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Elimination of Chlorinated Dibenzofurans Associated with Polychlorinated Biphenyls Fed to Mallards (*Anas platyrhynchos*)

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Elimination of Chlorinated Dibenzofurans Associated with Polychlorinated Biphenyls Fed to Mallards (*Anas platyrhynchos*) NORSTROM, R. J., RISEBROUGH, R. W., AND CARTWRIGHT, D. J. (1976). *Toxicol Appl. Pharmacol.* 37, 217–228. A method which allows chlorinated dibenzofurans (CDFs) to be extracted from lipid and separated from interfering polychlorinated biphenyls (PCBs) was tested. Greater than 90% recovery of tetra- to hexa-CDFs was found. Starting with 40 g of lipid, the detection limit for individual CDFs in lipid was of the order of 0.01 μg/kg. Lipid from mallards which had been fed Aroclor 1254 for approximately 1 year at levels from 5 to 100 mg/kg in the diet was found to contain 3750 mg/kg of PCB. The minimum concentration of individual CDFs (nine tetra- to hexa-CDFs) potentially in the lipid from this PCB concentration was in the order of several tenths μg/kg. It was concluded that less than 3% of the dosed CDFs were accumulated by the mallards, since CDFs could not be detected by a comparison of lipid to Aroclor 1254 extracts containing the same amount of PCBs. It appears unlikely that CDFs are sufficiently persistent in avian species to enable their detection in environmental samples.

The chlorinated dibenzofurans (CDFs) are structurally similar to the chlorinated dibenzo-*p*-dioxins (CDDs), some of which are both highly toxic and teratogenic (Schwetz *et al.*, 1973; Verrett, 1971). The effectiveness of several tetra- and penta-CDFs, particularly 2,3,7,8-tetra-CDF, in inducing aryl hydrocarbon hydroxylase activity has been found to be nearly as high as that of 2,3,7,8-tetra-CDD (A. Poland and A. S. Kende, personal communication), the latter being the most toxic of the CDDs. The potency of aryl hydrocarbon hydroxylase induction by CDDs has been shown to be strongly correlated with toxicity (Poland and Golver, 1973). A dietary level of 5 μg/kg of 2,3,7,8-tetra CDF was found to be highly toxic to chicks (Goldstein *et al.*, 1974).

Embryotoxicity of the polychlorinated biphenyls (PCBs) Clophen A-60 and Phenoclor DP-6 has been attributed to CDFs present as trace contaminants in the commercial preparations (Vos and Koeman, 1970; Vos *et al.*, 1970). Subsequently, tetra-, penta-, and hexa-CDFs were detected in the American preparations of PCB,

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Aroclor 1248, 1254, and 1260; concentrations of the individual CDFs were in the order of 0.1 μg/kg of the PCB (Bowes *et al.*, 1973). Therefore, CDFs are possible causes of embryonic mortality and birth defects observed in PCB-feeding experiments (Tumasonis *et al.*, 1973), and in PCB-contaminated populations of Common Terns (*Sterna hirundo*) in Long Island Sound (Hays and Risebrough, 1972) or Herring Gulls (*Larus argentatus*) in Lake Ontario (Gilbertson and Hale, 1974).

Although little is known of their environmental persistence or accumulation in food webs, substantial amounts of CDFs have entered the global environment associated with PCBs as well as with chlorinated phenols (Buser *et al.*, 1975). Attempts to detect CDFs in eggs of Common Terns (*Sterna hirundo*) and Ospreys (*Pandion haliaetus*) from Long Island Sound, in eggs of Herring Gulls from Lake Ontario (R. W. Risebrough and G. W. Bowes, unpublished results; Bowes *et al.*, 1973), and in several species of birds and fish from the Bay of Fundy region (Zitko, 1972) have been unsuccessful. In some cases, amounts of material used for lipid extraction, cleanup, and subsequent analysis may have been too small. Before starting on a more exhaustive search for residues in birds whose lack of reproductive success may be attributable to CDFs, it was considered important to establish a method for quantitative recovery, and to obtain information on the persistence of CDFs in avian species.

