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Session I:

HEALTH EFFECTS AND HUMAN EXPOSURE

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MONS 202550

INTRODUCTORY REMARKS

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The history of PCB's is a remarkable case study in the continuing story of toxic substances and the public health. From 1929, when it was first manufactured in the United States, until 1966, it was presumed that this substance was being used primarily in a closed environment. However, in 1966 PCB's were found in fish in the Baltic Sea. Then they were discovered in birds and other animals. Since then it has been demonstrated they have an almost global distribution. Three years after being discovered as an environmental contaminant, they were determined to be the causative agent in an outbreak of a disease in Japan now called "Yusho" or "oil" disease.

Thus, in a relatively short period of time, we saw a manmade compound introduced into commerce for a relatively narrow purpose, a closed use, broadened through new applications, and discovered to be hazardous to animals, birds, fish, and to man. In slighly more than two generations, we had closed the all-too-frequent circle of progress: product development, distribution, use, and resulting hazard to human health.

In 1971, we became concerned that the PCB's appeared to be an ever-expanding problem whose potential limits were essentially unknown. Thus, in December of 1971 we called a 2-day open meeting to discuss the entire range of current and potential health problems associated with the widespread use and dispersion of PCB's. The meeting was broadly attended by scientists and administrators throughout the world who were concerned with this problem.

The scientists at that meeting focused on the entire spectrum of problems that needed investigation. Their papers ranged from biological concerns: animal toxicology, mechanisms of action, and human body burden; to regulatory and control concerns: environmental transport, distribution, and alteration, and occurrance. In his "Where do we go from here" summary, Dr. Norton Nelson singled out two general areas of concern.(ref.1).

First, relative to environmental distribution patterns, including: (1) further refinement of sources, and (2) better quantitation of discharge amounts by route into water and air. Second, he pointed out that there were inadequate data to determine if the PCB's caused malignant tumors in laboratory animal studies and urged that we follow the ongoing studies with care. He also pointed out the potential deleterious affects of the PCB's on reproduction in mammals.

In following up these and the other points Dr. Nelson made, scientists, many of them in DHEW, have been engaged in attempting to develop the information required for the resolution of the biological aspects of this problem. Last month, in order to insure that our Department's efforts are as effective as possible, we established a Subcommittee on PCB's of the DHEW Committee to Coordinate Toxicology and Related Programs.

Through this Subcommittee we will:

1. Assemble, review, and interpret data that assess the health significance of polychlorinated biphenyls, and
2. Formulate recommendations as to future research needs.

In all of these efforts, we will be cooperating with those agencies which must carry out regulatory responsibilities in this difficult area. Continuing collaborative efforts by biological scientists here and abroad have brought us far toward the resolution of these problems. The reports which you will hear relating to Health Effects and Human Exposure in the session which follows should indicate the progress which we have made since the 1971 conference.

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REFERENCE

1. Norton Nelson, "Comments on Research Needs," *Environmental Health Perspectives 1* Vol. 1 (April 1972), pp. 181-186.

MONS 202551

SOME OF THE RECENT FINDINGS CONCERNING YUSHO

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Abstract

Analysis of Yusho disease, which was first detected in 1968, has been limited and analyses have produced varying results. Yusho oil has been determined to contain a high level of polychlorinated dibenzofurans (PCDF's). Nagayama et al. found that PCDF levels in the oil were especially high when the oil was contaminated with PCB's used as a heat transfer medium. Their studies also showed that the concentration of PCDF's is much closer to that of PCB's in liver than in adipose tissues in patients with Yusho.

The current clinical state of patients with Yusho is dicussed at length. Subjective symptoms, dermatological findings, serum triglyceride levels, liver conditions, mortality rates, and the effects on children born to mothers with Yusho are all reported.

INTRODUCTION

More than 7 years have passed since the outbreak of an epidemic of Yusho in 1968. According to the latest tabulation, Prof. Omae, current chief of the Study Group for the Therapy of Yusho, reported that a total number of 1,291 patients have been registered as Yusho in 22 prefectures of Western Japan by April 30, 1975 (ref. 1).

We would like to describe some of the recent findings concerning Yusho which we think to be particularly relavent for understanding of toxicity of PCB's. For more detailed information, readers are advised to refer to original papers appearing mainly in the fourth and fifth reports of the study on Yusho and PCB (ref. 3). Most of the patients described in these reports are those living in Fukuoka prefecture. Published information of patients living in other areas are unfortunately very few, so with a few exceptions no reference will be made to them.

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PCB's IN THE BODIES OF PATIENTS WITH YUSHO

1. *Tissues*

First of all, the concentration of PCB's retained in the tissues and fluids of patients with Yusho should be referred to before their current clinical state will be discussed.

Although no accurate estimation is feasible because of the very limited number of analyses so far made, the concentration of PCB's in adipose tissues of patients seems to have been fairly high soon after the occurrance of poisoning—that is, in November 1968, at least 1 month after the discontinued use of the toxic rice oil by patients, as shown in table 1. The corresponding concentrations were considerably lower in 3 patients who died in the next year, 1969. However, no such marked difference could be seen between those who died in 1969 and the subsequent decedents, although cases 7 and 10, who died in 1970 or 1975, showed quite low levels of PCB's in adipose tissues. As compared with the figures available from a nationwide survey on residual PCB's in autopsied tissues, the levels noted in the recent decedents are considered to be fairly close to the usual level of ordinary autopsied materials. Similar facts were also noted for PCB concentration in the skin and liver.

2. *Blood*

Since the analysis of PCB's in blood started only after 1972, no figures are available in regard to the blood levels of PCB's in patients in the earlier stage of poisoning. As shown in table 2, however, it is clear that the blood levels of patients had approached the level of ordinary parsons already in 1972, although they were still significantly higher than that level. This fact as well as the previous fact of lowered tissue levels of PCB's in recent decedents seem to be rather surprising if we consider also that the majority of patients are still showing various clinical symptoms, as will be discussed later.

3. *Gaschromatographic Patterns of PCB's in the Bodies of Patients*

Masuda first noted a peculier common gas-chromatographic pattern of PCB fractions isolated from various tissues, blood, and breast milk of patients with Yusho and called the attention of the Study Group for the Therapy of Yusho to it in early 1972. Figure 1 shows a typical example of such a pattern in comparison

MONS 202552

Table 1. PCB's concentration in tissues of patients with Yusho and other diseases

Case	Time of death, operation	PCB's (ppm)						Reference
		Skin		Adipose tissue		Liver		
		Whole basis	Fat basis	Whole basis	Fat basis	Whole basis	Fat basis	
Case 1 High school boy	Nov. 1968			76 (face) 13 (abdomen)				
Case 2, 3 Adult male, female	Nov. 1968			32, 46 (cheese-like substance from acneform eruptions)				10
Case 4 Boy, 13 yr	July 1969			1.3 (mesentery)	3.7	0.14	9.5	
Case 5 Male, 25 yr	July 1969	1.2	8.7	2.8 (mesentery)	15.1	0.2	10.4	
Case 6 Male, 73 yr	Nov. 1969	1.0	4.4	3.8 (mesentery)	8.4	0.07	3.1	11, 12
Case 7 Female, 48 yr	Dec. 1970	0.6	0.8	0.7 (mesentery)	0.9	0.07	1.3	
Case 8 Male, 46 yr	May 1972	1.8	3.2	4.3	6.5	0.08	8.4	
Case 9 Female, 33 yr	Sept. 1972			1.9 (subcutaneous)	2.9			
Case 10 Male, 72 yr	April 1975			0.19 (mesentery)	0.4	0.04	3.0	14
National survey Males and females 25 - 49 yr	1973	0.04 - 1.7 (n = 54)		0.2 - 4 (n = 47)	0.3 - 6.4 (n = 48)	0.01 - 0.6 (n = 51)	0.02 - 3.1 (n = 30)	13

MONS 202553

Table 2. PCB's in blood patients with Yusho, workers and ordinary persons

Material	No. of subjects	Time of examination	PCB's (ppb) Whole basis mean ± S.D.	Range	Reference
Whole blood					
Yusho patients	41	March - August 1973	7	2-26	7
Ordinary persons	37	1972	3	1-7	
Plasma					
Yusho patients	15		6.3 ± 4.0	2-15	
Normal persons	82		3.0 ± 1.3	1- 7	
Whole blood		Jan. 1972			8
Yusho patients	25		4.8 ± 2.9	1-12	
Normal persons	11		2.8 ± 1.5	1- 6	
Whole blood					
Workers[a]	23	1972	364 ± 262	60-920	9

[a]Workers engaged in the production of Kanechlor 200-600 in the air containing 0.05 to 0.2 mg/m^3 of PCB's [Kanechlor-300 + Kanechlor-400 (1:1)]. Two of them showed dermal signs.

with the common one seen in ordinary persons, which is very much close to that of a mixture of Kanechlor 500 + 600 (1 : 1). It is easily notable that the peak 1, which appears immediately after p,p'-DDE in the gas chromatogram in figure 1, is very low in patients with Yusho as compared with ordinary persons, while the peak 6 is much more prominent in Yusho than in ordinary persons. Masuda and his associates observed this peculiar pattern (designated as type "A") in blood of about 60 percent of patients with Yusho, and a somewhat similar pattern (designated as type "B") in about 37 percent (refs. 4,7,11,12).

Takamatsu et al. also observed the same peculiarity in the blood of patients with Yusho (ref. 8). It was further demonstrated that those patients who show the peculiar gas-chromatographic pattern as designated as pattern A by Masuda et al. have a higher average concentration of PCB's in their blood than do other patients (refs. 4,7). Abe et al. (ref. 23) examined in 1974 the PCB residues in the plasma of 30 children born to 18 mothers who had consumed Yusho oil at Goto Islands, Nagasaki prefecture. The specific gas-chromatographic pattern of type A was seen among 24 percent of the children and 44 percent of their mothers, but the peculiarity seemed somewhat less marked as compared with the one seen among patients in Fukuoka prefecture.

The chemical and toxicological nature of the compound or compounds yielding the above-mentioned peak 6 must be clarified but have not yet fully been examined. Masuda, however, considers the peak 6 to be 3,4,2',3',4',5'-hexachlorobiphenyl, judging from its retention time on the Apiezon L column developed by Jensen and Sundström (ref. 22).

MONS 202554

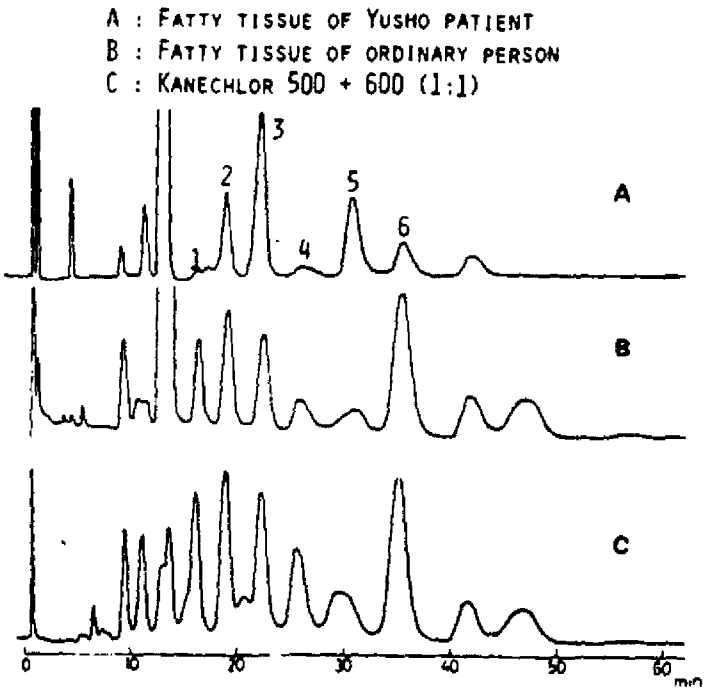

Figure 1. Gaschromatograms (ECD) of PCB's on SE-30.

POLYCHLORINATED DIBENZOFURANS IN KANECHLORS, "YUSHO OIL," AND TISSUES OF PATIENTS

1. *Polychlorinated Dibenzofurans in Kanechlors and "Yusho oil"*

Recently Nagayama et al. analyzed Kanechlors and three samples of toxic "Yusho oil" used by three independent families with Yusho for polychlorinated dibenzofurans (PCDF's) and polychlorinated dibenzo-p-dioxins (PCDD's) (ref. 18). They did a column chromatographic fractionation of PCDF's and PCDD's from a bulk of PCB's by using activated alumina as adsorbent, and n-hexane, n-hexane containing 20 percent carbon tetrachloride, or n-hexane containing 20 percent methylenchloride as eluent. The fractions thus obtained were subjected to gas-chromatographic and mass-spectrometric examination. Quantitative estimation of PCDF's and PCDD's was made by two methods, namely by measuring the gas chromatographic peak heights and by measuring the gas chromatographic peak area of perchlorinated derivatives of these compounds.

Although no PCDD's were found in any of these samples, PCDF's were found in all of them. As shown in table 3, KC-400 contained the highest concentration of PCDF's, about 18 ppm of PCDF's consisting of dichloro- up to pentachloro- dibenzofurans. A peak with the same retention time as that of 2,3,7,8-tetrachlorodibenzofurans, which was kindly provided by Dr. J. G. Vos, was noted in its gas chromatogram. All of the three samples of "Yusho oil" also contained about 5 ppm of PCDF's, the major constituents of which were tetra- and pentachlorodibenzofurans. Here again, the peak with the same retention time as 2,3,7,8-tetrachlorodibenzofuran was noted. Kashimoto also found 1.6 ppm of PCDF's in another batch of "Yusho oil," as shown in table 5 (ref. 18). Table 4 summarized the concentrations of PCDF's in Kanechlor-400, reported by several authors. As clearly noted, there is a fairly large discrepancy in their findings. Whether or not it was due to their analytical procedures or to batch difference cannot be decided at the present time.

