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Isomerically Pure Chlorobiphenyl Congeners and Hepatic Function in the Rat: Influence of Position and Degree of Chlorination

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Isomerically Pure Chlorobiphenyl Congeners and Hepatic Function in the Rat: Influence of Position and Degree of Chlorination. ECOBICHON, D. J. AND COMEAU, A. M. (1975). *Toxicol. Appl. Pharmacol.* 33, 94-105. Isomerically pure mono-, di-, tri-, tetra, penta, hexa-, and octachlorobiphenyls were injected ip into weanling rats at a dosage of 50 mg/kg/day for 3 consecutive days and the animals were killed 96 hr after the last injection. The influence of position and degree of chlorination of the biphenyl nucleus on hepatic function was compared to that produced by purified biphenyl, measuring hepatic *p*-nitroanisole *O*-demethylase, aniline hydroxylase, carboxylesterase, and sulfobromopthalein-glutathione conjugating enzyme activities in the 12,000*g* supernate. For those enzymes closely associated with the hepatic endoplasmic reticulum, enhanced induction of activity was observed with agents having chlorine atoms substituted at the 4- and 4'-positions, irrespective of chlorination at other positions. For those enzymes less discretely localized in the hepatocyte, the position of the chlorine atoms appeared to be less important; enzyme induction was stimulated by unsubstituted biphenyl though enhanced effects were observed following substitution with one or more chlorine atoms.

The toxicologic assessment of commercially available polychlorinated biphenyls has been complicated by the heterogeneity of the chlorobiphenyl congeners and by marked differences in physical and chemical properties that influence the rates of absorption, distribution, biotransformation, and excretion (Grant *et al.*, 1971; Sissons and Welti, 1971; Webb and McCall, 1972; Bush *et al.*, 1974; Ecobichon and Comeau, 1974). Recent evidence has confirmed that not all chlorobiphenyl congeners produce the same pharmacologic effects (Fujita *et al.*, 1971; Vos and Notenboom-Ram, 1972; Chen *et al.*, 1973; Johnstone *et al.*, 1974; Ecobichon and MacKenzie, 1974). Of particular interest is the role played by the position of the chlorine atom on the biphenyl nucleus (Johnstone *et al.*, 1974). The availability of a large number of relatively pure, isomerically separated chlorobiphenyls of known position and degree of chlorination prompted a more extensive investigation of the selective influence of these agents on several functionally diverse, drug-metabolizing enzymes in the rat.

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METHODS

Agents studied. The isomerically pure chlorobiphenyls were purchased from Analabs Inc., a division of New England Nuclear, Boston, Mass., and were used without further purification. Since it was important to study only the effects of position and degree of chlorination, biphenyl (Eastman Organic Chemicals, Rochester, New York) was used as the "control" agent. The biphenyl was purified by preparative thin-layer chromatography on 20 × 100 cm silica gel plates coated with Merck silica gel F-254. *n*-Heptane was employed as the developing solvent. The material was eluted and recrystallized from aqueous methanol (Hutzinger *et al.*, 1971).

The agents were dissolved in warm peanut oil by continuous stirring until solution was effected. The solution was adjusted with additional oil to a final concentration of 17.5 mg/ml. No solubility problems were encountered with the exception of 3,4,3',4'-tetrachlorobiphenyl, as was previously noted (Johnstone *et al.*, 1974). This tetrachlorobiphenyl was administered as a warm (approx. 37°C) solution.

Animal treatment. The animals used were young male Wistar rats (BioBreeding Laboratories) weighing 65–85 g at the beginning of the experiment. Each treatment group, composed of six animals, was housed three to a cage on hardwood shavings, and was provided food and water *ad libitum*. The agents were administered at a dosage of 50 mg/kg ip in a volume of 0.15–0.25 ml of peanut oil solution. Control animals ($n = 18$) were given purified biphenyl in peanut oil at the same concentration and in the same volume of solvent. Groups of biphenyl-treated animals were tested at intervals during the investigations to ensure reproducibility of the assay procedures. The animals were given daily doses for 3 consecutive days and killed 96 hr after the last injection (Schwark and Ecobichon, 1967; Johnstone *et al.*, 1974).

