Chem.-Biol. Interactions, 9 (1974) 341–350
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COMPARATIVE EFFECTS OF COMMERCIAL AROCLORS ON RAT LIVER ENZYME ACTIVITIES*

DONALD J. ECOBICHON AND ANNE M. COMEAU

Department of Pharmacology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia (Canada)

(Received February 4th, 1974)
(Revision received June 3rd, 1974)
(Accepted July 9th, 1974)

SUMMARY

Hepatic DNA, RNA, protein and *p*-nitroanisole *O*-demethylase, aniline hydroxylase, nonspecific carboxylesterase, bromosulphophthalein-glutathione (BSP-GSH) conjugating enzyme and *p*-nitrophenol UDPglucuronyl transferase activities were measured in young Wistar male rats which had received intraperitoneal injections (50 mg/kg) of biphenyl and Aroclors 1016, 1221, 1232, 1242, 1248, 1254 and 1260, dissolved in peanut oil, for 3 consecutive days and assayed 96 h after the last injection. Biphenyl and all the Aroclors caused the same degree of enhancement of BSP-GSH conjugating enzyme. Decreased DNA content, increased RNA and protein content and the other enzymatic activities were related to the percent weight of chlorine and the chlorobiphenyl composition of the Aroclors. More marked effects were observed with the highly chlorinated Aroclor 1248, 1254, and 1260 mixtures which contained predominantly tetra-, penta-, hexa-, and higher-chlorinated biphenyls.

INTRODUCTION

A statement by TUCKER AND CRABTREE[1] suggested that the sublethal effects of PCBs were directly correlated with the chlorine content while lethal effects were inversely correlated. BICKERS *et al.*[2] found that hepatic ethylmorphine *N*-demethylase activity was more pronounced in Aroclor 1254-treated rats than in those treated with Aroclor 1016. Similar observations were made by VILLENEUVE *et al.*[3] using Aroclor

*This project was supported by National Health Grant No. 602-7-162 made by the Department of National Health and Welfare of Canada.
Abbreviations: BSP, bromosulphophthalein; GSH, glutathione; PCB, polychlorinated biphenyl; TCA, trichloroacetic acid; UDPGA, uridine-5-diphosphoglucuronic acid.

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1221 and 1254, more enhanced effects being observed with the highly chlorinated mixture. While the ability of commercially available PCBs to enhance the induction of mammalian and avian hepatic mixed-function oxidases has been well documented, it has not been demonstrated that this property extends to other enzymatic functions of the liver[2-6]. It was decided to investigate the influence of a series of Aroclor mixtures on the induction of some functionally diverse hepatic enzymes in young male rats.

MATERIALS

Samples of Aroclor 1016, 1221, 1242, and 1254 were generously provided by the Monsanto Industrial Chemicals Company, St. Louis, Mo. Samples of Aroclor 1232, 1248 and 1260 were purchased from Analabs, Inc., North Haven, Conn. Biphenyl was purchased from Eastman Organic Chemicals, Rochester, N.Y. The animals used were young (4–5 weeks old) male Wistar strain rats (Bio Breeding Laboratories, Ottawa, Canada), weighing 70–85 g at the beginning of the experiment.

METHODS

The agents were dissolved in warm peanut oil by continually stirring until solution was effected. The solution was adjusted to volume, yielding a final concentration of 17.5 mg of agent per ml. The agents were administered intraperitoneally at a dosage of 50 mg/kg (0.20–0.26 ml) for 3 consecutive days and the rats were killed 96 h after the last injection[7]. Control animals (n = 18) received peanut oil on the same volume basis as did the experimental animals. Each treatment group (n = 6) was housed in plastic cages (3 rats per cage) on hardwood shavings and received food and water *ad libitum*.