Another important fact was disclosed by Nagayama et al. They determined the concentration of PCB's in "Yusho oil" as approximately 1,000 ppm or slightly less than 1,000 ppm, as shown in table 3. Since "Yusho oil" is known to have been contaminated with Kanechlor-400, the ratio of the concentration of PCB's to that of PCDF's in "Yusho oil" is expected to be about 1,000:0.018. The observed ratio, approximately 1,000:5,

MONS 202555

Table 3. PCDF's and PCB's in Kanechlors and Yusho oil

Sample		PCDF's (ppm)		PCB's (ppm)	
		Peak height method	Perchlorination method	Peak height method	Perchlorination method
Kanechlor	300	1	1.5	-	-
	400	18	16.6	-	-
	500	4	2.5	-	-
	600	5	2.7	-	-
Yusho oil	A	5	4.4	830	870
	B	4	5.1	900	920
	C	5	5.2	1030	980

Table 4. Reported concentrations of PCDF's in Kanechlor-400

Authors	Year	Concentration (PPM)
Roach et al.[a]	1974	1
Nagayama et al.[b]	1975	18
Kashimoto et al.[c]	1975	33

[a]Ref. 17.
[b]Ref. 16.
[c]Ref. 18.

was thus about 250 times higher than expected (table 5). The reasons for this great discrepancy are not clear yet. It should be noted, however, that the sample of Kanechlor-400 analyzed was an "unused" one, while the Kanechlor-400 present in "Yusho oil" was "used" as heat transfer medium. This fact suggests a practically important possibility that Kanechlor-400 and probably other commercial PCB's, too, will increase their PCDF concentrations when used as heat transfer medium.

2. *PCDF's in Tissues of Patients with Yusho*

Nagayama et al. (ref. 24) further examined the tissues of patients with Yusho for their possible content of PCDF's. Table 6 summarized their findings. Adipose tissues and liver from two ordinary persons who died of accidents contained no detectable amount (< 0.1 ppb) of PCDF's, but those from three patients with Yusho who died in 1969 or 1972 were all shown to contain PCDF's. Figures 2 and 3 show their gas-chromatograms and mass spectra. The concentration on a whole basis was 0.009 ppm on average for adipose tissues and 0.013 ppm for liver. This seems to be a rather surprising fact because in the case of PCB's the concentration on a whole basis is usually much lower in liver than in adipose tissues. When compared on fat basis, another interesting fact was noted that PCDF concentration was much higher in liver than in adipose tissue. Although the number

MONS 202556

Table 5. Concentrations of PCB's and PCDF's and their ratios in various materials

Materials		PCB's (ppm)	PCDF's (ppm)	PCB's / PCDF's	Reference
Kanechlor-400		1,000,000	ca. 20	50,000	16
Yusho oil	A[a]	ca. 1,000	5	200	
	B[b]	134	1.6	84	18
Patient with Yusho	Adipose tissue	1.3	0.009	144	24
	Liver	0.05	0.013	4	

[a]Samples of the rice oil produced on February 5 or 6, 1968.

[b]A sample of the rice oil produced on February 10, 1968.

Table 6. PCB's and PCDF's in tissues of patients with Yusho and ordinary persons

Subjects	Tissue	Case No.	Time of death	PCB's (ppm) Whole basis	PCB's (ppm) Fat basis	PCDF's (ppm) Whole basis	PCDF's (ppm) Fat basis	Ratio PCB's/PCDF's Whole	Ratio PCB's/PCDF's Fat
Yusho patients	Adipose	1	1969	1.4	3.4	0.013	0.03	108	113
		2	1969	1.3	8.5	0.006	0.04	217	213
		3	1972	1.2	2.1	0.007	0.01	171	210
		Avg.		1.3	4.7	0.009	0.003	144	157
	Liver	1	1969	0.05	4.7	0.025	2.3	2	2
		2	1969	0.06	5.6	0.010	1.1	6	5
		3	1972	0.03	3.5	0.003	0.3	10	12
		Avg.		0.05	4.6	0.013	1.2	4	4
Ordinary persons	Adipose	1	1975	1.0	1.4	ND	ND		
		2	1975	0.4	0.7	ND	ND		
	Liver	1	1975	0.08	1.3	ND	ND		
		2	1975	0.02	1.0	ND	ND		

MONS 202557

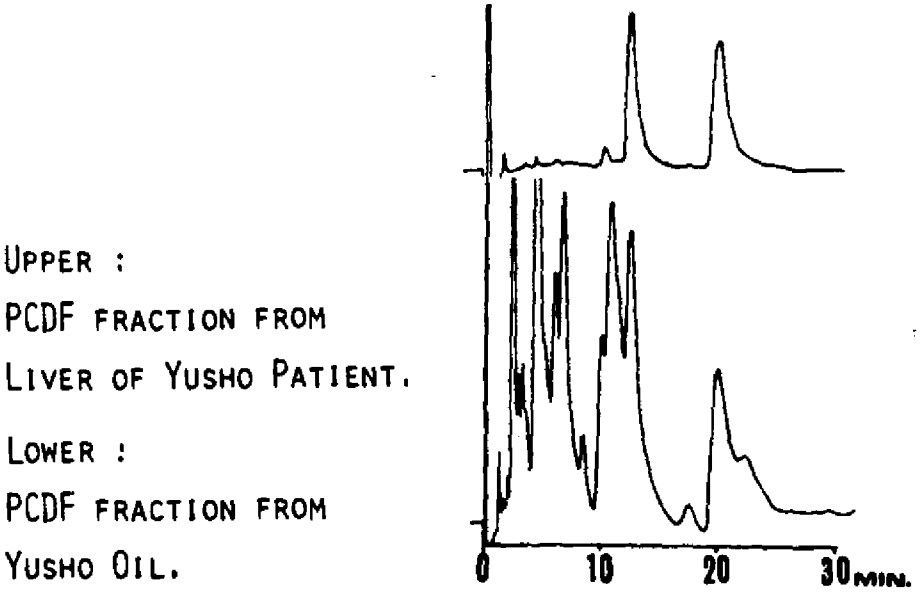

Figure 2. Gaschromatograms of PCDF fractions from liver of patient with Yusho and from "Yusho oil."

of analyses made is quite limited and nothing can be said with certainty, this fact seems to deserve attention. As shown in table 6, these different behaviors in tissue distribution of the compounds caused a remarkable difference in ratio of PCB's to PCDF's between adipose tissues and liver. It was thus demonstrated that the concentration of PCDF's is much closer to that of PCB's in liver than in adipose tissues in patients with Yusho. The PCDF's identified in liver were mainly penta- and hexachlorodibenzofurans, containing only a trace of tetrachloroisomers.

CURRENT CLINICAL STATE OF PATIENTS WITH YUSHO

In 1974, Prof. Urabe (ref. 2), former chief of the Study Group, reported that the dermal and mucosal signs that were most marked at the incipient stage of the poisoning had gradually been improved, while symptoms such as general fatigue, poor appetite, in constant abdominal pain, heavy headedness and headache, feeling of numbness and pain at the limbs, and cough and expectoration of sputum, all of which are considered to be due to some internal disturbances, have become more prominent year by year. In view of these tendencies together with the discovery of a characteristic gas-chromatographic pattern of PCB's remaining in the blood and tissues of patients, the diagnostic criteria for Yusho was revised in 1972 (ref. 2), as shown in table 7. As compared with the former one, the revised criteria describe briefly the dermal and mucosal lesions but newly refer to other noncutaneous objective signs and findings from several laboratory tests. It should be noted, however, that the new criteria do not refer to any specific liver function tests.

MONS 202558

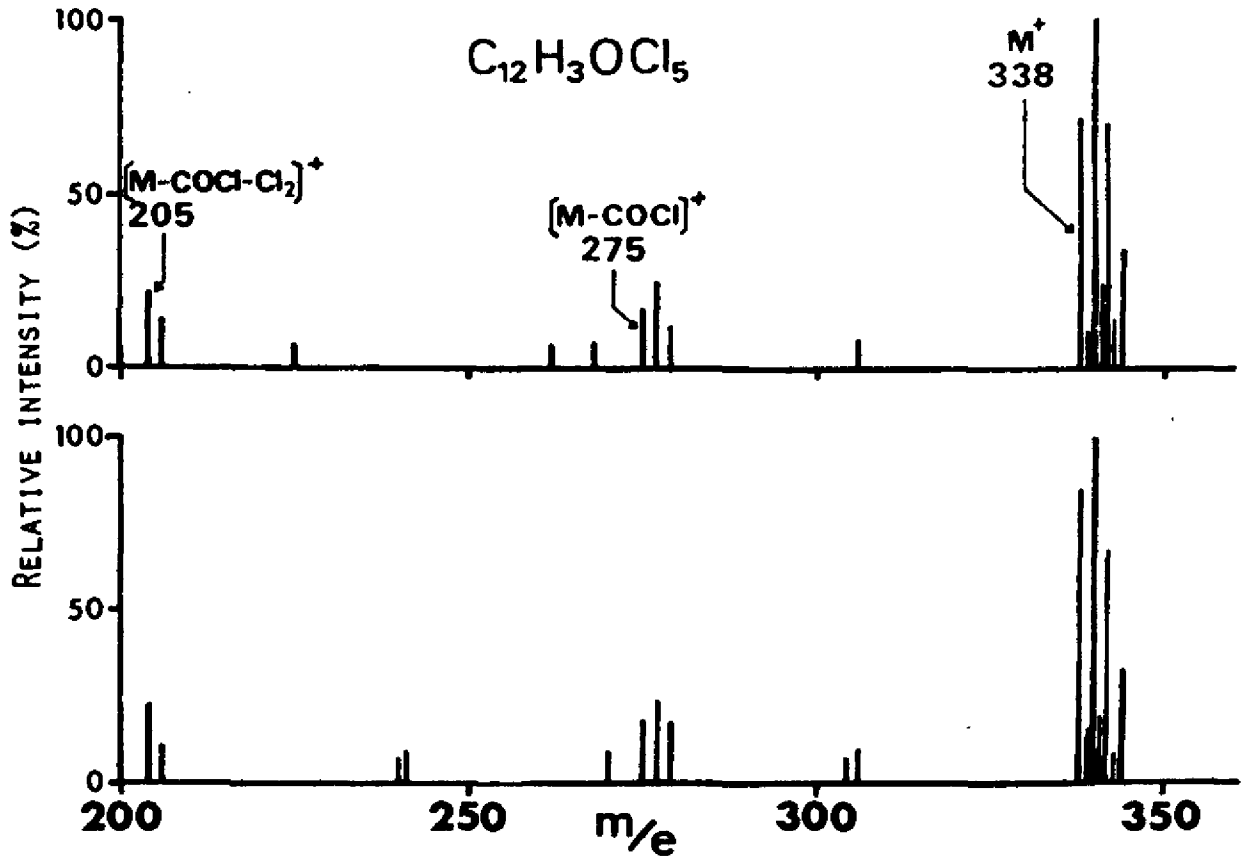

Figure 3. GC-MS of PCDF's fraction from liver of patient with Yusho (upper) and of synthesized PCDF's (lower).

MONS 202559

Table 7. The diagnostic criteria for Yusho (revised in October, 1972).

Yusho is considered as an acute or subacute poisoning with PCB. The general symptoms currently seen are retarded growth, neuroendocrine disturbances, phenomenon of enzyme induction, disturbances in the respiratory system, and abnormal lipid metabolism. As the local symptoms, acneform eruption and pigmentation as cutaneomucosal lesions and ocular symptoms are seen.

1. Conditions of attack
   Fact of ingestion of Kanemi rice oil contaminated with PCB and familial occurrence seen in most cases.

2. General symptoms
   a. Subjective symptoms
      1) general fatigue
      2) heavy headedness and headache
      3) inconstant abdominal pain
      4) feeling of numbness and pain at the limbs
      5) swelling and pain at the joints
      6) cough and sputum
      7) changes in menstruation

   b. Objective symptom
      1) bronchitis-like symptom
      2) sensory neuropathy
      3) bursitis
      4) inhibition in growth and abnormal teeth in children
      5) Small-For-Dates baby and pigmentation of the entire skin of newborns

   c. Results from clinical examination
      1) abnormal properties and concentration of PCB in blood
      2) increase of neutral lipids in blood
      3) anemia, lymphocytosis, hypoalbuminemia
      4) reduced velocity of the sensory nerve conduction and adrenocortical hypofunction

3. Cutaneomucosal signs
   a. Acneform eruption
      Black comedones and acneform eruptions which are seen at the face, buttocks, and other intertriginous sites and their suppurative tendency.

   b. Pigmentation
      Pigmentation of the face, palpebral conjunctiva, gingiva, and nails of the fingers and toes.

   c. Ocular signs
      Swelling and hypersecretion of the Meibomian gland and palpebral edema.

(Translation was made by Kuratsune)

MONS 202560

1. *Subjective Symptoms*

Table 8 shows that a considerable portion of the patients are still suffering from various subjective symptoms in recent years. Kōda and Masuda examined their possible association with PCB concentrations in blood, finding no positive association at all (ref. 4). Umeda also reported on various symptoms due to disturbances of higher nervous activities (e.g., forgetfulness) complained of by most patients, but no definite association between such symptoms and the blood levels of PCB's was observed (ref. 5).

2. *Dermatological Findings*

Kōda and Masuda examined 72 patients with Yusho for dermatological signs and PCB levels in the blood from April 1973 to March 1974 (ref. 4). As shown in table 9, the majority of patients were still suffering from skin lesions such as pigmentation, deformation of nail, and hypersecretion of Meibomian gland even 5 years after the poisoning. They also demonstrated another important fact that group A, consisting of patients whose blood shows the gas-chromatographic pattern A, had significantly higher prevalences of dermatologic signs such as pigmentation, acneform eruption, and deformed nails than did group B, which consisted of patients showing no such typical gas-chromatographic pattern.

Since group A had a higher average concentration of PCB's than group B, the dermal lesions seen among current patients, contrary to the subjective symptoms, seem to be causally associated with the current level or pattern of PCB's remaining in their blood. However, no conclusion could be readily made in this regard. First of all, the current excess of PCB's in the blood of patients is not remarkable in degree and is almost negligible as compared with the enormous elevation seen among the occupationally exposed workers, who nevertheless showed a rather low prevalence of dermal symptoms (table 2).

It seems rather hard, therefore, to explain the persisting dermal lesions by elevated PCB levels in blood alone. The chemical peculiarity of such PCB's and the presence of PCDF's in the bodies of current patients seem to be particularly important in this connection. However, our present knowledge does not allow us to continue discussion of the matter along this line without speculation. Furthermore, an entirely different explanation might also be possible. The skin lesions currently seen may merely be the persisting original skin lesions, the severity of which must have been determined primarily by the amount of intake of PCB's; such intake must in turn be reflected by the current PCB levels in blood of patients. According to this explanation, the

Table 8. Frequency of subjective symptoms complained by patients with Yusho from 1973 to 1974

Symptoms	Proportion[a] %
Fatigue	51.4
Headache	41.7
Phymata in articular region	8.3
Fever	2.8
Cough and sputum	56.9
Digestive disorder	40.3
Numbness of extremities	33.3
Menstrual disturbance	26.9 (7/26)

[a]Calculated by Kuratsune from original figures published by Kōda and Masuda (ref. 4).

