Hexachlorobenzene Treatment on Hepatic Mitochondrial Function Parameters and Intracellular Coproporphyrinogen Oxidase Location

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These studies try to elucidate why isocoproporphyrin appears in hexachlorobenzene-poisoned rats' feces. Chronic exposure of hexachlorobenzene to rats produces an experimental model for human porphyria cutanea tarda. After 8 weeks of treatment, rats showed high porphyrin excreta and 50% inhibition of liver uroporphyrinogen decarboxylase activity. Uroporphyrin plus heptacarboxylic porphyrin exceeded coproporphyrin in urine, whereas in feces, isocoproporphyrin, from abnormal pentacarboxylic porphyrinogen III oxidative decarboxylation by liver coproporphyrinogen oxidase, became the main porphyrin. Trypsin-treated mitochondria showed that the outer and inner membrane permeability barrier was highly conserved after hexachlorobenzene intoxication. In digitonin-treated hexachlorobenzene mitochondria, coproporphyrinogen oxidase was free in the mitochondrial intermembrane space, whereas in normal mitochondria, 30% to 50% remained anchored to the inner membrane. Hexachlorobenzene led to a decrease in respiratory control and ADP/O ratios (uncoupled mitochondria). Albumin restored oxidative phosphorylation, indicating no irreversible inner membrane damage. Normal and hexachlorobenzene mitochondria oscillatory studies exhibited similar damping factor values, showing that hexachlorobenzene had no significant effect on membrane fluidity and elasticity. Mitochondrial uncoupling could explain the free state of the enzyme within the intermembrane space. The free state of the enzyme makes it more flexible and would allow pentacarboxylic porphyrinogen III, whose levels are increased, to compete with coproporphyrinogen III and being transformed into dehydroisocoproporphyrinogen, the liver forerunner of fecal isocoproporphyrin.

Keywords
- Coproporphyrinogen Oxidase
- Experimental Porphyria
- Hexachlorobenzene
- Isocoproporphyrin
- Mitochondria

The formation of tetrapyrroles and their subsequent modification into heme is a multistep process that has been highly conserved throughout evolution (Beale and Weinstein 1990; Wyckoff and Kushner 1994). In eukaryotes, the biosynthetic pathway leading to heme involves a sequence of eight enzymatic steps. The fifth of these steps is catalyzed by the cytosolic enzyme uroporphyrinogen decarboxylase (URO-D; EC 4.1.1.37). URO-D catalyzes the sequential decarboxylation of uroporphyrinogen (Uro’gen) III to coproporphyrinogen (Copro’gen) III. Theoretically, this transformation may involve 24 possible routes with 14 porphyrin structures. We have demonstrated that URO-D takes only one route, and this involves three porphyrinogen intermediates: heptacarboxylic (Hepta’gen) d, hexacarboxylic (Hexa’gen) ad, and pentacarboxylic (Penta’gen) abd (the letters indicate the position of methyl groups) (Jackson et al. 1976a, 1976b; Jackson, Sancovich, and Ferramola de Sancovich 1980; Smith et al. 1976).

At this stage, Copro’gen III is transferred into the mitochondria and undergoes two stepwise oxidative decarboxylations by the enzyme coproporphyrinogen oxidase (CPO; EC 1.3.3.3). This enzyme catalyzes the removal of the carboxyl group and the two hydrogens from the propionic acid side chains of Copro’gen III to form vinyl groups at positions 2 and 4, leading to protoporphyrinogen (Proto’gen) IX. In intact mitochondria, CPO is loosely attached to the inner membrane surface surrounding the intermembrane space (Elder and Evans 1978b).

Porphyrias are disorders caused by marked enzymatic deficiencies in the heme pathway. Porphyria cutanea tarda (PCT) is the most common, and it is caused by a heterozygous deficiency of URO-D (Elder 1998). Estimates of its prevalence range from 1 in 25 000 in the U.S. population (Elder 1998) and 1 in 37 000 in Argentina (Méndez et al. 2005). PCT is biochemically characterized by a marked increase in porphyrin excretion: uroporphyrin (Uro) and heptacarboxylic porphyrin (Hepta) in urine, and isocoproporphyrin (Isocopro) and smaller amounts of coproporphyrin (Copro) in feces (Elder 1972).

