Biochemical Pharmacology, Vol. 27, pp. 361–363. Pergamon Press, 1978. Printed in Great Britain.

Induction of cytochrome P-450 by halogenated benzenes

(*Received* 13 *January* 1977; *accepted* 19 *May* 1977)

It has been recognized for a number of years that agents which cause induction of the hepatic microsomal enzyme systems responsible for the metabolism of drugs, pesticides and other xenobiotics can be divided into two classes [1]. One is often termed the phenobarbital-type and the other represented by a number of polycyclic hydrocarbons is called the benzo (a) pyrene- or 3-methylcholanthrene-type. These two groups differ in the spectrum of xenobiotic metabolizing reactions induced and the species of cytochrome P-450 which is increased. The former increases this carbon monoxide binding pigment giving a peak at 450 nm whereas in the latter case the peak is at 448, thus giving rise to the term P-448 [2] or P_1-450 [3]. Recent evidence indicates that there are multiple forms of cytochrome P-450 that are induced by phenobarbital [4, 5]. This simple division into two groups has been complicated by the findings of Alvares *et al.* [6] that polychlorinated biphenyls (PCB) cause the induction of a broad spectrum of enzymes similar to phenobarbital, but that the species of P-450 induced is really P-448. However, this may be due to some PCB isomers resembling phenobarbital in the species of cytochrome P-450 and the spectrum of enzymes activities induced and some isomers resembling 3-methylcholanthrene [7].

In the past 2 years, interest has developed in the ability of halogenated benzenes to induce xenobiotic metabolism. Several investigators have studied the effects of the fungicide hexachlorobenzene [8–10]. Our own investigations [11, 12] and those of others [13–15] have revealed that even the less halogenated benzenes cause induction, although they appear not to be as potent as hexachlorobenzene. The species of cytochrome P-450 induced by hexachlorobenzene has been reported as simply P-450 [8–13]. The species for the less halogenated compounds has also been reported as P-450 [11–15]. However, a recent paper by Stonard [16] reported that hexachlorobenzene administration resulted in a mixed type induction perhaps similar to that for the polychlorinated biphenyls [6]. The peak wavelength for the P-450 measurements was reported as 448.7 nm. In view of the apparent conflict between these findings and our results for 1,4-dichlorobenzene, 1,2,4-trichlorobenzene, 1,3,5-trichlorobenzene, 1,4-dibromobenzene, 1,2,4-tribromobenzene, 1,3,5-tribromobenzene, hexabromobenzene and hexachlorobenzene itself [11, 12], it was necessary to test simultaneously several of these compounds, in addition to phenobarbital and 3-methylcholanthrene as positive controls.

Additional studies were also performed to examine characteristics of these compounds with those known for the two classes of inducing agents. Microsomal NADPH–cytochrome *c* reductase activity was measured, since this is known to be increased by phenobarbital but is not increased or may actually be decreased by pretreatment with 3-methylcholanthrene [17]. Also, experiments were carried out on the ability of 1,2,4-tribromobenzene, which had been shown to be the most potent of the lesser halogenated benzenes [11, 12], to cause increases in xenobiotic metabolism additive with those of phenobarbital and 3-methylcholanthrene. It has been shown that inducers from the two classes can give additive results when each is given at doses which cause maximal stimulation, but that agents within the same class do not give such additivity [2, 18].

Groups of male Sprague–Dawley rats (Laboratory Supply Co., Indianapolis, IN) weighing 200–250 g were given p.o. daily 20 mg/kg of 1,2,4-trichlorobenzene (Eastman Organic Chemicals, reagent grade), hexachlorobenzene, or 1,2,4-tribromobenzene (Eastman Organic Chemicals, reagent grade) or 400 mg/kg of hexabromobenzene (Aldrich Chemical Co., 98%) in corn oil for 14 days. Although the hexachlorobenzene was practical grade (Eastman Organic Chemicals), gas chromatographic analysis revealed that it was at least 99+ per cent pure. Controls received an equivalent volume of corn oil (0.1% body weight). Phenobarbital (50 mg/kg, i.p., for 4 days) and 3-methylcholanthrene (40 mg/kg, i.p., 72 and 48 hr prior to sacrifice) were used as positive controls. The livers were perfused *in situ* with cold isotonic saline, and microsomal fractions were obtained as previously described [19]. NADPH–cytochrome *c* reductase activity and cytochrome P-450 content were determined using the method of Dallner [20]. A Beckman DB-G spectrophotometer was used for the former and a newly installed and calibrated Beckman Acta C-III spectrophotometer for the latter. Proteins were determined according to the method of Lowry *et al.* [21].