MONS 202561

Table 9. Prevalence of dermatological and other signs among patients with Yusho from April 1973 to to March 1974, in connection with concentration and gaschromatographic pattern of PCB's in blood

Signs		Prevalence (%)[c] Group A (43 cases)	Group B (26 cases)	Group C (33 cases)	Total (72 cases)
Pigmentation	Skin	51.2[b]	0	0	30.6
	Palpebra	72.1[b]	19.2[b]	0	50.0
	Gingiba	95.3[b]	57.7[b]	66.6	80.6
	Nail	74.4[b]	34.6[b]	0	56.9
Acneform eruption		34.9[b]	0[b]	0	20.8
Comedo		34.9	23.1	0	29.2
Infection of skin		32.6	11.5	0	23.6
Deformation of nail		65.1[a]	38.5[a]	0	52.8
Alopecia		0	3.8	0	1.4
Disorder in teeth		18.6	7.7	0	13.9
Dypersecretion of Meibomian gland		93.0	80.8	100.0	88.9
PCB's in blood	Conc. (ppb) Avg. ± S.D.	7.2 ± 4.9	4.3 ± 3.1	1.7 ± 0.2	5.9 ± 4.5
	Pattern	A[d]	B[d]	C[d]	

[a]Significant ($P < 0.05$) difference.

[b]Significant ($P < 0.01$) difference.

[c]Calculated by Kuratsune from figures published by Kōda and Masuda (ref. 4).

[d]"A" means the characteristic gaschromatographic pattern of PCB's remaining in the body of most patients with Yusho. "B" means gas chromatographic patterns somewhat similar to "A". "C" means patterns indistinguishable from those of normal persons.

observed association of the dermal lesions with current blood levels of PCB's is considered as a phenomenal one but not as a causal one. In order to evaluate these different possibilities, it seems essential to examine chemically and toxicologically the PCB's and PCDF's still remaining in patients' bodies.

3. *Serum Triglyceride*

One of the most dominant objective signs seen at the incipient stage of Yusho was a markedly increased concentration of serum triglyceride. Okumura and his associates reported recently the results of their extensive followup study on 40 patients who were examined for

MONS 202562

serum triglyceride at least once a year successively for 6 years from 1969 to 1974 (ref. 19). As shown in table 10, a group of 14 male patients has shown no significant change in serum triglyceride levels since 1969, still maintaining levels as high as 180 ± 118 mg/100 ml even in 1974. For 26 female patients, however, a significant decrease was seen in 1973 and 1974 when compared with the levels in the previous years. However, 42 percent of them still showed higher levels than 110 mg/100 ml in 1974.

Okumura et al. (ref. 20) examined the possible association between serum triglyceride levels and PCB concentrations in blood in patients. As shown in table 11, they observed a significantly higher mean level of serum triglyceride in a group of patients who showed the characteristic gas chromatographic pattern A, as compared with other patients who did not show such a typical pattern. They also observed a significantly positive correlation between serum triglyceride levels and PCB concentrations in blood ($r = 0.485$).

Table 10. Results of followup study on serum triglyceride levels in patients with Yusho[A]

Subjects	PCB's pattern	No. cases	Age mean	PCB's in blood (ppb)	Triglyceride mg/100 ml	Reference
Patients with Yusho	A	20	31.9	8.6 ± 5.2[b]	134 ± 60.0[a]	
	B	14				
	C	2	21.4	3.8 ± 2.2[b]	91 ± 39.8[b]	20
Controls	C	37	34.5	2.8 ± 1.6		
Controls					74 ± 29	19.21

[a] $P < 0.05$.

[b] $P < 0.005$.

Table 11. PCB's concentrations in blood and serum triglyceride levels in patients with Yusho in 1973

Patients			Triglyceride (mg/100 ml) Mean ± S.D.					
Sex	Age	No.	1969	1970	1971	1972	1973	1974
Male	11 - 73	14	159 ± 57	166 ± 55	169 ± 60	174 ± 69	164 ± 68	160 ± 118
Female	7 - 59	26	155 ± 75	161 ± 70	155 ± 80	153 ± 63	129 ± 50[b]	111 ± 56[b]

[a] Cited from a report by Okumura et al. (ref. 19).

[b] Significantly lower than in 1969, 1970, 1971, and 1972 ($P < 0.05$).

MONS 202563

Here again, a similar question can be raised in regard to such observed correlation, as already discussed in connection with the dermal lesions. Are the current elevated levels of PCB's in blood and their peculiarities in gas-chromatographic patterns causally connected with the abnormally high serum triglyceride levels found in patients? Since, as mentioned, the female patients started to decrease in serum triglyceride concentration in recent years, a followup examination of PCB's in their blood might give a good clue to answer the above question.

4. *Liver of Patients with Yusho*

Both PCB's and PCDF's are well-known toxic agents to the liver. PCDF's seem to be particularly toxic because a single oral administration of PCDF's as small as about 1 mg/kg could kill rabbits by severe liver necrosis (refs. 28,29). Therefore, it is reasonable to expect that patients with Yusho would have a severe liver damage. Okumura et al. (ref. 30) performed detailed medical examinations on 24 patients soon after the onset but, unexpectedly, obtained no objective findings to indicate definite liver disorders. No patients presented jaundice and only three of them had palpable livers. However, an electron microscopic examination of liver biopsy specimens conducted on a patient in February 1969 revealed a marked hypertrophy of smooth endoplasmic reticulum, indicating stimulated enzyme induction in the liver (ref. 31).

Okumura examined 38 patients with various subjective symptoms for serum enzymes, including isozymes from 1971 to 1972 (ref. 32). An increase in a fraction of lactate dehydrogenase (LDH-5) and high titers in thymol turbidity tests were observed in some of the severe cases but no definite evidence for liver disorders was obtained. Recently Hirayama et al. (ref. 33) examined 121 adult patients with Yusho and 257 healthy adult controls for serum bilirubin, demonstrating a significant lower average concentration in the patient group than in the control. They also showed significantly negative correlations between serum bilirubin and blood PCB's in concentration ($r = -0.349$, $p<0.025$) and similarly between serum bilirubin and serum triglyceride ($r = -0.215$, $p<0.05$). They considered that a lowered concentration of serum bilirubin in patients seemed mainly due to an accelerated bilirubin disposal from the blood.

Hirayama et al. investigated 125 patients for Australia antigen and antibody by the immunoelectro-osmophoresis in 1971 (ref. 34). The antigen was positive in three, while the antibody was negative in all of them, indicating no difference at all in the prevalences between the patients and healthy controls. This finding seems to be important in connection with the future risk of cancer which patients might experience.

In view of all these findings, liver function tests currently available do not readily detect serious liver lesions in the patients, but it is highly desirable that adequate caution will continuously be paid to this well-known target organ of chlorinated hydrocarbons.

CHILDREN BORN TO MOTHERS WITH YUSHO

The birth of unusual babies from mothers who took "Yusho oil" during pregnancy is already well known (refs. 25,26). Their clinical features were dark brown pigmentation of the mucous membrane and the entire skin, gingival hyperplasia with pigmentation, a tendency to be small for the date, eruption of teeth at birth, hypersecretation of the Meibomian gland, and edema of the orbital area. Pigmentation of the skin disappeared in 2 to 5 months, followed by growth similar to that of normal babies.

It seems noteworthy, however, that babies with the dark brown pigmented skin continued to be born for a few years after the intake of "Yusho oil" was discontinued by mothers. Yoshimura reported on nine babies with such skin who were born to mothers with Yusho in Nagasaki prefecture from 1969 to 1972 (ref. 27). Three of such babies had been delivered by a patient from 1969 to 1971. Abe et al. (ref. 23) recently reported on PCB levels in the plasma of 30 children (aged 0–7) born to 18 mothers with Yusho in Nagasaki prefecture. Their examination was made in 1974. As shown in table 12, the PCB levels of these children were significantly higher than those of ordinary children but lower than the levels of their mothers. Their gas-chromatographic patterns of PCB's in plasma were already referred to earlier in this paper. Children fed on breast milk from mothers with Yusho tended to show a higher plasma concentration of PCB's than those who were not fed on such milk.

Yoshimura also reported an interesting case, where a baby was thought to have suffered from Yusho due exclusively to intake of PCB's through breast milk from a woman with Yusho (ref. 27). Very few data are available in regard to the concentration of PCB's in breast milk of mothers with Yusho. Masuda et al. found 0.03 – 0.06 ppm of PCB's in 5 samples of breast milk collected from a woman with Yusho within 5 days after delivery in 1973 (ref. 12). Masuda also found about 0.03 ppm of PCB's in another sample of breast milk collected a few days after a woman with Yusho delivered a baby with no dermal signs (ref. 14). The PCB levels in breast milk from patients with Yusho were therefore just within the normal range. However, the gas-chromatographic patterns of these samples were quite unique, the same as that characteristic for Yusho.

MONS 202564

Table 12. Concentration of PCB's in plasma of children born to mothers with Yusho

Subjects	PCB's in plasma (ppb)			Reference
	Subjects	Range	Mean ± S.D.	
Mothers with Yusho	18	3-33	11.2 ± 7.32	
Children born to above mothers	30	1-20	6.7 ± 4.28	23
Ordinary children	14	1-8	3.7 ± 1.97	

Table 13. Deaths seen among patients with Yusho[A]

Cause of death	Number
Malignant neoplasms	9
Stomach cancer	2
Stomach cancer + liver cancer	1
Liver cancer + liver cirrhosis	1[b]
Lung cancer	1
Lung Tumor	1
Breast cancer	1
Malignant lymphoma	2
Cerebrovascular lesion	3
Amyloidosis	1[b]
Osteodystrophia fibrosa	1[b]
Myocardial degeneration + pericarditis	1[b]
Status thymicolymphaticus	1[b]
Liver cirrhosis	1
Suicide	1
Senility	1
Traffic accidents	3
TOTAL	22

[a]Cited from a report by Urage 1974 (ref. 2).

[b]Autopsied cases.

MONS 202565

MORTALITY OF PATIENTS WITH YUSHO

Omae reported in 1975 that 29 deaths occurred among 1,291 patients with Yusho up to April 30, 1975 (ref. 1). Causes of these deaths were not given, however. Urabe also reported on 22 deaths seen among 1,200 patients until September 13, 1973, and referred to their causes (ref. 2). As shown in table 13, 9 of 22 deaths were caused by malignant neoplasms, suggesting a possible excess of deaths from cancer. Since some essential information needed for epidemiological analysis is not available to us, no further reference can be made with certainty to such a possibility at the present time.

CONCLUSION

As mentioned earlier, we demonstrated the presence of PCDF's in "Yusho oil" at a much higher concentration than expected. We also showed that PCDF's are relatively more concentrated in liver. Although neither the chemical nature nor the toxicity of PCDF's contained in "Yusho oil" and in the bodies of patients are known yet, our findings clearly indicate the necessity to pay greater attention to PCDF's for clarification of the nature of Yusho. Furthermore, our studies suggested the possible formation of PCDF's from PCB's when used as heat transfer medium. Beside this, another possibility that PCDF's might be formed by heating PCB's with peroxides, which are well known to be formed during heating cooking oils, should also be investigated.

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MONS 202566

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DISCUSSION

MR. ALLEN GREY (IIT Research Institute, Chicago, Illinois): Do you have any feel whether dibenzofurans concentrating in the body are more rapid in metabolism than the result of polychlorinated biphenyls?

DR. KURATSUNE: Unfortunately I have no definite idea. It is a more potent compound than PCB, I guess. I really cannot say.

DR. ORVILLE PAYNTER (EPA, Washington, D.C): Are there any reproductive problems or irregularities continuing for many years after the ingestion of these materials?

DR. KURATSUNE: There is some disturbance of menstruation. There have been some disturbances and there have been some related problems.

MS. DEBORAH A. BARSOTTI (University of Wisconsin, Madison, Wisconsin): Were there are doings that suspected any widespread gastic ulcerations or an erosion in the patients—gastric ulcers?

DR. KURATSUNE: No, I do not think so. Some of the patients had very persistant disorders of the intestines, disorders of the digestive system, but I do not know if they were suffering as a result of this or not.

VOICE: Any residual changes in the sebaceous gland during the autopsy?

DR. KURATSUNE: No, I do not know very much about it.

VOICE: I would like to ask one more question. Have you been able to specifically identify any of the dibenzofurans other than those in the body?

DR. KURATSUNE: You are asking if we could identify any of the dibenzofurans? No.

NONS 202567

PATHOLOGICAL FINDINGS ASSOCIATED WITH CHRONIC EXPERIMENTAL EXPOSURE TO PCB's

Renate D. Kimbrough, M.D.*

Abstract

The principle organs effected by long-term exposure to commercial mixtures of polychlorinated biphenyls (PCB's) in rodents, mink, and monkeys are the gastrointestinal tract, the liver, and the lymphatic system. Lesions of the gastric mucosa have been described in monkeys, and ulcerations of the gastric mucosa have been observed in the rat. Pathological changes in the liver in many species, such as the monkey, rodent, and mink, consist of hepatomegaly, lipid accumulation, and liver cell necrosis, particularly at higher dietary levels. Hyperchromatic nuclei and mitotic figures are noted. Ultrastructural changes in the cytoplasm of effected hepatocytes consist of an increase in smooth endoplasmic reticulum, atypical mytochondria, and accumulation of concentrically arranged membranes, which usually surround lipid vacuoles. Particularly in rodents, accumulation of a brown pigment has been observed in Kupffer cells and macrophages (ceroid pigment and uroporphyrin).

In the mouse, (male Balb/cJ strain) neoplastic nodules (hepatomas, hyperplastic nodules) developed after dietary exposure to Aroclor 1254. Female Sherman strain rats fed 100 ppm Aroclor 1260 for 21 months developed hepatocellular carcinomas as well as neoplastic nodules (hyperplastic nodules) of the livers.

In the rat and the mouse, adenofibrosis of the liver occurred after exposing rodents to either Aroclor 1254 or Aroclor 1260. Adenofibrosis, a persistent lesion, does not disappear upon cessation of exposure to the PCB's.

In connection with the persistence of the different lesions mentioned in the rodents as well as in primates, it was noted that certain homologues of the PCB mixtures fed to the animals were not eliminated from adipose tissue and liver in any appreciable amount. In rats, 6 months feeding of Aroclor 1254 at a dietary level of 100 ppm followed by a 16-month recovery period resulted in total adipose tissue levels of 152 ppm penta, hexa-, and heptachlorobiphenyl. These animals still showed pathological changes in the liver, and liver PCB levels of 4.5 ppm.

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INTRODUCTION

For the past 45 years, polychlorinated biphenyls (PCB's) have been used in transformers, capacitors, as heat transfer fluids, and have had other industrial applications (ref. 1). The toxicity of these and related compounds has recently been reviewed (ref. 2). A number of new observations have been made since a conference was held on PCB's in 1971 and it is the purpose of this paper to outline additional findings that are associated with chronic experimental exposure of animals to PCB's.

