Drug-induced accumulation of uroporphyrin in chicken hepatocyte cultures

Structural requirements for the effect and role of exogenous iron

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1. The ability of drugs to cause uroporphyria in hepatocytes from 17-day-old chick embryos has been investigated and the response of the cells in culture compared with that of the intact liver of the embryos in ovo. 2. In this chick-embryo system, drugs that cause accumulation of uroporphyrin within 19–24 h can only do so in culture; in contrast, 2-allyl-2-isopropylacetamide and 3,5-diethoxycarbonyl-1,4-dihydrocollidine, which stimulate production of protoporphyrin, are effective both in culture and in ovo. 3. A role of exogenous iron in worsening drug-induced uroporphyria was demonstrated in cultures of hepatocytes; iron also caused preferential accumulation of uroporphyrin from added 5-aminolaevulinate in the absence of a porphyrogenic chemical. 4. Uroporphyria was induced in cultures of hepatocytes by drugs of widely different structures, suggesting that the primary molecular target with which they interact may be relatively aspecific in its binding characteristics. 5. These results are briefly discussed, and two alternative hypotheses for the drug-induced effect in uroporphyrinogen metabolism are considered.

Hexachlorobenzene and other polyhalogenated aromatic compounds induce in man and experimental animals a hepatic porphyria (henceforth referred to as 'uroporphyria') characterized by a defect of the liver enzyme uroporphyrinogen decarboxylase and by marked accumulation of uroporphyrin and other highly carboxylated porphyrins [reviewed by Elder (1978) and Smith & De Matteis (1980)]. The mechanism by which polyhalogenated chemicals induce the enzymic defect is not yet known, but a role for iron is suggested by the observations that, in rodents, concurrent administration of large doses of iron will worsen the metabolic disorder and the underlying enzymic defect (Taljaard et al., 1971; Smith & Francis, 1983), whereas a condition of iron deficiency will afford protection (Sweeney et al., 1979). An important step forward in the study of the underlying mechanisms has been the finding (Sinclair & Granick, 1974) that chicken-embryo hepatocytes in culture are very sensitive to polyhalogenated hydrocarbons and develop a marked uroporphyria within hours of exposure to the appropriate chemicals, whereas, in the intact rodent, continuous treatment for several days or weeks is usually necessary before a condition of uroporphyria becomes established. The reasons for the marked sensitivity of the chicken hepatocyte system (whether due to some intrinsic property of the chick-embryo liver, to the culture conditions in vitro, or to both) are not yet known. Also, although a defect of the uroporphyrinogen decarboxylase has been documented in chicken hepatocyte cultures treated with polyhalogenated chemicals (Sinclair et al., 1983; Swain et al., 1983; De Verneuil et al., 1983a), attempts by the latter authors to influence the degree of porphyria by iron overload have proved unsuccessful.

We have now investigated the ability of several drugs to induce uroporphyria in chicken hepatocyte cultures and compared the response of cells in culture with that of the intact liver of the embryos.
in ovo. Drugs that cause accumulation of uroporphyrin can only do so in culture; in contrast, drugs such as AIA and DDC, which stimulate production of protoporphyrin, are effective both in culture and in ovo. The structural requirements for the induction of uroporphyrinia by drugs is less clearly defined than originally suspected, suggesting that chemicals will inhibit the metabolism of uroporphyrinogen by first interacting with a relatively aspecific molecular target. A role of exogenous iron in worsening the uroporphyrinia caused by drugs is now demonstrated in cultures of hepatocytes, and iron is also found to cause preferential accumulation of uroporphyrin from added ALA in the absence of a porphyrogenic chemical.