Tissue preparation. The animals were killed by cervical dislocation, quickly opened, and the livers were excised, weighed, and placed in chilled 50-ml beakers in crushed ice. All subsequent steps of tissue homogenization and centrifugation were carried out at 0–4°C. A 5.0-g sample of liver was minced finely with scissors and was washed twice with cold 1.15% KCl to remove trapped blood. The minced tissue was completely homogenized with 20 ml of 1.15% KCl solution by 10 strokes of a loose-fitting, motor-driven Teflon pestle in a 50-ml Potter-Elvehjem glass homogenizer. The homogenate (25 ml) was centrifuged at 4°C for 242,000*g*-min (12,000*g* for 20 min) in a Sorvall RC2-B centrifuge. The postmitochondrial supernate was carefully removed, stored in crushed ice, and used within 2 hr of preparation.

Assays. The nucleic acid (DNA and RNA) content of the hepatic homogenate prior to centrifugation was determined on an aliquot of homogenate from each animal using the method of Schneider (1957) as described previously (Johnstone *et al.*, 1974).

Protein concentrations of the 242,000*g*-min supernate were determined by the method of Hartree (1972), using bovine serum albumin for the preparation of a standard curve.

The mixed function oxidases (*p*-nitroanisole *O*-demethylase and aniline hydroxylase) were assayed by the methods of Netter and Seidel (1964) and Kato and Gillette (1965), respectively. The reaction mixtures contained 0.5 ml of homogenate, 50 μmol of glucose-6-phosphate, 1.0 unit of glucose-6-phosphate dehydrogenase, 100 μmol of nicotinamide, 1.0 μmol NADP, substrate (5.0 μmol of *p*-nitroanisole or 15 μmol of aniline) and sufficient 0.05 M Tris buffer pH 7.4 to yield a final volume of 4.0 ml. For the

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aniline hydroxylase assay, 25 μmol of $MgCl_2$ were added. Incubations were carried out for 10 and 20 min at 37°C with continuous shaking in a Dubnoff metabolic shaker; the reactions were stopped by the addition of 2.0 ml of 20% TCA. Initial rates of product formation were calculated from the rectangular hyperbola plotted for the two incubation intervals.

Nonspecific carboxylesterase activity was determined on aliquots of the postmitochondrial supernatant fraction by a spectrophotometric technique which consisted of a continuous monitor at 322 nm of the formation of free alpha-naphthol from 1×10^{-3} M alpha-naphthyl acetate at pH 7.4 and 37°C (Ecobichon, 1970).

The conjugation of sulfobromophthalein (BSP) with reduced glutathione (GSH) was measured using an aliquot of the supernatant fraction which had been dialyzed overnight at 4°C against 0.1 M sodium pyrophosphate buffer pH 8.2. The method of Goldstein and Combes (1966) was used; the rate of conjugate formation was monitored for 1 min at 37°C and pH 8.2 as described previously (Johnstone *et al.*, 1974).

Enzymatic activities were presented as nanomoles (*O*-demethylase and hydroxylase) of product formed or as micromoles (carboxylesterase) of substrate changed per minute in the total weight of fresh liver per 100 g of body wt. The activity of the BSP–GSH conjugating enzyme was expressed in the same way but in terms of micrograms of conjugate formed per minute.

Statistical significance of the various parameters measured in treated and control groups was analyzed by Student's *t* test for two sample means based on independent samples. Significance was assessed at the 5% level, $p < 0.05$.

RESULTS

It was essential, before studying the effects of chlorination, to determine what effect the biphenyl nucleus had on hepatic enzymes. Table 1 shows the results of the ip

TABLE 1

THE EFFECTS OF INTRAPERITONEALLY ADMINISTERED PEANUT OIL, COMMERCIAL BIPHENYL, AND PURIFIED BIPHENYL DISSOLVED IN PEANUT OIL ON ENZYME ACTIVITIES OF RAT LIVER[a]

Enzyme	Activites (in total wt of fresh liver/100 g body wt)[b]		
	Vehicle	Commercial biphenyl	Purified biphenyl
p-Nitroanisole *O*-demethylase	168.0 ± 23.9	211.5 ± 38.7	195.6 ± 19.1
Aniline hydroxylase	102.7 ± 26.7	352.8 ± 102.8[c]	97.3 ± 15.1
Carboxylesterase	234.4 ± 53.5	336.7 ± 55.3[c]	236.2 ± 24.7
BSP–GSH conjugating enzyme	5.0 ± 0.9	14.4 ± 1.9[c]	8.[illegible] ± 0.9[c]

[a] The animals received 50 mg of biphenyl kg (0.15–0.25 ml of solution) for 3 consecutive days and were killed 96 hr after the last injection. Vehicle-treated animals received peanut oil on the same volume basis. Twelve animals were treated with vehicle, 11 with commercial biphenyl, and 18 with purified biphenyl.