A PCB-feeding experiment of mallards (*Anas platyrhynchos*) carried out in 1971 and 1972 showed both embryonic mortality and deformities among ducklings (D. Cartwright, unpublished results). Subsequently the Aroclor 1254 used in this experiment was examined for its CDF content, and the concentrations of tetra-, penta-, and hexa-CDFs were determined [preparation Aroclor 1254 (*1970*) of Bowes *et al.*, 1975a]. Total CDF concentration was 1.5 mg/kg of the PCB used. A high PCB concentration in the mallards at the end of the experiment and the ample amount of available material afforded an opportunity to determine the relative persistence of CDFs to PCBs in duck tissues during the feeding experiment.

METHODS

PCB Feeding Experiment

Aroclor 1254[3] was dissolved in corn oil and stirred into duck feed with a Hobart feed blender. Three groups of 22 birds, 11 females and 11 males, were fed diets containing 5, 50 and 100 mg/kg of Aroclor 1254 starting January 1, 1971. Progeny of these birds hatched in the spring of 1971 were fed on the same diet as their parents until June 22, 1972, when they were killed. The average dosage period of the progeny was 296 days.

A total of 23 progeny, about four males and four females from each of the dosage groups, was used for the current study. Beaks, long wing feathers, and feet were removed and discarded, and the remainder was thoroughly homogenized by three passes through a Hobart food chopper. After mixing and freeze drying, about 10% of the sample was placed in a preextracted paper thimble in a large Soxhlet apparatus and extracted with 5 liters of hexane/acetone azeotrope for 48 hr. The bulk of the solvent was removed in a large evaporating dish on a warm hot plate with successive additions of the extract to prevent oxidation of lipid, and the final solvent removed under a nitrogen stream, leaving 740 g of lipid.

[3] Canada Colours, Ltd., Toronto, Canada; purchased in 1970.

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Two 0.5-g aliquots of the lipid were cleaned up for PCB analysis on a 2% water-deactivated Florisil column (McLeod and Ritcey, 1973) using 300 ml of hexane as the eluant. All analyses were performed on a Hewlett-Packard 5700 A gas chromatograph equipped with a linear Ni-63 detector and a 6-ft × 2-mm i.d. glass column at 190°C packed with 3% SP-2100 on Supelcoport 100/120 mesh.[3] Other conditions were: injection port at 250°C, detector at 300°C, and flow rate at 30 ml/min of 10% methane in argon. Chromatograms of the Aroclor 1254 standard and the lipid extract are compared in Fig. 1. Using the peak with retention time approximately 10 min as reference, the concentration of Aroclor 1254 in the lipid was calculated to be 3750 mg/kg.

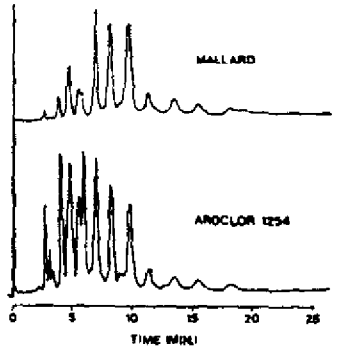


FIG. 1. Relative abundance of PCBs in Aroclor 1254 compared to those in mallard lipid after 296 days' administration of Aroclor 1254. The chromatograms have been normalized to the peak with retention time of 10 min.

Separation of CDFs from Lipids and PCBs

The method for separation of CDFs and PCBs from lipids (procedure 1, Table 1) was adapted from the column partitioning method of Porter and Burke (1973). The necessity of using large volumes of lipid ruled out adsorption column cleanup. The Florisil[4]/lipid column was prepared according to Porter and Burke (1973), but the eluting solvent was changed to 5% water/acetonitrile. The acetonitrile was shaken with 100 ml of hexane, then 50 ml of saturated sodium sulfate, 800 ml of water, and 15 ml of methylene chloride were added. After shaking again, the aqueous layer was discarded and the hexane layer was washed twice with 100 ml of water. The hexane layer was filtered through anhydrous sodium sulfate, evaporated to dryness in a rotary evaporator, and the residue was taken up in 5 ml of hexane. Previous experience in our laboratory had shown these modifications to give essentially 100% recovery of organochlorines such as Mirex which are poorly recovered by the original method, without significantly increasing the amount of co-eluting lipid.

The separation of PCBs from CDFs was the same as that of Bowes *et al.* (1975a). The bulk of the PCBs was removed on a column of 180 g of Florisil (deactivated with 2% water), as outlined in procedure 2, Table 1. Up to 2 g of PCBs may be placed on the column.

