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Biochemical Pharmacology 66 (2003) 35–42
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Tryptophan metabolism via serotonin in rats with hexachlorobenzene experimental porphyria

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Received 19 March 2001; accepted 2 December 2002

Abstract

One of the three pathways for the metabolisation of dietary tryptophan is the formation of serotonin. Tryptophan hydroxylase catalyses the formation of 5-hydroxytryptophan, the first and regulatory step of this biosynthesis. The aim of the present work is to study alterations in this tryptophan metabolism in rats with experimental Porphyria Cutanea Tarda induced by hexachlorobenzene. With this purpose, the content of tryptophan and its metabolites related to the serotonin pathway are determined by HPLC techniques, in tissues (brain, liver and gut) and in fluids (blood, plasma and urine) of controls and hexachlorobenzene-porphyric rats. In these experimental-porphyric animals, we determine a significant increase in the excretion of 5-hydroxyindole acetic acid in urine and a decrease in the content of serotonin in small gut, respect to controls. Significant increases in contents of serotonin in 24-hr urine and tryptophan in liver are also found. No other significant variations for the different metabolites are detected in any of the tissues and fluids studied. Brain and liver activities of the rate-limiting enzyme tryptophan hydroxylase can only be measured in porphyric rats. Our results agree with an increased turnover of gastrointestinal serotonin derived from dietary tryptophan and its excretion as urinary 5-hydroxyindole acetic acid, which is formed in liver. An increased serotonin pathway in porphyric livers is confirmed by the measured increase in the activity of hepatic tryptophan hydroxylase. The absence of neurological symptoms in patients with Porphyria Cutanea Tarda could be related to the absence of a statistically significant variation in serotonin content shown in brain.

Keywords: Tryptophan; Metabolites of tryptophan pathway via serotonin; Tryptophan hydroxylase; Hexachlorobenzene; Experimental hepatic porphyria; Porphyria Cutanea Tarda

1. Introduction

HCB produces in rat an experimental hepatic porphyria similar to human PCT [1–5]. Photosensitivity with a vesicular eruption on sun-exposed areas, hirsutism, impaired hepatic function, dark red urine, hyperpigmentation of exposed areas, are some of the clinical manifestations of this disease [6].

Bleavins *et al.* [7] reported the effects of dietary HCB exposure on the manifestations of behavioural alterations in mink and European ferrets, as well as on regional brain concentrations of some tryptophan metabolites. Jackson *et al.* [8] reported an increased excretion of two indigoid pigments of indolic structure derived from tryptophan in urine of patients suffering from PCT or Crohn's disease.

Dietary tryptophan can be metabolised in mammals by three different routes (Fig. 1): (1) conversion to indole [9] which is excreted in the urine as indoxylsulphate [10]; (2) formation of serotonin (5-hydroxytryptamine, 5-HT) via 5-HTRP [11]; (3) formation of formylkynurenine by the action of tryptophan pyrrolase (L-tryptophan oxygen 2,3-oxidoreductase (decyclising), EC 1.13.11.11) [12]. In the serotonin route, 5-HTRP is formed in the first step by action of tryptophan hydroxylase (L-tryptophan, tetrahydrobiopterin oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4) and then converted to 5-HT.

Peripheral serotonin is formed in enterochromaffin cells in the gastrointestinal tract, in the upper small intestine [13], secreted to plasma and stored after in the platelet dense granules in blood [11]. Platelet uptake is the final regulatory mechanism in maintaining the free concentration of 5-HT within certain limits to avoid unwanted

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Abbreviations: HCB, hexachlorobenzene; PCT, Porphyria Cutanea Tarda; 5-HT, serotonin; 5-HTRP, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindole acetic acid.