To study the long-term toxicity of the PCB's, commercial mixtures have usually been employed. This was done for two reasons: similar mixtures were found in the environment, and the individual constituents of the PCB's are very expensive and not readily available in the quantity necessary to conduct long-term studies. Table 1 gives the composition of some of the commercial PCB mixtures. If the PCB's are given as a single dose, they are of a low order of toxicity (table 2). The acute dermal toxicity is also low. It is the repeated and often long-term exposure to these compounds that may lead to a cumulative toxic effect.

HEPATOTOXICITY

Dietary exposure of rats to the PCB's produces enlargement of the liver cells in some adult rats at a dietary level of 5 ppm (0.4 mg/kg/day) (ref. 3). Liver weights were increased in 21-day-old F_1 male weanlings when the dams were fed as little as 1 ppm of Aroclor 1254 (ref. 3). At a dietary level of 20 ppm or above of Aroclor 1254 or 1260, which is equivalent to a dietary intake of 1.5 mg/kg/body weight per day, for an extended period of time, the hepatocytes in many rats had foamy cytoplasm consistent with lipid accumulation. In addition, hyperchromatic nuclei and binucleation was observed in many liver cells. Inclusions were present in the cytoplasm of the liver cells. These inclusions on electron microscopic examination were consistent with concentrically arranged membranes surrounding lipid vacuoles. Other ultrastructural changes observed at this and higher dietary levels in rats consisted of an increase in smooth endoplasmic reticulum and atypical mytochondria. These ultrastructural changes have also been described in primates (ref. 4) and are known to occur with a number of other xenobiotics. Similar observations were made in a study with smaller numbers of animals

MONS 202568

Table 1. Typical percentage composition of polychlorinated biphenyl products[a]

Homologue # Cl/biphenyl	Aroclor 1221	Aroclor 1016	Aroclor 1242	Aroclor 1254
0	11	<0.1	<0.1	<0.1
1	51	1	1	<0.1
2	32	20	16	<0.5
3	4	57	49	1
4	2	21	25	21
5	<0.5	1	8	48
6	ND[b]	<0.1	1	23
7	ND	ND	<0.1	6

[a]Percent (w/w) by GC/mass using area correction factors by homolog response, (Papageorge, written communication, June 1973).

[b]None detected; <0.01% = ND.

Table 2. The incidence and type of liver lesions in female sherman strain rats

	Incidence	
Type of Lesion	Controls	Experimental
Hepatocellular carcinoma	1/173	26/184
Neoplastic nodules	0/173	144/184
Foci or areas of cytoplasmic alteration	28/173	182/184

MONS 202569

where the rats were fed Aroclor 1242 and Aroclor 1016 (ref. 5).

A brown pigment representing ceroid pigment and uroporphyrin was observed in Kupffer cells and macrophages. Particularly at higher dietary levels, livers showed pink fluorescence under UV lights.

In addition, exposure of rats and mice to PCB's for 6 months or longer produced greyish white lesions in the liver. On microscopic examination, these lesions consisted of proliferation of glandular epithelial cells that formed ducts and were surrounded by very pronounced fibrosis (refs. 6,7). The ducts often contained cellular debris. This lesion, called adenofibrosis, was first described by Edwards and White (ref. 6) in rats fed the carcinogen butter yellow (P-dimethylaminoazobenzene).

When rats were exposed for 6 months to a dietary intake of 500 ppm Aroclor 1254 and then sacrificed first at monthly and then at bimonthly intervals, the adenofibrosis persisted through a 10-month observation period. Usually adenofibrosis in rodents occurs concomitantly with hepatocellular carcinomas and/or neoplastic nodules (hyperplastic nodules). This was true for the Balb/cJ mouse (ref. 7). When 50 Balb/cJ inbred male mice were fed 300 ppm Aroclor 1254 (49.8 mg/kg/bodyweight per day), a total of 10 neoplastic nodules (hyperplastic nodules) were found in 9 per 22 surviving mice after 11 months of exposure to the experimental diet. No tumors were found in 58 surviving controls.

In another study, 400 weanling 21- to 26-day-old Sherman strain COBS female rats were distributed into two groups of 200 animals according to a table of random numbers. The animals were housed 10 rats per cage. Two hundred rats were fed plain ground purina chow and 200 rats were fed the same diet containing 100 ppm Aroclor 1260. This study was only conducted at this dietary level and with female rats because in a preliminary feeding study, a bladder tumor was found in 1 of 10 female rats fed 100 ppm Aroclor 1260 (ref. 8). The food consumption of the rats and their bodyweights were determined at intervals. A slight decline in the rate of the weight gain of the test group compared with the control group began about 3 months after onset of the experiment. Mean final bodyweights were 420 grams for the control group and 392 grams in the test group. This difference was statistically significant ($P < 0.001$) since such a large group of animals was involved in this study. The food consumption of rats declines with age. Accordingly, PCB intake declined from 11.6 mg/kg/day during the first week of exposure to 6.1 mg/kg/day at 3 months of exposure and to 4.3 mg/kg/day at 20 months. We determined the PCB concentrations in the experimental diet at intervals and these ranged usually from 70 to 107 ppm. We also spot checked the diet for aflotoxins. Aflotoxins were never detected in either the control or the experimental diet. A total of 173 control and 184 experimental animals survived to the age of 21-23 months. At autopsy of these rats, a consistent difference in the appearance of the livers was observed between the experimental and the control groups. Almost all (170 of 184 livers) of the experimental animals had from a few to multiple elevated tan nodules on the liver surface. Additional nodules were usually seen on sectioning. These nodules varied from .1 to several centimeters in diameter. In contrast, the liver of only one control animal showed gross abnormalities and was markedly enlarged, nodular, tan, and firm. A variety of tumors of other organs was observed in both the experimental and the control group but a difference in the incidence of tumors in other organs in the experimental and the control group did not exist.*

Histologic examination of the liver showed that 26 experimental rats and 1 control rat had hepatocellular carcinomas. An additional 144 experimental animals had hepatocellular nodules consistent with neoplastic nodules (hyperplastic nodules). In addition, 182 treated and 28 control animals had areas of hepatocytes with altered cytoplasm. The cells in these areas were often similar to those that were observed in the neoplastic nodules but the architecture of these atypical areas in the liver was the same as that of normal liver tissue and the liver plates merged with the surrounding liver tissue rather than compressing it.

In classifying the different liver lesions, the recommendations made in a liver tumor workshop (ref. 9) were followed.

The results of this bioassay test illustrate that Aroclor 1260 produces hepatocellular carcinomas according to traditional histological criteria and neoplastic nodules, which are a part of the spectrum of a response to hepatocarcinogenic agents. Neoplastic nodules must be included in the evaluation of tumorigenesis. Neoplastic lesions of the liver have also been produced by some of the commercial Japanese PCB mixtures; in mice with Kanechlor 500, and in Donryu rats with Kanechlor 400 (refs. 10,11).

The effect on the gastric mucosa as it has been described in primates (ref. 4) does not seem to be as prevalent in rodents. Only very high dietary levels, such as single doses of 3,000 mg/kg, will cause alteration of the gastric and duodenal mucosa (ref. 2). Primates (ref. 4) receiving 100 to 300 ppm Aroclor 1248 developed thickened gastric mucosa that contained numerous large cysts filled with mucin. Extension of the atypical

*About 40 percent of the rats in each group had extrahepatic tumors. In a number of the rats, multiple tumors were present.

MONS 202570

appearing glandular epithelium into the muscularis mucosa and the underlying submucosa was also noted. This hyperplastic gastritis was quite persistent in the primate.

In conjunction with some of the feeding studies in rodents, tissue levels were determined in adipose tissue and the liver. It was noted that the gas chromatograms of the PCB's that were extracted from tissues was quite different from gas chromatograms of the standards. The PCB compounds remaining in adipose tissue as well as liver even after dietary exposure to PCB's has been terminated for as long as 10 or 16 months were the penta-, hexa-, and heptachlorobiphenyls, with molecular weights of 324, 358, and 392 (ref. 6). When rats were fed Aroclor 1254 at a dietary level of 100 ppm for 6 months, and were then removed from exposure to PCB's for 16 months, levels of 4.4 ppm of PCB-derived material was present in the liver and 152 ppm of PCB-derived material in adipose tissue on a wet weight basis. The livers of these rats showed some of the morphological changes already described (ref. 12). In primates that consumed Aroclor 1248 and reached adipose tissue levels of 127 ppm, after an 8-month recovery period the adipose tissue levels were 34 ppm of PCB-derived materials (ref. 4).

SUMMARY

The administration of PCB's to animals—particularly rodents but also monkeys and mink—produces a variety of responses in the liver. Early enlargement of the liver is primarily due to hypertrophy of the cells and an increase of the smooth endoplasmic reticulum of the cytoplasm of the liver cells. The smooth endoplasmic reticulum may condense in the liver cells and form hyalin inclusions, which on electron microscopic examination consist of concentrically arranged membranes containing lipid vacuoles. In addition to these lipid vacuoles, lipid accumulation may also occur throughout the liver cells. These changes are initially reversible. However, if the exposure to PCB's is continued, then liver damage, including liver cell necrosis, may also develop. This is accompanied by proliferation of the liver cells resulting in pleomorphism. Long-term exposure to PCB's in rodents produces a tumorigenic response which consists of the development of atypical areas, neoplastic nodules, and hepatocellular carcinoma. Adenofibrosis, which usually occurs concomitantly with hepatocellular carcinomas, has also been produced in the rat and the mouse. Pigment deposition within the macrophages and Kupffer cells has been noted (ref. 6). This pigment is composed of ceroid pigment (lipofuchsin) and uroporphyrin. Hemosiderin may also be present. In addition to liver changes, hyperplasia of the gastric mucosa has been observed in the primates (ref. 4) following the exposure to PCB's.

Because of these findings in experimental animals, ingestion of PCB's in humans must be curtailed. Exposure from all sources in the occupational environment needs to be reviewed and workers with long-term occupational exposure to PCB's should be studied. An effort should be made to establish all environmental sources of PCB's in order to assess exposure of the general population.

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MONS 202571

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DISCUSSION

MR. LAWRENCE ROY: You indicated after an animal has been exposed for 6 months you get regression, and if they did regress . . .

DR. KIMBROUGH: I don't know if I can answer all of that; you may have to repeat part of your question. But, first of all, I think in the studies that I did—and we really haven't got any time to go into all of this this morning—if you feed animals for only 6 months you do not see any tumors; you have to feed animals PCB's for much longer periods of time.

The first indication of any tumor development that I saw in reproduction studies we did was when the animals were exposed for at least 300 days. On the other hand, in the rat you do get what I call adenofibrosis if you feed the animals for about 6 months. That lesion is also known as cholangiofibrosis. Adenofibrosis does not regress. We did a feeding study where you feed the animals for 6 months, and then sacrifice them at bimonthly intervals, and the lesion will still be there 10 months later. So this is one problem with PCB's.

And at least some of the PCB homologs are retained for such a long period of time that I don't know whether you could also have what you might call internal exposure.

I think you had some other questions?

VOICE: First of all, do tumors cause death in these animals, and do these tumors cause an increase in size?

DR. KIMBROUGH: Some of the animals I think have died with large tumors at the end of the study. I don't think the tumors that are called hepatocellular carcinomas would regress. I also did not say that we had any regression in the animals we fed for 6 months.

MS. DIANE HORVAK (University of Wisconsin, Department of Pathology): What was the difference between the carcinoma and the nodule?

DR. KIMBROUGH: We did use histological criteria. In our classification, the tumors that we call neoplastic nodules were well circumscribed tumors that would extend over several lobules and some of them were actually quite large, and would compress the surrounding normal liver tissue, and because of that, they were circumscribed.

The lesions that were classified as carcinomas showed disorganized liver. Liver plates were two or three layers thick. You would have dilated sinusoids, would see mitotic figures and a great deal of pleomorphisms, but none of these tumors metastisized.

CHAIRMAN RALL: There's a question over there.

DR. ROBERT RISEBROUGH (University of California): Since we now know that these PCB preparations do contain dibenzofurans, I wonder if it is your plan to repeat these experiments with PCB's that do not contain the dibenzofurans. I wonder also if you would care to guess whether these tumors are caused by PCB or by the contaminants.

DR. KIMBROUGH: I have no plans at the moment to repeat these experiments. I do not know if I could get a completely clean Aroclor 1260.

The PCB that we used was Aroclor 1260 with the lot number AK3, which Dr. Bowes has analyzed. I think the level of the combined furans in that particular sample is less than 1 ppm. I may be wrong there, but if you recalculate that, you will find that the amount of furans consumed by the animals was very small. The amount of PCB's which the animals ingested was 5 mg/kg body weight/day, and the furans constituted less than 1 ppm of that. This amount is so small that I wonder whether it had any effect in this particular experiment.

CHAIRMAN RALL: We'll come back to the issue of dibenzofurans later.

MONS 202572

SUMMARY OF TOXICOLOGICAL STUDIES ON COMMERCIAL PCB's

J. C. Calandra, M.D., Ph.D.*

Abstract

A broad toxicological program was conducted to evaluate the biological effects of Aroclor 1242, 1254, and 1260 in mammalian as well as avian species. No-effect levels were defined which permit an assessment of the hazard these materials present to man and his environment.

The urgent need for additional toxicological data became apparent when the refinement of analytical techniques made possible the identification of PCB's in many portions of the ecosystem.

To assess the biological hazards of these materials, chronic feeding studies in rats and dogs were conducted according to accepted traditional procedures with Aroclors 1242, 1254, and 1260. It should be pointed out that Aroclor 1260 is no longer produced in this country since it does not meet the physical specifications for its restricted uses (ref. 1).

In addition, three-generation two-litter reproduction and teratology studies in albino rats as well as a dominant lethal mutagenic study in albino mice were considered to be necessary for the broad spectrum safety evaluation of these materials.

Effects such as decreases in eggshell thickness described in certain avian species suggested that toxicity and reproduction studies in chickens would be helpful in evaluating possible untoward effects in birds. A list of the studies conducted are presented in table 1.

The experimental design of the chronic rat and dog studies is given in tables 2 and 3. It should be noted that these studies were of typical Food and Drug Administration design for the evaluation of the safety of direct and indirect food additives.

The important results in the chronic rat studies which were conducted on Charles River rats are summarized briefly in tables 4, 5, and 6.

Food consumption, mortality, and hematologic, urine, and blood chemistry studies did not differ among treatment and control groups. The only weight depression observed was at the 24-month point in females fed 100 ppm of Aroclor 1254.