[3] Supelco, Inc., Bellefonte, Pa.
[4] Florisil PR grade; Floridin Corp., Pittsburgh, Pa.

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TABLE 1

COLUMN CHROMATOGRAPHY PROCEDURES USED FOR THE SEPARATION OF CDFs FROM PCBs AND LIPIDS[a]

Procedure No.	Column type	Eluant	Eluant volume	Fraction no.	Content of fraction
1	Partition[b]	5% water/acetonitrile	200	—	Lipid, biogenic hydrocarbons, PCBs, PCNs and CDFs
2	Florisil[c]	hexane	1200	1	PCBs, PCNs
		5% diethyl ether/hexane	400	2	PCBs, PCNs, di- and tri-CDFs
		5% diethyl ether/hexane	400	3	PCBs, PCNs, tetra- and penta-CDFs
		25% diethyl ether/hexane	400	4	PCBs, PCNs, tetra-, penta-, and hexa-CDFs
		25% diethyl ether/hexane	400	5	PCBs, PCNs, hexa-CDFs, lipid, and biogenic hydrocarbons
		acetone	500	6	lipid, biogenic hydrocarbons
3	Alumina[d]	1% methyl chloride/hexane	10	—	PCBs, PCNs
		20% methylene chloride/hexane	10	—	tetra- to hexa-CDFs, some PCNs, lipid and hydrocarbons
		50% methylene chloride/hexane	10	—	lipid, biogenic hydrocarbons

[a] CDF = chlorinated dibenzofuran; PCB = polychlorinated biphenyl, PCN = polychlorinated naphthalene

[b] Porter and Burke (1973) See text for modifications For mallard lipid, five partition column eluates, concentrated to 10 ml, were combined for procedure 2

[c] Vos *et al* (1970), Bowes *et al* (1975a) Fractions 1 and 2 contained the majority of the PCBs, and were discarded Fractions 3, 4, and 5, which were highly enriched in CDFs and PCNs, were evaporated just to dryness and taken up in 1 ml of hexane for procedure 3. Fraction 6 did not contain any CDFs

[d] Porter and Burke (1971) Fractions 3, 4, and 5 may be passed through procedure 3 either separately or combined The first eluate was discarded The second eluate was evaporated to dryness, then taken up in 1 ml of hexane and tested by GC for the presence of PCBs The second eluate was passed through procedure 3 up to two more times The third eluate potentially contained higher chlorinated CDFs, but no CDF present in PCB mixtures has been found in this eluate

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TABLE 2

RECOVERY OF TRI- TO HEXA-CDFS FROM THE CLEANUP AND PCB SEPARATION PROCEDURES[a]

Recovery test no.	Procedure no. from Table 1	Percent Recovery of: 2,3,8-tri-CDF	3,4,6,7-tetra-CDF	1,2,3,7,8-penta-CDF	1,2,3,6,7,8-hexa-CDF	2,3,4,6,7,8-hexa-CDF
1	2	92	95	95	40[b]	94
2	2 and 3	87	97	95	39	94[c]
3	1, 2, and 3	82	94	93	36	—

[a] The spiking levels and test solutions are described in the text. All recoveries are the mean of two results. The results were within ±2 percentage units from the mean in all cases.

[b] A large excess of acetone eluant did not recover any further amount.

[c] The recovery in the alumina procedure (No. 3, Table 1) was 39% in the 20% methylene chloride/hexane fraction. An additional 55% appeared in the 50% methylene chloride/hexane fraction.

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Further separation to remove last traces of PCBs was accomplished by passing fractions 3, 4, and 5 from the Florisil column through an alumina[5] microcolumn, as outlined in procedure 3, Table 1. Each of the three 20% methylene chloride/hexane eluates was passed two more times through procedure 3.

In order to have as direct a comparison as possible between CDFs in Aroclor 1254 fed to mallards and the amount present in the lipid, a solution of Aroclor 1254 was made in corn oil so that the PCB concentration was the same as that in the mallard lipid (3750 mg/kg). A series of five 8-g aliquots of both the corn oil solution and the mallard lipid were then carried through procedure 1 (Table 1) and combined for separation of the PCBs and CDFs by procedures 2 and 3, for a total of 40 g of lipid and 150 mg of PCB per sample. The series was done in duplicate. The final volume was 1 ml for each of the three final fractions used for GC analysis.