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doi:10.1016/S0006-2952(03)00241-7

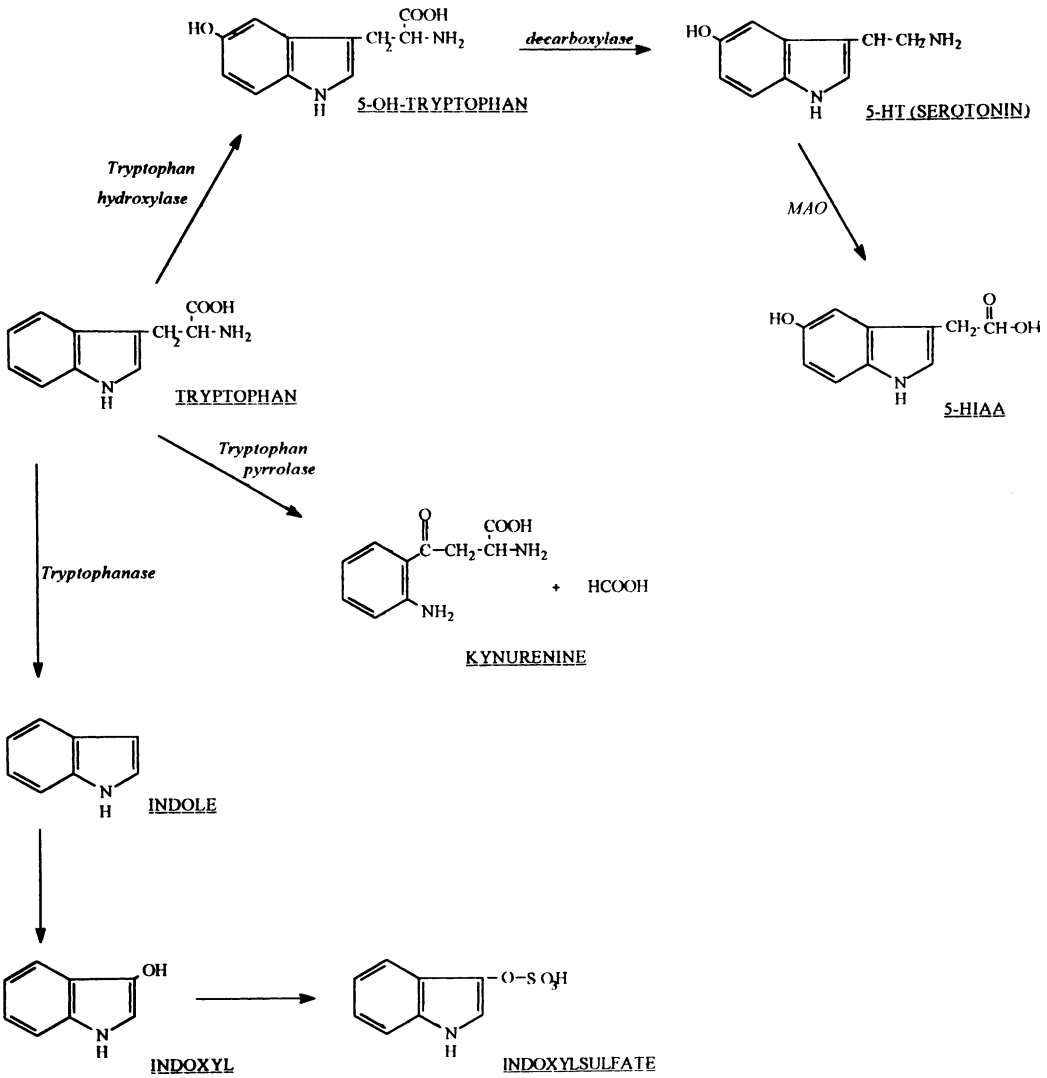

Fig. 1. Metabolism of tryptophan.

vasoactive effects. In kidney and liver, 5-HT is converted to 5-HIAA, the main metabolite, by the action of monoamine oxidase. 5-HIAA is then excreted in urine. Extra cellular plasma 5-HT is the fraction of total 5-HT released into circulation that is not oxidised to 5-HIAA. In some diseases, changes in the content of intermediates in the 5-HT pathway could be due to liver dysfunction [14].