Materials and methods

Chicken embryos

Embryos, 16 days of age, of Rhode Island Red × White Rock chickens, were obtained from a commercial supplier and after 16–24h in a humidified incubator at 37°C they were either injected with drugs or used to prepare hepatocytes for culture. Drugs, dissolved in 0.1 ml of DMSO, were injected into the fluids surrounding the embryo through a small hole in the shell and the air sac; the embryos were returned to the incubator for further 24 h before obtaining their liver for porphyrin analyses. Hepatocytes were isolated from finely chopped livers (which had been perfused in situ by injecting 5 ml of ice-cold HBSS through the heart). The livers were treated with collagenase (0.5 mg/ml) in HBSS without Ca²⁺ and Mg²⁺; the resulting hepatocytes were counted and, after determining their viability by Trypan Blue exclusion (usually > 90%), they were suspended at a density of 0.9 × 10⁶ cells/ml in Williams E medium containing glutamine (2 mm), gentamycin (50 μg/ml), insulin (5.7 μg/ml), cortisol (4.8 μg/ml) and foetal-calf serum (5%, v/v). The hepatocyte suspensions were seeded in either 150-mm-diameter dishes (20 ml/dish) or in eight-well multiplates (2 ml/well, each 26 mm × 33 mm) and incubated in a humidified atmosphere of CO₂/air (1:19) at 37°C. After 20h incubation the medium was replaced by fresh medium of identical composition but containing no foetal serum and the change of medium repeated at 22h after seeding, when any exogenous Fe was added. Drugs and/or exogenous ALA were added at 24h after seeding and cells left to incubate for various periods, usually for 19h.

Analytical techniques

At the end of incubation the medium was removed and diluted for porphyrin determination, with 8 vol. of 1 M HClO₄/methanol (1:1, v/v); this solvent mixture was also added to the cell monolayers to extract cellular porphyrins. The concentration and composition of porphyrins in the extracts were determined fluorometrically in Samco soda-glass tubes (6 mm × 50 mm) by using a Perkin-Elmer LS-5 luminescence spectrometer calibrated with a 100 nm-Copro standard. Cellular protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard after solubilization of the cell monolayer in alkali. The concentration of Uro, Copro, and Proto in the extract were obtained by the method of Grandchamp et al. (1980), involving measurement of fluorescence at three different excitation–emission wavelength pairs and use of a program containing a matrix constructed with standard solutions of the three porphyrins and run on a Apple microcomputer. Pentacarboxylate and heptacarboxylate porphyrins were found to be determined by this method as a mixture of Copro and Uro, Copro predominating (74%) with the former and Uro (76%) with the latter. When tested with porphyrin mixtures of known composition, this fluorometric technique proved reliable and accurate, and results obtained from hepatocyte cultures were in good agreement with those obtained when the accumulating porphyrins were separated by h.p.l.c. as the methyl esters. The latter were prepared by treating, at room temperature overnight in the dark, portions of the culture media or of the cell homogenates with 15 vol. of methanol/conc. H₂SO₄ (9:1, by vol.), a known amount of the copper complex of heptacarboxylate porphyrin being added to the methylaing mixture as internal standard. The methyl esters were transferred to chloroform, washed, dried and chromatographed on a h.p.l.c. silica column (4.6 mm × 250 mm) of Nucleosil 5, by using isocratic elution with methyl acetate/heptane (4:6, v/v) and a flow rate of 1 ml/min for the first 8 min, followed by 1.5 ml/min for a further 20 min. The porphyrin fluorescence of the eluate was monitored with a Gilson filter fluorimeter, and the A₄₅₀, due to the internal standard, with a Cecil spectrophotometer.

Source of special chemicals.