Recovery Experiments

The recovery of CDFs was studied in three tests employing spikes of authentic CDF standards. A tri-, tetra-, penta- and two hexa-CDFs, as indicated in Table 2, were chosen because their retention times on the SP-2100 column employed for GC analysis did not overlap those of any CDFs present in Aroclor 1254 (Bowes *et al.*, 1975b). Aroclor 1254 was spiked at the 0.1-mg/kg level with the first four authentic CDFs for recovery tests 2 and 3. The solutions for each of the recovery tests were made up as follows: test 1, approximately 15 mg of each of the five authentic CDFs dissolved in 10 ml of hexane; test 2, 2 g of spiked Aroclor 1254 dissolved in 100 ml of hexane; and test 3, 150 mg of spiked Aroclor 1254 dissolved in 8 g of corn oil.

RESULTS

Table 2 indicates the results of the recovery tests. With the exception of 1,2,3,6,7,8-hexa-CDF, the recovery of all CDFs was generally greater than 90%. Neither the absolute amount of CDF present, nor the presence of PCBs and lipid appeared to affect the recoveries. The tri-CDF was partially lost in the alumina chromatography step. The methods outlined in Table 1 can therefore be recommended for CDFs with four to six chlorine atoms per molecule. It is not known whether hepta- and octa-CDFs would be recovered by the methods. Complete recovery of 2,3,4,6,7,8-hexa-CDF from the alumina column required a 50% methylene chloride/hexane eluant.

The low recovery of 1,2,3,6,7,8-hexa-CDF could be ascribed to partial decomposition during storage as a solution in hexane. GC analysis of the standard showed the parent and a decomposition peak at longer retention time. The second peak was not recovered in any of the tests, indicating that it was a polar compound. Since the parent CDF was recovered in fractions 4 and 5, Table 1, but no more could be eluted with acetone in fraction 6, it is probable that during decomposition a compound formed which could react in the GC inlet to reform the parent CDF, but could not be eluted from Florisil.

The pattern of PCB accumulation by the mallards at the end of the 296-day dosing period is shown in Fig. 1. All peaks with retention time less than approximately 6 min were significantly reduced relative to the later peaks. Peaks with retention time greater than approximately 10 min remained essentially unchanged in height relative to one

[5] Cat. No. A540; Fisher Scientific, Fair Lawn, N.J.

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another when the mallard extract was compared to the Aroclor 1254 standard, as was found for White Carneau pigeons (deFreitas and Norstrom, 1974). This is strong indication that the later PCBs were not metabolized to any extent by the mallards.

Elimination of unmetabolized PCB did, however, occur in females, since egg production is an important route of excretion of unmetabolized PCBs from female birds (Dahlgren *et al.*, 1972). In the present study, the majority of females were producing eggs during the 3-month period prior to sacrifice As a result, the PCB concentration in the fat of individual dosage groups, which ranged from a low of 300 mg/kg for 5 mg/kg-fed females to 9988 mg/kg for 100 mg/kg-fed males, was two to three times higher for males than females in each dosage group (D. Cartwright, unpublished results)

The PCB concentration in the lipid of the composite whole body sample used for CDF analysis therefore gave the minimum amount of CDFs potentially in the lipid, that is, assuming that no metabolism or excretion of PCBs or CDFs had occurred. Using values given by Bowes *et al.* (1975a) for the CDF content of Aroclor 1254 (*1970*), and 3750 mg/kg of Aroclor 1254 in lipid, the minimum potential concentrations in the lipid were approximately 0.8 μg/kg of tetra-CDFs, 1 5 μg/kg of penta-CDFs, and 0.9 μg/kg of hexa-CDFs.

Figure 2 compares the CDF content of 150 mg of Aroclor 1254 standard to the CDF content of 40 g of mallard lipid. The lipid also contained 150 mg of Aroclor 1254, based on the PCB reference peak with retention time 10 min (Fig. 1) For the individual CDFs

TABLE 3

IDENTITY OF THE CDFs AND PCNs PRESENT IN AROCLOR 1254

Peak designation[a]	Compound type	No of chlorines	Relative retention time[b]	Reference[c]
A	CDF[d]	4	1 58	a
B	PCN	6	1 72	a
C	PCB	5	1 90	a
D	PCN	6	2.00	a
E	CDF	5	2.40	b
F	CDF	5	2.54	b
G	CDF	5	2.77	b
H	CDF[e]	5	2.95	a
I	PCN	7	3 44	a
J	CDF	6	4.11	c
K	CDF	6	4 25	c
L	CDF	6	4.68	b
M	CDF	6	4.98	b
N	PCN	8	5 68	c