Following cervical dislocation, the rats were quickly opened and the livers were excised and dropped into ice-cold 1.15 % KCl–0.02 *M* Tris buffer pH 7.4. The blotted tissue was weighed and the entire liver or a 5.0 g sample was minced finely with scissors and was washed twice with cold KCl–Tris solution to remove residual blood. The minced tissue was completely homogenized with KCl–Tris solution by 10 strokes of a loose-fitting, motor-driven Teflon pestle in a 50 ml Potter Elvehjem glass homogenizer. The volume was adjusted to yield a 20 % w/v homogenate which was then centrifuged at 4° for 240 000 *g* min (12 000 *g* for 20 min) in a Sorvall RC2-B centrifuge to remove nuclei, cell debris and mitochondria. This post-mitochondrial supernatant was removed by Pasteur pipette and was stored in crushed ice and used within 2 h of preparation.

The nucleic acid (DNA and RNA) content of an aliquot of the whole homogenate prior to centrifuging was determined by the method of Schneider as described previously[7]. The protein content of the post-mitochondrial supernatant was determined by the method of Lowry *et al.*[8], 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[9] and Kato and Gillette[10] respectively. The reaction mixtures for these assays contained 0.5 ml of

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enzyme, 50 μmoles of glucose-6-phosphate, 0.5 units of glucose-6-phosphate dehydrogenase, 100 μmoles of nicotinamide, 1.0 μmole NADP, substrate (5.0 μmoles of *p*-nitroanisole or 15 μmoles of aniline) and sufficient 0.05 *M* Tris buffer pH 7.4 to yield a final volume of 4.0 ml[7]. For the aniline hydroxylase assay, 25 μmoles of $MgCl_2$ was added. Incubations were carried out at 37° with continual shaking in a Dubnoff metabolic shaker, the reactions being stopped at 10 or 20 min by the addition of 2.0 ml of 20% TCA. Activities were presented as nmoles of substrate changed per min per mg of protein.

The glucuronidation of *p*-nitrophenol was assayed by the method of VORNE[11], the incubation mixture of 3.0 ml containing 0.5 ml of enzyme, 1.7 μmole of UDPGA (ammonium salt; Sigma Chemical Company, St. Louis, Mo.) and 1.0 μmole of *p*-nitrophenol. The reaction was stopped by the addition of 2.0 ml of 20% TCA. The disappearance of substrate was measured at 420 nm using a 3.0 ml volume of the centrifuged supernatant to which was added 0.2 ml of 40% NaOH. Initial rates of activity were calculated from the curves and the enzymatic activity was calculated in terms of nmoles of substrate changed per min per mg of protein.

Nonspecific carboxylesterase (EC 3.1.1.1) activity was determined on aliquots of the post-mitochondrial fraction by a spectrophotometric technique, continuously monitoring at 322 nm the formation of free alpha-naphthol from $1 \cdot 10^{-3}$ *M* alpha-naphthyl acetate at pH 7.4 and 37° (ref. 12). Activity was presented as μmoles of substrate hydrolyzed per min per mg of protein.

The conjugation of BSP with reduced GSH was measured using an aliquot of the postmitochondrial supernatant which had been dialyzed overnight at 4° against 0.1 *M* sodium pyrophosphate buffer pH 8.2. The method of GOLDSTEIN AND COMBES[13] was used, continuously monitoring the rate of conjugate formation for 1 min at 37° and pH 8.2 as was described previously[7]. Conjugated BSP for the preparation of a standard curve was synthesized by the method described by WHELAN *et al.*[14]. Activity was expressed as μg of conjugate formed per min per mg of protein.

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

RESULTS

The influence of the vehicle and each Aroclor preparation on liver weight, DNA and RNA content of the whole homogenate (20% w/v) and on the protein concentration of the post-mitochondrial supernatant is presented in Table I. Statistically significant decreases in DNA were observed with all Aroclors, suggesting increased cellular size, though a comparison of liver weight/body weight ratios showed statistically significant increases only with Aroclor 1248, 1254, and 1260. Increased RNA concentrations were observed with all agents tested. Protein concentration was significantly higher only when the results were presented in terms of the DNA content of the whole homogenate. The most significant changes in the cellular parameters were observed in rats treated with Aroclor 1254.