Chronic exposure to hexachlorobenzene (HCB) may generate a toxic cutaneous porphyria in rats. In several aspects, this form of porphyria resembles human PCT (Stormard 1974).
seems to be a dose-related response to the absorption of the porphyrigenic chemical, but it is not influenced by individual susceptibility (Elder 1972). HCB intake was followed initially by a moderate increase of Copro and later by an increase of highly carboxylated porphyrins, such as Uro and Hepta, in urine (Elder, Evans, and Matlin 1976) and the presence of Isocopro and smaller amounts of Copro in feces (Elder 1972).

Isocopro excreta is the result of increased quantities of Penta’gen III (Penta’gen abd) (Jackson et al. 1976a, 1976b; Jackson, Sancovich, and Ferramola de Sancovich 1980; Smith et al. 1976), which is formed in the cytosol and competes with Copro’gen III for CPO decarboxylation (Elder 1972; Kennedy et al. 1970), producing dehydroisocopro’gen, which is not further metabolized in the liver. The former porphyrinogen is then hydrogenated by intestinal bacteria and before being excreted is oxidized to Isocopro.

It is still not clear whether an increase in Penta’gen abd–Copro’gen III ratio, caused by URO-D impairment, is the only reason for this abnormal transformation. It may be speculated that alterations in mitochondria could play an essential role or, at least, contribute to dehydroisocopro’gen biosynthesis. To test this hypothesis, comparative studies on mitochondria properties and function parameters were carried out in normal (N) and HCB-intoxicated (HCB) rats.

Results emphasize the notion that abnormal dehydroisocopro’gen biosynthesis catalyzed by CPO depends not only on increased cytosol hepatocyte Penta’gen abd levels, but also on other mitochondrial alterations. It was observed that the main difference between N and HCB mitochondria was the location of CPO. In HCB mitochondria, CPO was mostly free in the mitochondrial inner membrane space (MIMS), whereas in N mitochondria, most enzyme activity was anchored to the outer face of the mitochondrial inner membrane (MIM) in the MIMS. Thus, HCB treatment would let CPO be more flexible, allowing Penta’gen abd to compete with Copro’gen III for the CPO catalytic site, and to be transformed into dehydroisocopro’gen, the liver precursor of fecal Isocopro.

**MATERIALS AND METHODS**

**Chemicals**

HCB (commercial grade) (95% HCB, 5% tetra- and pentachlorobenzene) was generously provided by Compañía Química S.A. (Buenos Aires, Argentina). Copro III and Uro III were obtained from Porphyrin Products (Logan, UT, USA). Porphyrinogens were prepared with sodium amalgam, according to Mauzerall and Granick (1958). l-malic acid, l-glutamic acid, l-malonic acid, ADP (grade I), valinomycin, rotenone, EDTA, digitonin, trypsin, soybean trypsin inhibitor, Percoll, DMSO, and crystalline bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden). All other reagents were of commercially available analytical grade.

**Animals**

Female Wistar rats, weighing 150 to 180 g at the beginning of the experiment, were fed with Purine 3 diet (Cabeca SCA, Argentina) and water ad libitum. Environmental conditions consisted of 12-h light–12-h dark cycles, 20°C to 24°C, and uncontrolled humidity. A 5-ml suspension of HCB (1 g/kg body weight) was administered daily by stomach tube. The fungicide was suspended in water (40 mg/ml) containing Tween 20 (0.5 ml/100 ml). Control (N) animals received equal volumes of the appropriate solvent by the same route. The dose of HCB employed was chosen based on previous studies from our laboratory and those of other researchers (Kleiman de Pisarev, Ferramola de Sancovich, and Sancovich 1995; van Raaij et al. 1993; Fernández Tomé et al. 2000; Randi et al. 1998, 2003; Loaiza Pérez et al. 1999), demonstrating that this dose elicited clear manifestations of hepatic porphyria and thyroid hormone metabolism alterations. The general health of the animals was not affected by the dose of HCB employed, as it was evaluated by the behavior and appearance of the rats, including examination of their coats, mucous membranes, and body weights, and their food and water consumption.