In the studies on the interactions of the enzyme-inducing abilities of the halogenated benzenes with those of the well-known inducing agents phenobarbital and 3-methylcholanthrene, rats were given either 10 mg/kg of 1,2,4-tribromobenzene or the corn oil vehicle for 14 days p.o. In one study, half of each group received phenobarbital (50 mg/kg, i.p.) for the last 2 days. In the other, half of each group received 3-methylcholanthrene (40 mg/kg, i.p.) 72 and 48 hr before sacrifice. The detoxification of EPN (*O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate) and *O*-demethylation of *p*-nitroanisole were determined according to the procedure of Kinoshita *et al.* [22]. Azoreductase activity of the 9000 *g* supernatant fraction was measured using the method of Smith and Van Loon [23]. UDP glucuronyltransferase activity was measured utilizing the method of Lucier *et al.* [24] with naphthol as the substrate.

In the studies on cytochrome P-450 and cytochrome *c* reductase, comparisons were made between the treated groups and the control using Student's *t*-test. In the interaction studies, groups were compared with controls and each other using Duncan's new multiple range finding test [25]. In both cases, the level of significance chosen was $P < 0.05$.

As expected from our previous experiments [11, 12], 1,2,4-trichlorobenzene, 1,2,4-tribromobenzene and hexachlorobenzene administration resulted in increases in NADPH–cytochrome *c* reductase activity (Table 1). Hexabromobenzene, despite the fact that it was given at a much higher dose level than the other compounds, was without effect. The positive controls also gave the expected results with a large increase being observed after phenobarbital pretreatment and no changes being seen with 3-methylcholanthrene pretreatment. Elevations occurred in the level of cytochrome P-450 with 1,2,4-tribromobenzene and hexachlorobenzene. The elevation due to 1,2,4-trichlorobenzene was probably real but was not statistically significant. Again hexabromobenzene was without effect, although in other studies it had shown small but significant increases

Table 1. Comparison of effects of halogenated benzenes on microsomal cytochrome *c* reductase and cytochrome P-450*

Compound	Dose (mg/kg)	NADPH-cytochrome *c* reductase (nmoles cyto. *c* reduced/ min/mg protein)	Cytochrome P-450 (nmoles/mg protein)	Wavelength of maximum absorbance (nm)
Corn oil (vehicle)	†	164 ± 14	1.09 ± 0.18	450.3 ± 0.05
1,2,4-Trichlorobenzene	20†	224 ± 13‡	1.65 ± 0.21	450.1 ± 0.04
1,2,4-Tribromobenzene	20†	261 ± 21‡	2.07 ± 0.14‡	450.2 ± 0.07
Hexachlorobenzene	20†	272 ± 27‡	2.31 ± 0.11‡	450.1 ± 0.09
Hexabromobenzene	400†	157 ± 13	1.33 ± 0.04	449.4 ± 0.10‡
Phenobarbital	50§	288 ± 42‡	2.80 ± 0.28‡	450.1 ± 0.10
3-Methylcholanthrene	40‖	131 ± 21	1.65 ± 0.14‡	448.2 ± 0.07‡

* Values are expressed as the mean ± S.E. for a group of five rats.
† Administered daily p.o. for 14 days.
‡ Significantly different from control ($P < 0.05$).
§ Administered i.p. 96, 72, 48 and 24 hr prior to sacrifice.
‖ Administered i.p. 72 and 48 hr prior to sacrifice.