[b] The activities of *O*-demethylase and aniline hydroxylase are expressed as nanomoles of product formed/minute. Carboxylesterase activity is expressed as micromoles of substrate hydrolyzed/minute, while the BSP–GSH conjugating enzyme activity is expressed as micrograms of conjugate formed/minute. Activities are presented in terms of the total liver/100 g of body wt.

[c] Values are statistically different from vehicle-treated control values at $p < 0.05$.

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administration of vehicle (peanut oil), commercially available biphenyl and purified biphenyl on the activities of hepatic *O*-demethylase, aniline hydroxylase, carboxylesterase, and BSP–GSH conjugating enzyme. Treatment with unpurified biphenyl resulted in statistically significant ($p < 0.05$) increases in activities of three of the enzymes over values observed in vehicle-treated controls. In contrast, chromatographically purified biphenyl caused a significant increase only in the BSP–GSH conjugating enzyme activity. Since relatively pure compounds were to be investigated, the values for the purified biphenyl administered in peanut oil were used as control data.

TABLE 2

CHANGES IN LIVER WEIGHT, DNA, AND RNA FOLLOWING INTRAPERITONEAL ADMINISTRATION OF PURE CHLOROBIPHENYLS TO RATS[a]

Agent		Liver weight /100 g body wt.	DNA (mg/g)	RNA (mg/mg DNA)
Biphenyl		5.33 ± 0.25	3.95 ± 0.49	3.99 ± 0.11
Monochloro:				
	2-	5.10 ± 0.12	3.26 ± 0.26	6.76 ± 0.19[b]
	3-	5.29 ± 0.18	3.62 ± 0.07	5.90 ± 0.19[b]
	4-	5.52 ± 0.46	3.73 ± 0.24	5.52 ± 0.13[b]
Dichloro:				
	2,2'-	4.90 ± 0.20	4.26 ± 0.36	4.48 ± 0.14[b]
	2,4'-	5.58 ± 0.38	3.39 ± 0.20	4.22 ± 0.17[b]
	3,3'-	5.39 ± 0.37	3.19 ± 0.33	6.50 ± 0.34[b]
	4,4'-	5.27 ± 0.18	3.36 ± 0.23	4.58 ± 0.24[b]
Trichloro:				
	2,4,4'-	5.74 ± 0.46	2.94 ± 0.21[b]	6.64 ± 0.28[b]
	2,5,2'-	5.29 ± 0.30	3.31 ± 0.29	5.90 ± 0.52[b]
	2,5,3'-	5.20 ± 0.18	3.18 ± 0.22	5.60 ± 0.24[b]
	2,5,4'-	6.28 ± 0.62[b]	2.78 ± 0.30[b]	5.96 ± 0.34[b]
	3,4,2'-	5.19 ± 0.29	3.53 ± 0.23	5.80 ± 0.29[b]
Tetrachloro:				
	2,4,2',4'-	5.94 ± 0.38	3.29 ± 0.12	4.76 ± 0.19[b]
	2,6,2',6'-	5.37 ± 0.15	3.67 ± 0.18	4.32 ± 0.33
	2,3,2',3'-	5.23 ± 0.62	3.53 ± 0.57	3.87 ± 0.40
	2,5,2',5'-	5.19 ± 0.34	3.58 ± 0.21	4.33 ± 0.26
	3,4,3',4'-	6.59 ± 0.36[b]	3.27 ± 0.27	4.50 ± 0.52
Higher congeners:				
	2,3,4,2',5'-	5.48 ± 0.21	3.51 ± 0.21	5.94 ± 0.27[b]
	2,4,5,2',4',5'-	6.17 ± 0.14[b]	3.65 ± 0.21	4.33 ± 0.14
	2,4,6,2',4',6'-	5.47 ± 0.45	3.21 ± 0.29	6.20 ± 0.25[b]
	2,3,4,5,2',3',4',5'-	5.60 ± 0.35	2.56 ± 0.10[b]	7.33 ± 0.27[b]

[a] Values presented are the mean ±SD of 18 biphenyl-treated (control) and 6 chlorobiphenyl-treated animals per group. Values are based on wet wt of tissue.