Animals received humane care and were treated in accordance with guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC). The experimental protocols were also approved by the Local Committee of Animal House in the School of Sciences, University of Buenos Aires, Argentina.

**Analysis of Porphyrins in Urine and Feces**

Analysis of porphyrins was carried out weekly in 24-h specimens of urine and feces, collected from each rat in individual metabolic cages. Porphyrin profiles were determined by high-performance liquid chromatography (HPLC) in aliquots of 1 to 3 ml urine and 1 to 2 g wet weight feces according to the method described by Lim and Peters (1984). Total porphyrins were determined spectrophotometrically using the correction formula of Rimington and Sveisson (1950).

**Tissue Fractions**

Animals were decapitated and livers were weighed and washed thoroughly with cold saline. Homogenates were made by adding 0.33 M isotonic sucrose, then centrifugated at 11 000 ×g for 20 min. Soluble fractions were used as URO-D enzyme source after (NH4)2SO4 fractionation. Pellets (30% to 70%) were suspended in 0.134 M potassium phosphate buffer (pH 6.8). Preparations from porphyric livers were filtered through a Sephadex G-25 column and eluates, with or without fluorescence traces, were pooled and used for URO-D activity determination.

**Enzyme Assays**

Each data point was calculated as the mean enzymatic activity, obtained from the liver or liver mitochondria of different
**TABLE 1**
Mitochondrial respiratory parameters in N and HCB-treated rats

<table>
<thead>
<tr>
<th>Respiratory parameters</th>
<th>N</th>
<th>None</th>
<th>+ BSA</th>
<th>HCB</th>
<th>None</th>
<th>+ BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Malate-glutamate as substrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>$V_{state3}$</td>
<td>132.17 ± 11.44</td>
<td>130.74 ± 10.37</td>
<td>135.67 ± 11.02</td>
<td>131.97 ± 12.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{state4}$</td>
<td>23.28 ± 2.11</td>
<td>23.34 ± 2.25</td>
<td>51.75 ± 4.85*</td>
<td>23.31 ± 2.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCR</td>
<td>5.67 ± 0.46</td>
<td>5.63 ± 0.48</td>
<td>2.66 ± 0.19*</td>
<td>5.66 ± 0.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP/O</td>
<td>2.60 ± 0.14</td>
<td>2.44 ± 0.12</td>
<td>1.32 ± 0.12*</td>
<td>2.58 ± 0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Succinate as substrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{state3}$</td>
<td>142.03 ± 13.57</td>
<td>143.74 ± 12.28</td>
<td>145.35 ± 10.75</td>
<td>142.38 ± 10.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{state4}$</td>
<td>25.57 ± 2.54</td>
<td>24.16 ± 2.43</td>
<td>66.92 ± 5.63*</td>
<td>24.89 ± 2.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCR</td>
<td>5.55 ± 0.47</td>
<td>5.94 ± 0.60</td>
<td>2.20 ± 0.11*</td>
<td>5.73 ± 0.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP/O</td>
<td>1.40 ± 0.09</td>
<td>1.48 ± 0.10</td>
<td>0.92 ± 0.08*</td>
<td>1.47 ± 0.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note.* Malate-glutamate and succinate were employed as substrates as described in text. Respiratory velocities of mitochondria at rest ($V_{state4}$) and during active metabolism ($V_{state3}$) were calculated as ng atom oxygen/min/mg mitochondrial protein, as described in Materials and Methods. Active state respiration was induced by adding ADP 500 µM (final concentration). In +BSA groups, 0.1% BSA (final concentration) was added to the incubation medium before the mitochondrial suspension. Values represent means ± SEM, n = 6. *Significantly different from N and N + BSA groups. The differences were analyzed by the Student's t test ($p < .05$).
Trypsin Treatment