Williams E medium and glutamine were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.; HBSS and foetal-calf serum from Gibco Europe, Paisley, Renfrewshire, Scotland, U.K.; gentamycin from Essex Chemie AG, Lucerne, Switzerland and collagenase (Clostridium histolyticum) from Boehringer, Lewes, East Sussex, U.K.; insulin, cortisol, trisodium nitritoltriacetate and protoporphyrin IX dimethyl ester from Sigma. Uroporphyrin I, coproporphyrin III, pentacarboxylate I and heptacarboxylate I porphyrins (all
Drug-induced uroporphyrin accumulation

methyl esters) were obtained from Porphyrin Products, Logan, UT, U.S.A., and desferrioxamine mesylate and metyrapone (2-methyl-1,2-di-3-pyrlylpropan-1-one) from Ciba Laboratories, Horsham, West Sussex, U.K. 2-Allyl-2-isopropylacetamide and 2-propyl-2-isopropylacetamide were gifts from Roche Products, Welwyn Garden City, Herts., U.K., Aroclor 1254 was from Monsanto Co., St. Louis, MO, U.S.A., and benoxaprofen [2-(2-(4-chlorophenyl)benzoxazol-5-yl)propionic acid] from Lilly Research Centre, Windlesham, Surrey, U.K.

The copper complex of the heptacarboxylate porphyrin was prepared by adding, to the solution of the porphyrin methyl ester in chloroform, 25 vol. of a 0.5% solution of CuAc acetate in chloroform/methanol (19:1, v/v) and heating at 90°C for 5 min. Stock ferric nitritrocate was prepared as described by Shedlofsky et al. (1983) by dissolving ferric citrate and nitritrocate in water to final concentrations of 5mM and 10mM respectively. DDC and DDCox were prepared as described by De Matteis & Gibbs (1975) and N-methylprotoporphyrin-IX dimethyl ester (a mixture of the four structural isomers) was prepared by the method of De Matteis et al. (1981), hydrolysed by treatment with 6M-HCl and stored dissolved in ethanol at −20°C.

Results

Induction of uroporphyrin in chicken hepatocyte cultures by several chemically unrelated drugs

The effect of treating hepatocytes in culture with several drugs on the concentration and composition of the accumulating porphyrins is shown in Table 1(a). Two groups of chemicals can be distinguished according to whether Uro or Proto is the main porphyrin that accumulates, typical examples of the two groups being AIA and DDC on the one hand, and Aroclor 1254 and Lindane on the other. This difference between drugs in the pattern of porphyrin accumulation that they induce has already been reported by Sinclair & Granick (1974). Table 1(a) also shows that drugs which cause accumulation of Uro in chicken hepatocyte cultures are of diverse molecular structure as they include, in addition to Aroclor and Lindane, another halogenated chemical, 1,1′-(2,2,2-trichloroethylide)bis(4-chlorobenzene) (‘DDT’), and also several non-halogenated drugs of various molecular size and shape. Among these are phenobarbitone and DDCox [see also De Verneuil et al. (1983a) and Marks (1978)], 2-phenyl-2,2-diethylacetamide, metyrapone, benoxaprofen and 2-propyl-2-isopropylacetamide. It will be noted that although an increase in Uro was observed with all these chemicals, a considerable variation in potency was nevertheless found between them; some, for example 2-propyl-2-isopropylacetamide, being only moderately active.

The activity of uroporphyrinogen decarboxylase was studied by the indirect method of Sinclair et al. (1983), involving incubation of intact cells with exogenous ALA (in order to bypass the rate-limiting enzyme of porphyrin synthesis, ALA synthase) and analysis of composition of the accumulating porphyrins. This technique has the

Table 1. Effect of several drugs added to chicken hepatocyte cultures on concentration and composition of porphyrins accumulating in the absence (a) or presence (b) of exogenous ALA

<table>
<thead>
<tr>
<th>Drug added (µg/ml of medium)</th>
<th>Total porphyrins (pmol)</th>
<th>Percentage of total</th>
<th>Uro</th>
<th>Proto</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>110</td>
<td>—</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>DDC (10)</td>
<td>540</td>
<td>—</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>AIA (50)</td>
<td>2500</td>
<td>13</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>N-MePP (0.2)</td>
<td>720</td>
<td>—</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Aroclor 1254 (1)</td>
<td>2860</td>
<td>74</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Lindane (5)</td>
<td>1830</td>
<td>71</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>DDT (5)</td>
<td>220</td>
<td>21</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>DDCox (10)</td>
<td>2600</td>
<td>62</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>2-Phenyl-2,2-diethylacetamide (10)</td>
<td>2700</td>
<td>66</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Sodium phenobarbitone (200)</td>
<td>1300</td>
<td>65</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Benoxaprofen (12)</td>
<td>150</td>
<td>44</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Metyrapone (40)</td>
<td>1500</td>
<td>28</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>2-Propyl-2-isopropylacetamide (50)</td>
<td>1500</td>
<td>28</td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>