The production and metabolism of 5-HT in the brain and peripheral organs are believed to be independent although Pietraczek *et al.* proposed that midbrain may regulate the synthesis and/or release of 5-HT from intestinal enterochromaffin cells (cf [15]).

The aim of our work is to find alterations in tryptophan metabolism in chronic hepatic porphyria and to relate them to some clinical manifestations of this disease.

In the present report, alterations in the tryptophan via serotonin metabolic pathway are evaluated in experimental PCT induced in rats by HCB. Thus, variation respect to controls, in the contents of 5-HTRP, 5-HT, tryptophan and 5-HIAA are determined in different tissues and fluids. Brain and liver activities of the rate-limiting enzyme tryptophan hydroxylase were also determined.

2. Materials and methods

2.1. Materials

Rats were fed with Purina Diet 3 (Cabeca). Standards used were from Sigma–Aldrich. Methanol was HPLC grade from Sintorgan. All the other reagents were analytical grade.

2.2. Animal treatment

Randomly bred female Wistar rats, weighing 160–180 mg at the beginning of each experiment, were used. All procedures were performed in accordance with international guidelines for the care and use of laboratory animals (Guide for the Care and Use of Laboratory Animals, National Research Council, USA, 1996, and Council

of European Communities Directive 86/609/ECC). Rats were kept in a 12-hr light/dark cycle, with free access to tap water and food. Rats were made porphyric with 1 g-HCB/kg body weight, given by gastric tubing in a single daily dose for 5 days per week, during 6 weeks. The drug was suspended in water (40 mg/mL) containing Tween 20 (0.5 mL/100 mL of suspension). Controls were treated with this suspension media, without HCB, in the same conditions. This protocol caused no death, detectable neurological syndromes or animal suffering [16]. For 24-hr urine collection, rats were individually placed in stainless steel metal cages. Fed rats were killed by decapitation, and the organs were immediately removed and used.

2.3. Standards samples preparation

New standard solutions in the corresponding $HClO_4$ dilution were prepared for each experiment, and kept at 4°, in the dark, until use.

2.4. Preparation of plasma and urine samples

Blood and urine from normal and porphyric rats were used. Rats were bled from the retro-orbital venous plexus, using EDTA as an anticoagulant. Twenty-four hour urine was collected at room temperature in the dark. Plasma and urine samples were prepared according to Bearcroft *et al.* [11]. Plasma was prepared by extraction with 6% (v/v) perchloric acid containing 2 mM EDTA; supernatants from 12,000 *g* were used. Urine extractions were made with 2% (v/v) perchloric acid and 2 mM EDTA, and immediately used.

2.5. Preparation of blood samples

Samples of whole blood from normal and experimental porphyric rats were prepared according to Xiao *et al.* [17] by extraction with 0.8 M perchloric acid containing 0.1 M ascorbic acid and 0.01 M EDTA.

2.6. Tissues samples preparation

Samples of brain, liver and small intestine from normal and HCB-treated rats were prepared according to Wolf and Kuhn [18]. 12,000 *g* supernatants of extractions with 0.16 M perchloric acid plus 0.1% EDTA and 0.1% ascorbic acid were used.

Samples and extracts were kept at 4°, in the dark, until use. All samples were filtered through Millipore filters SJHV013NS, 0.45-μm pore-size (Millipore Corp.) before injecting.

2.7. Apparatus

A SpectraSERIES (Thermo Separation Products) Model P200 liquid chromatograph and a FL2000 fluorescence detector set at excitation and emission wavelengths of 290 and 330 nm, respectively. Samples (250 μL) were injected with a Rheodyne 7125 injector fitted with a 50 μL loop. The separation was carried out on a C_{18} reverse-phase 3.9 mm × 300 mm μBondapak column (Waters) with an isocratic solution of 40 mM sodium acetate pH 3.5, as described by Moran and Fitzpatrick [19]. These solutions and the water used were filtered through 0.45-μm filters and degassed by bubbling with helium. The flow rate was 1.0 mL/min.