Trypsin treatments were carried out according to La Piana et al. (2005) with some modifications. Intact and disrupted N and HCB mitochondria (0.50 mg protein/ml) were incubated for 5 min with 10 µg trypsin (25 U). Then, soybean trypsin inhibitor, in at least threefold weight excess (10 µg inhibit 100 U trypsin), was added, and suitable aliquots were used for the determination of CPO and marker enzyme activities of intermembrane and matrix spaces.

Digitonin Method for Mitochondrial Fractionation

Portions of ice-cold digitonin (10 mg/ml) in isolation medium at different pHs (7.4 to 6.4) were added to the same volume of mitochondrial suspension (50 mg/ml) in isolation medium at the assayed pH (medium pH was adjusted with 1 M KOH immediately before use). The resulting suspension was gently mixed for 10 min at 0°C, diluted with 2 volumes of isolation medium at the pH previously used, mixed again, and centrifuged at 12,000 ×g for 10 min (Janski and Cornell 1980). The supernatant was collected and the pellet (inner membrane–matrix complex) was resuspended in the same volume of isolation medium. Further studies of the inner membrane–matrix complex included assays carried out after mitoplast disruption.

For each fraction, enzyme activity recovery was calculated as the sum of total activity in each fraction in relation to total activity in nonfractioned material (disrupted mitochondria). Enzyme activity of each fraction was expressed as a percentage of total recovered activity.

Mitochondrial Respiration Assay

Oxygen consumption (OC) was measured polarographically, according to a modification of Toyomizu et al. (2000) with a Gilson Medical Electronics oxygraph at 30°C using a vibrating platinum electrode. The reaction mixture (1.9 ml) contained 0.24 M sucrose, 34 mM KCl, 5 mM MgCl₂, 2 mM EDTA, 9 mM Tris-HCl, 6 mM KH₂PO₄–K₂HPO₄, and 1.4 mg mitochondrial protein, pH 7.4. When present, 0.1% BSA, fatty acid free (final concentration), was added to the incubation medium before adding the mitochondrial suspension. The following substrates and additional chemicals were used: 6 mM L-malate, 6 mM L-glutamate, and 3 mM malonate, pH 7.4, or 5 mM succinate (plus 0.2 µM rotenone), pH 7.4. Respiratory velocities of mitochondria at rest ($V_{state4}$) and during active metabolism ($V_{state3}$), as ng atoms oxygen/min/mg of mitochondrial protein were obtained plotting OC (ng atoms) versus time (min). Active state respiration was induced adding 500 µM adenosine diphosphate (ADP) (final concentration). The respiratory control ratio (RCR) was calculated as the relation of $V_{state3}$ to $V_{state4}$.

Damping Factor Calculation

The damping factor is defined as the ratio of successive oscillation amplitudes of peaks and troughs of tracing damped harmonic oscillatory variations. Peak damping factors were obtained from the a/b, b/c, c/d, and d/e ratios, and trough damping factors from the 1/2, 2/3, and 3/4 ratios (Figure 1).

Protein Concentration

Protein concentration was determined by the method of Bradford (1976) using BSA as standard.