Table 2. Interaction of 1,2,4-tribromobenzene and phenobarbital on the induction of xenobiotic metabolism*

Treatment	$\frac{\text{Liver wt}}{\text{Body wt}} \times 100$	EPN detoxification (μg *p*-nitrophenol/ 50 mg/30 min)	Glucuronyl-transferase (nmoles/min/mg protein)	Azoreductase (ng/min/mg protein)
Control	3.66 ± 0.15†	4.2 ± 0.4†	4.8 ± 0.9†	58.5 ± 15.0†
Phenobarbital	4.19 ± 0.10‡	10.5 ± 0.8‡	5.5 ± 0.6†,‡	73.5 ± 13.3†
1,2,4-TBB	4.57 ± 0.18‡	14.2 ± 1.8§	6.5 ± 0.4†,‡	73.2 ± 10.7†
Phenobarbital + 1,2,4-TBB	4.55 ± 0.13‡	13.1 ± 0.8‡,§	6.9 ± 0.7‡	79.3 ± 10.1†

* Rats received corn oil or 10 mg/kg/day of 1,2,4-tribromobenzene (TBB) p.o. for 14 days. On the last 2 days, half of each group received phenobarbital (50 mg/kg/day, i.p.). Values are expressed as the mean ± S.E. for a group of six rats.
†–§ Values with different superscripts are significantly different from one another ($P < 0.05$).

in P-450 content [12]. The positive controls, phenobarbital and 3-methylcholanthrene, gave the expected increases. When the wavelength maximum for the cytochrome P-450 peak was determined, the control was 450.3 ± 0.05 nm (Table 1). 3-Methylcholanthrene had its characteristic peak at 448.2 ± 0.07 nm. The only other value significantly different from the control value was for hexabromobenzene with a peak at 449.4 ± 0.10 nm.

When phenobarbital and 1,2,4-tribromobenzene were given separately and in combination (Table 2), all three treated groups were significantly different from the control in the liver to body weight ratio but were not different from one another. There were no differences among the control or treated groups in azoreductase activity. In EPN detoxification, all three treatments increased activity, but the value for the combination of phenobarbital and 1,2,4-tribromobenzene was intermediate between the values for the single agent treatments. Even for glucuronyltransferase where the combination value was significantly different from the control, it was not different from either phenobarbital or 1,2,4-tribromobenzene alone. However, when similar studies were carried out to determine the

Table 3. Interaction of 1,2,4-tribromobenzene and 3-methylcholanthrene on the induction of xenobiotic metabolism*

Treatment	*O*-demethylation of *p*-nitroanisole (μg *p*-nitrophenol/ 50 mg/30 min)	Azoreductase (ng/min/mg protein)
Corn oil–corn oil	4.7 ± 0.4†	37.6 ± 3.3†
Corn oil–3-MC	24.1 ± 3.9‡	48.7 ± 2.0‡
1,2,4-TBB–corn oil	16.3 ± 1.0§	66.4 ± 4.5§
1,2,4-TBB–3-MC	45.6 ± 3.4‖	77.0 ± 3.3‖

* Rats were given either 10 mg/kg of 1,2,4-tribromobenzene (1,2,4-TBB) in corn oil or an equivalent volume of corn oil p.o. for 14 days. On days 12 and 13, half of each group received 40 mg/kg of 3-methylcholanthrene (3-MC), i.p. Values are expressed as the mean ± S.E. for a group of six rats.
†–‖ Values with different superscripts are significantly different from one another ($P < 0.05$).

additivity of effect of 1,2,4-tribromobenzene with 3-methylcholanthrene (Table 3), both treatments resulted in increases in the *O*-demethylation of nitroanisole and in azoreductase activity, but the combination resulted in increases which were very closely additive.

The results of these studies support those previously reported in that the chlorinated and brominated benzenes are inducers of xenobiotic metabolism. Moreover, it would appear that these compounds are of the phenobarbital-type rather than the 3-methylcholanthrene-type or mixed type. This is based on the finding that the species of cytochrome P-450 induced has a maximum which is not different from the control. This was found to be true even for hexachlorobenzene. The results seen for hexachlorobenzene are not due to contamination by small amounts of extremely potent inducers such as dibenzo-*p*-dioxins or dibenzofurans, since they induce P-448 rather than P-450 [26, 27]. The reason for the discrepancy between our data and those of Stonard [16] is not known. Also the reason for the peak maximum wavelength for the hexabromobenzene-treated animals being different from the control is unclear at the present time. It should be noted that in this study there was no increase in the amount of the cytochrome.

