UROPORPHYRIN FORMATION INDUCED BY CHLORINATED HYDROCARBONS (LINDANE, POLYCHLORINATED BIPHENYLS, TETRACHLORODIBENZO-p-DIOXIN). REQUIREMENTS FOR ENDOGENOUS IRON, PROTEIN SYNTHESIS AND DRUG-METABOLIZING ACTIVITY

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Summary: The chlorinated hydrocarbons, lindane (gamma-hexachlorocyclohexane), polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin, induce chick embryo liver cells in culture to rapidly accumulate uroporphyrin III. This is in marked contrast to the delayed effect in animals. The induction is dependent on protein synthesis (cycloheximide inhibited), endogenous iron (chelators inhibited) and perhaps cytochrome P-450 (SKF-525A inhibited). Evidence was obtained for an unstable intermediate that is generated in the liver from chlorinated hydrocarbons which inhibits uroporphyrinogen decarboxylase thus causing uroporphyrin to accumulate. The data suggest that the intermediate may be a hydroxylated derivative of the chlorinated hydrocarbon.

Many chemicals, including barbiturates, steroids and chlorinated hydrocarbons will induce an increase in porphyrin accumulation by chick embryo liver cells in culture (1,2). This increase in accumulation is preceded by an increase in the activity of the enzyme, delta-aminolevulinic acid synthetase.

Humans, other mammals and some birds accumulate large amounts of uroporphyrin in their livers after treatment with chlorinated hydrocarbons (3,4,5,6,7). However, the effect is delayed, varying from several days in the case of birds, to several weeks for mammals. In this paper we will show that the cultured cells respond rapidly to the chemicals to specifically accumulate uroporphyrin. This accumulation is dependent on both protein synthesis and endogenous iron. Our results suggest that these cells may metabolize the chlorinated hydrocarbons to active metabolites which inhibit the enzyme, UROgen decarboxylase

Materials and methods: The chlorinated hydrocarbons used were: lindane (gamma-hexachlorocyclohexane) 99.5% from Shell Company; Aroclor 1254, a mixture of

Abbreviations: URO, uroporphyrin; COPRO, coproporphyrin; PROTO, protoporphyrin; ALA, delta-aminolevulinic acid.

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chlorinated biphenyls predominantly 4,5 and 6 chlorines per molecule, from Monsanto; 2,4,5,2',4',5'-hexachlorobiphenyl from Analabs; and 2,3,7,8-tetrachlorodibenzo-p-dioxin from Dow Chemical Company. All except the dioxin were dissolved in ethanol. The dioxin was dissolved in acetone. One to two microliters of solvent were added per ml of culture medium. Desferrioxamine was Desferal from Ciba and was freshly prepared in saline from the ampoule; the activity decreases when the powder is exposed to moist air but less so in a dessicator at 4°. Rhodotorulic acid was a gift from Dr. H. Akers of this university. SKF-525A (2-diethylaminoethyl 2,2-diphenylvalerate HCl) was a gift from Smith Kline and French laboratories. Piperonyl butoxide was 96% and was a gift from FMC Corporation.

The composition and quality of porphyrins extracted from cells or homogenates in 1N perchloric acid (aqueous):methanol (1:1) were determined fluorometrically on the Hitachi-Perkin Elmer spectrofluorometer using the filters and phototube described previously (8). This procedure yields a 2-banded spectrum with peaks at about 605 and 660nm. The ratios of the peak heights at these wavelengths permit PROTO to be simply distinguished from URO and/or COPRO. The actual identification of URO was confirmed by thin layer chromatography on silica gel by the kerosene/chloroform (2:8) system. After determining the ratios of the peak heights, and the fluorescence yield at 660nm of standard solutions, this method can be used to easily give the % composition of PROTO vs. URO and/or COPRO and the quantity of porphyrins in mixtures. The method will be described in detail in a subsequent publication.

To determine the isomer composition of the URO's they were decarboxylated to COPRO (9) and chromatographed on Eastman Chromagrams (silica gel) (10).

RESULTS

The chlorinated hydrocarbons cause the cells to accumulate uroporphyrin. Iron is involved. Table I shows that 2 of the tested chlorinated hydrocarbons, lindane and 2,4,5,2',4',5'-hexachlorobiphenyl, cause the accumulation of URO. This is in contrast to allylisopropylacetamide and other inducers which cause the accumulation of mainly PROTO. Incubation of the cells with any tested inducer together with a chelator of iron (e.g. desferrioxamine) caused the accumulation of PROTO rather than URO.