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1. Accumulation of porphyrins in cultured hepatocytes exposed to various doses of 2-phenyl-2,2-diethylacetamide and DDC\textsubscript{ox} either in the absence or in the presence of exogenous ALA

Values are those determined by fluorimetry in combined cells and media after 19 h of treatment and are shown as nmol/mg of protein in panels (a) and (c) (○, ●, total porphyrins; △, ▲, Uro) and as a percentage of total porphyrins (△, ▲, Uro; ○, ●, Copro; □, ■, Proto) in panels (b) and (d). In all cases open symbols and closed symbols represent values seen in the absence or the presence of exogenous ALA (25 μg/ml of culture) respectively.

Advantage of being simpler to perform, and, according to Sinclair \textit{et al.} (1983), may also be more sensitive than the conventional assays of uroporphyrinogen decarboxylase, as it may detect in the intact cells a reversible inhibition of the enzyme that may be lost when the cells are homogenized and diluted. Under these conditions a marked prevalence of Uro was still demonstrable with all chemicals associated with uroporphyria (Table 1b), suggesting that they all cause a partial block of haem biosynthesis at the level of uroporphyrinogen decarboxylase. In contrast, cells incubated with either AIA or DDC in presence of exogenous ALA accumulated predominantly Proto, indicating that, with these drugs, ferrochelatase, rather than the decarboxylase, was rate-limiting. An interpretation supported by the finding that a similar porphyrin profile was obtained with N-MePP (Table 1) and with desferrioxamine (Table 4 below), two inhibitors of the conversion of Proto to haem.

Fig. 1 shows the result of dose–response experiments, in which the effect of increasing doses of either 2-phenyl-2,2-diethylacetamide or DDC\textsubscript{ox} on concentration and percentage composition of porphyrins was studied. With both drugs a gradual percentage increase in Uro was accompanied (both in presence and in absence of exogenous ALA) by a corresponding decrease in Proto and, to a lesser extent, in Copro; when exogenous ALA was present, Uro increased and Proto decreased also in absolute values. This finding is also compatible with a dose-dependent inhibition of uroporphyrinogen decarboxylase. Similar findings were obtained with Aroclor (results not shown), except that with this drug the concentration required to produce maximum accumulation of Uro was at least ten times lower.

\textit{Experiments in the intact chick embryo}

Attempts to reproduce uroporphyria by administration of large doses of Aroclor or DDC\textsubscript{ox} to chicken embryos \textit{in ovo} (of the same age and strain as those providing hepatocytes for culture) were not successful. When given on its own, Aroclor did not cause liver porphyrin accumulation \textit{in ovo} over
Table 2. Effect of AIA, DDC, DDC<sub>ox</sub> and Aroclor (given on their own or in combination) on the liver porphyrin content of the intact chick embryo in ovo

Embryos (17 days of age) were treated as indicated in the Experimental section and their liver porphyrins determined 24h later by the fluorimetric technique of Grandchamp et al. (1980) and related to the liver protein content. Results are given as averages (± S.E.M.) for the number of observations given in parentheses. *P < 0.05; **P < 0.02, when compared with corresponding value obtained with DDC alone.