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TABLE I

EFFECT OF ACUTE AROCLOR TREATMENT ON WEIGHT AND DNA, RNA AND PROTEIN CONTENT OF RAT LIVER[a]

Treatment	*n*	*Liver wt/body wt (g/100 g)*	*DNA (mg/g)*	*RNA (mg/mg DNA)*	*Protein (mg/mg DNA)*
Vehicle	18	5.3 ± 0.3	4.94 ± 0.50	2.62 ± 0.17	3.77 ± 0.27
Aroclors:					
1016	6	5.5 ± 0.2	3.41 ± 0.07[b]	3.17 ± 1.10[b]	5.78 ± 0.29[b]
1221	6	5.3 ± 0.4	3.40 ± 0.26[b]	3.30 ± 0.15[b]	5.70 ± 0.24[b]
1232	10	5.3 ± 0.3	3.50 ± 0.13[b]	3.12 ± 0.13[b]	5.48 ± 0.18[b]
1242	6	5.6 ± 0.5	3.39 ± 0.22[b]	3.40 ± 0.16[b]	6.70 ± 0.32[b]
1248	6	5.7 ± 0.2[b]	3.02 ± 0.15[b]	3.74 ± 0.22[b]	7.16 ± 0.37[b]
1254	6	6.1 ± 0.2[b]	2.66 ± 0.05[b]	4.05 ± 0.27[b]	8.10 ± 0.36[b]
1260	6	6.4 ± 0.3[b]	3.30 ± 0.22[b]	3.90 ± 0.23[b]	5.87 ± 0.31[b]

[a] Values presented are mean ± S.D. of the numbers of animals indicated. See text for details of treatment.

[b] $p < 0.05$ comparing Aroclor *vs.* vehicle treatment.

Since the Aroclors possess the biphenyl nucleus, it was important to determine what influence biphenyl might have on hepatic functions. No significant changes were observed in weight, DNA, RNA or protein concentration of livers from animals treated with biphenyl. A comparison of hepatic enzymatic activities of animals treated with peanut oil and biphenyl in peanut oil revealed, as is shown in Table II, that only BSP-glutathione conjugating enzyme was significantly affected by biphenyl.

Fig. 1 summarizes the influence of the Aroclor mixtures on the hepatic mixed-function oxidases *p*-nitroanisole *O*-demethylase and aniline hydroxylase. For *O*-demethylase, significant increases in activity were observed following treatment with all the Aroclors except Aroclor 1221. As the percent weight of chlorine increased,

TABLE II

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

Enzyme	*Activities*[b]	
	Vehicle	*Biphenyl*
p-Nitroanisole *O*-demethylase	0.35 ± 0.06	0.44 ± 0.08
Aniline hydroxylase	0.21 ± 0.05	0.20 ± 0.03
Carboxylesterase	0.54 ± 0.12	0.49 ± 0.05
BSP-glutathione conjugating enzyme	11.1 ± 2.2	18.2 ± 2.2[c]
p-Nitrophenol UDPglucuronyl transferase	0.23 ± 0.07	0.20 ± 0.06

[a] The animals received 50 mg of biphenyl/kg body wt (0.20–0.26 ml of solution) for 3 consecutive days, and were killed 96 h after the last injection. Vehicle-treated animals received peanut oil on the same volume basis. 18 control animals and 6 biphenyl-treated rats were used. Values are mean ± S.D.

[b] The activities of *O*-demethylase, aniline hydroxylase and *p*-nitrophenol UDPglucuronyl transferase are expressed as nmoles of product formed/min/mg of protein. Carboxylesterase activity is expressed as μmoles of substrate hydrolyzed/min/mg of protein. The BSP-glutathione conjugating enzyme activity is expressed as μg of conjugate formed/min/mg of protein.