Liver weight increases were noted in males and females fed 100 ppm Aroclor 1254 or Aroclor 1260, and in females only in the 100 ppm group fed Aroclor 1242.

As expected, the important histopathologic changes in the rat study were present in the livers of the 24-month sacrifice animals and consisted of hepatocellular alterations such as focal hypertrophy, cytoplasmic lipid changes and in some animals at 100 ppm, hepatomas or cholangiohepatomas. No evidence of hepatocellular carcinogenicity of the Aroclors was found in this study.

The conclusion that "no evidence of hepatocellular carcinogenicity of the Aroclors was found in this study" was reached only after extensive reevaluation of the original liver slides as well as additional liver sections from all of the animals after the Kimbrough results on Aroclor 1260 became known to us (ref. 2).

The slides were read independently and separately by Dr. Donovan Gordon (ref. 3), Professor Ward Richter (ref. 4), and Professor P. Pour (ref. 5) of Industrial BIO-TEST Laboratories, the University of Chicago, and the Eppley Institute for Cancer Research, respectively.

The interpretation of pathologic changes that occur in the liver as a result of absorption of chlorinated hydrocarbons is a key issue that needs resolution by the community of pathologists (ref. 6, 7). The problem is highlighted in the context of PCB's since there appears to be disagreement as to whether Aroclor 1260 is or is not a hepatocellular carcinogen (ref. 8). Pour reevaluated the Kimbrough slides and does not agree with the reported findings.

The findings in the chronic dog study are summarized in table 7. The feeding of Aroclor 1260 at 100 ppm showed an increase in serum alkaline phosphatase activity and liver weights. A slight decrease in body weight gain was noted at the 100 ppm dose level in males and at 10 and 100 ppm in females. No remarkable histopathologic changes were found.

The design of the dominant lethal mutagenic study is presented in table 8. No effects related to Aroclor treatment were seen in the parameters listed in table 9—mortality, mating index, number of implantation and resorption sites, number of viable embryos, preimplantation loss or mutation rates. This finding is in agreement with that of Green et al. (ref. 10).

No effects related to Aroclor treatment were seen in the parameters listed in table 9—mortality, mating index, number of implantation and resorption sites, number of viable embryos, preimplantation loss or mutation rates. This finding is in agreement with that of Green et al. (ref. 10).

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MONS 202573

The standard design for a three-generation two-litter reproduction study in albino rats is given in table 10 and the teratology study in table 11. A brief summary of the results (table 12) indicates that none of the Aroclors studied produced adverse effects in the two litters of the first generation.

All progeny delivered by female rats in each of three generations exposed to dietary levels of up to 100 ppm of either Aroclor 1242, 1254, or 1260 were structurally normal. A reduction in the mating index was observed in the second and third generations of animals fed either 10 or 100 ppm. The ability of females to conceive, carry the delivery process to parturition, and to successfully nourish the young was not effected by the three Aroclors. It should be stressed that no changes in the reproductive tract of either male or female rats were produced by any of the three Aroclors. Findings in the third generation were similar to those in the first with no suggestion of any alterations in response as a function of succeeding generations.

Teratologic studies in which albino rats were exposed to either Aroclor 1242, 1254, or 1260 at doses of up to 30 mg/kg during rapid organogenesis—gestation days 6 through 15—were conducted in our laboratories. No evidence of embryotoxicity as reflected by an increase of fetal resorption or teratogenicity as measured by complete external, skeletal, and internal evaluation of fetuses obtained from the Aroclor-treated females, was obtained. Other experimental data pertaining to the potential teratogenicity of polychlorinated biphenyls supports the lack of adverse effects. Mizunuya (ref. 11) and his colleagues found no evidence of teratogenicity in the rat when fed at dietary levels of up to 260 ppm. In the mouse, Toeruek (ref. 12) found that doses of up to 500 mg/kg were nonteratogenic although when given on gestation days 1 through 6 a decrease in implantation sites and fetal weights was observed. These doses given on gestation days 7 through 11 failed to produce any changes with respect to either reproductive parameters or teratogenicity. Further, no evidence of malformation was obtained in infant monkeys delivered to females fed either 2.5, 5, or 25 ppm Aroclor 1248 although the birth weights were reported to be reduced (ref. 9).

The last study in this series was a toxicity/reproduction study in white leghorn chickens (tables 13, 14, and 15).

Aroclor 1260 at all test levels did not produce adverse effects on the various parameters investigated. Egg production in hens fed 100 ppm Aroclor 1242 or 1254 was decreased as was egg hatchability. In fact, poor hatchability of eggs from hens fed 8 ppm Aroclor 1242 was found (table 15).

In addition, Aroclor 1242 at 10 and 100 ppm and Aroclor 1254 at 100 ppm were associated with reduced eggshell thickness. Chick viability was affected by both substances at the 10 ppm dose level.

CONCLUSIONS

1. The no-effect level for the Aroclors in the chronic rat and dog studies is about 10 ppm.
2. No teratogenic or mutagenic effects were found.
3. No hepatocellular carcinomas were present.
4. The no-effect level in the rat reproduction study is between 1 and 10 ppm and is the result of low mating indices.
5. In the chicken reproduction and teratology studies, effects were more severe with Aroclor 1242 with the no-effect level being 2 to 4 ppm.

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MONS 202574

DISCUSSION

MR. PAUL AGENTINE: You didn't find any affects or pathological agents in the dog?

DR. CALANDRA: Would you repeat that?

MR. ARGENTINE: How did you arrive at the no-affects level in canine at 10 ppm? In studies you showed no pathological agents and no toxicity?

DR. CALANDRA: As I indicated, this is a very brief and rapid summary. We have a question about liver weights and one or two other parameters and to be conservative, we have used the number of 10 ppm. In other words, we are not in any way indicating that PCB's are nontoxic materials, and this is a conservative estimate.

DR. ALLEN GREY (I.I.T. Research, Chicago, Illinois): Are you able to relate the differences in biological effects on the various Aroclors to their composition?

DR. CALANDRA: Only generally. As everyone knows, the higher chlorinated materials appear to be more persistant in the mammalian system. And there appears to be a target organ—the liver—which appears to be more susceptible to the higher chlorinated materials. I think these are the only generalizations you can make at this time.

Table 1. Toxicity studies conducted with Aroclors 1242, 1254 and 1260

Type of test	Test animal
Two-year chronic oral	albino rats
Two-year chronic oral	beagle dogs
Three-generation reproduction	albino rats
Teratology	albino rats
Dominant lethal mutagenic	albino mice
Toxicity/reproduction	white leghorn chickens

NONS 202575

Table 2. Two-year chronic oral toxicity study--albino rats and beagle dogs

Test material	Dietary levels (ppm)			Number of animals per dietary level			
				rats		dogs	
				M	F	M	F
None	-	-	-	50	50	4	4
Aroclor 1242	1,	10,	100	50	50	4	4
Aroclor 1254	1,	10,	100	50	50	4	4
Aroclor 1260	1,	10,	100	50	50	4	4

Table 3. Two-year chronic oral toxicity study--albino rats and beagle dogs

Parameters investigated:

- Body weight
- Food consumption
- Hematology
- Clinical blood chemistry (BUN, SAP, SGPT, fasting blood glucose, SGOT--dogs only)
- Urinalyses
- Pathology (gross, organ weights, microscopic)

Table 4. Ingestion of Aroclor 1242 by albino rats--liver affects

Increase in liver weights--100 ppm--females

Primary liver lesions

None at 3-, 6-, 12-month sacrifice

24-month sacrifice--100 ppm--increased incidence of:

Nodular hyperplasia	(8/20)
Hepatoma	(2/20)
Chologiohepatoma	(1/20)
Hepatocellular carcinoma	(0/20)

MONS 202576

Table 5. Ingestion of Aroclor 1254 by albino rats--liver affects

Increase in liver weights--100 ppm	
Primary liver lesions	
None at 3-, 6-, 12-month sacrifice	
24-month sacrifice--100 ppm--increased incidence of:	
Nodular hyperplasia	(13/27)
Hepatoma	(4/27)
Cholangiohepatoma	(2/27)
Hepatocellular carcinoma	(0/27)

Table 6. Ingestion of Aroclor 1260 by albino rats--liver effects

Increase in liver weights--100 ppm	
Primary liver lesions	
None at 3-, 6-, 12-month sacrifice	
24-month sacrifice--100 ppm--increased incidence of:	
Nodular hyperplasia	(7/27)
Hepatoma	(5/27)
Cholangiohepatoma	(2/27)
Hepatocellular carcinoma	(0/27)

Table 7. Effects of ingestion of Aroclors--beagle dogs

Test material	Effect
Aroclor 1242	no effects
Aroclor 1254	slight decrease in body weight gain --100 ppm
Aroclor 1260	slight decrease in body weight gain --100 ppm, males --10 and 100 ppm, females
	increase in SAP--100 ppm
	increase in liver weights--100 ppm

Table 8. Dominant lethal mutagenic study--albino mice

Test material	Dose*
Corn oil	0.9 ml/kg
Methyl methane sulfonate (MMS)	100 mg/kg
Aroclor 1242	500 or 1000 mg/kg
Aroclor 1254	500 or 1000 mg/kg
Aroclor 1260	500 or 1000 mg/kg

*Single dose given i.p. to 12 males/group.

MONS 202577

Table 9. Dominant lethal mutagenic study--albino mice

No effects related to Aroclor treatment seen in:
• Mortality
• Mating index
• Number of implantation sites
• Number of resorption sites
• Number of viable embryos
• Preimplantation loss
• Mutation rates

Table 10. Three-generation reproduction study[a]--albino rats

Test material	Dietary levels (ppm)			Number of animals per dietary level	
				M	F
None	-	-	-	8	16
Aroclor 1242	1,	10,	100	8	16
Aroclor 1254	1,	10,	100	8	16
Aroclor 1260	1,	10,	100	8	16

[a]Two litters per generation.

MDNS 202578

Table 11. Teratology study--albino rats

Test material	Dose[a] (mg/kg/day)	Number of gravid rats
Corn oil	-	26
Aroclor 1242	10 or 30	26
Aroclor 1254	10 or 30	26
Aroclor 1260	10 or 30	26

[a]Administered on gestation days 6 through 15 (10 doses).

Table 12. Effect of Aroclors on reproduction/teratology--albino rats

1. Reproduction study
   - First generation--no effects
   - Second and third generations--10 and 100 ppm
   - – decrease in mating index
   - – decrease in incidence of pregancy
   - (Aroclor 1242--no third generation with 100 ppm).
2. Teratology study--no effects.

Table 13. Toxicity/reproduction study--white leghorn chickens

Test material	Dietary levels (ppm)	Number of animals per dietary level	
		M	F
None	-	8	40
Aroclor 1242	1, 2, 4, 8, 10, 100	4	20
Aroclor 1254	1, 10, 100	4	20
Aroclor 1260	1, 10, 100	4	20

MONS 202579

Table 14. Toxicity/reproduction study--white leghorn chickens

Parameters investigated:
Body weight
Food consumption
Egg production
Egg quality
Egg hatchability
Eggshell thickness
Chick body weight
Chick viability
Pathology

Table 15. Effects of Aroclors on chickan raproduction

Parameter	Dietary level 1242	Causing effect 1254
Egg production	100	100
Egg hatchability	8	100
Shell thickness	100	100
Chick viability	10	10
Aroclor 1260--no effects at any dose level		

MONS 202580

PATHOBIOLOGICAL RESPONSES OF PRIMATES TO POLYCHLORINATED BIPHENYL EXPOSURE

J. R. Allen, D.V.M., Ph.D., and D. H. Norback*

Abstract

Male and female rhesus monkeys received varying levels of polychlorinated biphenyls (PCB's) and were evaluated for toxic effects, reproductive dysfunctions, and metabolism of the compounds. Female rhesus monkeys exposed to dietary levels as low as 2.5 and 5.0 ppm of PCB (Aroclor 1248) developed facial acne, erythema, subcutaneous edema, conjunctivitis, and loss of eyelashes. Reproductive dysfunctions were manifested by irregular menstrual cycles, early abortions, and stillbirths. As a result of transplacental migration of the compounds, all infants born of PCB-exposed animals contained PCB's in their tissues at birth. The infants, which continued to be exposed to PCB's by ingestion of milk from their lactating mothers, developed skin lesions and 50 percent expired within 4 months.

Metabolic studies demonstrated 90 percent absorption of the PCB's from the gastrointestinal tract and distribution in organs of high lipid content. Hydroxylated metabolites were formed in the liver and excreted through the biliary and urinary routes. Lower chlorinated congeners were more rapidly metabolized and excreted, while concentrations of the highly chlorinated biphenyls persisted in the adipose tissue in excess of 2-1/2 years. The detection of the urinary metabolite trans-3,4-dihydro-3,4-dihydroxy-tetrachlorobiphenyl suggests that the mechanism of metabolism is through an arene oxide intermediate. In vivo and in vitro studies demonstrated binding of PCB's with macromolecules.

INTRODUCTION

Even though the polychlorinated biphenyls (PCB's) have been used extensively for various industrial purposes for the past 40 years, the health significance of human exposure to these compounds only recently has become of widespread concern. The scientific community was alerted to the potential environmental health problem by Jensen in 1966 (ref. 1) after PCB's were identified in tissue extracts of birds experiencing reproductive difficulties in Sweden. The magnitude of the problem was brought to the forefront by the "Yusho" incident when over 1,000 Japanese suffered prolonged ill effects from exposure to PCB-contaminated rice oil (ref. 2). Further concern about the potential danger of PCB's on human health followed disclosure of increasing levels of these compounds in various foods. The contamination has been attributed to incorporation of these compounds within the food chain or from packaging of food products in PCB-impregnated paper containers (ref. 3). Increasing levels of PCB's in human tissue samples attest to the magnitude of the human exposure (refs. 4,5).

Within this laboratory various animal models, including the nonhuman primate and rodent, have been evaluated following exposure to PCB's for the development of lesions which parallel those recorded in man. Emphasis has been placed on determining pathophysiological alterations that arise in animal models as a result of exposure to several levels of PCB's for variable periods of time. The absorption, tissue distribution, and rate of excretion of PCB's have been determined. The interaction of the PCB's or their metabolites with cellular macromolecules has also been a major area of investigation. The following report, which includes previously unpublished observations, presents a summary of the progress that has been made in this laboratory on the above mentioned areas of PCB research.