<table>
<thead>
<tr>
<th>Treatment and dose (mg/embryo)</th>
<th>Liver porphyrin content (pmol/mg of protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total porphyrins</td>
<td>Proto</td>
</tr>
<tr>
<td>Control, DMSO</td>
<td>3.8 ± 0.36 (11)</td>
<td>2.51 ± 0.2 (11)</td>
</tr>
<tr>
<td>AIA (2)</td>
<td>227 ± 59 (8)</td>
<td>&gt;90%†</td>
</tr>
<tr>
<td>DDC (0.3)</td>
<td>143 ± 41 (14)</td>
<td>&gt;90%†</td>
</tr>
<tr>
<td>Aroclor (2.5)</td>
<td>4.5 ± 0.7 (9)</td>
<td>3.41 ± 0.4 (9)</td>
</tr>
<tr>
<td>DDC&lt;sub&gt;ox&lt;/sub&gt; (10)</td>
<td>38.4 ± 19.1 (11)</td>
<td>36.6 ± 19.1 (11)</td>
</tr>
<tr>
<td>DDC (0.3) + Aroclor (2.5)</td>
<td>622 ± 171 (8)**</td>
<td>&gt;90%†</td>
</tr>
<tr>
<td>DDC (0.3) + DDC&lt;sub&gt;ox&lt;/sub&gt; (10)</td>
<td>433 ± 111 (6)*</td>
<td>&gt;90%†</td>
</tr>
</tbody>
</table>

† With these treatments only Proto could be detected.

a 24h period, and even though DDC<sub>ox</sub> was slightly effective [in agreement with previous findings (Racz & Marks, 1972)], the majority of porphyrin accumulating was, nevertheless, Proto, not Uro. The embryos readily accumulated Proto in response to DDC or AIA, and, when primed with DDC, could respond to either DDC<sub>ox</sub> or Aroclor with massive increases in Proto accumulation (Table 2), as originally reported in intact rodents (De Matteis, 1973; De Matteis & Gibbs, 1975).

Role of iron in uroporphyrina

A role of iron in cultured hepatocytes has been suggested by previous findings that desferrioxamine, an iron chelator, could reduce the block at the uroporphyrinogen decarboxylase (Sinclair & Granick, 1974; De Verneuil et al., 1983a), but attempts to demonstrate in cell cultures a direct potentiation effect of exogenous iron had not been successful. We now confirm the protective effect of desferrioxamine and also find that when iron is added to cultures in a form that can be readily taken up by cells (White & Jacobs, 1978; Shedlovsky et al., 1983), i.e. complexed with nitrilotriacetate, it can potentiate the uroporphyrin caused by Aroclor. This was shown by direct fluorimetric determination of porphyrin composition (results not given) and confirmed by extracting the porphyrin methyl esters and separating them on h.p.l.c. (Table 3); the increase in Uro and heptacarboxylate porphyrin, characteristic of uroporphyrin, was found to be exacerbated by iron and partially prevented by desferrioxamine. The uroporphyrin caused by a non-halogenated drug, metyrapone, was also exacerbated by iron; the dose-dependent accumulation of Uro and the corresponding decrease in Proto were both significantly greater in presence of iron than in its absence (Fig. 2), suggesting that iron added directly to cells in culture can potentiate the inhibition of uroporphyrinogen metabolism caused by drugs, as shown by Smith & Francis (1983) in chronic-administration experiments involving intact rodents. Therefore a direct cellular effect of iron appears to be involved.

An unexpected finding was the ability of desferrioxamine and iron to produce changes in Uro accumulation from added ALA in the absence of a porphyrigenic chemical (Table 4). The most pronounced changes observed after desferrioxamine and iron concerned the amounts of Proto accumulating from exogenous ALA, which were markedly increased and decreased respectively. Desferrioxamine also caused a significant decrease in Uro accumulating from added ALA, not only as a percentage of total porphyrins (as might have been expected; Sinclair & Granick, 1975), but also in absolute amounts, an effect confirmed by measuring Uro and heptacarboxylate porphyrin by h.p.l.c. In contrast, Fe caused a preferential accumulation of Uro from added ALA, but the effects of Fe were less consistent; in most experiments, like the one described in Table 4, Uro increased both as a percentage of total porphyrins and in absolute amount, in others a similar percentage increase was seen, but the actual amount of Uro remained unchanged. These effects of Fe, as well as the potentiation of drug-induced uroporphyrina, could not be reproduced by equivalent amounts of sodium nitrilotriacetate.