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

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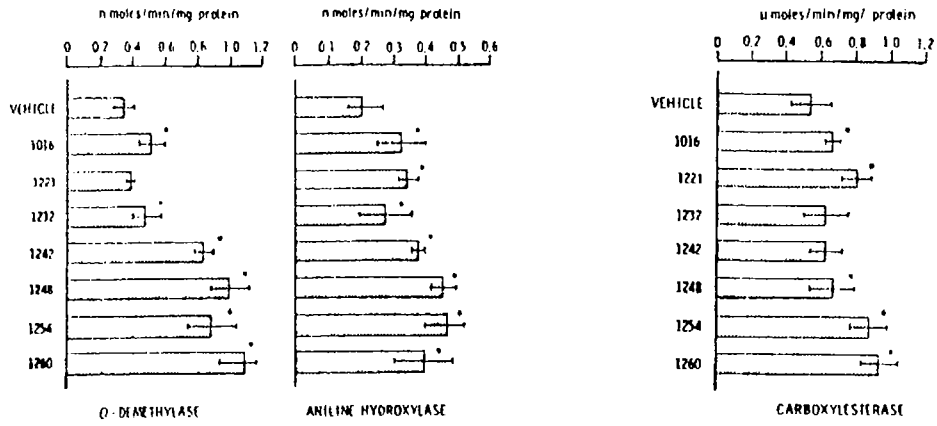

Fig. 1. The effects on hepatic *p*-nitroanisole *O*-demethylase and aniline hydroxylase of young male rats treated with a series of commercial Aroclor preparations. The animals were injected intraperitoneally for 3 consecutive days at a dose of 50 mg of agent/kg body wt. and were killed 96 h after the last injection. Activities are expressed as nmoles of product formed/min/mg of protein. The values (bars) represent the mean enzymatic activities, the horizontal lines, the standard deviations of the means of 18 control, vehicle-treated animals and 6 animals per treated group. The asterisk (*) signifies statistically significant differences from control values at $p < 0.05$.

Fig. 2. The effects on hepatic nonspecific carboxylesterase of young male rats treated with a series of commercial Aroclor preparations. For other details, see the legend of Fig. 1.

more pronounced effects were observed though there were no statistically significant ($p < 0.05$) differences between Aroclors 1248, 1254, and 1260. Compared to control activity, statistically significant ($p < 0.05$) increases in aniline hydroxylase activity were observed for all agents tested. More pronounced effects were observed with the more highly chlorinated Aroclors.

Fig. 2 is a summary of the influence of the Aroclors on the hepatic carboxylesterase activity in young male rats. The enhancement of activity was not as great as that observed for the mixed-function oxidases, the maximum being only 2-fold higher than the control activity. Of the Aroclors tested, statistically significant ($p < 0.05$) increases were observed for all, with the exception of Aroclor 1232 and 1242. The greatest responses were observed following treatment with Aroclor 1254 and 1260.

Fig. 3 summarizes the changes observed for two quite different "synthetic" conjugating enzymes, BSP-GSH conjugating enzyme and *p*-nitrophenol UDP-glucuronyl transferase following Aroclor treatment. As was observed (Table I), biphenyl caused a marked increase in the BSP-GSH conjugating enzyme. The results following Aroclor treatment demonstrated that, while levels were higher than those of vehicle-treated controls, they were not significantly ($p > 0.05$) different from the levels detected in biphenyl-treated rats. In contrast, the response of hepatic UDPglucuronyl transferase following Aroclor treatment was markedly different. Significantly ($p < 0.05$) higher levels of enzyme activity were observed only following treatment with Aroclor 1221, 1242, 1248, 1254 and 1260. A highly significant increase was

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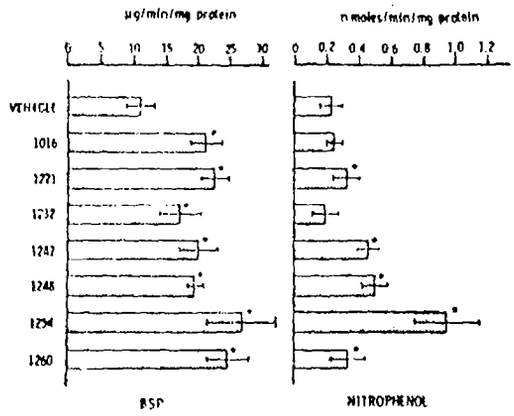

Fig. 3. The effects on hepatic BSP-glutathione conjugating enzyme and *p*-nitrophenol UDPglucuronyl transferase of young male rats treated with a series of commercial Aroclor preparations. For other details, see the legend of Fig. 1.

observed in Aroclor 1254-treated rats. The activity being 4-fold higher than that of control animals and 2-fold higher than that observed in Aroclor 1248- and 1260-treated animals.