GENERAL EFFECTS OF PCB'S ON NONHUMAN PRIMATES

The investigation of toxicity produced by PCB's in various animal species demonstrated the limitations of rodents as animal models. Male Sprague-Dawley rats were able to survive for 1 year on diets containing 100 ppm PCB (Aroclor 1248, 1254, or 1262) without showing signs of illness (ref. 6). These observations substantiated those of Keplinger et al. (ref. 7). Increasing the PCB content of the rat diets to 1,000 ppm did not produce skin lesions; however, death occurred within 6 to 8 weeks due to widespread hepatic degeneration (ref. 8).

Male rhesus monkeys developed many of the signs

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MONS 202581

experienced by humens that hed been inadvertantly exposed to PCB's. Monkeys fed diets containing 100 end 300 ppm PCB (Aroclor 1248) (table 1) developed facial edema, erytheme, ecne, end elopecia within 3 weeks. The lesions became progressively more severe with increased length of exposure (refs. 9,10). In addition, the enimels developed enorexie, loss in weight, hypoproteinemia, hypolipidemie, end enemie. Within 3 months the mejority of the monkeys had died or were moribund. Necropsies of these enimels revealed decided mucosel gestric hyperplesie with penetretion of the glendular epithelium into the underlying submucose (ref. 10). Numerous ulcerations of the hyperplestic gestric mucose were elso present (ref. 11). In eddition, there was e decided hypertrophy of the liver.

Femele monkeys fed 25 ppm PCB (Aroclor 1248) (teble 1) in the diet developed facial lesions simller to those observed in the enimels receiving higher levels of PCB's (ref. 12). After 2 months on the PCB diet it wes necessery to discontinue the exposure due to the severity of intoxicetion. One of the six experimentel enimels died 4 months efter the initiel exposure to PCB's. Necropsy evaluetion demonstreted severe gestric hyperplesie end ulceretion. The surviving edult femele monkeys continued to be devoid of eyeleshes end displeyed facial ecneform lesions 2 yeers following exposure to the PCB's. Infents born to these femeles were smell (350 vs. 460 g) and contelned PCB's in their tissues et birth.

Femele monkeys given 2.5 end 5.0 ppm PCB (Aroclor 1248) (teble 2) in their diets developed facial edeme, swollen eyelids, erytheme, loss of heir, end ecne within 2 months (ref. 13). By the fourth month, irregularities in the menstruel cycles and an increased level of urinery ketosteroids were recorded (ref. 14). Following 6 months of PCB exposure the femele monkeys were bred to control males. Six of eight enimels on the 5.0 ppm diet conceived (teble 2). The remaining two were bred on five seperete occesions without conceiving. Four of the six femeles experienced abortion early in gestetion. Eight of eight of the 2.5 ppm PCB fed enimels conceived; however, only five were eble to carry their infents to term. As was the case with infents of enimels given the higher levels of PCB's, all the infants were smell end et birth their skin contelned detectable levels of PCB's.

The infants were permitted to nurse their mothers for 4 months. Within 2 months focel areas of hyperpigmentetion, swollen lips and eyelids, loss of eyeleshes, end ecneform lesions of the face developed. The skin of these infants showed e decided increase in the PCB level over this period. Within 4 months, 3 of the 6 infants died due to PCB intoxicetion. After weening, the remeining three heve shown improvement of the skin lesions during the 4-month period.

Four edult mele rhesus monkeys were elso exposed to e diet contelning 5.0 ppm PCB's (Aroclor 1248) (table 1) for 17 months (everege totel intake of PCB's 460 mg). They began to develop e slight periorbitel edeme efter 6 months of exposure; however, it was much less severe than in the femele monkeys receiving e simller level of PCB. The morphologicel feetures end viebility of the spermetozoe es well es the ebility to fertilize control femele rhesus monkeys was uneffected during the initiel 12 months of PCB exposure. Subsequently one of the four meles lost weight end developed elopecie, ecne, periorbitel edeme end decreased libido. A testiculer biopsy of this enimel showed e decided hypoactivity of the seminiferous tubules. There was en ebsence of meture spermetozoe end e predominence of Sertoli cells of the tubules. The remaining three meles heve remained healthy end sexually active (ref. 15).

ABSORPTION, METABOLISM, TISSUE DEPOSITION, AND EXCRETION OF PCB's

Over 90 percent of e single orel dose (1.5 or 3.0 g per kg) of PCB's (Aroclor 1248) given to edult rhesus monkeys was ebsorbed from the gestrointestinel tract. Chrometogrephic enelysis of the tissues 14 deys efter exposure reveeled e predominence of higher chlorine isomers thet hed e predilection for the edipose tissue and organs containing e high fet content (ref. 16).

Rhesus monkeys fed 25 ppm PCB (Aroclor 1248) in the diet etteined levels of 127 μg/g within the edipose tissue efter 2 months. Eight months efter discontinuetion of exposure to PCB's the levels were 34 μg/g within the edipose tissue. After 33 months, the levels within the edipose tissue ranged from 3 to 14 μg/g; the residues contained greetly increased proportions of highly chlorinated congeners. Transplecentel movement of the PCB's was demonstrated by the presence of PCB's in the infants born to exposed females. The tissues of en infent, born to e female 8 months following the discontinuetion of PCB's in the diet, contained 25 μg/g of PCB in the fet end edrenel tissues et the time of birth. In en infant born to e female 29 months following the discontinuetion of PCB's, the levels within the edipose tissue were 3.38 μg/g et 4 months of age.

Female monkeys given 5.0 ppm PCB in their diets ettained maximum levels of PCB's within their edipose tissue et 6 months (141 to 177 μg/g edipose tissue). However, it required approximetely 14 months on the 2.5 ppm diet for the monkeys to reech similer maximum PCB levels in their edipose tissue (126 to 144 μg/g). Males which received 5.0 ppm PCB's etteined levels

MONS 202582

Table 1. Experiments on exposure of primates to PCB's (Aroclor 1248*)

Level of PCB in diet (ppm)	No. of animals	Length of exposure (months)	Total PCB intake
300	6 males	3	3.6 to 5.4 g
100	6 males	2	0.8 to 1.0 g
25	6 females	2	250 to 400 mg
2.5	8 females	16-19	243 to 303 mg
5.0	8 females	16-19	460 to 614 mg
5.0	4 males	17	530 to 692 mg

*Monsanto Co., Inc., St. Louis, Missouri.

Table 2. Modification in reproduction in primates that were exposed to Aroclor 1248 in the diet

	Control	2.5 ppm	5.0 ppm
Total impregnated (no./no. animals)	12/12	8/8	6/8
Resorptions or abortions (no./no. animals)	0/12	3/8	4/8
Stillborn (no./no. animals)	0/12	0/8	1/8
Normal births (no./no. animals)	12/12	5/8	1/8

MONS 202583

ranging from 128 to 200 μg per g adipose tissue at 14 months. Infants born of mothers exposed to 2.5 and 5.0 ppm PCB's within the diet contained concentrations of PCB's ranging from 1.0 to 4.8 μg/g within the skin at birth. While nursing from mothers consuming PCB diets, the infants continued to accumulate the compound. At 3 months, the levels within the tissues ranged from 86 to 136 μg/g. The concentration of PCB's within the milk ranged from 0.16 to 0.40 μg/g. The tissues of the infants which died while nursing PCB-fed mothers contained high levels of PCB's within the thymus, ovaries, brain, kidneys, adrenal glands and pancreas (20-48 μg/g tissue). Lower levels were found in the liver, lymph nodes, and bone marrow (8-16 μg/g).

Monkeys and rats demonstrate species variation in the metabolic response to the PCB congener 2,5,2',5'-tetrachlorobiphenyl (TCB). Over 66 percent of the single dose (500 mg/kg) administered to rats was recovered from the feces, and an additional 10 percent was present in the urine within the initial 72 hours (ref. 17). The material present in the body was concentrated within the adipose tissue. There was a transient high level of TCB within the blood at 24 hours. Other organs which contained significant quantities of TCB, however at lower concentrations, included the liver, skin, and muscle. The major urinary metabolite was identified as 3-OH-2,5,2',5'-tetrachlorobiphenyl. Other monohydroxy TCB metabolites were present in minor quantities (ref. 17).

Over 90 percent of an oral dose (500 mg/kg) of ^{3}H TCB administered to infant rhesus monkeys was absorbed from the gastrointestinal tract and was highly concentrated in the skin, adrenal gland, liver, and adipose tissue. At 72 hours, less than 2 percent of the dose had been eliminated in the urine and 1 percent in the feces. Monohydroxy TCB, a major metabolite present in the rat urine, was a minor metabolite in the urine of the monkeys. The two major metabolites were dihydroxy-TCB and trans-3,4-dihydro-3,4-dihydroxy TCB (ref. 18). A second minor metabolite was hydroxy-3,4-dihydro-3,4-dihydroxy TCB.

Following the oral dose of ^{3}H-TCB (1 g/kg) to juvenile monkeys, a major percentage of the material was absorbed from the gastrointestinal tract and was highly concentrated in the adrenal, adipose tissue, and skin. Significant quantities were present within the liver, muscle, and uterus. After 4 weeks, 14.8 percent of the dose was secreted into the bile. Approximately 75 percent of the biliary material was reabsorbed by the gut and the nonabsorbed material was recovered from the feces during this period. Over 90 percent of the PCB's excreted in the bile was in the form of water soluble glucuronic acid conjugates. An additional 8 percent of the total dose was recovered from the urine.

Administration of the higher chlorine congener ^{3}H 2,4,5,2',4',5'-hexachlorobiphenyl (HCB) to rats or monkeys demonstrated low levels of excretion into the bile. An oral dose of HCB (1 g/kg) administered to rats resulted in excretion of 0.3-0.7 percent of the dose per day in the bile over a period of 14 days. The urine was free of detectable levels of radioactivity (ref. 19). At 14 days, 85 percent was recovered from the body tissues. The compound was highly concentrated within the adrenal glands, adipose tissue, and skin and in the female within the ovaries and uterus. Following the administration of a single dose of HCB (1 g/kg) to juvenile rhesus monkeys, less than 2 percent was excreted via the biliary-fecal route over a 3-week period. There was no detectable radioactivity within the urine. The organs with the highest concentration of the material included the adrenal, adipose tissue, and skin. Due to the large mass of muscular tissue, this was a major reservoir of the compound.

INTERACTION OF METABOLITES WITH CELLULAR MACROMOLECULES

Following the administration of ^{3}H 2,5,2',5'-tetrachlorobiphenyl (TCB) to infant rhesus monkeys, interaction of TCB and macromolecules of cells and of serum was evaluated (ref. 20). Separation of the serum constituents by polyacrylamide gel electrophoresis demonstrated association of the TCB primarily with serum albumin. Over 90 percent of the radioactivity of liver homogenates eluted from a Sephadex G-25 column was in the protein and nucleic acid fractions. The majority of the macromolecular-associated HCB apparently was bound by hydrophobic association. Extraction of liver homogenates with hexane, precipitation of the hexane-extracted homogenate with TCA, and subsequent extraction of the TCA precipitate with methanol resulted in extraction of the majority of the radioactivity. In the extracted residue, 1.1 percent of the radioactivity remained which may represent covalently bonded material. Recent in vitro studies employing monkey microsomes incubated with an NADPH generating system demonstrated 20 percent of the metabolized ^{3}H-TCB was bound to microsomal protein and RNA in a nonextractable form. Binding of ^{3}H-TCB was prevented by heating the microsomes to 100°C prior to incubation (ref. 21).

DISCUSSION

These experiments employing nonhuman primates have demonstrated development of parallel signs and

MONS 202584

lesions of PCB intoxication in humans and rhesus monkeys exposed to similar levels over comparable periods of time. Acne, subcutaneous edema of the face, and edema of the eyelids were observed in man (ref. 22) and lower primates (ref. 15) exposed to PCB's. The facial signs of PCB exposure appear to be a sensitive indicator of PCB intoxication. The rhesus monkeys after exposure for 2 months to dietary levels of PCB's (2.5 and 5.0 ppm) developed facial alterations after a total consumption of 32-60 mg of PCB's. It is noteworthy that PCB levels of 5.0 ppm are presently permitted in foods destined for human consumption.

The most debilitating lesions in the monkeys were the severe hyperplasia and ulceration of the stomach. Whether similar changes occur in the stomach of man exposed to PCB's remains to be clarified. Nausea and anorexia described by the human subjects suggest potential gastric alterations. Liver hypertrophy, proliferation of the endoplasmic reticulum, and increased hepatic microsomal enzyme activities were observed in man (ref. 23) and in lower primates (ref. 11).

Menstrual irregularities, decreased libido, occurrence of stillborns, reduced birth weights, and transplacental movement of PCB's in humans and rhesus monkeys have been recorded (refs. 13,24). The reproductive failures of monkeys exposed to PCB's were due to inability to maintain a pregnant state. The majority of the animals did not experience appreciable difficulty in conception; however, a large percentage of the animals aborted during the first 45 days of pregnancy. These observations suggest an inability of implantation or inability to maintain the implanted embryo during the early stages of pregnancy.

Although the mechanism of reproductive dysfunction has not been clarified, there is some indication of hormonal modifications. Alterations in the urinary ketosteroids were reported in humans exposed to PCB's (ref. 22). Increased levels of urinary ketosteroids have been observed in the nonhuman primates that experienced reproductive failures (ref. 14). One mechanism of altered steroid metabolism may be secondary to the increase in the hepatic mixed function oxidases that are present in the hypertrophic livers of exposed animals. It has also been a consistent observation that the organs associated with steroid production, the adrenals and ovaries (particularly the corpora lutea), have contained relatively high concentrations of PCB's in exposed animals. Thus the compounds may possibly have a direct effect on these organs.

The presence of PCB's in the milk of other species has been previously reported (ref. 3). This avenue of infant exposure and the potential morbidity and mortality was vividly demonstrated in the infant monkeys who nursed from mothers exposed to 2.5 and 5.0 ppm in the maternal diets. The presence of relatively low levels of PCB's in the diets of lactating females represents a potential source of PCB intoxication to nursing infants.

Metabolic studies of the rhesus monkey demonstrate over 90 percent absorption of the PCB's from the gastrointestinal tract following oral administration. The material is concentrated in organs with high lipid content, including the adipose tissue, skin, adrenal, corpora lutea of the ovaries, and brain. Within the liver the greatest portion of the material is associated with the membranes of the endoplasmic reticulum.

Studies with the single congener TCB demonstrate the metabolism of the compound to hydroxylated forms which are conjugated with glucuronic acid and excreted into the bile. Enterohepatic circulation of the PCB's undoubtedly occurs as only 20 percent of the material secreted into the bile was recovered from the feces. The greatest portion of the metabolized TCB was excreted through the urinary system. The more highly chlorinated biphenyl HCB was more slowly eliminated from the body via the biliary-fecal route; HCB or metabolites were not detected within the urine. The facilitated metabolism and excretion of the lower chlorine congeners was also indicated by the relative decreased storage of these compounds, and conversely the accumulation of higher chlorinated congeners, within the adipose tissue.