[a] As shown on Fig. 2.
[b] Relative to dieldrin on a 6-ft., 3% SP-2100 (OV-1) column.
[c] a, Bowes *et al* (1975a); b, Bowes *et al.* (1973); c, G. W Bowes, personal communication.
[d] Positional isomer identified as 2,3,7,8-tetrachlorodibenzofuran
[e] Positional isomer identified as 2,3,4,7,8-pentachlorodibenzofuran

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in Aroclor 1254, the peak height reproducibility between duplicates was within 10%. The lettered peaks and their retention times relative to dieldrin are listed in Table 3. Only two of the nine CDFs have been structurally identified (Bowes *et al*, 1975b).

With minor exceptions, there were no detectable peaks in the mallard lipid extract that matched a CDF present in Aroclor 1254. Based on a 2-mm peak height, less than

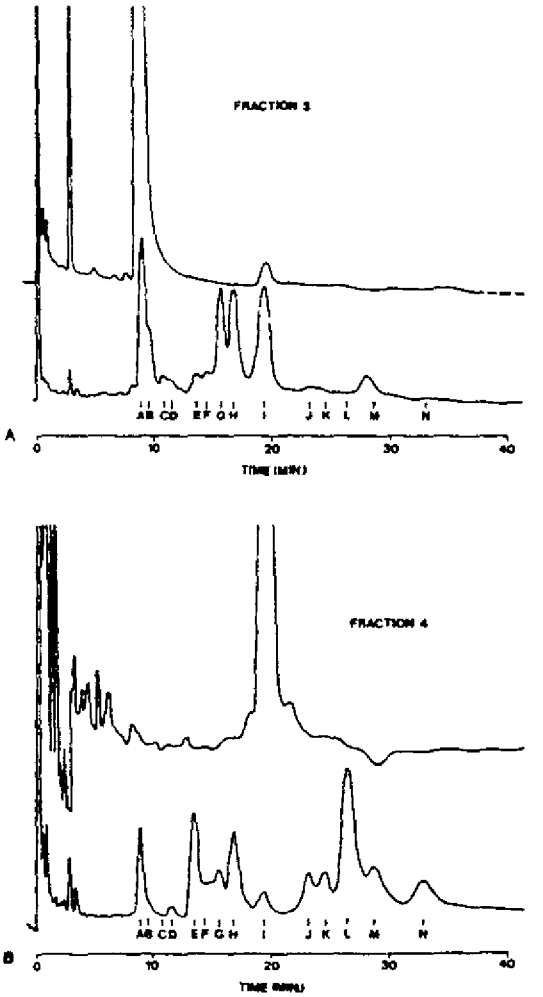


FIG. 2 Relative abundance of CDFs in Aroclor 1254 (lower chromatograms) compared to those in mallard lipid (upper chromatograms) after 296 days' oral administration of Aroclor 1254. Upper panel (A) is Fraction 3, Table 1; middle panel (B) is Fraction 4, Table 1; panel (C) is Fraction 5, Table 1. The amount of Aroclor 1254 represented is the same for all chromatograms, based on the peak with retention time 10-min, Fig. 1.

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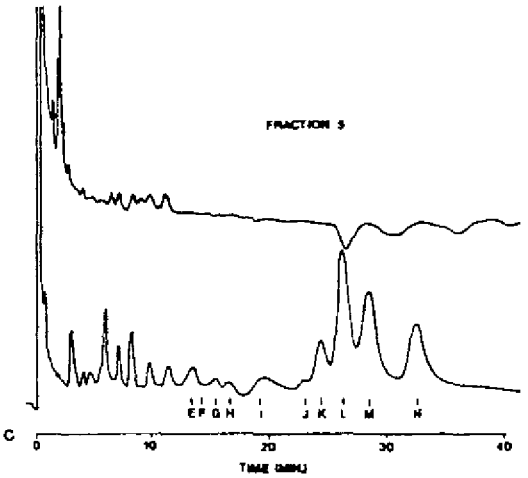


FIG 2 *continued*

3% of the minimum potential concentration of CDFs was estimated to be present. The actual concentration may have been much lower. The detection limit for individual CDFs was estimated to be in the order of 0.01 μg/kg starting with a 40-g lipid sample.

