min per mg of protein) are expressed as means ± S.E.M. of five experiments with percentage decrease compared with normal rats in parentheses. Uroporphyrinogen decarboxylation was measured as hepta- + hexa- + penta- + tetra-carboxylic porphyrins; phyriaporphyrinogen decarboxylation as hexa- + penta- + tetra-carboxylic porphyrins; hexaporphyrinogen decarboxylation as penta- + tetra-carboxylic porphyrins; coproporphyrinogen formation as tetracarboxylic porphyrin. The tissues of two or three animals were pooled in each experiment. Probabilities of differences from corresponding controls are indicated thus: †, $P < 0.1$; ‡, $P < 0.01$; *, $P < 0.001$.

Tissue		Porphyrinogens (%)					Enzyme activities (nmol of porphyrins/30 min per mg of protein)			
		Octa-carboxylic	Hepta-carboxylic	Hexa-carboxylic	Penta-carboxylic	Tetra-carboxylic	Uroporphyrinogen decarboxylation	Phyriaporphyrinogen decarboxylation	Hexaporphyrinogen decarboxylation	Coproporphyrinogen decarboxylation
Liver										
Normal		38±2	27±3	4±0.3	4±0.3	27±2	0.56±0.02	0.33 ±0.02	0.29 ±0.02	0.26 ±0.02
Hexachloro-	(*a*)	85±1	10±1	5±0.5	0	0	0.15±0.01* (73)	0.052±0.005* (84)	0 (100)	0 (100)
benzene	(*b*)	75±2	17±1	4±0.5	2±0.5	2±0.5	0.14±0.01* (75)	0.046±0.005* (86)	0.024±0.005* (92)	0.015±0.002* (94)
Kidney										
Normal		24±1	23±1	4±0.5	4±0.5	45±1	0.26±0.01	0.18 ±0.01	0.16 ±0.01	0.15 ±0.01
Hexachlorobenzene		49±2	25±1	6±0.5	4±0.5	16±2	0.23±0.01† (11)	0.12 ±0.01‡ (33)	0.093±0.006* (42)	0.072±0.006* (52)

According to the results and considerations mentioned above, the enzyme preparations used for the determination of porphyrinogen carboxy-lyase activities in each of the different tissues studied were as indicated in the legends of Tables 3 and 5 (i.e. preparations from porphyric livers and kidneys were only filtered through Sephadex G-25). Corrections for endogenous or remaining porphyrins in the enzyme preparations were made for porphyrinogen carboxy-lyase determinations in all porphyric tissues and normal Harderian gland, as described under 'Methods'. In this way determinations of porphyrinogen carboxy-lyase activities in liver, spleen, kidney, erythrocytes and Harderian glands from normal and porphyric rats were made. The results obtained fo liver and kidney with uroporphyrinogen III as substrate are shown in Table 3. The percentages of the substrate that remained and the products are shown, and the enzymic activities expressed as: (*a*) uroporphyrinogen decarboxylation, the elimination of the first carboxyl group from urophyrinogen (first stage, octacarboxylic → heptacarboxylic) (García *et al.*, 1973); (*b*) phyriaporphyrinogen decarboxylation, the elimination of a second carboxylic group from urophyrinogen (Tomio *et al.*, 1970), i.e. the first carboxyl group from biosynthesized phyriaporphyrinogen; (*c*) hexaporphyrinogen decarboxylation, the elimination of the third carboxyl group from uroporphyrinogen; (*d*) coproporphyrinogen formation, the elimination of three carboxyl groups from the biosynthesized phyriaporphyrinogen (second stage; heptacarboxylic →→→ tetracarboxylic) (García *et al.*, 1973). As shown in Table 3, hexachlorobenzene treatment produces a strong decrease in the activity of the liver enzyme that affects both steps of decarboxylation, the second step being affected to a greater extent. When a similar amount of protein from normal and porphyric tissues was used in enzyme determinations (*a* in Table 3), decreases of 73% for the first and 100% for the second step were found for the porphyric tissue. To discover if the amount of coproporphyrin formed so was present but in undetectable amounts under these conditions, assays with twice the amount of protein from a porphyric-liver preparation were carried out. The results obtained (*b* in Table 3) show that small amounts of coproporphyrin (mean 2%) and penta- and hexa-carboxylic intermediates (6% total) could be detected when the incubated protein concentration was increased. Coproporphyrin decreased more than the intermediates, compared with normal rats. Thus the first step (octacarboxylic → heptacarboxylic) was decreased by 75% and the heptacarboxylic → hexacarboxylic step (phyriaporphyrinogen decarboxylation) by 86%, whereas the formation of coproporphyrin was strongly diminished (94%).