Statistical Analysis

The values in the figures and Table 1 are expressed as means ± SEM. Statistical comparisons were made as indicated in the text, using one-way analysis of variance (ANOVA), followed by Tukey’s test (Statistica 6.0). Other statistical analyses were

![FIGURE 1](image-url) Damped oscillation in rat liver mitochondria. Typical tracing of damped oscillation obtained with N rat liver mitochondria (M) showing different possible measurement parameters: a, b, c, d, and, e represent peaks; and 1, 2, 3, and 4 represent troughs. I = initiator of mitochondrial oscillations; A = absorption scale at 520 nm; t = time scale.
ISOCOPROPORPHYRIN IN HCB POISONED RAT FECES

RESULTS

Effect of HCB on Porphyrin Rat Excreta

To detect the onset of HCB intoxication, urine and feces were collected weekly from animals in N and HCB groups. The evolution of porphyrin excreta (Figures 2 A and 3A) showed that HCB intoxication increased porphyrin content of both urine and feces in function of time of the treatment. During the first 5 weeks of treatment, both HCB and N rats showed very low porphyrin content in urine and feces, and Copro III as the main tetrapyrrrole component in excreta. From the 6th week onwards, an increase in porphyrin excreta was observed. In urine, a decrease of Copro III percentage was paralleled to a concomitant increase of highly carboxylated porphyrins, mainly Uro III and Hepta III (Figure 2B). From the 7th week onwards, Uro III plus Hepta III proportions exceeded Copro III content. Between weeks 7 and 11, the pattern of porphyrin excreta remained nearly constant. In feces, concomitant with a high increase in porphyrin excreta, Copro III and Isocopro, another tetracarboxylated porphyrin, were accompanied by smaller amounts of penta-, hexa-, and heptacarboxylated porphyrins (Figure 3B). After the 8th week, the highly carboxylated porphyrin fraction was barely noticeable and Isocopro became the main product.

Time Course of Uro-D Inhibition

Rat liver URO-D activity was monitored over 11 weeks of HCB treatment (Figure 4). Liver URO-D activity seemed to fall from the beginning of the experiment. It was significantly inhibited from the 2nd week of HCB intoxication, but maximum inhibition (over 50%) was not observed until 5 to 6 weeks later. Then, the level of URO-D activity remained nearly constant until the end of the treatment. No significant differences (NSD) in values were observed from week 8 until the end of the experiment.

Trypsin Treatment and Digitonin Action on Isolated Mitochondria

Figure 5 shows that, in HCB-treated rats, CPO and marker enzyme activities, with different location in mitochondria, were not affected by trypsin treatment. After mitochondrial digitonin treatment, nearly 100% CPO, AK, and SO activities were recovered in the supernatant. In supernatant and sediment trypsin treatments, no CPO, AK, and SO activities were detected in any preparation. However, CS and MD (matrix enzyme) activities were not modified (Figure 5B to F).

Marker enzyme activities in N rats (data no shown) showed NSD with HCB data in all the treatments assayed. The only difference was observed in the digitonin treatment, in which 28% CPO activity appeared joined to the sediment (mitoplasts), whereas the remnant CPO activity was found in the supernatant. After trypsin treatment, CPO activity disappeared from both fractions (supernatant and sediment) (Figure 5A).

When N intermembrane mitochondrial space proteins were released, modifying the pH during digitonin treatment, different results for CPO location were obtained. Figure 6 shows the percentages of mitochondrial enzyme activities in the sediment of digitonin treatments at pHs 7.4 to 6.4. No differences were found in matrix marker enzyme activities of N (Figure 6A) and HCB (Figure 6B) rats at all the pHs studied. However, N mitochondria showed that when pH decreased in digitonin treatment,
FIGURE 3
Effect of HCB on daily fecal porphyrin excreta: (A) total, (B) relative distribution. Female Wistar rats were treated as indicated in Figure 2. Porphyrin analysis was carried out weekly on 24-h feces collected in individual metabolic cages. Total porphyrins were determined as indicated in Materials and Methods. Porphyrin profiles in 1 to 2 g wet weight feces aliquots of samples for A were treated and analyzed by HPLC, as described in Materials and Methods. Data are expressed as mean ± SEM for six rats. Statistical comparisons were made with ANOVA. p < .05 when compared with N.