The large peak in the mallard extract with a similar retention time to peak A (Fig 2A) was shown not be 2,3,7,8-tetra-CDF by means of differing retention times on a 5-ft 3% OV-1/5% OV-210, 0.5-ft 3% OV-1/6% OV-225 column. The efficiency of PCB and polychlorinated naphthalene (PCN) separation from CDFs in mallard fraction 3 (Fig 2A) was not as good as, and more variable than that of pure Aroclor 1254, probably due to the presence of biogenic hydrocarbon or lipid materials. The heptachloronaphthalene, peak I, derived from Aroclor 1254 was present in large quantities in the mallard lipid (Fig 2B), indicating that it was not easily metabolized or excreted. The negative peaks in the chromatogram of mallard fraction 5 (Fig 2C) were shown to be due to hydrocarbons when Florisil cleanup and treatment with concentrated sulfuric acid did not remove them. GC analysis using a flame ionization detector showed a continuous series of peaks with retention times from 5 min to 2 hr.

DISCUSSION

Little is known about the environmental persistence and bioaccumulation potential of CDFs. They are chemically stable (e.g., towards strong acids and bases) like many of the more persistent organochlorines, but also more photochemically labile than PCBs (Hutzinger *et al.*, 1973). There is some evidence that Atlantic salmon (*Salmo salar*) accumulated di-, tri-, and tetra-CDFs from food to increasingly higher levels as the number of chlorines/molecule increased (Zitko *et al.*, 1973). Explanations for the increase included easier metabolism, faster excretion and decreased efficiency of uptake from the GI tract of the lower chlorinated CDFs. The accumulated concentrations of all CDFs were very low. Curley *et al.* (1975) produced tentative mass spectral evidence that a

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tetra-CDF was eliminated in the urine of rats fed Aroclor 1254 The presence of 2,3,7,8-tetra-CDF in Aroclor 1254 has been confirmed (Bowes *et al*, 1975b) The ability to detect CDFs in urine suggests that they are more rapidly eliminated from the rat than PCBs, since the total tetra-CDF intake of all rats throughout the study was approximately 2 4 μg,[a] and the urine sample represented only a small time period in the PCB-ingestion experiments.

Studies with the chemically similar CDDs may be indicative of CDF bioaccumulation potential. Microbial metabolism and model ecosystem studies with 2,3,7,8-tetra-CDD (TCDD) indicated that it was more stable towards metabolic breakdown than most organochlorine compounds (Matsumura and Benezet, 1973, Isensee and Jones, 1975) However, unlike DDE and the more highly chlorinated PCBs, which are also relatively resistant to metabolism, TCDD appeared to be less efficiently absorbed from the GI tract, and was cleared from organisms more rapidly as the unchanged molecule (Vinopal and Casida, 1973, Piper *et al*., 1973).

The evidence, therefore, seems to point to a faster elimination of CDFs than of PCBs, which is strongly reinforced for birds by the present study which has shown that mallards eliminated >97% of all CDFs associated with Aroclor 1254 in the long-term feeding experiment. The PCB concentrations reached were similar to those found in the most highly contaminated wild birds, such as Herring Gulls in Lake Ontario (Gilbertson, 1974; Frank *et al*., 1975) A large body burden of PCBs may contribute to more rapid clearance of CDFs by inducing a higher level of hydroxylase activity.

Further attempts to find CDF residues in the environment, even in birds with exceptionally high PCB concentrations, appear destined to fail The practical difficulties of environmental CDF analysis can be seen from the present study. To achieve the CDF detection limit of 0 01 μg/kg in lipid, approximately 500 g wet weight tissue, containing a potential CDF concentration of 0 8 ng/kg (ppt), was required for each analysis The use of mass fragmentography may eliminate time-consuming cleanup steps (Buser, 1975), but is unlikely to give much better detection limits The contribution of chlorinated phenols to environmental contamination by CDFs may be as high as that of PCBs (Firestone *et al*., 1972), but given the apparent lack of CDF bioaccumulation, this contribution is also unlikely to result in measurable residues at the top of a food web.

ACKNOWLEDGMENTS

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[a] Three grams total Aroclor consumption times 0 8 mg/kg tetra-CDFs (Bowes *et al*, 1975a).

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