DISCUSSION

Polychlorinated biphenyls are produced by the controlled chlorination of biphenyl at one or more of 5 possible positions on each phenyl ring, resulting in complex mixtures of congeners. Aroclor 1254 was found to contain 18 congeners (1 trichlorobiphenyl, 4 tetrachloro isomers, 4 pentachloro isomers, 5 hexachloro isomers and

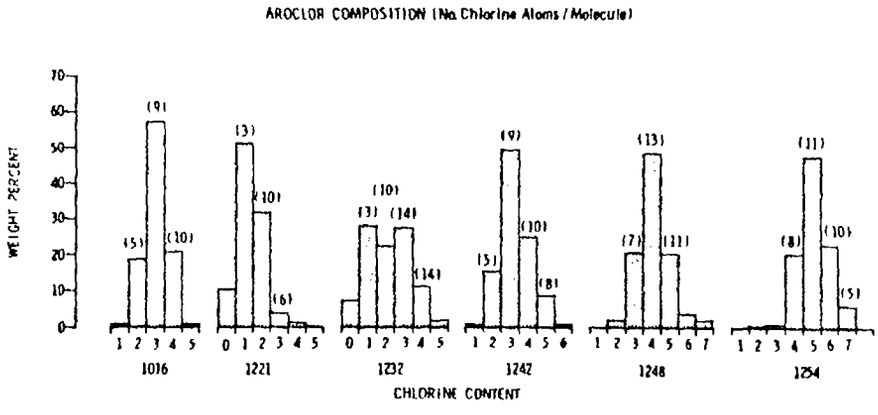

Fig. 4. The congener composition of commercially available Aroclors, based on the weight percent of biphenyls bearing different numbers of chlorine atoms/molecule. The numbers in parentheses represent the number of isomers found for any one chlorobiphenyl. Data were obtained from reports by WEBB AND MCCALL[17] and SISSONS AND WELTI[18] and from information supplied by the Monsanto Industrial Chemicals Company.

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4 heptachloro isomers)[15]. KOLMAN *et al.*[16] demonstrated that Aroclor 1260 was composed of 11 congeners (5 hexachloro isomers, 5 heptachloro isomers, and an octachlorobiphenyl). As techniques of definitive analysis have developed, studies have revealed that the Aroclor mixtures are even more complex than was previously reported. Fig. 4 summarizes data obtained for the congener composition of Aroclors from reports by WEBB AND MCCALL[17] and SISSONS AND WELTI[18] and from information kindly provided by the Monsanto Industrial Chemicals Company. With the exception of Aroclor 1016 and 1232, it can be seen that one predominant congener, composed of a number of isomers, is found in each Aroclor preparation and, as the percent chlorine increases, this congener shifts from a mono- to a tri- to a tetra- to a penta-chlorobiphenyl. The composition of Aroclor 1260 was not available but it is understood to be composed of 70 % of hexachlorobiphenyls and higher homologues.

Some European polychlorinated biphenyl mixtures (Phenoclor DP6 and Clophen A60) have been found to be contaminated with chlorinated dibenzofurans which contributed markedly to their toxicity[19]. In contrast, Aroclor mixtures appear to contain little, if any, chlorinated dibenzofurans or dibenzo-*p*-dioxins[19,20]. Only one study has reported possible contamination of Aroclor 1254 by dibenzofurans and this remains unconfirmed[21].