[b] Values are significantly different from control values, $p < 0.05$.

The influences of each chlorobiphenyl on (1) liver weight in terms of 100 g body wt, (2) DNA and, (3) RNA content of whole homogenates (20% w/v) are presented with the results for control, biphenyl-treated animals in Table 2. Statistically significant ($p <$

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0.05) increases in liver weight were observed for only a very few agents (2,5,4′-trichloro-, 3,4,3′,4′,-tetrachloro-, and 2,4,5,2′,4′,5′-hexachlorobiphenyls). All agents tested caused a reduction in DNA content, which suggested increased cellular size, though these were only significantly different from biphenyl-treated controls in a few instances (Table 2). Increased RNA concentrations were observed with all of the chlorobiphenyl agents tested though significant increases were observed only for some of the agents.

The influence of monochlorobiphenyls on the hepatic *O*-demethylase, hydroxylase, carboxylesterase, and BSP–GSH conjugating enzyme activities are compared in Table 3 with the effects following treatment with purified biphenyl. No significant changes in microsomal *O*-demethylase were noted, though microsomal aniline hydroxylase activities were significantly increased over control values. Carboxylesterase activity was significantly ($p < 0.05$) increased following treatment with the monochlorobiphenyls. No significant ($p > 0.05$) differences were observed for BSP-GSH conjugating enzyme activity between biphenyl- and the chlorobiphenyl-treated animals.

TABLE 3

EFFECTS OF ACUTE INTRAPERITONEAL ADMINISTRATION OF BIPHENYL AND MONOCHLOROBIPHENYLS ON HEPATIC ENZYME ACTIVITY

Treatment	n	Activities (in total wt of fresh liver/100 g body wt)[a] OD[c] (nmol/min)	AH (nmol/min)	CE (μmol/min)	BSP (μg/min)
Biphenyl	18	195.6 ± 19.1	97.3 ± 15.7	236.2 ± 24.7	8.8 ± 0.9
2-Chlorobiphenyl	6	184.8 ± 17.2	188.3 ± 30.4[b]	309.2 ± 35.1[b]	7.5 ± 1.5
3-Chlorobiphenyl	6	193.6 ± 28.1	225.8 ± 55.3[b]	381.7 ± 64.3[b]	9.8 ± 1.5
4-Chlorobiphenyl	6	233.4 ± 32.5	168.1 ± 37.4[b]	306.2 ± 24.5[b]	9.8 ± 1.6

[a] Values presented are the mean ±SD of the number of animals per group.
[b] Values are significantly different from values obtained from biphenyl-treated animals, $p < 0.05$.
[c] The enzymes investigated include *p*-nitroanisole *O*-demethylase (OD), aniline hydroxylase (AH), carboxylesterase (CE), and sulfobromophthalein-glutathione conjugating enzyme (BSP).

Figure 1 shows the influence of a series of isomerically pure dichlorobiphenyls on hepatic *O*-demethylase, aniline hydroxylase, carboxylesterase, and BSP–GSH conjugating enzyme activities in comparison with the effect produced by purified biphenyl. Statistically significant ($p < 0.05$) increases in the activities of the mixed function oxidases (*O*-demethylase and hydroxylase) were observed only with 4,4′-dichlorobiphenyl, whereas carboxylesterase activity was markedly increased by 2,2′-, 2,4′- as well as by 4,4′-dichlorobiphenyl. The BSP–GSH conjugating enzyme was increased only following treatment with 2,2′- and 4,4′-dichlorobiphenyl. No enhancement of activity was observed following treatment with 3,3′-dichlorobiphenyl; the activities of all four hepatic enzymes were lower than those of control, biphenyl-treated animals.

