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The Influence of Pure Polychlorinated Biphenyl Compounds on Hepatic Function in the Rat[1]

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The Influence of Pure Polychlorinated Biphenyl Compounds on Hepatic Function in the Rat. JOHNSTONE, G. J., ECOBICHON, D. J. AND HUTZINGER, O. (1974). *Toxicol. Appl. Pharmacol.* 28, 66–81. Pure biphenyl and isomerically pure mono-, di-, tetra-, hexa-, and octachlorobiphenyls of known chemical composition were injected ip (50 mg/kg/day) into young male Wistar rats for 3 days; the animals were killed 96 hr after the last injection. The potency of the pure PCBs was compared to that of *o,p'*-DDT and *p,p'*-DDT and commercial Aroclors (1254, 1260) administered at the same concentrations. Hepatic function was assessed by pentobarbital sleeping times and in vitro assays of hepatic microsomal *O*-demethylase, *N* demethylase, aniline hydroxylase, nitroreductase, carboxylesterase and the cytoplasmic bromosulfophthalein-glutathione conjugating enzyme. Biphenyl and 4-monochlorobiphenyl did not cause induction of hepatic drug-metabolizing enzymes. Microsomal mono-oxygenases were induced by hexa- and octachlorobiphenyls and by di- and tetrachlorobiphenyls with chlorines substituted at the 3 and 4 positions. Nitroreductase and carboxylesterase activities were affected only by the highly chlorinated compounds whereas all agents, including biphenyl, caused a marked induction of the bromosulfophthalein-conjugating system.

The ability of the commercially available, polychlorinated biphenyls (Aroclors, Phenoclors, Clophens, Kanechlors, etc.) to influence hepatic function has been well documented (Lincer and Peakall, 1970; Fujita *et al.*, 1971; Villeneuve *et al.*, 1971; Bickers *et al.*, 1972; Litterst *et al.*, 1972). Toxicologic assessment of these mixtures has been complicated by the heterogeneity of the PCB isomers, by marked differences in physical and chemical properties influencing the rates of absorption, distribution, metabolism and excretion and by the possible presence of such toxic impurities as chlorinated dibenzofurans and dibenzo-*p*-dioxins (Vos *et al.*, 1970; Sissons and Welti, 1971; Zitko and Choi, 1971; Greig, 1972; Hutzinger *et al.*, 1972; Webb and McCall, 1972). A statement by Tucker and Crabtree (1970) suggested that sublethal effects were directly correlated with the chlorine content, while lethal effects were inversely correlated. Because of the uncertainty surrounding the interpretation of toxicologic results and the inability to determine which of the many isomers were toxic, it was decided to

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study the influence of a series of isomerically pure chlorobiphenyl compounds on a number of functionally diverse, hepatic, drug-metabolizing enzymes of the rat. With this series of pure chlorobiphenyl compounds of varying degree and known position of chlorination, several questions could possibly be answered. Are the alterations in hepatic function due to the biphenyl nucleus itself, to the position(s) occupied by individual chlorine atoms, or to the number of chlorines present on the biphenyl nucleus?

METHODS

Synthesis of chlorobiphenyls. The various chlorobiphenyl compounds were synthesized from suitable starting materials by different procedures as described previously (Hutzinger *et al.*, 1971). The synthesis of 2,4′-dichlorobiphenyl was accomplished by the reduction of 2,4′-dinitrobiphenyl (Columbia Organic Chemicals) with zinc and acetic acid followed by conversion of the crude 2,4′-diaminobiphenyl to 2,4′-dichlorobiphenyl via a diazotization reaction and treatment with cuprous chloride (Sandmeyer reaction) (Hutzinger *et al.*, 1971).

TABLE 1

PHYSICAL PROPERTIES OF SOME ISOMERICALLY PURE CHLOROBIPHENYLS

Compound	Melting point (°C)	$R_f \times 100$[a]	Water solubility (μg/ml)
4-Chloro	77–78	43	1.18
2,2′-Dichloro	59–60	38	1.50
2,4′-Dichloro	24–25	50	1.87
4,4′-Dichloro	148–149	41	0.075
2,5,2′,5′-Tetrachloro	86.5–87	65[b]	0.046
2,4,2′,4′-Tetrachloro	41–42	58	0.0675
3,4,3′,4′-Tetrachloro	172–173	44	0.175
2,4,5,2′,4′,5′-Hexachloro	262–263	74	0.0088
2,3,5,2′,3′,5′-Hexachloro	128–129	70[b]	—
2,4,6,2′,4′,6′-Hexachloro	112–113	69	—
2,3,4,5,2′,3′,4′,5′-Octachloro	156–157	82[b]	0.0070
2,3,4,5,6,2′,3′,4′,5′,6′-Decachloro	305–306	76	0.015

[a] In heptane on commercially prepared thin-layer plates (Merck silica gel F-254).
[b] In hexane on preparative (20 × 100 cm) thin-layer plates (Merck silica gel F-254).

The agents were purified by preparative thin-layer chromatography on 20 × 100 cm silica gel plates coated with Merck Silica gel F-254 (layer thickness of 0.9 mm) using *n*-hexane or *n*-heptane as the developing solvent. The chlorobiphenyls were recrystallized from aqueous methanol or ethanol (Hutzinger *et al.*, 1971). All the agents prepared were homogeneous on thin-layer chromatograms and gave single peaks when analyzed by gas-liquid chromatography using a 4.0% SE-30 column (Zitko *et al.*, 1971). Mass spectra, obtained for all compounds, confirmed the molecular composition (Safe and Hutzinger, 1972). Table 1 presents some physical characteristics of the isomerically pure chlorobiphenyls investigated.