TABLE I

Effect of chlorinated hydrocarbons on chick embryo liver cells in culture

Treatment	Porphyrin pmole/mg protein	Type of porphyrin
None	12	PROTO
Desferrioxamine (1.5mM)	18	PROTO
Allylisopropylacetamide (200µg/ml)	900	80% PROTO*
Allylisopropylacetamide + Desferrioxamine	1700	PROTO
Lindane (3µg/ml)	280	URO
Lindane + Desferrioxamine	1100	PROTO
Hexachlorobiphenyl (2µg/ml)	90	URO
Hexachlorobiphenyl + Desferrioxamine	250	PROTO

* and 20% URO + COPRO
Primary chick embryo liver cells were cultured as described (11). The last change of medium was to the completely defined Ham-F12 medium containing insulin. Chemicals were added and porphyrin assayed in cells and medium after a further 16 hr incubation.

In other words, addition of the chelator removed the block in the biosynthetic chain so that PROTO accumulated. The other chlorinated hydrocarbons described in this paper also block porphyrin synthesis at the URO step. Of the chelators tested, effective and non-toxic ones in appropriate concentrations were: desferrioxamine (1.5mM), CaMg EDTA (equimolar Ca^{2+}, Mg^{2+}, EDTA) (10mM), 8-hydroxyquinoline sulfonate (0.1mM), and rhodotorulic acid (1.5mM).

Another effect of the chelator of iron, independent of the effect on the kind of porphyrin accumulated, is to increase the total porphyrin, i.e. to cause a potentiation. This is due to an increase in the total activity of the enzyme, delta-aminolevulinic acid synthetase (11).

Active metabolism of the cells is required for URO-production. In these experiments

TABLE II

Requirements for URO accumulation in the presence of different chlorinated hydrocarbons* in chick embryo liver cells in culture

Expt.	Addition	Porphyrin	Required for URO accumulation
1	ALA**	PROTO	
2	ALA + Chlorinated HC	URO	Chlorinated HC
3	ALA + Chlorinated HC + Chelator	PROTO	Endogenous iron
4	ALA + Chlorinated HC + Cycloheximide	PROTO	Protein synthesis
5	ALA + Chlorinated HC + SKF-525A	PROTO	Cytochrome P-450 dependent metabolism
6	ALA + Chlorinated HC + Piperonyl butoxide	PROTO	Cytochrome P-450 dependent metabolism
7	ALA + Chlorinated HC + Heme	URO	Induction of ALA-synthetase not required

* The individual chlorinated hydrocarbons in μg/ml that were used were: lindane (3); 2,4,5,2',4',5'-hexachlorobiphenyl (2); Aroclor 1254 (3); tetrachlorodibenzo-p-dioxin (0.03).

** The concentrations in μg/ml used were: for ALA, 25; for cycloheximide, 0.1; for SKF-525A, 5; for piperonyl butoxide, 5; for heme, 5. Culture conditions and chelator concentrations were as described in legend to Table 1.

(Table II) exogenous ALA was given so that porphyrin production was not dependent on the activity of the cellular ALA-synthetase. The total porphyrin from ALA whether URO or PROTO was about the same regardless of the treatment. 1. The accumulation of URO by the cells was dependent on protein synthesis because cycloheximide (and emetine) blocked URO formation (expt 4). 2. Endogenous iron is required because in the presence of chelators of iron, PROTO, rather than URO is formed (expt 3). 3. The inhibition by SKF-525A and piperonyl butoxide (expts 5,6) suggests that the compounds act via metabolism possibly involving cytochrome P-450 since these are compounds known to inhibit drug metabolism (12). 4. The chlorinated hydrocarbons

have 2 effects: they cause induction of synthesis of ALA-synthetase, an effect which is blocked by heme (11); and they cause URO accumulation from added ALA, an effect which is not blocked by heme (expt 7).