Other evidence also suggests that the inducers are of the phenobarbital-type. There were increases in cytochrome *c* reductase activity similar to that for phenobarbital and not to 3-methylcholanthrene which in this and other studies was found not to increase this activity [17]. Finally the data in Tables 2 and 3 indicate that the inducing ability of 1,2,4-tribromobenzene is additive with that of 3-methylcholanthrene and not with phenobarbital which is in agreement with other investigators regarding the additivity of induction between the two classes [2, 18].

Acknowledgements—The author gratefully acknowledges the technical assistance of Shelley Steva and Anne Gilroy. This study was supported by EPA Grant R804328 and the contents do not necessarily reflect the views of EPA nor does the mention of trade names or commercial products constitute endorsement or recommendation for use.

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REFERENCES

1. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
2. A. P. Alvares, G. Schilling, W. Levin and R. Kuntzman, *Biochem. biophys. Res. Commun.* **29**, 521 (1967).
3. N. E. Sladek and G. J. Mannering, *Biochem. biophys. Res. Commun.* **24**, 668 (1966).
4. M.-T. Huang, S. B. West and A. Y. H. Lu, *J. biol. Chem.* **251**, 4659 (1976).
5. D. A. Haugen, M. J. Coon and D. W. Nebert, *J. biol. Chem.* **251**, 1817 (1976).
6. A. P. Alvares, D. R. Bickers and A. Kappas, *Proc. natn. Acad. Sci. U.S.A.* **70**, 1321 (1973).
7. J. A. Goldstein, P. Hickman, H. Bergman, J. D. McKinney and M. P. Walker, *Fedn Proc.* **36**, 941 (1977).
8. M. D. Stonard and P. Z. Nenov, *Biochem. Pharmac.* **23**, 2175 (1974).
9. J. C. Turner and R. S. Green, *Biochem. Pharmac.* **23**, 2387 (1974).
10. H. M. Mehendale, M. Fields and H. B. Matthews, *J. agric. Fd Chem.* **23**, 261 (1975).
11. G. P. Carlson and R. G. Tardiff, *Toxic. appl. Pharmac.* **36**, 383 (1976).
12. G. P. Carlson, *Ann. N.Y. Acad. Sci.*, (in press).
13. T. Ariyoshi, K. Ideguchi, Y. Ishizuka, K. Iwasaki and M. Arakaki, *Chem. pharm. Bull., Tokyo* **23**, 817 (1975).
14. T. Ariyoshi, K. Ideguchi, K. Iwasaki and M. Arakaki, *Chem. pharm. Bull., Tokyo* **23**, 824 (1975).
15. T. Ariyoshi, K. Ideguchi, K. Iwasaki and M. Arakaki, *Chem. pharm. Bull., Tokyo* **23**, 831 (1975).
16. M. D. Stonard, *Biochem. Pharmac.* **24**, 1959 (1975).
17. A. von der Decken and T. Hultin, *Archs Biochem. Biophys.* **90**, 201 (1960).
18. J. R. Gillette, *Prog. Drug Res.* **6**, 11 (1963).
19. G. P. Carlson, *Toxicology* **4**, 83 (1975).
20. G. Dallner, *Acta path. microbiol. scand.* **166** (suppl.), 7 (1963).
21. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
22. F. K. Kinoshita, J. P. Frawley and K. P. DuBois, *Toxic. appl. Pharmac.* **9**, 505 (1966).
23. E. J. Smith and E. J. Van Loon, *Analyt. Biochem.* **31**, 315 (1969).
24. G. W. Lucier, O. S. McDaniel and H. B. Matthews, *Archs Biochem. Biophys.* **145**, 520 (1971).
25. R. G. D. Steel and J. H. Torrie, *Principles and Procedures of Statistics*, p. 107. McGraw-Hill, New York (1960).
26. J. B. Greig and F. DeMatteis, *Environ. Hlth Perspect.* **5**, 211 (1973).
27. A. Poland and E. Glover, *Molec. Pharmac.* **9**, 736 (1973).