Discussion

The present work has confirmed the marked sensitivity of chicken hepatocytes to drugs that induce accumulation of Uro and also shown that...
Table 3. Protective effect of desferrioxamine and potentiation by exogenous iron of the accumulation of Uro and heptacarboxy-
late porphyrin caused by Aroclor in hepatocyte cultures

Cultures of hepatocytes were prepared in 150mm-diameter dishes; ferric nitroltriacetate was added to a final
collection of 50 μM-Fe at 22h, and desferrioxamine (1.5 mM) and Aroclor at 24h; 19h later, porphyrins were
extracted from both cells and media and separated as the methyl esters by h.p.l.c. Results are those of an individual
experiment and were confirmed at least once.

<table>
<thead>
<tr>
<th>Treatment and dose (μg/ml of culture)</th>
<th>Proto</th>
<th>Copro</th>
<th>Pentacarboxylate</th>
<th>Hexacarboxylate</th>
<th>Heptacarboxylate</th>
<th>Uro</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>–</td>
<td>150</td>
<td>80</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Aroclor (0.1)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>320</td>
<td>350</td>
<td>–</td>
</tr>
<tr>
<td>Cells</td>
<td>–</td>
<td>145</td>
<td>70</td>
<td>–</td>
<td>230</td>
<td>110</td>
</tr>
<tr>
<td>Medium</td>
<td>–</td>
<td>160</td>
<td>135</td>
<td>167</td>
<td>700</td>
<td>290</td>
</tr>
<tr>
<td>Aroclor (0.1) + Fe</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1300</td>
<td>2500</td>
<td>–</td>
</tr>
<tr>
<td>Cells</td>
<td>–</td>
<td>50</td>
<td>240</td>
<td>280</td>
<td>1700</td>
<td>1000</td>
</tr>
<tr>
<td>Medium</td>
<td>1300</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1050</td>
<td>2030</td>
</tr>
<tr>
<td>Aroclor (5)</td>
<td>2000</td>
<td>440</td>
<td>500</td>
<td>390</td>
<td>1300</td>
<td>670</td>
</tr>
<tr>
<td>Desferrioxamine</td>
<td>2060</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cells</td>
<td>2010</td>
<td>180</td>
<td>40</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

this response can only be produced in culture. Even though Aroclor and DDCox did not cause uroporphyra in ovo, they could both potentiate markedly the accumulation of porphyrins caused by DDC, but here again Proto, not Uro, accumulated. This indicates that both Aroclor and DDCox must be able to reach the liver of the intact embryo, where they can stimulate liver porphyrin formation by the ‘aspecific effect’ related to lipid solubility (De Matteis & Gibbs, 1975; Maxwell & Meyer, 1978). However, they cannot elicit in ovo their primary effect on uroporphyrinogen decarboxylation, which is such a characteristic feature of their effect in culture. We conclude that either the mechanism involved in the specific defect of uroporphyrino-
gen metabolism is activated in culture, or alternatively, that the liver cell possesses in ovo a protective mechanism against this effect of drugs that is then lost on culturing.