Can the hepatic enzyme induction observed following acute exposure to Aroclors be explained on the basis of the congener composition of the Aroclor mixtures? It has been shown that morphological alterations such as proliferation of the smooth endoplasmic reticulum, lipid inclusions, microbodies, *etc.* were more extensive in hepatic tissue of animals treated with Aroclor 1254 than with Aroclor 1260 even though the latter compound has higher chlorine content[21–23]. VOS AND NOTENBOOM-RAM[24] found that 2,4,5,2′,4′,5′-hexachlorobiphenyl caused more severe hepatic injury than did Aroclor 1260. A similar situation was found using isomerically pure chlorobiphenyls, 2,3,5,2′,3′,5′-hexachlorobiphenyl causing marked alterations similar to those caused by Aroclor 1254 and greater than those caused by biphenyl, 2,2′- or 4,4′-dichlorobiphenyl[23]. FUJITA *et al.*[5], studying the effects of oral administration of pure chlorobiphenyls to rats, observed increased hepatic weights, microsomal protein, mixed-function oxidase activities, *etc.*, the greatest changes being observed with penta- and hexa-chlorobiphenyls. BENTHE *et al.*[25] found that equimolar doses of tetrachlorobiphenyls were distinctly more effective inducers of hepatic mixed-function oxidases than were dichlorobiphenyls. Studies in this laboratory, with a series of isomerically pure mono-, di-, tetra-, hexa-, and octa-chlorobiphenyls, demonstrated marked enzymatic induction following treatment with hexa- and octa-chlorobiphenyls and marked induction by di- and tetra-chlorobiphenyls only if chlorines occupied the 3 and 4 positions on the rings[7].

Feeding rats for 4 weeks with Aroclors 1242, 1248, 1254 and 1260, LITTERST *et al.*[6] demonstrated a good correlation between hepatic mixed-function oxidase activities and chlorine content. VILLENEUVE *et al.*[3] demonstrated a marked difference in the hepatic carboxylesterase activity of Aroclor 1254-treated rabbits and those given Aroclor 1221. They showed that Aroclor 1221 treatment had little effect on hepatic aniline hydroxylase and aminopyrine *N*-demethylase in contrast to marked increases

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in these activities following treatment with Aroclor 1254 (ref. 3). BICKERS *et al.*[2] found Aroclor 1254 to be a much more potent inducer of hepatic enzymes than Aroclor 1016, the latter mixture having predominantly low-chlorine containing biphenyls (Fig. 4).

In spite of the inability to determine which of the many isomers of each congener is toxic, it can be seen from Figs. 1–4 that, with the exception of BSP-GSH conjugating enzyme, there is a correlation between congener content, the DNA, RNA and protein concentrations and ability to induce functionally diverse enzymes. Induction of *O*-demethylase and aniline hydroxylase (Fig. 1) was greatest with Aroclor 1248, 1254 and 1260, mixtures having tetra-, penta- and hexa-chlorobiphenyls as the predominant compounds. This trend was equally good for *p*-nitrophenol UDPglucuronyl transferase (Fig. 3), marked induction being observed with Aroclor 1254 containing a high concentration of penta- and more highly chlorinated biphenyls. CHEN *et al.*[26] found no induction of UDPglucuronyl transferase following administration of two tetrachlorobiphenyls. The relationship between congener composition and hepatic carboxylesterase induction (Fig. 2) was less distinct but this may be due to the fact that carboxylesterases are localized in both the inducible microsomal fraction and in the cytoplasm[27].

An interesting facet of this study was the response of the hepatic enzymes to treatment with Aroclor 1232. This mixture, containing a large number of mono-, di-, tri-, and tetra-chlorobiphenyl isomers (Fig. 4), caused no induction of carboxylesterase or of UDPglucuronyl transferase activities. Where induction occurred with this compound, the activities were lower or were not significantly different from that obtained after treatment with Aroclor 1221. It would appear that the isomeric forms of the different congeners may play an important role in the induction phenomenon. Certain positions on the ring, primarily the 3 and 4 positions occupied by chlorine, caused induction as marked as that obtained with highly chlorinated hexa- or octachlorobiphenyls[7]. A good correlation was observed between morphological alterations and enzymatic activities within the same rats treated with isomerically pure chlorobiphenyls differing in degree and position of chlorine[7,23]. The variety of isomers possible for any one chlorobiphenyl makes toxicological assessment of the Aroclor mixtures extremely difficult.

ACKNOWLEDGEMENTS

We are grateful to W. B. PAPAGEORGE of the Monsanto Industrial Chemicals Company for providing te samples of Aroclors and the information about their composition. The technical assistance of Miss P. CAMERON is gratefully acknowledged.

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