The column was operated at room temperature and the detector was set on the 0–20 range. All chromatograms, retention times and peak areas were recorded with a Data Jet Integrator (Thermo Separation Products). The integrator was programmed to have an attenuation of 8 and a chart speed of 0.25 cm/min.

Samples with an endogenous content of free uroporphyrin III could be accurately measured by the method described above since peaks corresponding to this porphyrin were not detected under our conditions.

2.8. Tryptophan hydroxylase activity

Tryptophan hydroxylase activities were determined by the discontinuous chromatographic method described by Moran and Fitzpatrick [19] and expressed as (mean ± SEM) of four determinations.

2.9. Protein determination

Protein determination was performed by the method of Lowry *et al.* [20].

2.10. Quantification and validation of the method

Every peak eluted from the high-performance liquid chromatography (HPLC) was confirmed by co-elution with the corresponding standard. The peaks were quantified by measuring the peak area on the chromatogram. Calibration curves were made under the same conditions described, using different amounts of each standard sample, in aqueous solution, for injection in the HPLC. The system was found to be linear, at least up to a concentration of: 2000 ng/mL ($r = 0.9997$) for 5-HTRP; 9685 ng/mL ($r = 0.9977$) for 5-HT; 12500 ng/mL ($r = 0.9775$) for tryptophan; 8000 ng/mL ($r = 0.9911$) for 5-HIAA. Detection limit was defined as the lowest injected amount that produced a signal-to-noise ratio of 3. Detection limits, expressed in ng/mL, were 36 for 5-HTRP, 98 for 5-HT, 320 for tryptophan, and 404 for 5-HIAA. Recoveries were calculated by comparing the measured values of supplemented samples of each medium with those of aqueous calibration solutions for, at least, four analyses. Endogenous contain of every metabolite in each sample was considered. Mean recoveries (%), standard deviations and coefficients of variations are shown in Table 1. No corrections in data shown in Table 2 were made as

Table 1
Precision of the assay

Contents	Concentration (ng/50 μL)	Sample	Recoveries[a] (%)	CV[b] (%)
5-HTRP	10.3	Whole blood	185 ± 24.0	13.0
	21.5	Plasma	84 ± 13.9	16.5
	14.2	Urine	126 ± 8.0	6.3
	11.1	Gut	124 ± 14.0	11.2
	5.7	Liver	74 ± 7.3	9.9
	10.0	Brain	107 ± 14.6	13.6
5-HT	41.5	Whole blood	75 ± 11.0	14.6
	41.5	Plasma	122 ± 17.0	14.0
	41.5	Urine	99 ± 3.0	3.0
	17.4	Gut	101 ± 14.0	13.1
	16	Liver	81 ± 11.8	14.6
	17.4	Brain	106 ± 12.2	11.5
Tryptophan	21.6	Whole blood	172 ± 12.8	7.4
	41.5	Plasma	119 ± 15.0	12.6
	27.4	Urine	60 ± 3.5	5.8
	28.0	Gut	92 ± 9.6	10.4
	29.4	Liver	157 ± 5.6	3.6
	28.8	Brain	106 ± 9.3	8.8
5-HIAA	43.2	Whole blood	156 ± 16.0	10.0
	43.2	Plasma	118 ± 10.0	8.4
	108.5	Urine	85 ± 2.9	3.4
	30.3	Gut	200 ± 4.9	2.5
	32.5	Liver	128 ± 18.8	14.7
	29.8	Brain	96 ± 2.2	2.3

Values of recoveries are means ± SD for at least four determinations.
[a] Within-run recoveries.
[b] Coefficients of variation.

reproducible similar results for the recoveries were obtained for normal and for experimental-porphyric samples, and we are comparing variations but not absolute values. For these reasons, different recoveries obtained for different tissues and values over 100% do not affect our results.

