EFFECTS OF CHLORINATED BENZENES ON THE ACTIVITIES OF δ -AMINOLEVULINIC ACID SYNTHETASE AND HEME OXYGENASE AND ON THE CONTENT OF HEMOPROTEIN IN THE LIVER OF RATS*

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(Received July 16, 1980)

Effects of chlorinated benzenes on the activities of δ -aminolevulinic acid (δ -ALA) synthetase and heme oxygenase, the rate-limiting enzyme in heme biosynthesis and degradation respectively, and on the incorporation of ^3H - δ -ALA into hemoprotein in the liver of male rats were investigated.

Monochlorobenzene (MCB) and 1,2,4-trichlorobenzene (TRCB) stimulated significantly the activities of above both hepatic enzymes.

After a single injection of 200 mg/kg, heme oxygenase activity was enhanced rapidly and sustained markedly for at least 48 hr by MCB treatment, while that was also enhanced but restored nearly to control levels at 48 hr by TRCB. δ -ALA synthetase activity was once decreased at 6 hr and restored within 12 hr and then reached peak levels (about 2–3 times to control levels) at 24 hr by MCB or TRCB treatment. However, this activity was sustained for 48 hr by TRCB treatment, whereas returned again to nearly control levels by MCB at 48 hr. Cytochrome P-450 content at 48 hr was significantly decreased by MCB (63% to control), in contrast, increased by TRCB (200% to control).

When MCB or TRCB injected once daily throughout the study, biphasic disappearance of radioactivity incorporated into CO-binding particles was shown in control and MCB- or TRCB-treated rats. The half-life of the fast phase was about 8 hr in control and about 6 or 13 hr in MCB- or TRCB-treated rats respectively.

Keywords—monochlorobenzene; 1,2,4-trichlorobenzene; δ -aminolevulinic acid synthetase; heme oxygenase; cytochrome P-450; incorporation of ^3H - δ -aminolevulinic acid; CO-binding particles

Halogenated benzenes, which are used in the industry, agriculture, and even home, have been shown to influence the metabolism of foreign organic compounds.^{1–7)} The biochemical properties of hexachlorobenzene, in particular, has been extensively studied and identified to be a potent inducer of xenobiotic metabolism.^{2,7–9)} Our previous studies^{3–5)} indicated that a number of chlorinated benzenes except monochlorobenzene also induce microsomal drug-metabolizing

enzymes in the liver of rats in relatively higher doses used and shorter time periods, and that especially 1,2,4-trichlorobenzene is a most potent inducer among these compounds. Two compounds, monochlorobenzene (MCB) and 1,2,4-trichlorobenzene (TRCB), were of particular interest because both chlorinated benzenes enhanced markedly δ -aminolevulinic acid (δ -ALA) synthetase activity, the initial and rate-limiting enzyme in porphyrin biosynthetic path-

* Parts of this work were presented at the 96th Meeting of Pharmaceutical Society of Japan, Nagoya, April 1976, and at the 100th Meeting, Tokyo, April 1980.

way. Cytochrome P-450 content, however, was decreased by the treatment with MCB, whereas increased significantly by TRCB. Since induction of cytochrome P-450 by various drugs and chemicals is associated with an increase in δ -ALA synthetase activity,¹⁰⁻¹⁴⁾ the effect of MCB on cytochrome P-450 content might be due to the stimulation of heme degradation or to the inhibition of porphyrin utilization.

In this study, therefore, we have determined the activities of δ -ALA synthetase and heme oxygenase which is the rate-limiting enzyme in heme degradation, and have made an attempt to clarify the relationship between cytochrome P-450 content and the enzyme activities in heme metabolic pathway.