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Agents studied. Analytical grade *o,p'*-DDT and *p,p'*-DDT (Geigy Chemical Corp., Ardsley, New York) and Aroclor 1254 and 1260 (Monsanto Chemical Company, St. Louis, Missouri)[3] were used as "control" inducing agents of hepatic drug-metabolizing enzymes for comparison with biphenyl (Eastman Organic Chemicals, Rochester, New York) and the series of isomerically pure chlorobiphenyls shown in Table 1. The agents were dissolved in warm peanut oil by continual stirring until solution was effected. The solution was adjusted to a final volume of 10 ml with additional oil, yielding a final concentration of 17.5 mg/ml.

Solubility problems were encountered with 2 compounds. Decachlorobiphenyl was insoluble in peanut oil and could not be included in the investigation. The 3,4,3',4',-tetrachlorobiphenyl was soluble in peanut oil when heated slightly above 40°C but, on cooling to room temperature, a portion of the material precipitated from solution. In order that this isomer could be studied, the mixture was heated until a solution was formed, and it was injected as a warm solution (approximately 37°C).

Animal treatment. The animals used were young male Wistar rats (Woodlyn strain) weighing 50–65 g at the beginning of the experiment. Each treatment group, composed of 7 animals, was housed in wire-floored, metabolic cages (2–3 rats per cage) during and after drug administration. The animals were provided food and water ad libitum. For comparison purposes, the "control" inducing agents, biphenyl and the pure chlorobiphenyls were administered at a dosage of 50 mg/kg ip in a volume of 0.15–0.20 ml of peanut oil solution. Control animals ($n = 19$) were given peanut oil on the same volume basis as were the experimental animals. Two groups ($n = 5$ per group) of vehicle-treated controls were tested during the investigations with the various agents to ensure reproducibility of the assay procedures. The dosage schedule used by Schwark and Ecobichon (1967) was used, the animals being injected once daily for 3 days and killed 96 hr after the last injection.

Prior to killing the rats, a pentobarbital sleeping time was determined by injecting 40 mg/kg of sodium pentobarbital (0.4–0.6 ml ip) and placing them in a thermally neutral environment under a heating lamp at 32–34°C. The animals were continually handled and turned during the period of drug effect. The sleeping time was measured from the loss of the righting reflex until it was regained. On recovery, they were killed by $CHCl_3$ anesthesia, quickly opened and the livers and kidneys were excised, weighed and placed in chilled 50-ml beakers in crushed ice.

Tissue preparation. All subsequent steps of tissue homogenization and fractionation were carried out at 0–4°C. The entire liver or a 5.0-g sample was minced finely with scissors and was washed twice with cold 1.15% KCl to remove residual 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 (10,000 rpm for 20 min) in a Sorvall RC2-B centrifuge. The postmitochondrial supernatant was recentrifuged at 4,621,500 *g*-min (34,000 rpm for 50 min) on an International B-60 centrifuge. The supernatant was carefully removed and dialyzed against buffer in

[3] Abbreviations used: *o,p'*-DDT, 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; *p,p'*-DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane. The nomenclature of the chlorobiphenyl compounds follows the accepted form, the position of chlorines on one phenyl being indicated by numbers from 2 to 6 while chlorine positions on the second ring are designated by a number with a superscript prime, i.e., 2,2'-dichlorobiphenyl.

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preparation for a subsequent enzyme assay (see below). The combined pellets (microsomes) were resuspended and washed in cold 1.15% KCl, recentrifuged for 2,295,200 *g*-min (34,000 rpm for 30 min), and the pellet was resuspended by homogenization in 1.15% KCl sufficient to yield a suspension in which 1.0 ml contained the microsomes of 500 mg liver. This crude microsomal fraction was used in the following enzymatic assays, being stored not longer than 2 hr in crushed ice.

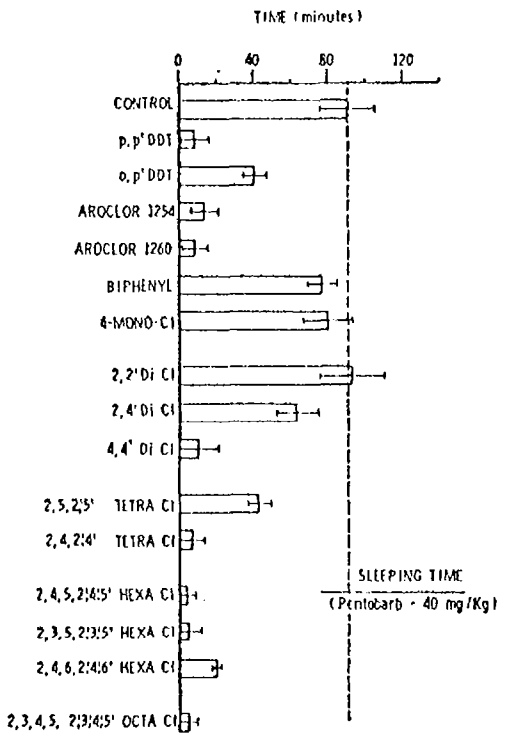


FIG. 1. The effect of pretreatment of young male rats with DDT (*o,p'*- and *p,p'*-isomers), Aroclor 1254 and 1260, biphenyl and a series of isomerically pure chlorobiphenyls on the sleeping time produced by an injection of 40 mg/kg sodium pentobarbital. Animals were treated by ip injection for 3 consecutive days, and the sleeping time was determined 96 hr after the last injection. The values represent the mean sleeping time (min) ± SD for 19 control animals and 7 animals per treated group.