Effect of HCB on Rat Liver Mitochondrial Respiration
Table 1 shows NSD between N and HCB mitochondrial $V_{state 3}$ with malate-glutamate or succinate as substrate. Likewise, no changes were detected in the presence of BSA (1 mg/ml). HCB treatment with malate-glutamate or succinate as substrate triggered a significant increase in $V_{state 4}$ and a significant decrease of the means of RCR and the ADP/O ratio. When BSA was used in these determinations, respiration at rest (state 4) significantly fell in relation to values obtained without this compound. Likewise, RCR and ADP/O mean ratios were higher than in studies without BSA, and similar to data from N rats.

Effect of HCB on Mitochondrial Oscillation
Figure 7 shows the tracing damped harmonic oscillation variation obtained with N and HCB-intoxicated rat liver mitochondria. Both curves were clearly similar. The damping factor, defined as the ratio of successive oscillation amplitudes of peaks and troughs, was calculated. Figure 8 shows the mean values of damping factors of the two groups of rats treated (N and HCB). It is evident that HCB mitochondria showed parameter values similar to those of N mitochondria.

DISCUSSION
The time of onset of HCB experimental porphyria showed that increase in porphyrin excreta was a function of time of the intoxication (Figures 2A and 3A). In urine, Uro III plus Hepta III proportions exceeded Copro III content from the 7th week onwards (Figure 2B), and this pattern remained nearly constant up to the end of the treatment. In feces, Isocopro became the main excreta porphyrin after the 8th week (Figure 3B). HCB seemed to have no effect on rat erythrocytes, because only traces of Copro were detected in N and HCB-intoxicated rat erythrocytes (data not shown).
Mitochondrial enzyme location in N and HCB-treated rats. The percentage of total recovered enzyme activities (CPO and marker location enzymes [AK, SO, CS, MD]) was calculated as indicated in Materials and Methods. Results are mean ± SEM for five rat values, in sediments (sed) and supernatants (sup) of intact liver mitochondria, before and after trypsin (T) and/or digitonin (D) treatments, as described in Materials and Methods. (A) N rat liver mitochondria. (B to F) HCB rat liver mitochondria.
Mitochondrial CPO and marker enzyme activities in sediments after digitonin treatment at different pH values. The percentage of total CPO and mitochondrial marker localization enzyme (AK, SO, CS, MD) activities was calculated as indicated in Materials and Methods. Results are mean ± SEM for five rat activity values of sediments from (A) N and (B) HCB-treated rats, after Digitonin treatment at different pHs. Statistical comparisons were made with ANOVA. $p < .05$ when compared with pH 7.4 (#).

Kinetic studies with HCB (Figure 4) demonstrated that liver URO-D activity fell from the beginning of the experiment. However, maximum inhibition (over 50%) was not observed until the 7th week. Such decrease seemed not to be caused by lower liver URO-D synthesis (Elder and Sheppard 1982).

Smith and Francis (1983) reported that rodents exposed to long HCB treatment developed massive accumulation of porphyrins in the liver. This accumulation was detected at the early intoxication stages (Masini et al. 1984). Uro and Hepta predominated, but considerable amounts of Copro, Hexa, and Penta were also observed (Doss, Schermuly, and Koss 1976).

The aim of this work was to find why Isocopro appear in HCB poisoned rat feces. It is still not clear whether the increase in the ratio between Penta’gen abd and Copro’gen III, caused by URO-D impairment, would be the only reason for CPO to develop an abnormal Penta’gen abd transformation. It may be speculated that mitochondrial alterations would play an essential role in dehydroisocopro’gen biosynthesis or at least contribute to it.

To test this hypothesis, comparative studies on mitochondrial properties and function parameters were carried out in N and HCB-intoxicated rats. According to the results obtained, all further studies were performed on HCB-treated rats for at least 2 months (Figures 2 to 4).

HCB increases lipid metabolism of the lipogenic enzymes glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme (Kleiman de Pisarev, Ferramola de Sancovich, and Sancovich 1995; Loaiza-Pérez et al. 1999), and lipid peroxidation levels (Fernández Tomé et al. 2000). Mitochondrial functions depend on membrane phospholipid and fatty acid composition, and membrane-bound enzyme functions and interactions within the membrane depend on the physical properties of phospholipids or, at least, on the nature of their fatty-acyl components (McMurchie et al. 1983).