MATERIALS AND METHODS

Chemicals — Monochlorobenzene (MCB) and 1,2,4-trichlorobenzene (TRCB) were obtained from Nakarai Chemicals Ltd., Kyoto, Japan. δ -aminolevulinic acid (δ -ALA) and hemin were purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo and Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan, respectively. Nicotinamide adenine dinucleotide phosphate, glucose 6-phosphate disodium salt, and glucose 6-phosphate dehydrogenase were obtained from Boehringer Man-

nheim Yamanouchi Co., Ltd., Tokyo, Japan. δ -[3,5-³H(N)]-Aminolevulinic acid hydrochloride (³H- δ -ALA, 5.05 Ci/mmol, New England Nuclear Corporation) was purchased from Nippon Isotope Kyokai, Tokyo, Japan. Other chemicals of reagent grade were obtained from commercial sources and used without further purification.

Animals — Male rats of the Wistar strain weighing 110 to 130g were used throughout the study. They were housed in an air-conditioned room with free access to food and water.

Assay Procedures — Chlorinated benzenes were dissolved in olive oil, and were injected intraperitoneally to animals. The control animals received the appropriate vehicle. For a dose-response experiments, animals were injected with MCB at a single dose of 50, 100 and 200 mg/2 ml/kg 24 hr before sacrifice. The time course after treatment with MCB or TRCB at a single dose of 200 mg/2 ml/kg was examined by sacrificing animals at 6, 12, 24 and 48 hr following administration. For a incorporation of ³H- δ -ALA into homoprotein, animals were treated with MCB or TRCB (200 mg/2 ml/kg) once daily for 3 days prior to the injection of ³H- δ -ALA (50 μ Ci/2 ml/kg in 0.9% NaCl), and killed either 1 or 4 hr after the isotope injection. For a disappearance of

TABLE I. Dose-Related Effect of MCB on the Activities of Hepatic δ -ALA Synthetase and Heme Oxygenase and on the Content of Cytochrome P-450

Treatment	Enzyme activity		Content of Cytochrome P-450 ^{c)}
	δ -ALA synthetase ^{a)}	Heme oxygenase ^{b)}	
Control	35.0 $\pm$ 1.1	1.49 $\pm$ 0.16	1.04 $\pm$ 0.08
MCB			
50 mg/kg, i.p.	42.0 $\pm$ 4.6	4.38 $\pm$ 0.24 ^{d)}	1.00 $\pm$ 0.13
100 mg/kg, i.p.	50.5 $\pm$ 0.8 ^{d)}	6.66 $\pm$ 0.59 ^{e)}	0.96 $\pm$ 0.07
200 mg/kg, i.p.	73.2 $\pm$ 4.0 ^{e)}	12.93 $\pm$ 0.89 ^{e)}	0.73 $\pm$ 0.04 ^{d)}

Animals were injected with monochlorobenzene (MCB) 24 hr before sacrifice.

Each value represents the mean $\pm$ S.E. of 4 rats.

a) nmol δ -ALA/g liver/hr.

b) nmol bilirubin/mg protein/hr.

c) nmol/mg protein.

d) Significantly different from control, $p < 0.05$.

e) Significantly different from control, $p < 0.01$.

labeled hemoprotein from CO-binding particles, animals treated with each chlorinated benzene (200 mg/2 ml/kg) once daily for 7 days, and on the 4th day were injected with ^3H - δ -ALA (50 $\mu\text{Ci}/2\text{ ml/kg}$) and killed at 6, 12, 24, 48, 72 and 96 hr later. The rats were killed by decapitation, and the livers were perfused *in situ* with a cold 0.9% NaCl solution, and then homogenized with 3 volumes of 0.25 M sucrose containing 1mM EDTA in a Potter-Elvehjem homogenizer with a teflon pestle. Preparation of microsomes was carried out by procedures described previously.¹⁵⁾ Protein concentration was measured by the method of Lowry *et al.*¹⁶⁾ using bovin serum

albumin as standard. δ -ALA synthetase activity of liver homogenates was determined by the method of Marver *et al.*¹⁷⁾ Heme oxygenase activity of microsomes was determined by the method of Maines and Kappas.¹⁸⁾ Cytochrome p-450 content was estimated as described by Omura and Sato.¹⁹⁾ The radioactivity incorporated into CO-binding particles was measured by the method of Levin and Kuntzman.²⁰⁾

RESULTS

As shown in Table I, MCB produced a roughly dose-related increase in activities of δ -ALA synthetase and heme oxygenase in the liver of rats when measured 24 hr after the administration. A single injection of MCB at a dose of 200 mg/kg caused the highest increase in activities of δ -ALA

FIG. 1. Time Course of Induction of δ -ALA Synthetase Activity in the Liver from Rats Injected with MCB or TRCB

Animals were injected intraperitoneally with MCB or TRCB at single dose of 200 mg/kg, and were killed at the indicated times after the administration. Each point represents the mean $\pm$ S.E. of 4 rats.