Evidence for a labile intermediate generated in vivo, detected in liver homogenates, and required for URO formation. Liver homogenates from untreated chick embryos when incubated with ALA accumulated PROTO (Table III). Addition of chlorinated hydrocarbons plus ALA to the homogenate from untreated embryos did not affect the porphyrin produced. However, as seen in Table III, when chick embryos were previously treated with Aroclor 1254 and their livers were homogenized and incubated with ALA, a short-lived component (or activity) appeared to be present in the homogenate which caused URO to accumulate. The short life of this component was shown by preincubating the fresh liver homogenate for 1 hr. before addition of ALA. This preincubation caused the loss of the ability of the homogenate to form URO from ALA, i.e.

TABLE III

Evidence for labile compound that causes URO accumulation by liver homogenates from chick embryos pretreated with Aroclor

Pretreatment of embryo	Preincubation of homogenate before addition of ALA	Porphyrin accumulated after addition of ALA
-	-	PROTO
-	+	PROTO
AROCLOR	-	URO
AROCLOR	+	PROTO

16-day chick embryos were injected with 15mg Aroclor 1254 in 0.2ml dimethyl sulfoxide. After 1.5 days homogenates were prepared in 0.25M sucrose, 50mM tris chloride (pH 7.4) and preincubated or not for 1hr, 37°. Incubation volume was 0.2ml containing 125µg wet weight of liver. ALA (3µg) was added to all tubes after preincubation period and tubes were incubated for another 1hr, 37°.

when ALA was added 1 hr. after preincubation, the porphyrin that was formed was PROTO. Thus once the blocking effect caused by the short-lived component has been removed, synthesis to PROTO proceeds. The fact that PROTO is formed indicates in addition that no damage of enzymes from ALA to PROTO has been caused by the chlorinated hydrocarbon or its metabolites.

The liver homogenates from control and Aroclor-treated embryos both contained very little porphyrin and it was PROTO. In the embryos, excess ALA is evidently required to observe the effect of the short-lived component in blocking at the URO step.

Effect of chlorinated phenols on URO production in liver homogenates. Rats and mice completely metabolize lindane to a mixture of di-, tri-, tetra- and penta- chlorophenols (13,14,15) and in part metabolize chlorinated biphenyls to the hydroxylated derivatives (16,17). The possibility occurred to us that a phenol metabolite might be

TABLE IV

Effect of phenols on URO production from ALA by liver homogenates

Addition	Porphyrin accumulated
ALA	PROTO
ALA + 2,4,6-trichlorophenol	PROTO
ALA + tetrachlorophenol	45% PROTO*
ALA + pentachlorophenol	URO
ALA + 2,4-dinitrophenol	URO
ALA + pentachlorophenol + chelator	URO
ALA + pentachlorophenol + Mn^{2+}	URO
ALA + pentachlorophenol + diphenylamine	URO

* and 55% URO

Liver homogenates from untreated embryos were prepared as in legend to Table III. Phenols were 1mM added in 1µl of acetone. Chelators were desferrioxamine (1.5mM); EDTA (10mM); and α,α-dipyridyl (0.5mM). Diphenylamine and Mn^{2+} were 1mM.

the actual blocking compound or related to it. We therefore tested certain phenols (1mM) plus ALA (0.15mM), added directly to liver homogenates from untreated chick embryos prepared as described in the legend to Table III. The 2,4,6-trichlorophenol caused PROTO accumulation. In contrast, the tetrachlorophenol caused 45% PROTO + 55% URO formation. Pentachlorophenol and 2,4-dinitrophenol caused only URO accumulation. Perhaps the electron-withdrawing groups such as chloro- or nitro- on the aromatic ring function to stabilize some intermediate required for blocking at the URO step.

URO formation in the homogenate containing ALA and pentachlorophenol is not affected by the addition of a number of chelators of iron (1.5mM desferrioxamine, 10mM EDTA, 0.5mM α,α-dipyridyl). Therefore iron is not necessary for this particular reaction. This reaction is different from the reaction that occurs in the cultured cells in which the chlorinated hydrocarbons require the presence of iron to produce URO (Table II, expt 3). Attempts to seek for the presence of a radical generated by the chlorinated phenols were unsuccessful. Manganese ions and diphenylamine have been used to block lipid peroxidation that is generated by free radicals in rat liver microsomes (18). When Mn^{2+}(1mM) or diphenylamine (1mM) were incubated together with the chlorophenols in a liver homogenate they did not prevent URO accumulation from ALA.