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 by the method described by Schneider (1957). Suitable blanks were prepared with the TCA solution and standard curves were prepared for the nucleic acids using known concentrations of purified calf thymus DNA type I and yeast type XI RNA (Sigma Chemical Company, St. Louis, Missouri).

Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin for the preparation of a standard curve.

The mixed function oxidases (*N*-demethylase, *O*-demethylase and aniline hydroxylase) were assayed under the same conditions, incubating for 30 min in an air environ-

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ment at 37°C and using 25-ml Erlenmeyer flasks in a Dubnoff metabolic shaker (115 rpm). The reaction mixtures for each assay consisted of the following: (1) 0.5 ml of microsomes equivalent to 250 mg liver; (2) 50 μmol of glucose 6-phosphate; (3) 0.5 unit of glucose 6-phosphate dehydrogenase (Torula yeast, type XII, Sigma Chemical Company) (4) 100 μmol of nicotinamide; (5) 1.0 μmol of NADP; (6) a variable volume of 0.05 M Tris·HCl buffer, pH 7.4; (7) substrate solution. The volume of the incubation mixture was 5.0 ml. For the *N*-demethylase and aniline hydroxylase assays, 25 μmol of

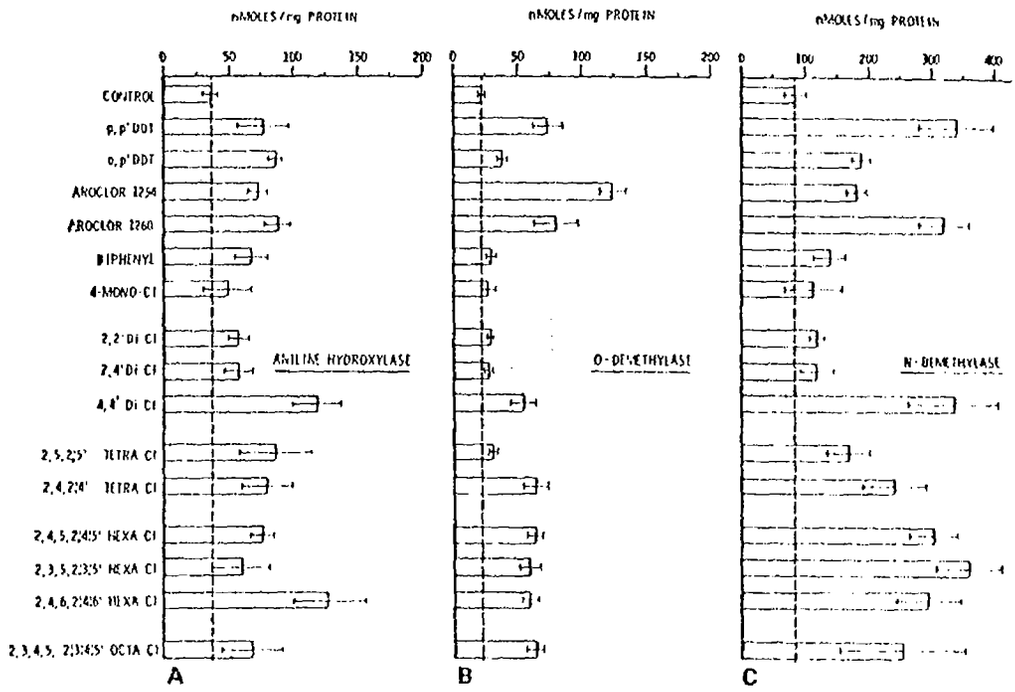


FIG. 2. The effects of pretreatment of young male rats with DDT (*o,p'*- and *p,p'*-isomers) Aroclor 1254 and 1260, biphenyl and a series of isomerically pure chlorobiphenyls on hepatic microsomal aniline hydroxylase (A), *p*-nitroanisole *O*-demethylase (B) and aminopyrine *N*-demethylase (C) activities. 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 nmol of product formed/mg of microsomal protein/30 min incubation. The values (bars) represent the mean enzymatic activities, ±SD of the means of 19 control animals and 7 animals per treated group.

$MgCl_2$ were added. The assay of microsomal nitroreductase activity was done in the same manner except that the incubation period was 60 min and the reaction was carried out under a nitrogen atmosphere (Fouts and Brodie, 1957). The demethylation of *p*-nitroanisole (5 μmol) to *p*-nitrophenol (*O*-demethylase) was assayed by the method of Netter and Seidel (1964). The dealkylation of aminopyrine (15 μmol) (*N*-demethylase) was measured colorimetrically using the Hansch reaction to detect the formaldehyde released (Cochin and Axelrod, 1959). The hydroxylation of aniline (15 μmol) was measured by the method of Kato and Gillette (1965). The reduction of *p*-nitrobenzoic acid (20 μmol) to *p*-aminophenol was measured by the method of Kato and Gillette (1965).

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The activities were calculated in terms of the nmol of substrate changed/mg of protein/30 min (mixed function oxidases) or 60 min (nitroreductase).

Nonspecific carboxylesterase activity was determined on aliquots of the postmitochondrial fraction by a spectrophotometric technique, continuously monitoring the formation of free α-naphthol from 1×10^{-3} M α-naphthyl acetate at pH 7.4 and 25°C (Ecobichon, 1970). A Pye Unicam SP500 recording spectrophotometer equipped with a thermostatted cell compartment and a Perkin Elmer model 165 recorder were used. Activity was presented as nmol of substrate hydrolyzed/mg of protein/min.