Metabolic studies that have been conducted on nonhuman primates suggest mechanisms of interaction of PCB's with tissues and, more importantly, indicate potential mutagenic and carcinogenic effects of the PCB's. Metabolites have been isolated from rhesus monkeys exposed to the PCB congener 2,5,2',5'-tetrachlorobiphenyl that are formed through an arene oxide intermediate (ref. 18). Similar metabolites of the PCB's and the potential for arene oxide formation has been demonstrated in rabbits (refs. 25,26). Arene oxides formed by the metabolism of other aromatic hydrocarbons have been shown to covalently bind with macromolecules and produce mutagenic and carcinogenic changes in mammalian cells (ref. 27). Dechlorination of the more highly chlorinated biphenyls, demonstrated by dechlorination of 2,4,5,2',4',5'-hexachlorobiphenyl (ref. 28), provides a mechanism through which metabolism of highly chlorinated biphenyls through an arene oxide intermediate would be facilitated.

Evidence demonstrating the association of PCB's with liver macromolecules supports the theoretical potential of the PCB's for covalent binding with cellular macromolecules. Thus, it appears that alkylation of macromolecules is one mechanism by which the PCB

MONS 202585

metabolites cause widespread injurious effects. Further credence for the ebility of the compound to produce elterations in the macromolecules is presented in recent reports of hepetocelluler tumors developing in rets end mice exposed to PCB's (refs. 29-31).

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DISCUSSION

VOICE: I'm from the Massachusetts Society. Have you examined the samples for minute contaminants?

DR. ALLEN: That is a good question. I presume your primary interests are in the dibenzofurans. The Monsanto Company has volunteered to analyze the Aroclor 1248 used in our experiments for the furans. In our discussion last week they were hopeful of having these data available for this conference. If Dr. Wright is in the audience perhaps he would give us a progress report on the subject. (No answer from the audience.) I can say that we have done some preliminary work in this area and have found undetectable levels of furans in the samples. However, more detailed studies to clarify this question are underway at the present time.

VOICE: How can you determine that the PCB's are covalently bound to macromolecules?

DR. ALLEN: Repeated extractions carefully monitored for radioactivity are the best methods of removing any absorbed material from the protein. Standard gel chromatography methods do not differentiate between adsorption and covalently bound materials. We hope to be able to generate enough PCB bound to macromolecules to permit the determination of the exact covalent nature of the bond following macromolecular digestion.

VOICE: Which macromolecule did you use?

DR. ALLEN: We were using protein and RNA from monkey microsomes. These microsomes were incubated with ^{3}H PCB in a NADPH generating system. The protein and RNA were isolated subsequently and their radioactivity determined.

VOICE: Could you tell us roughly how much PCB your animals consumed per kilogram of body weight?

DR. ALLEN: The female animals on the PCB experiments weighed between 6 and 7 kilograms. Table 1 gives the average total intake of PCB's by these animals during the various experiments.

MONS 202587

PCB CHLORINATION VERSUS PCB DISTRIBUTION AND EXCRETION

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Abstract

The distribution and excretion of selected ^{14}C-labeled polychlorinated biphenyls (PCB's) were studied in the male rat. The distribution and excretion of each of the PCB's was studied after either i.v. or oral administration. Following administration, each of the PCB's was rapidly removed from the blood and stored in the liver and muscle. The rates of PCB redistribution from liver and muscle to skin and adipose tissue and/or elimination in urine was related to the degree of chlorination. The subsequent rates of PCB removal from skin and adipose tissue, excretion in urine, and total excretion were related to the degree and position of chlorination of the biphenyl molecule. None of the PCB's studied was excreted to a significant extent prior to metabolism to more polar compounds. It appears as if the degree and position of chlorination controls the rate of metabolism. The limiting factor in metabolism may be the facility of arene oxide formation as a metabolic intermediate. The toxicologic implications of arene oxide formation versus PCB accumulation in animal tissues are discussed.

INTRODUCTION

Due to the complexity of commercial polychlorinated biphenyl (PCB) formulations and the difficulty experienced by others in their efforts to interpret the results of toxicological studies in which these mixtures were used, we have chosen to study selected individual PCB's. We believe that detailed studies of individual PCB's, chosen to represent industrial PCB formulations, may not only afford an insight into the biological fate of the more complex commercial formulations, but may also be used in the construction of pharmacokinetic models of the distribution and excretion of these and other chlorinated hydrocarbons. Our ultimate goal is to have pharmacokinetic models which will permit the accurate extrapolation of animal data to man.

Four of the PCB's studied were 4 chloro-, 4,4'-dichloro-, 2,4,5,2',5'-pentachloro-, and 2,4,5,2',4',5'-hexachlorobiphenyl. These PCB's have degrees of chlorination similar to, and are constituents of Aroclors 1221, 1232, 1254, and 1260, respectively. Each of the PCB's studied was labeled with carbon-14.

METHODS

All of the data presented in this paper were obtained with an i.v. dose of 0.6 mg PCB/kg body weight; however, studies with the pentachlorobiphenyl at doses ranging from 0.06 to 6.0 mg/kg failed to show any effect of dose on the distribution and excretion of this PCB (ref. 1). The results presented in this paper were obtained by i.v. injection of the PCB's; however, these results have been repeated without different results by studies in which the PCB's were administered by oral intubation (ref. 2). In these studies, the time points of sampling ranged from 15 minutes to 7 days for each of the PCB's and up to 42 days for the penta- and hexachlorobiphenyls. Three animals were treated and sacrificed at each time point and the data presented represent the average values obtained. The total radioactivity in the tissue samples was determined by oxidation and liquid scintillation counting.

RESULTS

Following i.v. injection, approximately 90 percent of the total dose of each PCB was removed from the blood within 15 minutes. Initially, most of the administered dose was stored in the liver and muscle. Within 15 minutes after administration, the liver contained 15 to 30 percent of the total dose of each of the PCB's (figure 1). Removal of mono- and dichlorobiphenyl from liver was primarily via excretion in the bile in the form of several metabolites; only trace amounts of the parent compounds were excreted in the bile. The pentachlorobiphenyl was removed from the liver by metabolism and excretion in the bile and by redistribution to other tissues, whereas the primary mechanism for the removal of hexachlorobiphenyl from the liver was redistribution. Due to the relatively large mass of muscle, total storage of the PCB's in muscle was similar to that observed in liver (figure 2). It is assumed that the only mechanism of PCB removal from muscle was by redistribution to tissues having a higher affinity for these compounds (ref. 2).

Most of the long-term storage of PCB's in the body was in the skin and adipose tissue. Since one of the earliest symptoms of chronic intoxication by PCB's and certain other chlorinated hydrocarbons is chloracne, a skin disorder (ref. 3), data on the accumulation of these

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MONS 202588

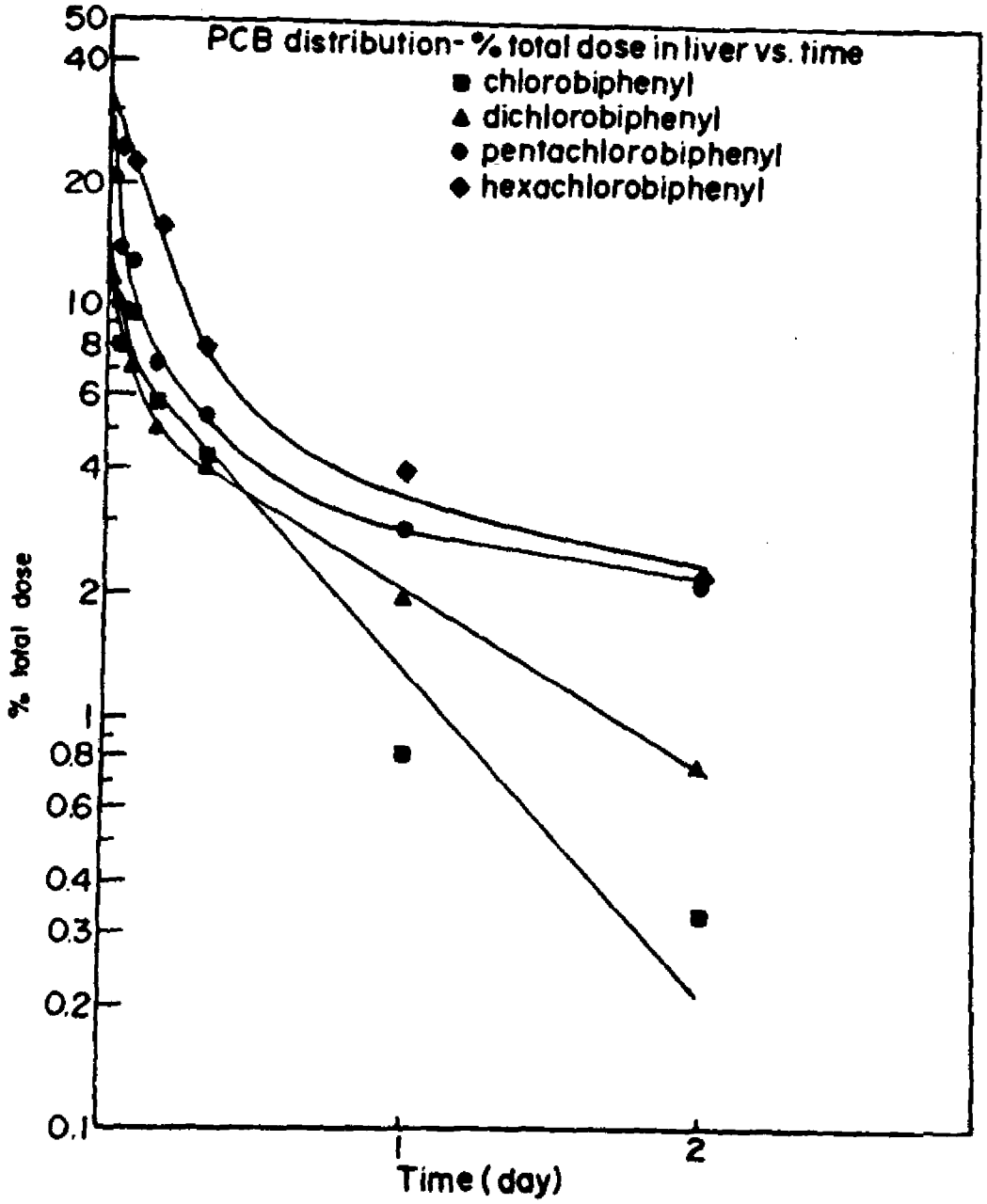

Figure 1. Rate of removal of PCB's from liver.

MONS 202589

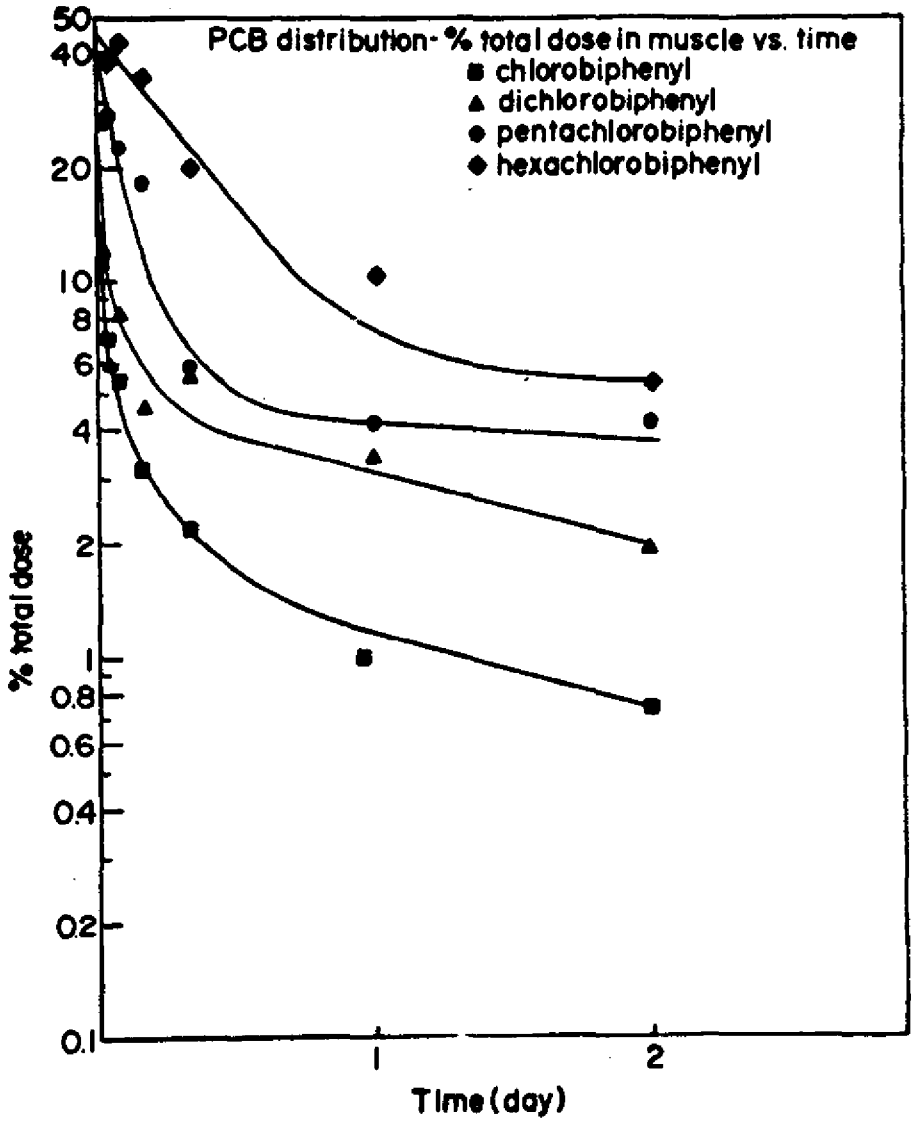

Figure 2. Rate of removal of PCB's from muscle.

MONS 202590

compounds in skin was of particular interest (figure 3). It may not be obvious in figure 3, due to the long time scale of 8 hours to 42 days, but the uptake of PCB's by skin was slower than the uptake by liver and muscle (ref. 2). The rate of PCB removal from skin decreased as the degree of chlorination of the PCB's increased, and following the removal of approximately 25 percent of the hexachlorobiphenyl, little further decay from skin was observed during the remainder of the 42-day study.