Figure 2 summarizes the influence of a group of isomerically pure trichlorobiphenyls on the hepatic enzymes as compared to the activities detected in biphenyl-treated animals. On the basis of *O*-demethylase activity, significant increases were observed

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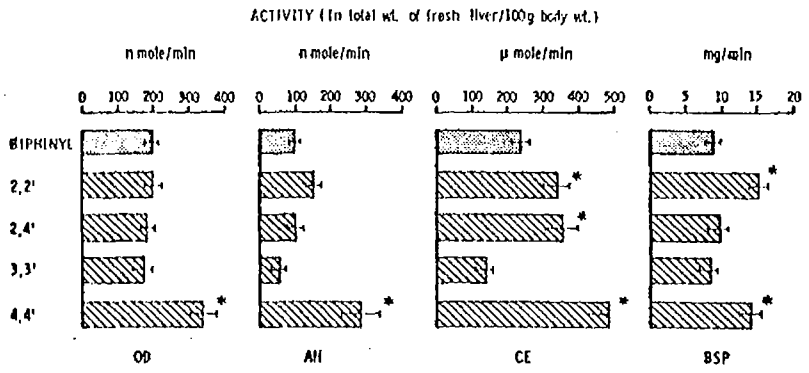


FIG. 1. The effects of pretreatment of young male rats with biphenyl and a series of isomerically pure dichlorobiphenyls on hepatic *p*-nitroanisole *O*-demethylase (OD), aniline hydroxylase (AH), carboxylesterase (CE), and sulfobromophthalein-glutathione conjugating enzyme (BSP) activities. Animals were treated by ip injection for 3 consecutive days and were killed and assayed 96 hr after the last injection. Activities are expressed as nanomoles of product formed/minute (OD and AH), micromoles of substrate hydrolyzed/minute (CE), or as micrograms of conjugate formed/minute (BSP), based on the total weight of fresh liver/100 g body wt. The values (bars) represent the mean enzymatic activities ±SD (lines) of 18 control biphenyl-treated animals and 6 animals per treated group. The asterisk (*) indicates values statistically different ($p < 0.05$) from the biphenyl-treated controls.

only following treatment with 2,5,4′- and 2,4,4′-trichlorobiphenyl. The response of microsomal aniline hydroxylase activity was somewhat different; significant increases were observed following treatment with all but 2,5,3′-trichlorobiphenyl. Significant increases in carboxylesterase and BSP–GSH conjugating enzyme activities were observed with all agents tested though the most marked response was observed for 2,5,4′- and 2,4,4′-trichlorobiphenyl.

The effects of a series of tetrachlorobiphenyls on the various hepatic enzyme activities

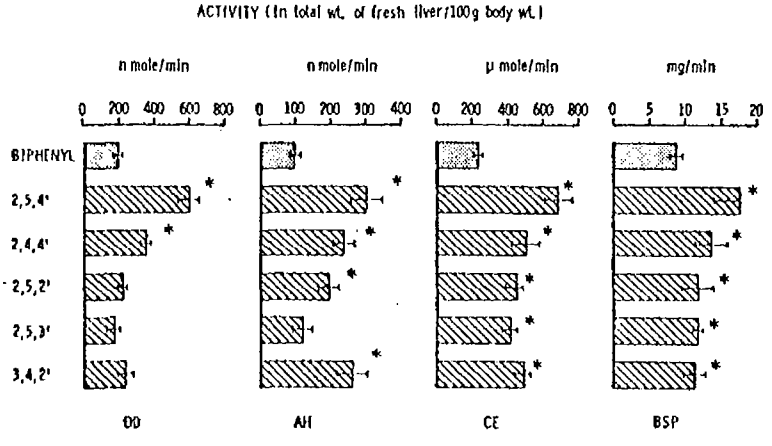


FIG. 2. The effects of pretreatment of young male rats with biphenyl and a series of isomerically pure trichlorobiphenyls on hepatic *p*-nitroanisole *O*-demethylase (OD), aniline hydroxylase (AH), carboxylesterase (CE), and sulfobromophthalein-glutathione conjugating enzyme (BSP) activities. For other details, see Fig. 1.