Hexachlorobenzene also produced a decrease in the activity of the kidney enzyme *in vivo* that was

Table 4. *Stepwise decarboxylation of uroporphyrinogen III and phyriaporphyrinogen III by liver porphyrinogen carboxy-lyase from normal and hexachlorobenzene-porphyric rats*

Incubation mixtures contained the usual components indicated in Table 3, 2.1 μM-uroporphyrinogen III or 1.8 μM-phyriaporphyrinogen III and (12 mg of protein) homogenate supernatant (11000 g for 20 min) for normal liver or (16 mg of protein) homogenate supernatant filtered through a Sephadex G-25 column for porphyric liver. Other conditions and details are as indicated in Table 3. Each value represents the average of two experiments in which two livers were pooled in each case.

Substrate	Enzyme preparation	Porphyrinogens (%)					Enzyme activities (nmol of porphyrins/30 min per mg of protein)			
		Octa-carboxylic	Hepta-carboxylic	Hexa-carboxylic	Penta-carboxylic	Tetra-carboxylic	Uro-porphyrinogen decarboxylation	Phyria-porphyrinogen decarboxylation	Hexa-porphyrinogen decarboxylation	Copro-porphyrinogen decarboxylation
Uroporphyrinogen III	Normal	31	27	4	4	34	0.49	0.30	0.26	0.24
	Porphyric	77	17	2	1	3	0.12 (76)	0.030 (90)	0.020 (92)	0.015 (94)
Phyria-porphyrinogen III	Normal		47	7.5	7.5	38		0.32	0.28	0.23
	Porphyric		91	4	2	3		0.39 (89)	0.022 (92)	0.013 (94)

significant (decreases of 52% and 11% for the second and the first stage respectively), though less than that for liver (Table 3).

The high inhibition found in the second stage of decarboxylation caused by hexachlorobenzene treatment could be attributed to the inhibition of the first stage, producing a deficiency of phyriaporphyrinogen, the substrate for the second step, or it could be attributed to a direct inhibition of the enzyme in the second stage. To differentiate between these two effects, the action of hexachlorobenzene on liver porphyrinogen carboxy-lyase was further studied by using phyriaporphyrinogen III as substrate. A comparably large decrease in the enzyme activity in the different steps of decarboxylation was observed with either phyriaporphyrinogen III or uroporphyrinogen III as substrate (Table 4). This result suggests that the second stage catalysed by the porphyric-liver enzyme was directly inhibited and that the effect is not due to a deficiency of the substrate, since the specific activity was very low, in spite of the substrate being present in sufficient amount (91% of remaining phyriaporphyrinogen III; Table 4).

The study of the reaction intermediates from uroporphyrinogen III or phyriaporphyrinogen III with porphyric-liver enzyme showed that the relative proportion of hexacarboxylic porphyrinogen was always greater than the pentacarboxylic intermediate (Tables 3 and 4). These results are different from those of normal liver, where both intermediates were in the same proportion. This would imply that the hexacarboxylic → pentacarboxylic step was also affected by the hexachlorobenzene treatment. A similar analysis of the relative percentage of tetra- and penta-carboxylic porphyrinogen suggests that the pentacarboxylic → tetracarboxylic step is also affected.