2.11. Statistics

Medians and interquartile ranges (IQR) used in each determination are shown in Table 2. Contents of the measured indoles in each sample from normal and HCB rats are expressed as medians, in the units corresponding to each sample as detailed in Table 2. The Mann–Whitney *U*-test compares medians. *P* values less than or equal to 0.05 were considered statistically significant.

3. Results

3.1. HPLC elution chromatograms

In Fig. 2A–C, typical HPLC elution diagrams for whole blood, plasma and urine extracts obtained from normal rats are respectively shown. Figure 3A–C depicts typical chromatograms obtained after injection of gut, liver and brain extracts, respectively. Retention times for the standards are: 5.19 (5-HTRP), 5.52 (5-HT), 8.70 (tryptophan) and 10.05 (5-HIAA).

Table 2
Medians and interquartile-ranges in contents of 5-HTRP, 5-HT, tryptophan and 5-HIAA in samples obtained from normal and experimental-porphyric rats

Contents	Sample	Units	Medians N/P	IQR N/P
5-HTRP	Whole blood	–	N.D.	–
	Plasma	–	N.D.	–
	Urine	ng/24-hr urine	107964/83622	140999–84674/181440–57792
	Gut	–	N.D.	–
	Liver	–	N.D.	–
	Brain	–	N.D.	–
5-HT	Whole blood	ng/mL sample	4208/4784	4616–4168/5924–4560
	Plasma	ng/mL sample	5004/3838	7708–2292/5296–2304
	Urine	ng/24-hr urine	193180/334620[a]	252800–181656/499296–278784
	Gut	ng/g sample	40224/29984[a]	44248–39608/31796–21176
	Liver	ng/g sample	2104/2112	2128–1856/2424–2016
	Brain	ng/g sample	3368/3152	3568–2200/3328–3016
Tryptophan	Whole blood	ng/mL sample	13052/11684	17216–5948/12900–9432
	Plasma	ng/mL sample	25726/19428	27300–23984/27992–11280
	Urine	ng/24-hr urine	N.D.	N.D.
	Gut	ng/g sample	21728/26616	25096–17912/30400–18760
	Liver	ng/g sample	8064/9648[a]	8200–7800/10360–9488
	Brain	ng/g sample	4928/4368	5040–4856/4480–3904
5-HIAA	Whole blood	–	N.D.	–
	Plasma	–	N.D.	–
	Urine	ng/24-hr urine	N.D./866880[a]	N.D./1796720–420000
	Gut	–	N.D.	–
	Liver	–	N.D.	–
	Brain	–	N.D.	–

Five to six normal and five to six porphyric rats were used for each determination. IQR: interquartile-range; N/P: normal/porphyric; N.D.: values under limit of detection.
[a] Significant differences between values determined in samples from normal and hexachlorobenzene-porphyric rats ($P < 0.05$).