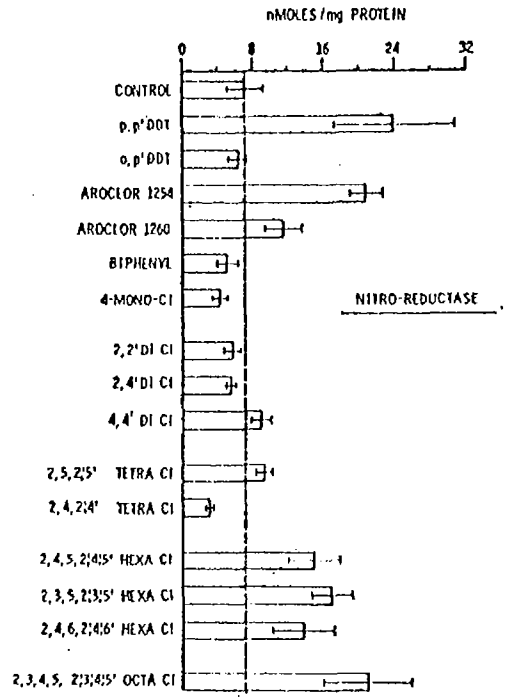


FIG. 3. The effect of pretreatment of young male rats with DDT (*o,p'*- and *p,p*-isomers), Aroclor 1254 and 1260, biphenyl and a series of isomerically pure chlorobiphenyls on the activity of hepatic microsomal *p*-nitrobenzoic acid nitroreductase. Activities are expressed as nmol of product formed/mg of microsomal protein/60 min. For other details, see Fig. 2.

The conjugation of bromosulfophthalein (BSP) with reduced glutathione was measured using the postmicrosomal supernatant (cytoplasmic fraction) which had been dialyzed overnight at 4°C against 0.1 M sodium pyrophosphate buffer, pH 8.2. The assay (Goldstein and Combes, 1966), was based on the observation that the conjugated form of BSP has a greater absorbance at 330 nm than does free BSP. An aliquot (0.1 ml) of dialyzed cytoplasmic fraction was added to 3.0 ml 0.1 M sodium pyrophosphate buffer pH 8.2 in a 25-ml Erlenmeyer flask. One milliliter of BSP solution (1.0 μmol/ml) was added and the thoroughly mixed contents were poured into a second 25-ml flask containing 0.3 ml of reduced glutathione solution (310 μmol/ml). After a 30-sec interval of mixing, the reaction mixture was poured into a cuvette and the enzymatic activity was measured spectrophotometrically, continuously monitoring the rate of conjugate

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formation for 10 min at 25°C. The changes in absorbance were related to a standard curve prepared with the conjugated form of BSP. Since this compound was not commercially available, it was synthesized and purified in our laboratory by the method described by Whelan *et al.* (1970).

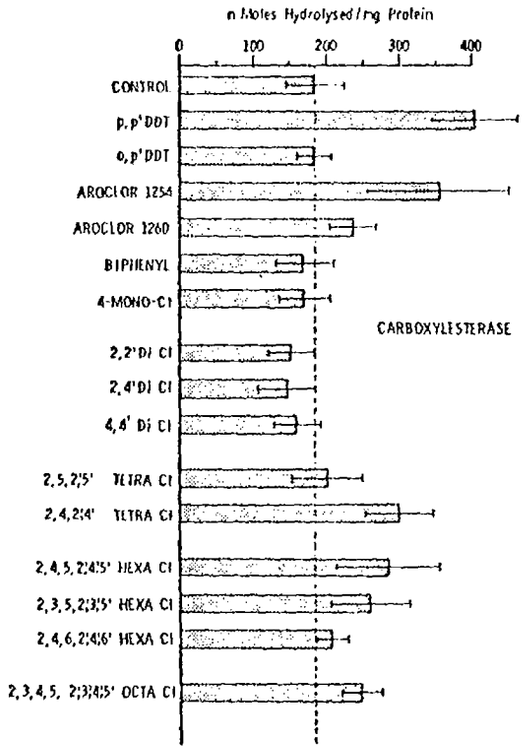


FIG. 4. The effect of pretreatment of young male rats with DDT (*o,p'*- and *p,p'*-isomers), Aroclor 1254 and 1260, biphenyl and a series of isomerically pure chlorobiphenyls on the activity of hepatic carboxylesterase activity. Activities are expressed as nmol of α-naphthyl acetate hydrolyzed/mg of protein/min. The fraction used in the assay was a postmitochondrial supernatant containing both microsomal and soluble carboxylesterases. For other details, see Fig. 2.

Statistical analysis of significance of the various parameters measured in treated and control groups was assessed by a Student's *t* test for 2 sample means based on independent samples. Statistical significance was assessed at the 5% level of probability ($p < 0.05$).

RESULTS

Figure 1 shows the influence of the DDT isomers, commercial Aroclors, biphenyl and the series of isomerically pure chlorobiphenyl compounds on pentobarbital-induced sleeping time. This histogram, as well as the subsequent diagrams, is arranged to show the results obtained for control, vehicle-treated animals followed by those treated with "control" inducing agents (*o,p'*-DDT, *p,p'*-DDT, Aroclor 1254, Aroclor 1260) and for the animals treated with the individual pure compounds, arranged from low to high

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chlorination. The mean sleeping time observed for control animals was 87 min. Highly significant reductions in sleeping time were observed with the commercial Aroclors and DDT isomers, though *o,p'*-DDT was not as effective. Biphenyl and 4-chlorobiphenyl caused no significant ($p > 0.05$) changes in sleeping time. Of the dichloro-isomers, a significant reduction was observed only with the 4,4'-isomer. Both tetrachlorobiphenyls caused a significant reduction in sleeping time though the 2,5,2',5'-isomer was less effective. All the hexa- and octachlorobiphenyls tested, caused statistically significant ($p < 0.05$) reductions in sleeping time, being comparable to *p,p'*-DDT, Aroclor 1254 and 1260 in effect.