The mechanism by which the characteristic block in uroporphyrinogen metabolism is pro-
duced is still obscure. There is evidence from the related drug-induced uroporphyrin of the rat that although the catalytic activity of uroporphyrino-
gen decarboxylase is markedly impaired, the concent-
ration of immunoreactive enzyme remains unchanged (Elder & Sheppard, 1982). A direct inhibitory effect of polychlorinated biphenyls on the activity of uroporphyrinogen decarboxylase has been demonstrated in vitro at relatively high concentrations (Kawanishi et al., 1981). However, further studies on the purified enzyme and in intact cells suggest that, at the dose levels which are effective in vivo, the porphorygenic chemicals are not themselves inhibitory, but interact with en-
yzymatic systems in the liver to generate an active inhibitor (De Verneuil et al. 1983a,b). The latter has been postulated to be a metabolite of the poly-
halogenated compounds (Sinclair & Granick, 1974; Debets et al., 1980; Swain et al., 1983; De Verneuil et al., 1983a; Cantoni et al., 1984), sufficiently reactive to become covalently bound to thiol groups at the enzyme active centre (Debets et al., 1980). This hypothesis will explain why pheno-
obarbitone, an inducer of cytochrome P-450, increases the inhibition of uroporphyrinogen decarboxylase due to 2,3,7,8-tetrachlorodibenzo-p-
dioxin (by producing more of the inhibitory metabolites; De Verneuil et al., 1983a), but does not readily explain why phenobarbitone itself is inhibitory. It is in fact difficult to visualize a reactive metabolite common to the various chemi-
icals now shown to be active and to postulate a mechanism by which iron will facilitate its production, as previously suggested (Sinclair & Granick, 1974). Nevertheless, the active-metabo-
lite hypothesis has attractive features and should be further explored.

An alternative hypothesis that we favour at present is that drugs may cause the metabolic block

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Fig. 2. Potentiation by ferric nitrilotriacetate of the accumulation of uroporphyrin caused by metyrapone in hepatocyte cultures
Ferric nitrilotriacetate was added to cultured hepatocytes 4 and 22h after seeding (each time at a dose of 50nmol of Fe/ml of culture); at 24h metyrapone was added and porphyrins were determined fluorimetrically in cells and media 19h later. Values are given as mol/mg of protein in the top panel (○, ●, total porphyrins; △, ◆, Uro) and as a percentage of total porphyrins (△, ◆, Uro; ○, ●, Copro; □, ■, Proto) in the bottom panel. Closed symbols represent values observed in the presence of exogenous iron in all cases.

indirectly, by stimulating production of reactive species, for example, peroxides and free radicals (De Matteis & Stonard, 1977; Sweeney, 1982). These could then lead to the enzymic block either by producing a long-lived inhibitor such as that detected in porphyric livers by Rios de Molina et al. (1980) and Cantoni et al. (1984), or by modifying the enzyme itself. A contributing factor could also be the oxidation by peroxides of the porphyrinogen substrates to the corresponding porphyrins, as the latter cannot be decarboxylated but can still bind the enzyme (Elder et al., 1983) and inhibit the decarboxylation reaction (Smith & Francis, 1981). The synergistic role of iron on the uroporphyrinogen decarboxylase could then result from the ability of ferrous iron to participate in peroxidative and free-radical reactions (Crichton, 1979).

A metabolic pathway that gives rise in the liver to superoxide radical and hydrogen peroxide and which can be stimulated by lipid-soluble drugs is the microsomal electron-transport chain (containing the NADPH:cytochrome P-450 reductase–cytochrome P-450 complex; White & Coon, 1980). Reactive oxygen species are produced particularly when the electron transport becomes uncoupled from the mono-oxygenation function of cytochrome P-450, as found with drugs that bind the cytochrome effectively but cannot be readily metabolized (Ullrich & Diehl, 1971). Whether this
pathway is involved in the induction of uroporphyra by drugs remains to be determined.

The present work has also shown that iron can cause preferential accumulation of Uro from added ALA in the absence of an exogenous chemical. This effect cannot yet be interpreted unequivocally, as iron also caused a marked decrease in total porphyrin concentration. Nevertheless this finding may be relevant to the observation that iron can also worsen the course of human uroporphyra in cases where no exposure to chlorinated chemicals is involved but a history of hepatitis is found (Kushner et al., 1975). Perhaps in the damaged liver of certain individuals a free radical (or peroxide-producing) mechanism becomes activated in the absence of a porphyrogenic drug, and this can then likewise interact with iron to produce a defect in uroporphyrinogen metabolism.

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