Homogenates of embryonic heart treated directly with pentachlorophenol accumulated just as much URO in the presence of ALA as did the liver homogenates. This shows that the pentachlorophenol reaction is not specific to the liver. Cytochrome P-450 is probably very low in heart homogenate. If cytochrome P-450 is low it would suggest that cytochrome P-450 may not be involved in the pentachlorophenol reaction to block the synthesis of heme at the URO step.

DISCUSSION

Figure 1 summarizes the possible actions of the chlorinated hydrocarbons in the blocking of porphyrin formation at the URO step: 1. There is an induction of a metabolizing system - this requires protein synthesis since it is blocked by cycloheximide. 2. The chlorinated hydrocarbon is converted to a metabolite by a metabolizing system containing cytochrome P-450, since known inhibitors of cytochrome P-450 action (e.g. SKF-525A) prevent URO formation. 3. Iron is required at some reaction

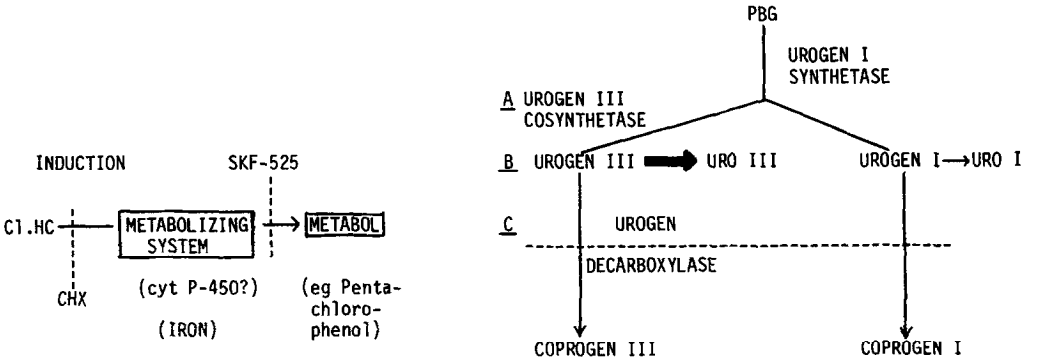

Fig. 1. Summary of actions of chlorinated hydrocarbons to cause URO accumulation.

step for the formation of the metabolite that causes URO accumulation, and 4. The metabolite may be a hydroxylated derivative of the chlorinated hydrocarbons since pentachlorophenol caused URO accumulation in the homogenate.

Three ways may be considered by which the chlorinated hydrocarbons can act probably via a metabolite (Fig. 1):

A) Inhibition of UROgen III cosynthetase. If this were the case then mainly the URO I isomer would have accumulated which is reported to occur when supernatants of homogenates of pig or human liver are incubated with ALA plus ferrous iron (19). However, the URO isomer accumulated by the cultured cells or the liver homogenates we found to be mainly the III isomer as well as some I. Therefore inhibition of cosynthetase as a mechanism of URO formation may be rejected. This conclusion is consistent with the fact that rats treated with hexachlorobenzene excrete URO III in the urine (20).

B) There may be a very rapid direct oxidation of the reduced UROgen III to URO III. In the case where pentachlorophenol was added to liver homogenates in the presence of ALA, the product formed was UROgen as indicated by the fact that no fluorescent porphyrin appeared, except after extraction and oxidation by iodine or in the presence of light. This result does not support the idea that the metabolite of chlorinated hydrocarbons generates a mechanism for rapid autoxidation of UROgen to URO.

C) Only a third possibility remains: the inhibition of the

enzyme, UROgen decarboxylase. We have not as yet directly demonstrated that this enzyme is inhibited. Inhibition of this enzyme would be consistent with 1) the findings of Taljaard et al (21) that the enzyme is completely deficient in rats treated for several weeks with both hexachlorobenzene and iron and 2) that lower levels of the enzyme have been found in rats treated with hexachlorobenzene alone (M. Tomio and S. San Martin de Viale, personal communication).

These findings support the conclusion that the chlorinated hydrocarbons are converted by liver metabolism to products that inhibit the enzyme, UROgen decarboxylase. Such products have tentatively been identified as the hydroxyl derivatives of the parent compounds which are known metabolites. However no metabolites have yet been reported for the very chemically inert dioxin. It has therefore been assumed that dioxin acts directly. The rapid effect on chick embryo liver cells seen with dioxin suggests that this may be a very good system for investigating whether it acts directly or via some metabolite.

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