—●— control, —○— MCB, —△— TRCB.

FIG. 2. Time Course of Elevation of Heme Oxygenase Activity in the Liver from Rats Injected with MCB or TRCB

Conditions were as described in Fig. 1. Each point represents the mean $\pm$ S.E. of 4 rats.

—●— control, —○— MCB, —△— TRCB.

synthetase and heme oxygenase, about 210 and 870% of control levels respectively, while the content of cytochrome P-450 was only 70% of control.

Figure 1 shows the time course of the effect of MCB or TRCB at a single dose of 200 mg/kg on hepatic δ -ALA synthetase activity. This enzyme activity was decreased approximately 30% when measured 6 hr after the administration, and subsequently restored and thereafter reached to peak values at 24 hr. This increased enzyme activity was again returned nearly to control levels 48 hr after the administration of MCB, whereas the activity of TRCB continued to increase to about two-fold of the control levels at 48 hr.

Figure 2 shows the time course of induction of heme oxygenase after a single treatment at 200 mg/kg with MCB or TRCB. A significant increase was observed 6 hr after the injection of MCB or TRCB. This elevated enzyme activity continued for 24 hr after the treatment with TRCB, and was sustained for 48 hr after the administration of MCB.

FIG. 3. Time Course of Loss and Induction of Cytochrome P-450 Content in the Liver from Rats injected with MCB or TRCB

Conditions were as described in Fig. 1.

Each point represents the mean $\pm$ S.E. of 4 rats.

—●— control, —○— MCB, —△— TRCB.

TABLE II. Effect of MCB or TRCB Treatment on Incorporation of ^3H - δ -ALA into CO-Binding Particles

Time after ^3H - δ -ALA		Treatment		
		Control	MCB	TRCB
1	Co-Binding particles			
	dpm/mg protein	3290 $\pm$ 210	2710 $\pm$ 230	2870 $\pm$ 60
	dpm/g liver	74660 $\pm$ 7790	60580 $\pm$ 7040	75490 $\pm$ 2090
	dpm/100g b.w.	361050 $\pm$ 33910	340410 $\pm$ 30470	496210 $\pm$ 23040 ^{a)}
4	Cytochrome P-450	0.95 $\pm$ 0.07	0.78 $\pm$ 0.03	1.98 $\pm$ 0.25 ^{a)}
	(n mol/mg protein)			
	Co-Binding particles			
	dpm/mg protein	2960 $\pm$ 320	2060 $\pm$ 270	2670 $\pm$ 310
4	dpm/g liver	62980 $\pm$ 6840	42780 $\pm$ 5670	68380 $\pm$ 4010
	dpm/100g b.w.	309760 $\pm$ 34420	240400 $\pm$ 27190	429850 $\pm$ 33450
	Cytochrome P-450	0.96 $\pm$ 0.07	0.84 $\pm$ 0.07	2.06 $\pm$ 0.13 ^{a)}
	(n mol/mg protein)			

Animals were injected intraperitoneally with monochlorobenzene (MCB) or 1,2,4-trichlorobenzene (TRCB) at a single dose of 200mg/kg once daily for 3 days.

On the 4th day, ^3H - δ -ALA (50 $\mu\text{Ci/kg}$) was injected intraperitoneally to rats, and the animals were killed either 1 or 4 hr after the injection of the isotope.

Each value represents the mean $\pm$ S.E. of 4 rats.

a) Significantly different from control, $p < 0.05$.

As shown in Figure 3, a remarkable decrease of cytochrome P-450 content was noted 24 hr after the injection of MCB, and this significant depression was still noted at 48 hr. On the contrary, TRCB showed about two-fold increase of cytochrome P-450 content as compared to control levels at 48 hr.