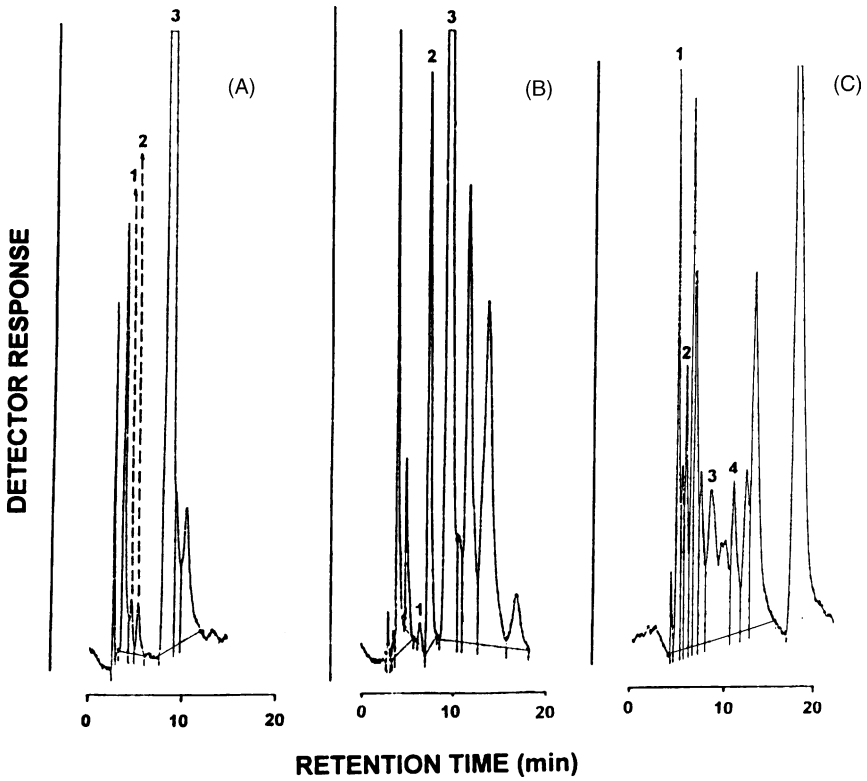

Fig. 2. A typical chromatogram obtained from the HPLC analysis of 5-HTRP (1), 5-HT (2), tryptophan (3) and 5-HIAA (4) in whole blood (A), plasma (B) and urine (C) extracts from normal rats.

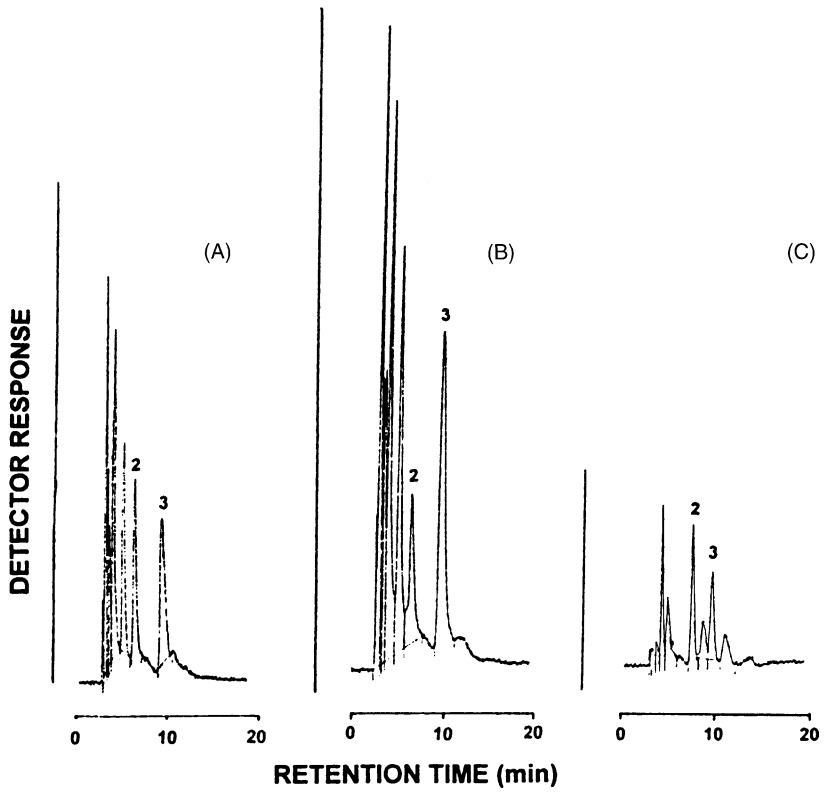

Fig. 3. A typical chromatogram obtained from the HPLC analysis of 5-HT (2) and tryptophan (3) in gut (A), liver (B) and brain (C) extracts from normal rats. Gut extracts were diluted 1:10 before injecting.