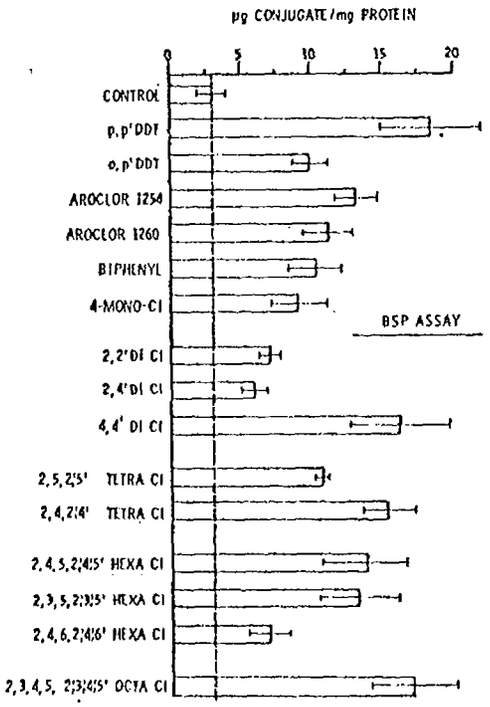


FIG. 5. The effect of pretreatment of young male rats with DDT (*o,p'*- and *p,p'*-isomers), Aroclor 1254 and 1260, biphenyl and a series of isomerically pure chlorobiphenyls on the hepatic cytoplasmic enzyme which conjugates BSP with reduced glutathione. Activities are expressed as μg of conjugate formed/mg of protein/min. For other details, see Fig. 2.

The influence of each agent on (1) liver weight, (2) DNA and RNA content of whole homogenate (25% w/v) and (3) microsomal protein extracted per g of liver are presented with the results for control vehicle-treated animals in Table 2. Statistically significant ($p < 0.05$) increases in liver weight were observed only following treatment with *p,p'*-DDT, Aroclor 1254 and Aroclor 1260. The quantity of DNA extracted from the whole homogenates of treated animals was relatively constant, no significant differences being observed from the control values. In contrast, many of the agents tested caused significant increases in hepatic RNA. No increases in hepatic RNA were observed with *p,p'*-DDT, Aroclor 1260, biphenyl, 4-chlorobiphenyl while 3 agents, 4,4'-di-, 3,4,3',4'-

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TABLE 2

CHANGES IN LIVER WEIGHT, DNA, RNA AND MICROSOMAL PROTEIN AS A RESULT OF INTRAPERITONEAL ADMINISTRATION OF DDT AND PCB ISOMERS TO RATS[a]

Group treatment	Liver weight (g)	DNA (mg/g)	RNA (mg/g)	Microsomal protein (mg/g)
Control.	4.77 ± 1.14	3.08 ± 0.11	8.02 ± 0.40	6.76 ± 1.40
p,p'-DDT	5.59 ± 0.89[b]	2.84 ± 0.83	8.28 ± 0.94	9.36 ± 2.32[b]
o,p'-DDT	4.91 ± 0.46	3.34 ± 0.26	13.37 ± 0.74[b]	7.70 ± 0.98
Aroclor 1254	6.23 ± 0.30[b]	2.69 ± 0.14	10.17 ± 0.79[b]	8.42 ± 1.54[b]
Aroclor 1260	6.15 ± 0.49[b]	2.94 ± 0.44	9.04 ± 1.43	9.52 ± 1.02[b]
Biphenyl	4.87 ± 0.11	3.34 ± 0.17	8.08 ± 0.51	6.22 ± 0.60
4-Chloro	4.76 ± 0.69	3.17 ± 0.43	7.63 ± 0.69	5.58 ± 1.26
2,2-Dichloro	4.85 ± 0.96	3.60 ± 0.49	13.50 ± 1.98[b]	6.56 ± 1.78
2,4'-Dichloro	4.69 ± 0.34	3.39 ± 0.12	13.89 ± 0.80[b]	7.78 ± 0.94[b]
4,4'-Dichloro	4.21 ± 0.44	3.40 ± 0.36	6.44 ± 0.36	5.54 ± 1.92
2,5,2',5'-Tetrachloro	4.68 ± 0.69	3.43 ± 0.39	6.70 ± 0.93	6.06 ± 1.44
2,4,2',4'-Tetrachloro	5.03 ± 0.48	2.90 ± 0.21	11.86 ± 1.07[b]	9.14 ± 1.30[b]
3,4,3',4'-Tetrachloro	4.73 ± 0.47	2.86 ± 0.12	6.78 ± 0.92	5.70 ± 0.93
2,4,5,2',4,'5'-Hexachloro	4.18 ± 0.91	3.64 ± 0.70	11.44 ± 0.71[b]	12.06 ± 1.10[b]
2,4,6,2',4',6'-Hexachloro	3.82 ± 1.29	3.80 ± 0.85	11.47 ± 1.07[b]	7.10 ± 0.92
2,3,5,2',3',5'-Hexachloro	5.80 ± 0.78[b]	3.31 ± 0.26	11.83 ± 1.08[b]	11.76 ± 2.32[b]
2,3,4,5,2',3',4',5 -Octachloro	4.53 ± 1.46	3.49 ± 0.64	11.51 ± 0.93[b]	8.34 ± 0.96[b]

[a] Values presented are the mean ± SD of 14 control and 7 treated animals per group. Values are based on wet weight of tissue.
[b] Values are significantly different from control values, $p < 0.05$.