Table II shows the incorporation of radioactivity into microsomal hemoprotein either 1 or 4 hr after the injection of ^3H - δ -ALA (50 $\mu\text{Ci/kg}$). The radioactivity incorporated into the CO-binding particles was observed to have a tendency to decrease by pretreatment with MCB (200 mg/kg/day) for 3 days prior to the isotope injection, whereas the radioactivity per 100g body

weight after the treatment with TRCB increased 1 hr after the isotope injection.

Figure 4 shows the results of disappearance of radioactivity with time from CO-binding particles in control and chlorinated benzenes-treated rats. The data indicated a biphasic pattern of disappearance of radioactivity from the CO-binding particles obtained from both control and MCB- or TRCB-treated rats. A corrected half-life of the fast phase was about 8 hr in control and approximately 6 or 13 hr in MCB- or TRCB-treated animals respectively, as shown in inset of Figure 4. This corrected half-life of the fast phase was obtained by subtracting the radioactivity contributed by the slow phase after extrapolating

FIG. 4. Disappearance of Labeled Hemoprotein from CO-Binding Particles obtained from MCB- or TRCB-Treated Rats

Animals were injected intraperitoneally with MCB or TRCB (200 mg/kg/day) for 3 days prior to the injection of ^3H - δ -ALA (50 $\mu\text{Ci/kg}$), and were sacrificed at various times after the administration of the isotope. The injection of MCB or TRCB continued every 24 hr throughout the study. Each point represents the mean $\pm$ S.E. of 3 to 4 rats. Values used for the determination of the corrected half-life of the fast phase (inset) were obtained by first extrapolating the slow phase to zero time and then subtracting the values of the extrapolated slow phase from values of the uncorrected fast phase.

—●— control, —○— MCB, —△— TRCB.

back to zero times. The half-life of the slow phase hemoprotein was about 48 hr in control or TRCB-treated rats, but about 54 hr in MCB-treated animals.

DISCUSSION

In the present study, we observed that MCB significantly decreased cytochrome P-450 content, but markedly enhanced the δ -ALA synthetase activity. These findings agreed with our previous results³⁾ and further confirmed our earlier speculation on the ability of MCB which would accelerate the degradation of cytochrome P-450 heme, as well as lower the utilization of synthesized porphyrin. Therefore, we measured the heme oxygenase activity, the rate-limiting enzyme in heme degradation. This increase in enzyme activity was clearly dose-related after a single administration of MCB, and the elevated activity was continued for at least 48 hr.

In addition, we found a relatively rapid disappearance of radioactivity in labeled hemoprotein from CO-binding particles as compared to control, namely about 8 or 6 hr in corrected values of half-life at fast phase in control or MCB-treated rats, respectively. This half-life value in control rats coincided well with the value reported by Levin and Kuntzman.²⁰⁾ Furthermore, pretreatment of rats with MCB for 3 days showed a tendency to depress the incorporation of ^3H - δ -ALA into hemoprotein either 1 or 4 hr after the isotope injection. These findings suggest that MCB could induce a rapid degradation of heme or cytochrome P-450 heme, and furthermore it seems to inhibit, in part, the heme biosynthetic pathway somewhere.

On the other hand, a single injection of TRCB showed an increase of two-fold in δ -ALA synthetase activity as compared to control even 48 hr after the injection, whereas heme oxygenase activity at 48 hr nearly restored to control levels. In the disappearance of labeled hemoprotein from CO-binding particles, in addition, the half-life value at fast phase was about 13 hr in TRCB-treated rats. These findings suggest that TRCB would con-

tinuously increase hepatic heme synthesis through the prolonged induction of δ -ALA synthetase, while it may not affect the decrease in cytochrome P-450 heme because of rapid restoration of the heme oxygenase. Consequently, TRCB may lead to the increase in cytochrome P-450 content seen at 48 hr after its administration. These different actions on the δ -ALA synthetase or heme oxygenase and on the incorporation of ^3H - δ -ALA into hemoprotein or the values of half-life in labeled hemoprotein from CO-binding particles seems to have resulted in the overall difference on the cytochrome P-450 content by the treatment with MCB or TRCB.