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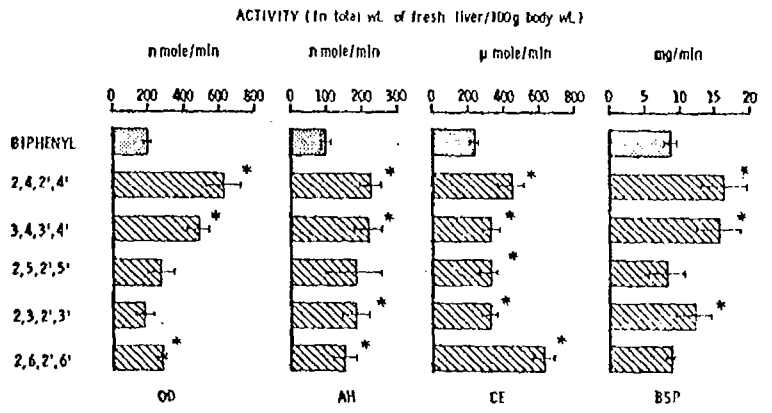


FIG. 3. The effects of pretreatment of young male rats with biphenyl and a series of isomerically pure tetrachlorobiphenyls on hepatic *p*-nitroanisole *O*-demethylase (OD), aniline hydroxylase (AH) carboxylesterase (CE), and sulfobromophthalein-glutathione conjugating enzyme (BSP) activities. For other details see Fig. 1.

as compared to those of biphenyl are shown in Fig. 3. While all the agents tested caused significant ($p < 0.05$) increases in hepatic carboxylesterase activity, the most marked effects were observed with 2,4,2′,4′- and 2,6,2′,6′-tetrachlorobiphenyl. In contrast, the mixed function oxidase, *O*-demethylase, was markedly increased by 2,4,2′,4′- and 3,4,3′,4′-tetrachlorobiphenyl. A small though significant increase was observed in the activity of the latter enzyme following treatment with 2,6,2′,6′-tetrachlorobiphenyl. As was observed in Fig. 2, aniline hydroxylase responded somewhat differently following treatment with tetrachlorobiphenyls. While significant induction was observed with 2,6,2′,6′- and 2,3,2′,3′-tetrachlorobiphenyl, the most marked increases were observed with the 2,4,2′,4′- and 3,4,3′,4′-isomers. Significant ($p < 0.05$) increases in the BSP–GSH conjugating enzyme were observed only with 2,4,2′,4′-, 3,4,2′,4′-, 3,4,3′,4′-, and 2,3,2′,3′-tetrachlorobiphenyl.

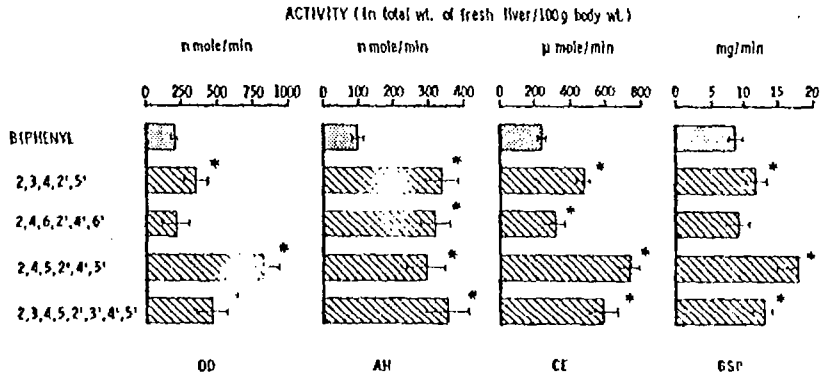


FIG. 4. The effects of pretreatment of young male rats with biphenyl and selected penta-, hexa, and octachlorobiphenyls on hepatic *p*-nitroanisole *O*-demethylase (OD), aniline hydroxylase (AH), carboxylesterase (CE), and sulfobromophthalein-glutathione conjugating enzyme (BSP) activities. For other details, see Fig. 1.

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Figure 4 summarizes the influence of treatment with a pentachloro-, two hexachloro-, and an octachlorobiphenyl on hepatic enzymes compared to the activities detected in biphenyl-treated rats. With the exception of 2,4,6,2′,4′,6′-hexachlorobiphenyl, all of the agents tested caused significant increases in the four enzymatic functions assayed. In contrast, 2,4,6,2′,4′,6′-hexachlorobiphenyl treatment resulted in significant ($p < 0.05$) increases in aniline hydroxylase and carboxylesterase activities whereas the *O*-demethylase and BSP–GSH conjugating enzyme activities were not significantly different from activities in biphenyl-treated animals.