Similar observations were made for kidneys (Table 3).

Results obtained for enzymic activities in erythrocytes, spleen and Harderian gland with uroporphyrinogen III are shown in Table 5; enzyme preparations from erythrocytes of porphyric animals exhibited unchanged porphyrinogen carboxy-lyase activities for the different steps of uroporphyrinogen decarboxylation.

Moreover, hexachlorobenzene poisoning promoted a slight increase in the enzyme activities of the spleen and a slight decrease in those of Harderian gland compared with normal tissue. Although these increases and decreases observed were not statistically significant at the *P* 0.05 level, the differences from the corresponding controls were more significant for the Harderian gland ($P<0.3$) than for spleen ($P<0.7$).

It is noteworthy that Harderian glands showed the highest porphyrinogen carboxy-lyase specific activity of all the tissues of normal rats (Tables 3 and 5),

Table 5. *Porphyrinogen carboxy-lyase activities in other tissues of normal and hexachlorobenzene-porphyric rats with uroporphyrinogen III as substrate*

Incubation mixture contained the usual components indicated in Table 3, 2μM-uroporphyrinogen III and with, as enzyme preparation, haemolysate supernatant for normal (10mg of protein) and porphyric (10mg of protein) erythrocytes, or homogenate supernatant for normal (15mg of protein) and porphyric (16mg of protein) spleen and for normal (1.3mg of protein) and porphyric (1.3mg of protein) Harderian gland. The results are expressed as means±S.E.M. of five experiments with relative activities compared with normal rats in parentheses. The tissues of two or three animals were pooled in each experiment. Other details were as given in Table 3.

	Enzyme activities (nmol of porphyrins/30min per mg of protein)			
Tissue	Uroporphyrinogen decarboxylation	Phyriaporphyrinogen decarboxylation	Hexaporphyrinogen decarboxylation	Coproporphyrinogen decarboxylation
Spleen				
Normal	0.36±0.02 (1)	0.15±0.01 (1)	0.13±0.01 (1)	0.10±0.01 (1)
Hexachlorobenzene	0.39±0.03 (1.08)	0.18±0.05 (1.20)	0.14±0.05 (1.07)	0.12±0.04 (1.20)
Erythrocytes				
Normal	0.48±0.03 (1)	0.30±0.02 (1)	0.26±0.02 (1)	0.22±0.01 (1)
Hexachlorobenzene	0.49±0.03 (1)	0.30±0.01 (1)	0.26±0.02 (1)	0.22±0.02 (1)
Harderian gland				
Normal	4.42±0.25 (1)	3.02±0.21 (1)	2.71±0.23 (1)	2.39±0.25 (1)
Hexachlorobenzene	4.31±0.01 (0.97)	2.56±0.25 (0.84)	2.25±0.27 (0.83)	1.94±0.25 (0.81)

the order of activity being: Harderian gland > liver > erythrocytes > spleen and kidney. Spleen showed higher uroporphyrinogen-decarboxylation activity and lower coproporphyrinogen-synthetic activity than kidney (i.e. kidney exhibits the highest activity for the formation of the end product of decarboxylation).

Discussion

The results suggest that the porphyrinogen carboxy-lyase activities in liver and kidney are actually diminished by the hexachlorobenzene treatment, since the enzymes were separated from the porphyrins by Sephadex G-25 filtration. In this way, difficulties caused by the presence of endogenous porphyrins, such as substrate oxidation, enzyme inhibition or decreased sensitivity of the enzyme assay, were avoided. Moreover, our results give the amount of the different products (hepta-, hexa-, penta- and tetracarboxylic porphyrinogens) for the expression of the porphyrinogen carboxy-lyase activity rather than merely coproporphyrinogen as other authors do [Mauzerall & Granick (1958), Romeo & Levin (1971), Taljaard *et al.* (1972)]. Our procedure eliminates the possibility of obtaining no decarboxylating activity at all, as was confirmed by our experiments with increased protein amounts (Table 3).