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tetra- and 2,5,2′,5′-tetrachlorobiphenyls, caused slight reduction in RNA content. Significant increases ($p < 0.05$) in hepatocellular RNA were observed with 2,2′-di-, 2,4′-di-, 2,4,2′,4′-tetra-, the hexa- and octachlorobiphenyls tested. Significant increases ($p < 0.05$) in microsomal protein were observed in animals treated with *p,p′*-DDT, Aroclor 1254, Aroclor 1260, 2,4,2′,4′-tetra-, 2,4,5,2′,4′,5′-hexa-, 2,3,5,2′,3′,5′-hexa- and the octachlorobiphenyls. The other agents caused no changes in microsomal protein concentration from that observed for the control animals.

Figure 2 summarizes the influence of the various agents tested on the hepatic mixed function oxidases aniline hydroxylase (A), *O*-demethylase (B) and *N*-demethylase (C).

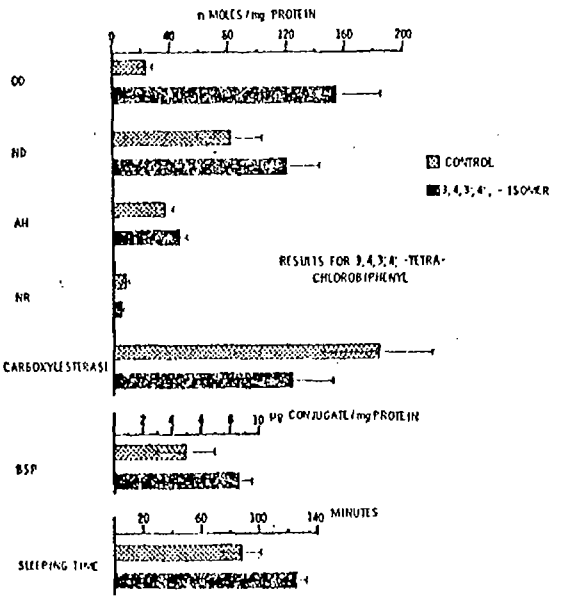


FIG. 6. The effect of pretreatment of young male rats with 3,4,3′,4′-tetrachlorobiphenyl on the hepatic *O*-demethylase (OD), *N*-demethylase (ND), aniline hydroxylase (AH), nitroreductase (NR), carboxylesterase, BSP conjugating enzyme and pentobarbital sleeping time. The animals were treated as described in the text. The values presented are the means ± SD for 7 control, vehicle-treated and 7 agent-treated animals.

For aniline hydroxylase, significant increases ($p < 0.05$) in activity were observed for all agents with the exception of 4-chloro- and 2,3,5,3′,5′-hexachlorobiphenyl. Marked increases were observed following treatment with 4,4′-di- and 2,4,6,2′,4′,6′-hexachlorobiphenyl. Compared to control activity, significant increases ($p < 0.05$) in *O*-demethylase activity were observed for all agents tested with the exception of biphenyl, 4-chloro-, 2,2′- and 2,4′-dichloro- and 2,5,2′,5′-tetrachlorobiphenyls. The influence of the various agents on hepatic *N*-demethylase were similar to those observed for *O*-demethylase, marked induction being observed with 4,4′-di-, 2,4,2′,4′-tetra-, the hexa- and octachlorobiphenyls. Aroclor 1260 had a more pronounced effect on *N*-demethylase than did Aroclor 1254, the reverse being observed for *O*-demethylase activity.

The effects of the various agents on hepatic microsomal nitroreductase activity are presented in Fig. 3, the results being somewhat different compared to those observed

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for the mixed function oxidases (Fig. 2). Marked increases were observed only in those animals treated with *p,p'*-DDT, Aroclor 1254, the 3 hexachloro-isomers and the octachlorobiphenyl. Statistically significant ($p < 0.05$) increases were observed following treatment with Aroclor 1260 and 4,4'-dichlorobiphenyl while the remainder of the agents (*o,p'*-DDT, biphenyl, 4-chloro-, 2,2'- and 2,4'-dichloro and 2,4,2',4'-tetrachlorobiphenyl) caused reductions of the nitroreductase activity.

Figure 4 is a summary of the influence of the various agents tested on the hepatic carboxylesterase activity in young male rats. The fraction used for this assay was the 242,000 *g*-min postmitochondrial supernatant since carboxylesterases have been found in both particulate (microsomal) and cytoplasmic (soluble) fractions (Schwark and Ecobichon, 1967). Of the agents tested, only *p,p'*-DDT, Aroclor 1254 and 2,4,2',4'-tetrachlorobiphenyl caused increases in activity which were significantly different from control values ($p < 0.05$). The other agents either caused nonsignificant ($p > 0.05$) increases in activity or reduced the activity below that observed in control animals.

Figure 5 presents the results observed for the cytoplasmic enzyme system involved in conjugating BSP with reduced glutathione following exposure to DDT, Aroclor 1254 and 1260, biphenyl and various pure chlorobiphenyl compounds. Significant ($p < 0.05$) increases in activity were observed with all the agents tested. Even agents such as biphenyl and 4-chlorobiphenyl, which characteristically had little or no influence on the other enzymatic activities, markedly increased the activity of this hepatic enzyme.