DISCUSSION

The ability of commercial chlorobiphenyl mixtures to alter hepatic function by ultrastructural changes and induction of a variety of hepatic enzymes which act on biotransformation, has been well documented (Nishizumi, 1970; Vos and Beems, 1971; Fujita *et al.*, 1971; Villeneuve *et al.*, 1971; Kimbrough *et al.*, 1972; Bickers *et al.*, 1972; Litterst *et al.*, 1972; Bruckner *et al.*, 1973; Hansell and Ecobichon, 1974). The heterogeneity of these mixtures has hindered toxicologic evaluation and has raised several pertinent questions. Do dichlorobiphenyls produce the same effects as tetra- or hexachlorobiphenyls? Do all isomers of the same degree of chlorination produce comparable toxicologic responses? Are the pathologic and toxicologic alterations observed due to the biphenyl nucleus, to the position(s) occupied by individual chlorine atoms or to the number of chlorines present in the structure? Are hepatic enzymes other than those associated with the endoplasmic reticulum affected by these agents? It is now known that not all chlorobiphenyl congeners possess the same physicochemical and pharmacologic properties (Johnstone *et al.*, 1974). A high priority has been suggested for studies using individual pure compounds (Hammond *et al.*, 1972), though to date such studies have been limited (Fujita *et al.*, 1971; Vos and Notenboom-Ram, 1973; Chen *et al.*, 1973).

The hepatic enzymes, *p*-nitroanisole *O*-demethylase and aniline hydroxylase, that were investigated in this study were selected as representative of endoplasmic reticulum-bound enzymes showing characteristic type I and type II spectral changes involving substrate interaction with cytochrome P450 (Remmer *et al.*, 1966; Schenkman *et al.*, 1967). Hepatic carboxylesterases were selected as examples of enzymes localized predominantly in the endoplasmic reticulum though a small quantity of cytoplasmic enzyme may be present due to a possible solubilization of the membrane-bound enzyme (Schwark and Ecobichon, 1968; Ljungquist and Augustinsson, 1971; Chow and Ecobichon, 1973). The enzyme which catalyzes the conjugation of BSP with GSH is a soluble cytoplasmic enzyme (Combes and Stakelum, 1961). The administration of such well-known inducing agents as phenobarbital and DDT results in markedly enhanced activities of each of these enzymes (Conney, 1967; Schwark and Ecobichon, 1968; Johnstone *et al.*, 1974).

As was shown (Table 1), biphenyl significantly increased the activity of BSP–GSH conjugating enzyme. Compared to the enzyme activity in biphenyl-treated animals, there were no statistically significant ($p > 0.05$) differences observed following treatment with monochlorobiphenyls but, with the exception of 2,4′- and 3,3′-di-, 2,5,2′,5′-, and 2,6,2′,6′-tetra-, and 2,4,6,2′,4′,6′-hexachlorobiphenyls, all of the chlorobiphenyl

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congeners produced significantly ($p < 0.05$) higher though somewhat comparable values. Position and degree of chlorination did not appear to play an important role, though the presence of two or more chlorine atoms on the biphenyl nucleus did have a marked influence on BSP conjugation. A similar situation was observed for the hepatic carboxylesterase activity. Statistically significant increases in activity above that measured following biphenyl treatment were found for all agents tested with the exception of 3,3′-dichlorobiphenyl. Certain of the other congeners, i.e. 4,4′-di-, 2,5,5′-tri-, 2,4,2′,4′-, and 2,6,2′,6′-tetra-, 2,4,5,2′,4,5′-hexa-, and 2,3,4,5,-2′,3′,4′,5′-octachlorobiphenyls, produced much more marked effects than did other chlorobiphenyls.