We conclude that in hexachlorobenzene-induced porphyria there is a large decrease in the activity of the liver porphyrinogen carboxy-lyase and that this decrease affects both stages of the catalysis (uroporphyrinogen III → phyriaporphyrinogen III → coproporphyrinogen III), the larger decrease being in the first stage. This response of the enzyme to hexachlorobenzene treatment is similar to that observed *in vitro* with other agents that always affect the second stage of decarboxylation to a greater extent than the first (San Martín de Viale *et al.*, 1969; Aragonés *et al.*, 1972). From the study of the second stage it is clear that the removal of each of the three carboxyl groups is affected. These findings of decreased porphyrinogen carboxy-lyase activity in hexachlorobenzene-treated rats were corroborated by Elder *et al.* (1976) but differ from the results of Taljaard *et al.* (1972). The present report of massive liver accumulation of uroporphyrin and phyriaporphyrin caused by hexachlorobenzene treatment parallel our previous results (San Martín de Viale *et al.*, 1970) and agree with those of Taljaard *et al.* (1972). This accumulation can be explained not only by the large inhibition of porphyrinogen carboxy-lyase activities, mainly in the phyriaporphyrinogen →→→ coproporphyrinogen steps, as reported in the present paper, but also by a significant increase in the activities of porphobilinogenase, δ-aminolaevulinate synthetase and δ-aminolaevulinate dehydratase (San Martín de Viale *et al.*, 1975).

Studies of kidney indicate that there is an accumulation of porphyrins in response to hexachlorobenzene poisoning, in agreement with the results of Gajdos & Gajdos-Török (1961). This effect can be attributed to the remarkable inhibition of kidney porphyrinogen carboxy-lyase activity, mainly in the second stage, that accounts for the uroporphyrin and phyriaporphyrin accumulation in this tissue.

In the Harderian gland, the decrease in the porphyrin content in hexachlorobenzene-porphyric rats could be explained by the inhibition in the δ-

aminolaevulinate dehydratase (San Martín de Viale *et al.*, 1975). The slight decrease in porphyrinogen carboxy-lyase activity produced by hexachlorobenzene in the Harderian gland agrees with the slight increase in uroporphyrin observed in this tissue.

In our previous studies on hexachlorobenzene-induced porphyria (San Martín de Viale *et al.*, 1970), there was an increase in urinary excretion of coproporphyrin. This indicates that coproporphyrin is synthesized in appreciable amounts in a tissue other than the liver, where its synthesis is almost nil, possibly in erythropoietic tissue. The present results show that spleen and erythrocytes from porphyric rats synthesize coproporphyrin at a rate equal to or slightly higher than normal cells. These findings, together with the observed increase in spleen porphobilinogenase and δ-aminolaevulinate dehydratase (San Martín de Viale *et al.*, 1975), could explain the source of the urinary coproporphyrin.

Therefore, although the porphyria produced by hexachlorobenzene affects the liver, kidney and spleen, the liver is the principal site of action of this drug, when the effect is measured quantitatively, i.e. massive porphyrin accumulation and high inhibition of the porphyrinogen carboxy-lyase in all decarboxylation steps. It is noteworthy that the kidney responds similarly but to a smaller extent. On the other hand, the erythropoietic tissue does not respond to hexachlorobenzene at all.

The hepatic character of hexachlorobenzene-induced porphyria and the observed decrease in hepatic porphyrinogen carboxy-lyase activity provides more evidence of the resemblance between this useful experimental model and the human cutanea tarda porphyria.

We thank Miss H. Gasparoli for assistance with the animals, Mrs. C. Aldonatti de Olivar for assistance with several experiments and Public Translator Mrs. S. J. Biasi for revising the manuscript. We are also grateful to Compañía Química S. A. for the gift of hexachlorobenzene. This work was partly supported by a research grant from Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

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