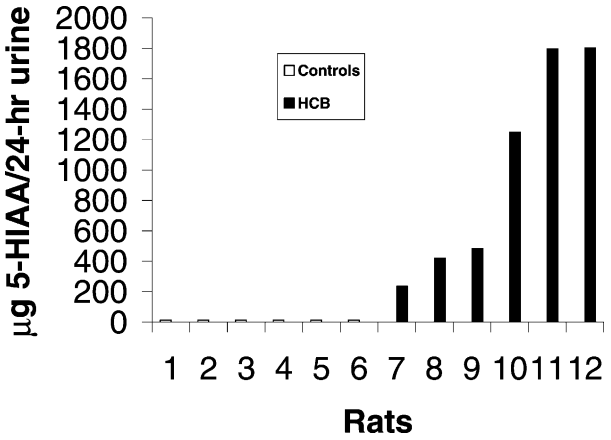

Fig. 4. µg 5-HIAA/24-hr urine in normal and hexachlorobenzene-porphyric rats.

3.2. Whole blood and plasma determinations

As shown in Table 2, concentrations of 5-HTRP in plasma and whole blood obtained from normal and from experimental-porphyric rats were under the limits of detection. No significant variations in the content of 5-HT and tryptophan were found in plasma and blood from these porphyric rats; 5-HIAA could not be detected in these fluids obtained from normal and treated rats, using the methods described in Section 2.

3.3. Chromatograms obtained from urine extracts

As depicted in Table 2 and Fig. 4, significant amounts of 5-HIAA could only be measured in 24-hr urine obtained from porphyric rats; values in controls were under the limits of detection. Thus, the 5-HIAA contents in HCB samples were statistically higher than controls. Significant increases ($P < 0.05$) in the content of 5-HT were also observed in porphyric urines (Table 2 and Fig. 5). Non-significant variations were observed in the content of 5-HTRP in 24-hr urine when rats were made porphyric, while tryptophan concentrations were under the limits of detection in normal and porphyric urine (Table 2).

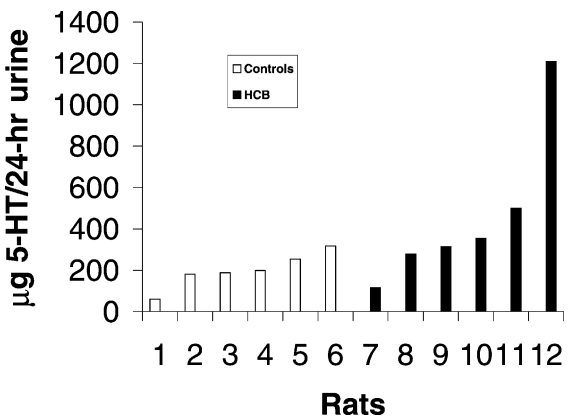

Fig. 5. µg 5-HT/24-hr urine in normal and hexachlorobenzene-porphyric rats.

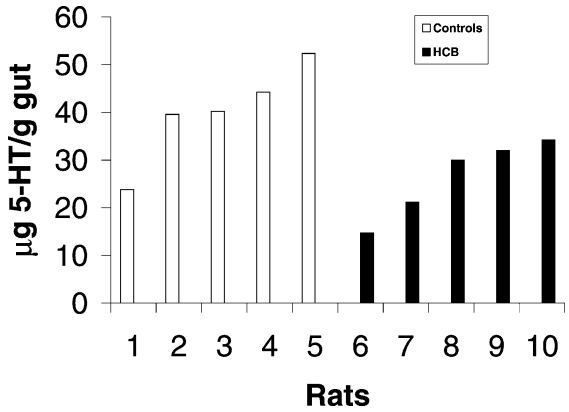

Fig. 6. µg 5-HT/g gut in normal and hexachlorobenzene-porphyric rats.

3.4. Gut, liver and brain extract measurements

As shown in Table 2 and Fig. 6, a significant decrease, with respect to controls, was observed in 5-HT content in extracts obtained from gut of porphyric rats ($P < 0.05$). By treatment with HCB, an increase in the amount of tryptophan was observed in livers ($P < 0.05$) (Table 2). 5-HTRP and 5-HIAA could not be detected in the tissues extracts obtained from normal or porphyric rats (Table 2).