It was reported²¹⁻²⁴⁾ that allylisopropylacetamide and other compounds containing allyl radical markedly increased the δ -ALA synthetase activity, but these compounds decreased the cytochrome P-450 content at early phase after the injection, and that these results seemed to indicate a destruction of cytochrome P-450 heme induced by active metabolites of the above compounds *in vivo*. Chemicals which promoted destruction of liver heme also may stimulate more effectively δ -ALA synthetase, since heme is known to exercise a negative feed back control on the activity of porphyrin biosynthetic pathway at the level of δ -ALA synthetase. Therefore, the regulation of δ -ALA synthetase and of heme oxygenase in the liver are closely interrelated. In this study, we found the different effects on the δ -ALA synthetase and heme oxygenase with time when administered MCB or TRCB to animals, and these might be caused by the difference in the formation of active metabolites in both chlorinated benzenes. Further investigation for metabolism or for active metabolites of both chlorinated benzenes will be needed to clarify the relationship between cytochrome P-450 content and both enzyme activities.

Cobalt as well as other metals, such as iron, cadmium and lead, induced markedly heme oxygenase activity^{18,25-27)} in addition, both chlorinated benzenes also enhanced noteworthy enzyme activities as shown in this study. This

appears to be toxicological problems, in consideration of the problem of contamination by chlorinated benzenes as one of the environmental pollutants, since these compounds remarkably affect both rate-limiting enzymes on the heme biosynthesis and degradation. Further study on a very low dose of chlorinated benzenes is being investigated.