Both in intact N and HCB mitochondria, the insensitivity to the proteolytic attack of MIMS enzymes (CPO, AK, and SO) exerted by trypsin was higher than 98% and lower than 2% in disrupted samples (Figure 5A to D). This demonstrated that in both treated groups, mitochondrial outer membranes were preserved. The permeability barrier of the external membrane against the release of MIMS enzymes, particularly CPO, and the accessibility of trypsin remained highly conserved even after HCB treatment.

Digitonin, which reacted, and formed complexes, with cholesterol molecules, was useful to selectively enhance the permeability of the external membrane of mitochondria, leaving the MIM, virtually devoid of cholesterol, almost completely intact (Janski and Cornell 1980).
phosphorylation in liver mitochondria. During HCB administration, porphyria resulted in partial and constant uncoupling of oxidative phosphorylation, and membrane potential would be dissipated. Masini et al. (1984) reported that induced experimental porphyria in HCB mitochondria was not completely coupled to the respiration process. A significant decrease in RCR and ADP/O mean ratio was observed after HCB treatment (Table 1). Thus, respiratory parameters (Table 1) were also very similar (Figure 8). This would indicate that HCB treatment had no marked influence on mitochondrial membrane fluidity and elasticity.

Studies showed that the main difference between HCB and N mitochondria after digitonin was CPO location. In HCB mitochondria, the enzyme was entirely located in the soluble fraction of MIMS (Figure 5B), and was not altered at the different pHs assayed (Figure 6B). In N mitochondria and depending on the pH assayed, 30% to 50% CPO was anchored to the outer surface of the MIM (Figures 5A and 6A). Matrix enzyme activities (CS and MD) from submitochondrial particles (mitoplasts) were not affected by trypsin treatment (Figure 7A, B, and C). Thus, even after digitonin exposure, MIM from N and HCB mitochondria also kept their permeability barrier.

In order to assess other damages caused by HCB treatment to the MIM, certain mitochondrial function properties from HCB rats were compared with those from N animals. RCR values have been important in the study of mitochondrial integrity with respect to oxidation and phosphorylation coupling. Effects on electron transport and oxidative phosphorylation in HCB-treated rats were compared with those of N animals (Table 1). During active metabolism (state 3), NSD in respiratory velocities were detected between N and HCB mitochondria. Data obtained may be considered normal standard values for this parameter. In N rats, BSA did not impair mitochondrial respiration parameters (Table 1). HCB treatment led to a substantial modification in the efficiency of the oxidative phosphorylation process. A significant decrease in RCR and ADP/O mean ratio was observed after HCB treatment (Table 1). Thus, respiration in HCB mitochondria was not completely coupled to phosphorylation, and membrane potential would be dissipated. Masini et al. (1984) reported that induced experimental porphyria resulted in partial and constant uncoupling of oxidative phosphorylation in liver mitochondria. During HCB administration, pentachlorophenol was the main metabolite recovered in rat tissues (Debets, Strik, and Olie 1980). HCB uncoupling can be attributed to the action of this metabolite, but albumin can substantially reverse this uncoupling (Masini et al. 1984; Trenti et al. 1986).

Table 1 also demonstrated that BSA fully reversed the uncoupling of the oxidative phosphorylation process in liver HCB mitochondria. These results indicated that no irreversible damage occurred, and that MIM potential could be reestablished.

Alterations in membrane lipid fatty-acid composition resulted in a shift in transition temperatures, suggesting that membrane fluidity can be modulated, altering fatty-acyl chain components of membrane phospholipids (Innis and Clandinin 1981). To assess HCB effects on mitochondrial fluidity and elasticity, changes in mitochondrial oscillations were studied.