The inductive effects of the tetrachlorobiphenyls on hepatic enzymes was of particular interest since lipid solubility appeared to be a problem with one, the 3,4,3',4'-isomer. It should also be noted that this particular compound exhibited greater water-solubility than the other two tetrachloro-isomers examined (Table 1). In this study, the agent was injected as a warm (37°C) solution. It is not known to what extent the agent was absorbed from the peritoneal cavity though there were no visible residual depots of material when the animals were killed 96 hr after the last injection. The effects of this isomer on the hepatic functions are presented separately in Fig. 6, since the results observed were much different than those obtained with the other tetrachloro-isomers. Only 1 of the enzymatic functions, *O*-demethylase, was significantly ($p < 0.05$) increased. Slight, though nonsignificant increases in activity were observed for *N*-demethylase, aniline hydroxylase and BSP-conjugating enzyme activities. Both nitroreductase and carboxylesterase activities were lower than control values and there was a statistically significant ($p < 0.05$) prolongation of the pentobarbital sleeping time.

DISCUSSION

Commercial PCB mixtures have been shown to cause a marked reduction in barbiturate-induced sleeping time (Komatsu and Tanaka, 1971; Bickers *et al.*, 1972). Most of the enzyme inductive effects have been demonstrated using enzymatic reactions coupled to the mixed function oxidase system including *O*-demethylase, *N*-demethylase and aniline hydroxylase, all of which showed marked increases in activity (Fujita *et al.*, 1971; Villeneuve *et al.*, 1971; Bickers *et al.*, 1972; Litterst *et al.*, 1972). Increases in hepatic cytochrome P-450 in rats have been observed following the administration of Aroclor 1016, 1242, 1248, 1254 and 1260 (Bickers *et al.*, 1972; Litterst *et al.*, 1972). Hepatic microsomal nitroreductase activity was increased in rats as was carboxylesterase activity

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in rabbits (Villeneuve *et al.*, 1971; Litterst *et al.*, 1972). The present results confirmed those reported in the literature, the commercial Aroclors (1254 and 1260) causing marked induction of these enzymatic functions. Data have not been reported to date on the induction of hepatic, synthetic (conjugating), enzymatic reactions by PCBs, but the present results indicate that, for at least one conjugative enzyme system (Fig. 5), marked induction occurred following the administration of Aroclor 1254 and 1260.

Few studies have been published concerning the effects of isomerically pure chlorobiphenyl compounds on hepatic enzyme activities. Fujita *et al.* (1971), studying the effects of oral administration of 4,4′-di-, 3,4,3′,4′- and 3,4,2′,4′-tetra-, 2,4,5,3′,4′-penta-

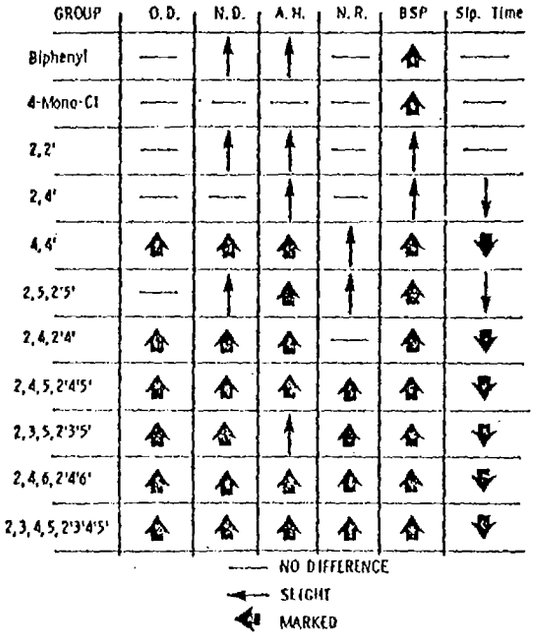


FIG. 7. A summary of the effects of pretreatment of young male rats with biphenyl and a series of isomerically pure chlorobiphenyls on hepatic microsomal *O*-demethylase (O.D.) *N*-demethylase (N.D.), aniline hydroxylase (A.H.), nitroreductase (N.R.), the BSP conjugating system and the pentobarbital sleeping time. The data of the previous figures are simplified to show that there were (1) no differences, (2) slight differences, or (3) marked differences from control values.

and 3,4,5,3′,4′,5′-hexachlorobiphenyls to rats, observed increased liver weights, increased microsomal protein, decreased barbiturate-induced sleeping times and marked increases in hepatic *O*-demethylase and aniline hydroxylase activities. The greatest changes were observed with the penta- and hexachlorobiphenyls, the former being the most active. Of the 2 tetrachloro-isomers studied, the 3,4,3′,4′-isomer exerted a more marked influence than did the 3,4,2′,4′-tetrachlorobiphenyl. Fujita *et al.* (1971) concluded that the positions of the chlorine atoms on the rings was a factor in determining the intensity of the inductive effects. In contrast to the results of Fujita *et al.* (1971), who found that orally administered 4,4′-dichlorobiphenyl scarcely altered the activity of hepatic enzymes, the present study employing ip injections, revealed that, with the

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exception of carboxylesterase and nitroreductase activities, the 4,4′-dichloro-isomer was as potent an inducing agent as the commercial mixtures or the isomers of high chlorine content when given ip.