3.5. Tryptophan hydroxylase activities

Tryptophan hydroxylase activities could only be determined in porphyric brains and livers. Formation of 5-HTRP was not detected in normal tissues using the methods described in Section 2. The 5-HTRP formed in porphyric brain incubations was confirmed by co-elution with the correspondent standard. Tryptophan hydroxylase specific activities, expressed in ng 5-HTRP formed/min/mg protein, were 111.4 ± 14.8 and 102.3 ± 13.2 for porphyric brains and livers, respectively.

4. Discussion

5-HT is found throughout the gastrointestinal tract, located predominantly in enterochromaffin cells. Virtually, all the 5-HT in the blood is derived from the gastrointestinal tract, and platelets have an avid uptake system to extract 5-HT from plasma and store it in dense granules. In the kidney and the liver, 5-HT is converted to 5-HIAA that is excreted in the urine [21]. In the present report, we show a significant increase, respect to controls, in the urinary excretion of tryptophan as 5-HIAA, its main metabolite, in rats made porphyric by treatment with HCB. An increase in the urinary excretion of 5-HIAA reflects an increased turnover of gastrointestinal 5-HT [22]. We here show this as a diminution in the measured

content of 5-HT in small gut of experimental-porphyric rats.

Notwithstanding the values for 5-HT in plasma and whole blood of HCB treated animals were not statistically different from controls at the $P < 0.05$ level of significance, the medians and the majority of the individual values show a decrease in plasma 5-HT and an increase in whole blood 5-HT. This assumption is confirmed by statistically significant decreases of individual plasma/whole blood ratios for samples from porphyric rats respect to those from normal ones. This tendency would suggest that HCB would augment the avidity exerted by the platelets thus impoverishing the plasma. The increase of the urinary 5-HT overcomes the decreases seen in gut and plasma. Perhaps the contribution of the kidneys and the urinary tract to this increase must be explored. The increase in 5-HT would be in accordance with the augmented tryptophan concentration since it has been reported that the rate of synthesis of 5-HT is directly regulated by tryptophan availability [23].

A possible blockage of the kynureninic route at the level of tryptophan pyrrolase by HCB treatment could be taken into account for the increase of tryptophan in liver. This hypothesis is based on the typical and sharp decrease of uroporphyrinogen decarboxylase activity, the target of HCB in the haem pathway [3,16] and on the fact that tryptophan pyrrolase is a haem protein. At this respect, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a drug mechanistically related with HCB, produced a sharp decrease of liver tryptophan pyrrolase with a concomitant increase of tryptophan levels [24].

The lack of a statistically significant variation in serotonin content in brain could account for the absence of neurological symptoms in patients with this hepatic type of porphyria.

The present results show that HCB treatment increases the tryptophan via serotonin metabolism by increasing tryptophan hydroxylase, the rate-limiting enzyme. Concomitantly, this fungicide increases the contents of urine 5-HT and of the final product 5-HIAA in urine, and decreases levels of 5-HT in gut. The raised daily urinary excretion of 5-HIAA in porphyric animals agrees with the observed increase of the serotonin pathway in liver, where 5-HIAA is formed.

On the other hand, these results contribute to better understand the metabolic action of HCB, a polychlorinated hydrocarbon and lipophilic compound, which not only disturbs haem and lipid metabolism [3,25], carbohydrate pathway [26] polyamine biosynthesis [27] but also amino acid biotransformation as it is now shown for the metabolisation of tryptophan via serotonin.

Acknowledgments

We wish to thank Professor Moisés Grinstein for his helpful advice. We also thank Dr. Javier Calcagno for his help in statistical calculations. Grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and University of Buenos Aires, Argentina, supported this research. These experiments comply with the current laws of the country in which the experiments were performed.

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