REFERENCES

- 1) F.Oesch, D.M.Jarina, J.W.Daly and J.M.Rice: Induction, activation, and inhibition of epoxide hydrazase: An anomalous prevention of chlorobenzene-induced hepatotoxicity by inhibitor of epoxide hydrazase, *Chem. - Biol. Interact.*, **6**, 189–202 (1973).
- 2) H.M.Mehendale, M.Fields and H.B.Matthews: Metabolism and effects of hexachlorobenzene on hepatic microsomal enzymes in the rats, *J. Agr. Food Chem.*, **23**, 261–265 (1975).
- 3) T.Ariyoshi, K.Ideguchi, Y.Ishizuka, K.Iwasaki and M.Arakaki: Relationship between chemical structure and activity. I. Effects of the number of chlorine atoms in chlorinated benzenes on the components of drug-metabolizing system and the hepatic constituents, *Chem. Pharm. Bull.*, **23**, 817–823 (1975).
- 4) T.Ariyoshi, K.Ideguchi, K.Iwasaki and M.Arakaki: Relationship between chemical structure and activity. II. Influences of isomers in dichlorobenzene, trichlorobenzene and tetrachlorobenzene on the activities of drug-metabolizing enzymes, *Chem. Pharm. Bull.*, **23**, 824–830 (1975).
- 5) T.Ariyoshi, K.Ideguchi, K.Iwasaki and M.Arakaki: Relationship between chemical structure and activity. III. Dose-response or time-course of induction in microsomal enzymes following treatment with 1,2,4-trichlorobenzene, *Chem. Pharm. Bull.*, **23**, 831–836 (1975).
- 6) G.P.Carlson and R.G.Tardiff: Effect of chlorinated benzenes on the metabolism of foreign organic compounds, *Toxicol. Appl. Pharmacol.*, **36**, 383–394 (1976).
- 7) G.P.Carlson: Induction of cytochrome P-450 by halogenated benzenes, *Biochem. Pharmacol.*, **27**, 361–363 (1978).
- 8) M.D.Stonard and P.S.Nenov: Effect of hexachlorobenzene on hepatic microsomal enzymes in the rat, *Biochem. Pharmacol.*, **23**, 2175–2183 (1974).
- 9) J.C.Turner and R.S.Green: Effect of hexachlorobenzene on microsomal enzyme systems, *Biochem. Pharmacol.*, **23**, 2387–2390 (1974).
- 10) O.Wada, Y.Yano, G.Urata and K.Nakao: Behavior of hepatic microsomal cytochromes after treatment of mice with drugs known to disturb porphyrin metabolism in liver, *Biochem. Pharmacol.*, **17**, 595–603 (1968).
- 11) J.Baron and T.R.Tephly: The role of heme synthesis during the induction of hepatic microsomal cytochrome P-450 and drug metabolism produced by benzpyrene, *Biochem. Biophys. Res. Commun.*, **36**, 526–532 (1969).
- 12) A.Poland, J.Goldstein P.Hickman and V.W.Burse: A reciprocal relationship between the induction of δ -aminolevulinic acid synthetase and drug metabolism produced by *m*-dichlorobenzene, *Biochem. Pharmacol.*, **20**, 1281–1290 (1971).
- 13) F.De Matteis and A.Gibbs: Stimulation of liver 5-aminolaevulinic acid synthetase by drugs and its relevance to drug-induced accumulation of cytochrome P-450: Studies with phenylbutazone and 3,5-diethoxycarbonyl-1,4-dihydrocollidine, *Biochem. J.*, **126**, 1149–1160 (1972).
- 14) C.Rajamanickam, J.Amrutavalli, M.R.S.Rao and G.Padmanaban: Effect of hexachlorobenzene on haem synthesis, *Biochem. J.*, **129**, 381–387 (1972).
- 15) T.Ariyoshi, E.Takabatake and H.Remmer: Drug metabolism in ethanol induced fatty liver, *Life Sci.*, **9**, Part II, 361–369 (1970).
- 16) O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall: Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, **193**, 265–275 (1951).
- 17) H.S.Marver, D.P.Tschudy, M.G.Perlroth and A.Collins: δ -Aminolevulinic acid synthetase. I. Studies in liver homogenates, *J. Biol. Chem.*, **241**, 2803–2809 (1966).
- 18) M.D.Maines and A.Kappas: Studies on the mechanism of induction of haem oxygenase by cobalt and metal ions, *Biochem. J.*, **154**, 125–131 (1976).
- 19) T.Omura and R.Sato: The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature, *J. Biol. Chem.*, **239**, 2370–2378 (1964).
- 20) W.Levin and R.Kuntzman: Biphasic decrease of radioactive hemoprotein from liver microsomal CO-binding particles, *J. Biol. Chem.*, **244**, 3671–3676 (1969).
- 21) U.A.Meyer and H.S.Marver: Chemically induced porphyria: Increased microsomal heme turnover after treatment with allylisopropylacetamide, *Science*, **171**, 64–66 (1971).
- 22) W.Levin, E.Sernatinger, M.Jacobson and R.Kuntzman: Destruction of cytochrome P-450 by secobarbital and other barbiturates containing allyl groups, *Science*, **176**, 1341–1343 (1972).
- 23) G.Abbritti and F.De Matteis: Decreased levels of cytochrome P-450 and catalase in hepatic porphyria caused by substituted acetamides and barbiturates: Importance of the allyl group in the molecule of the active drugs, *Chem. -Biol. Interact.*, **4**, 281–286

- (1971/72).
- 24) W.Levin, M.Jacobson, E.Sernatinger and R.Kuntzman: Breakdown of cytochrome P-450 heme by secobarbital and other allyl-containing barbiturates, *Drug Metab. Dispos.*, **1**, 275–285 (1973).
- 25) F. De Matteis and A.H.Gibbs: Inhibition of haem synthesis caused by cobalt in rat liver: Evidence for two different sites of action, *Biochem. J.*, **162**, 213–216 (1977).
- 26) H.C.Kransny and D.J.Holbrook, Jr.: Effects of cadmium on heme oxygenase and hemoproteins in smooth and rough endoplasmic reticulum of rat liver, *Biochem. Pharmacol.*, **27**, 364–366 (1978).
- 27) N.G.Ibrahim, S.T.Hoffstein and M.L.Freedman: Induction of liver cell haem oxygenase in iron-overloaded rats, *Biochem. J.*, **180**, 257–263 (1979).