Gooch and Packer (1974) found that in vitro mitochondria showed oscillatory phenomena with concomitant volume changes, and they suggested the damping factor was a good parameter to measure mitochondria oscillatory responses. The higher ratios corresponded to smaller damped mitochondrial oscillatory responses, usually due to lower membrane elasticity caused by volume changes. Studies with N and HCB-intoxicated rat liver mitochondria showed a clearly visible similarity in their oscillatory variations (Figure 7). Mean damping factor values for both groups of mitochondria were also very similar (Figure 8). This would indicate that HCB treatment have no marked influence on mitochondrial membrane fluidity and elasticity.

It has been reported that oxidative phosphorylation uncouplers such as 2,4-dinitrophenol enhance the damping factor, increasing passive membrane permeability to protons (Gooch and Packer 1974). Our data do not agree with these results, maybe because the HCB metabolite (pentachlorophenol) had a weaker uncoupling effect at the concentration levels in the experiments.

The final steps of the heme biosynthetic pathway in higher eukaryotes are catalyzed by three enzymes: CPO, protoporphyrinogen oxidase (PPO), and ferrochelatase, which are either bound to the MIM or associated with it. These enzymes may participate of the multienzyme complex in a stable or dynamic manner, following a spatially close orientation in the MIM that does not allow significant accumulation of intermediates to occur (Grandchamp, Pung, and Nordmann 1978; Deybach et al. 1985; Ferreira and Dailey 1988; Ferreira et al. 1988; Proulx, Woodard, and Dailey 1993; Olsson et al. 2002).

Peripheral (extrinsic) proteins like CPO may bind reversibly to the outer face of the MIM through electrostatic interactions and hydrogen bonding to membrane lipids and intrinsic proteins. They can be released by relatively mild treatments that interfere with electrostatic interactions or by breaking hydrogen bonds.

The techniques used to locate CPO could influence our results, affecting the actual CPO location. Data obtained, as previously stated by Grandchamp, Pung, and Nordmann (1978), do not rule out the possibility that CPO from in vivo N mitochondria could be entirely bind to the external face of the MIM. Thus, there would be a direct transfer of Proto'gen and Proto...
IX between catalytic sites. Should this not happen and due to their poor solubility in water, they could be intercalated into the surrounding mitochondrial membrane, rather than diffused into the bulk aqueous phase (Proulx, Woodard, and Dailey 1993).

In HCB-treated rat livers, data clearly demonstrated that CPO would be mainly free in the MIMS, as AK and SO are (Figure 5B to D). Thus, Copro’gen III would have a better accessibility to CPO catalytic site than in N mitochondria, in which it would be attached to the MIM, partially impeding porphyrinogen traffic into the CPO catalytic center. As Grandchamp, Pung, and Nordmann (1978) proposed, when Copro’gen III crosses the outer membrane, a free enzyme-substrate complex would be formed, which would induce and/or help it to bind to a specific MIM destination, near or on PPO.

The binding of an enzyme and a substrate involves conformational changes, which are essential to enzymatic functions. HCB treatment would grant CPO more flexibility. Even subtle changes in CPO three-dimensional structure would allow Penta’gen abd, whose levels are increased in liver, to compete with Copro’gen III for the CPO catalytic site and for being transformed into dehydroisocopropo’gen, the liver forerunner of fecal Isocopropo. This hypothesis is based on the notion that experimental Penta’gen abd oxidative decarboxylation by CPO was only achieved in disrupted mitochondria (Elder and Evans 1978a) or highly purified human CPO (Cooper et al. 2005).

CPO is synthesized outside the mitochondria and must be imported to its final location. A proton-motive force across the MIM (with electric and pH gradients provided by the electron transport chain) would be required for its translocation and could be indeed essential for CPO-MIM interactions. Because mitochon-dria from HCB-intoxicated rats are uncoupled, the electrochemical potential of the membrane is dissipated. Therefore, it would be important to keep CPO free in the MIMS.

REFERENCES