The effects of the different chlorobiphenyls on the mixed function oxidases should be considered separately since the responses were markedly different. Much greater inductive effects on the response of hepatic *O*-demethylase were observed following treatment with di-, tri-, tetra-, hexa-, and octachlorobiphenyls with chlorine atoms substituted at the 4,4′-positions. No significant increase was observed for 4-chlorobiphenyl. The exception to this rule was 2,4,6,2′,4′,6′-hexachlorobiphenyl where the activity was not different from control values. The poor response to this particular isomer in comparison to other hexachlorobiphenyls tested has been noted previously (Johnstone *et al.*, 1974). Treatment with chlorobiphenyls substituted in other positions resulted in either nonsignificant ($p > 0.05$) or significant ($p < 0.05$) differences though at a much lower level. There were less distinct differences in activities of aniline hydroxylase following chlorophenyl administration, though the same positional phenomenon was observed, i.e., chlorination at the 4- and 4′-positions elicited more marked effects.

Can the above enzyme inducting effects with isomerically pure chlorobiphenyls be explained on the basis of biodegradability? Investigators have shown that, following administration of Aroclor mixtures, the low chlorine analogs were eliminated more rapidly from animal tissues than were the highly chlorinated biphenyls (Grant *et al.*, 1971; Bailey and Bunyan, 1972; Bickers *et al.*, 1972). Block and Cornish (1959) demonstrated that biphenyl and 4-chlorobiphenyl were hydroxylated prior to excretion as conjugated products. Hutzinger *et al.* (1972) showed that, in the rat, biphenyl and 4-chlorobiphenyl were mono- and dihydroxylated, respectively, and excreted in the urine. Monohydroxylated derivatives were formed from 4,4′-di- and from 2,5,2′,5′-tetrachlorobiphenyl. The position of the substituted hydroxy group on 4-chlorobiphenyl has been established at the 4′-position and that on 4,4′-dichlorobiphenyl at the 3-position (Safe *et al.*, 1974). Gardner *et al.*, (1973) have established that metabolism of 2,5,2′,5′-tetrachlorobiphenyl probably occurs via an arene oxide intermediate; the metabolites 3-hydroxy-2,5,2′,5′-tetrachlorobiphenyl, 4-hydroxy-2,5,2′,5′-tetrachlorobiphenyl, and trans3,4-dihydro-3,4-dihydroxy-2,5,2′,5′-tetrachlorobiphenyl were isolated and characterized from rabbit urine. Recently, Bush *et al.* (1974) have demonstrated the influence of 4,4′-chlorination on the persistence of chlorobiphenyls in avian species, and have suggested that substitution at these positions is far more important than the degree of chlorination. Metabolism of chlorobiphenyls via arene oxide intermediates would require the presence of unsubstituted adjacent or vicinal carbon atoms in the biphenyl nucleus (Daly *et al.*, 1972). For chlorobiphenyls of low degree of chlorination, substitution at the 4- and 4′-positions would interfere with possible vicinal carbon interactions between the 3—4 and 4—5 positions and effectively retard biotransformation. The recent evidence of Jensen and Sundstrom (1974) suggests the

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presence of a different hydroxylation mechanism for highly chlorinated agents having isolated unsubstituted positions. The rate of hydroxylation for the latter is much lower than that observed for chlorobiphenyls with unsubstituted vicinal carbons. When 2,4,5,2′,4′,5′-hexachlorobiphenyl was given to rats it was slowly converted and excreted as a monohydroxy derivative, substituted at either the 3- or 6-position. The exact structure awaits confirmation. The inability to rapidly hydroxylate low chlorine-containing agents chlorinated in the 4- and/or 4′-positions and those highly chlorinated agents with isolated unsubstituted positions would contribute markedly to their persistence in animals and result in more marked induction of hepatic enzymes and other toxic effects. These pieces of evidence are supported by the hepatic enzyme induction studies herein, the most marked induction being observed with chlorobiphenyls substituted in the 4- and/or 4′-positions.

To conclude by answering the questions raised earlier, it would appear that for enzymes closely associated with the hepatic endoplasmic reticulum, the induction of enzyme activity was linked not to the biphenyl nucleus but to the position of chlorine substitution on the biphenyl structure. Isomers of the same degree of chlorination did not produce the same toxic effects, and dichlorobiphenyls may be quite similar to hexachlorobiphenyls depending on whether chlorine substitution occurs at the critical 4- and 4′-positions, thereby interfering with biotransformation. For enzymes less discretely localized in the hepatocyte (i.e., carboxylesterases and the BSP–GSH conjugating enzyme), the position of the chlorine atoms appears of less importance; enzyme induction was stimulated by biphenyl though enhanced effects were observed when one or more chlorine atoms were present.

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