Vos and Notenboom-Ram (1972), using 2,4,5,2′,4′,5′-hexachlorobiphenyl in a chronic study, showed that hepatic injury was more severe with this isomer than that produced by Aroclor 1260, a marked proliferation of hypertrophic, hypoactive smooth endoplasmic reticulum being observed. In the present study, 2,4,5,2′,4′,5′-hexachlorobiphenyl caused marked induction of all enzymatic functions tested, Benthe *et al.* (1972) reported that equimolar doses of tetrachlorobiphenyls were more effective than dichlorobiphenyls in inducing rat hepatic enzymes, but it must be pointed out that Aroclor 1248 and Aroclor 1232 were used as the source of tetra- and dichlorobiphenyls, respectively. These commercial compounds contain a number of other biphenyls of different degree of chlorination (Webb and McCall, 1972). Recently, Chen *et al.* (1973) reported the effects of the 2,3,4,5,- and 3,5,3′,5′-tetrachlorobiphenyl isomers on hepatic microsomal enzymes following short-term oral administration to rats. Ethylmorphine *N*-demethylase, aniline hydroxylase and *p*-nitroanisole *O*-demethylase activities were all markedly increased, especially the *O*-demethylase, which appeared to be extremely sensitive to these agents (Chen *et al.*, 1973). Our investigations demonstrated that, administered ip, 2,4,2′,4′-tetrachlorobiphenyl was a more potent inducer of enzyme activities than was the 2,5,2′,5′-isomer. The 3,4,3′,4′-tetrachloro-isomer caused marked induction only of *p*-nitroanisole *O*-demethylating activity, though the unusual solubility problems of this compound should not be disregarded.

Despite the available information regarding the effects of the PCBs, there still remains the problem of interpreting and relating effects with chemical structure. Is the biphenyl nucleus essential for induction? Is it the position of chlorine atoms on the molecule, or is it the number of chlorines, which determines the nature and the intensity of effect? Figure 7 presents, in summary fashion, the results found in Figs. 1–3 and 5 for the various isomerically pure chlorobiphenyls, indicating whether there was no effect, a slight (possibly significant or nonsignificant) effect or a marked effect on the enzymatic functions and sleeping times.

The biphenyl nucleus alone played little role in inducing hepatic drug-metabolizing enzymes. While the slight increases in *N*-demethylase and aniline hydroxylase were statistically significant from control values, pure biphenyl and 4-monochlorobiphenyl were the least effective inducing agents when compared with DDT, commercial Aroclors or the other chlorobiphenyls tested.

For the two DDT isomers, the position of the chlorine on the phenyl rings was important since greater inductive effects were observed when the 4- (para) positions were both occupied (*p,p′*-DDT) than when the 2- (ortho) and 4- (para) positions were occupied (*o,p′*-DDT). This quantitative difference was also observed for the PCB isomers. Considering the dichlorobiphenyls, when the 4- and 4′- positions were occupied, there was a much greater inductive effect for all enzymatic activities than was observed with 2,2′- and 2,4′-dichlorobiphenyls. The same positional phenomenon was observed for the tetrachlorobiphenyls studied, the induction caused by 2,4,2′,4′-tetrachlorobiphenyl being much greater than that observed for the 2,5,2′,5′-tetrachloro-isomer. Fujita *et al.* (1971) found that orally administered 3,4,3′,4′-tetrachlorobiphenyl caused a more marked induction of *O*-demethylase, *N*-demethylase and aniline hydroxy-

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lase than did the 3,4,2′,4′-isomer. While the rate of administration was different in the present study, 2,4,2′,4′-tetrachlorobiphenyl caused a 2-fold increase in *O*-demethylase activity (Fig. 2) whereas treatment with the 3,4,3′,4′-isomer resulted in a 7-fold increase (Fig. 6).

The results for the analogs containing more than 4 chlorines suggested that, with these higher chlorinated compounds, the positions of the chlorine atoms were not as important. Fujita *et al.* (1971) found that the penta-, hexa-, and octa chlorobiphenyls had greater enzyme-inducing potential than did the tetrachlorobiphenyl isomers, a result which, with the exception of 4,4′-dichlorobiphenyl, was confirmed in the present study. It has been demonstrated that more marked induction was obtained with commercial Aroclors with a greater proportion of highly chlorinated biphenyls (Aroclors 1254, 1260) than with those having predominantly low chlorine content (Aroclors 1016, 1221) (Villeneuve *et al.*, 1971; Bickers *et al.*, 1972). It would appear that positional specificity disappears when more than 4 chlorine atoms are incorporated on the rings since the 4- and 4′-positions are usually occupied.

An important factor governing the ability of any foreign chemical to cause induction of hepatic enzymes is the rate at which it is degraded in the body. Several investigators have shown that, after the administration of Aroclor mixtures, the low chlorine analogs were eliminated more rapidly from tissues than were the highly chlorinated biphenyls (Grant *et al.*, 1971; Bailey and Bunyan, 1972; Bickers *et al.*, 1972; Rehfeld *et al.*, 1972). Evidence has been presented that biphenyl and 4-chlorobiphenyl are hydroxylated and conjugated into readily excreted, water-soluble products (Block and Cornish, 1959). More recently, Hutzinger *et al.* (1972) showed that, in the rat, biphenyl and 4-chlorobiphenyl were mono- and dihydroxylated and excreted rapidly in the urine. Monohydroxylated derivatives were formed from 4,4′-dichlorobiphenyl, from 2,4,2′,4′- and 2,5,2′,5′-tetrachlorobiphenyl, but not from the 3,4,3′,4′-isomer. Chlorobiphenyls with more than 4 chlorine atoms were excreted only in the feces, no trace of the agents being detected in the urine. On the basis of these observations, biphenyl and 4-chlorobiphenyl might be less effective inducing agents than the highly chlorinated analogs because of rapid biotransformation and elimination from the body whereas the more lipid-soluble, highly chlorinated biphenyls persist in vivo.

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