The accumulation of PCB's in adipose tissue was a slower process than that observed in skin (figure 4). The peak concentrations of mono-, di-, penta-, and hexachlorobiphenyl in adipose tissue were reached at 1 hour, 2 hours, 4 hours, and 7 days after administration, respectively. The magnitude of the peak concentrations in fat also tended to increase with increasing chlorination, whereas the rates of PCB removal from adipose tissue decreased with increasing chlorination of the PCB. Hexachlorobiphenyl concentrations in fat never showed a decline from peak concentrations.

The rates of PCB removal from tissues would be expected to be reflected in their rates of excretion. All rats kept for 1 day or longer were held in individual metabolism cages and fed food and water *ad libitum*. Both urine and feces were collected daily. The most dramatic effect of increasing chlorination of the biphenyl molecule was seen in the percent of the total dose excreted in the urine (figure 5). Cumulative excretion in urine for 7 days accounted for approximately 60, 34, 8, and less than 1 percent of the total mono-, di-, penta-, and hexachlorobiphenyl dose, respectively. Greater than 90 percent of the material excreted in the urine was in the form of one or more conjugated metabolites of the given PCB (ref. 2).

Cumulative excretion of the PCB's in feces was a more consistent process which, with the exception of the hexachlorobiphenyl, did not appear to be greatly affected by degree of chlorination (figure 6). Animals treated with penta- or hexachlorobiphenyl were held for up to 42 days. Extrapolation of the daily rates of total PCB excretion showed that, with the exception of hexachlorobiphenyl, excretion would eventually account for approximately 100 percent of the administered dose of each PCB. The excretion of hexachlorobiphenyl was so slow that extrapolation to infinite time indicated that less than 20 percent of the administered dose would ever be excreted.

DISCUSSION

Work in our laboratory and elsewhere indicates that only about 10 percent of each of these PCB's is excreted as the parent compound. We also know from extraction and analysis of the radioactivity in the tissues that appreciable amounts of the metabolites are not stored in the tissues (ref. 2). We have thus assumed that metabolism is a prerequisite to the excretion of PCB's. We have also shown that the rates of excretion and the rates of removal from skin and adipose tissues of mono-, di-, and pentachlorobiphenyl are inversely proportional to their degrees of chlorination (ref. 2). On the other hand, the very slow excretion of hexachlorobiphenyl would not have been predicted by this relationship. Therefore, an additional factor appeared to be affecting the rate of metabolism of hexachlorobiphenyl.

An examination of the molecular structures of these four PCB's shows that there is only one obvious difference, other than chlorination, among the structures of the hexachlorobiphenyl and the three other PCB's. The difference is that this hexachlorobiphenyl does not have two adjacent unsubstituted carbon atoms.

It was demonstrated in Williams' laboratory in the 1950's that chlorinated benzens that had two adjacent unsubstituted carbon atoms were metabolized and excreted 3 to 20 times more rapidly than benzenes with similar degrees of chlorination that did not have adjacent unsubstituted carbon atoms (refs. 4,5). Schulte and Acker suggested that a similar substitution pattern is required for the metabolism of PCB's (ref. 6). This suggestion was partially confirmed by Jensen and Sundstrom (ref. 7) when they showed that PCB's which did not have two adjacent unsubstituted carbon atoms were found in the highest concentrations in the tissues of higher animals and man. The hexachlorobiphenyl used in our studies was found in the highest concentrations of any PCB in human tissues. Our data on the distribution and excretion of this PCB and the fact this particular PCB is one of the more common constituents of the more highly chlorinated commercial PCB formulations explain why such high concentrations were found in human and animal tissues.

As a point of reference, we have also studied the distribution and excretion of similar doses of several chlorinated pesticides in the rat. The initial half-life of dieldrin was quite similar to that of pentachlorobiphenyl. The initial half-life of DDT was five- to six-fold longer than that of pentachlorobiphenyl and only Mirex and hexachlorobenzene, of the pesticides tested, had infinitely long half-lives such as that observed for hexachlorobiphenyl (ref. 8).

Two adjacent unsubstituted carbon atoms are important to the metabolism of the PCB's because their presence facilitates the formation of arene oxides. Arene oxides are formed by the hepatic mixed-function oxidases as intermediates in the metabolism of a number of lipophilic compounds, and these oxides have been

MONS 202591

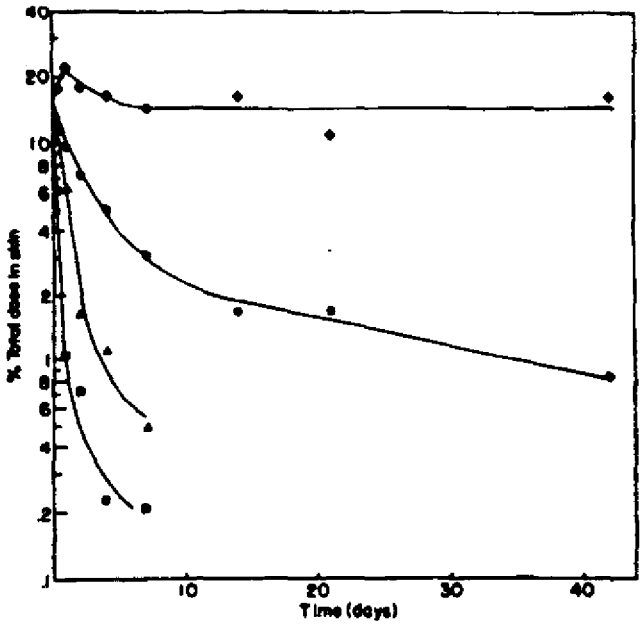

Figure 3. Rate of removal of PCB's from skin.

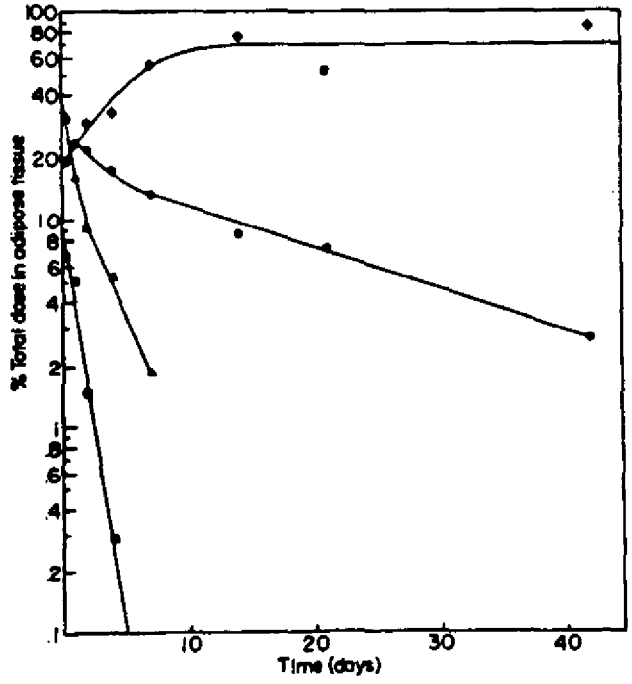

Figure 4. Rate of removal of PCB's from adipose tissue.

MONS 202592

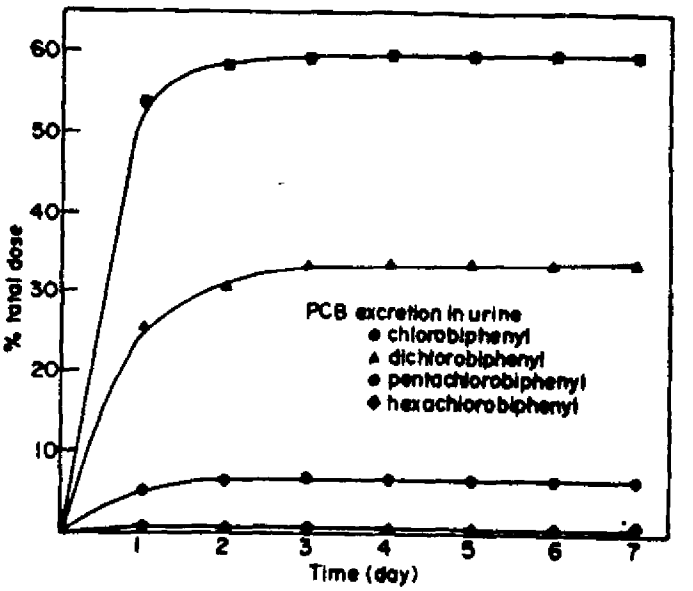

Figure 5. Cumulative excretion of PCB's in urine.

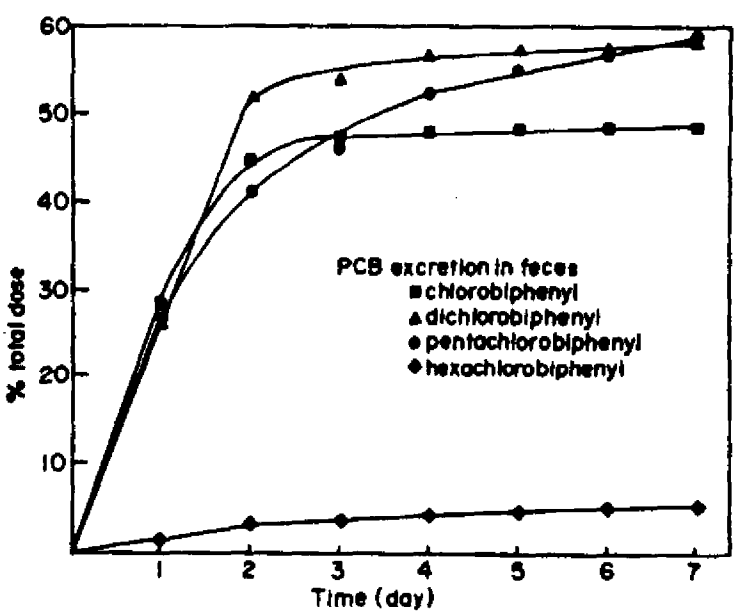

Figure 6. Cumulative excretion of PCB's in feces.

MONS 202593

implicated as potential carcinogens (refs. 9,10). Evidence for arene oxides as intermediates in the metabolism of implicated as potential carcinogens (refs. 9,10). Evidence for arene oxides as intermediates in the metabolism of PCB's has been provided by Gardner et al. (ref. 11), Safe et al. (ref. 12), and in our own laboratory (ref. 13).

Thus, we are given a dilemma. Those PCB's which can be metabolized and excreted may be metabolized via a carcinogenic intermediate and those PCB's which are not readily metabolized have an extremely long biological half-life. Several researchers have provided evidence that the PCB's may be carcinogenic (refs. 14-16). On the other hand, Vos et al. (ref. 17) have shown the very slowly metabolized hexachlorobiphenyl used in this study to be acnegenic, to cause liver damage, and to induce hepatic porphyrie. It is not yet known if it is the parent PCB's or their metabolites which account for the primate reproductive failures described by Allen (ref. 18). The only way that we are going to establish which of the PCB's or their metabolites are carcinogenic or are going to cause any of the other toxicological problems and at what levels of exposure the problems are likely to arise is through systematic pharmacokinetic studies, which will allow us to extrapolate the results of chronic low-dose environmental exposures from laboratory animals to man. Until such data are available, it is my opinion that every effort should be made to avoid environmental contamination by any type of PCB's.

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MONS 202594

ENZYMATIC AND OTHER BIOCHEMICAL RESPONSES TO SELECTED PCB's

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Abstract

Isomerically-pure mono-, di-, tri-, tetra-, hexa-, and octa-chlorobiphenyls were injected i.p. into weanling male rats at a dosage of 50 mg/kg/day for 3 consecutive days, the animals being 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. Hepatic function was assessed by pentobarbital sleeping times and in vitro assays of p-nitroanisole O-demethylase, aniline hydroxylase, aminopyrine N-demethylase, carboxylesterase and sulfobromophthalein-glutathione conjugating enzyme activities. For the mono-oxygenases closely associated with the hepatic endoplasmic reticulum, enhanced induction of activity was observed with highly chlorinated biphenyls and by low chlorine-containing congeners 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. Interrelationships with other hepatic functions and the rate of chlorobiphenyl biotransformation is discussed.

Toxicologic assessment of commercial chlorobiphenyl mixtures has been complicated by the heterogeneity of the congeners, by marked differences in physical and chemical properties which, undoubtedly, influence rates of absorption, distribution, metabolism and excretion and by the possible presence of toxic impurities and byproducts (refs. 1-4). As techniques of definitive analysis have developed, the complexity of commercial chlorobiphenyls (Aroclors, Monsanto Industrial Chemicals, St. Louis, Mo.) has been revealed showing that, with the exception of Aroclor 1016 and 1232, one predominent congener composed of a number of positional isomers is found in each preparation (fig. 1). As the percentage of chlorine increases, the predominant congener shifts from a mono- to a tri- to a tetra- to a penta-chlorobiphenyl (refs. 5,6).

If it was possible to separate these complex mixtures, one could examine several facets of the toxicology of these compounds, and several questions could be posed and, perhaps, answered. Do the various congeners possess the same toxicological properties: i.e., do dichlorobiphenyls have the same effect as hexachlorobiphenyls? Do all of the tetrachlorobiphenyl isomers produce the same toxicologic responses? Are the pathologic and toxicologic alterations observed due to the biphenyl nucleus itself, to the positions occupied by individual chlorine atoms, or to the number of chlorines present on the biphenyl nucleus. Since hepatic enzyme induction has been a well-characterized phenomenon of adaptation to these chemicals, we attempted to answer some of the above questions using changes in hepatic ultrastructure and in enzyme activity as indices of structure-activity relationships.

Our first study, completed in 1972, and published in 1974, used a small series of isomerically-pure chlorobiphenyls of known position and degree of chlorination, synthesized and purified by my colleagues, Dr. O. Hutzinger and Dr. S. Safe (refs. 3,7). The objective of these experiments was to elicit responses (induction of hepatic drug-metabolizing enzymes) which, hopefully, could be related to the structures of the pure chlorobiphenyls. We injected young male Wistar strain rats intraperitoneally with 50 mg/kg of the agent, dissolved in peanut oil, for 3 consecutive days, the animals being killed 96 hr after the last injection. The livers were quickly removed and samples were taken and stained for light and electron microscopy. The remaining hepatic tissue was used for the preparation of microsomes and soluble supernatant for the enzymatic assays. These assays included representative functions of the microsomal mono-oxygenases (p-nitroanisole O-demethylase, aniline hydroxylase, aminopyrine N-demethylase), hydrolases (nonspecific carboxylesterase) and the conjugation of sulfobromophthalein (BSP) with reduced glutathione (GSH). Some of the results of that study are shown in the next few figures.

Figure 2 presents the results observed for the cytoplasmic enzyme system involved in conjugating BSP following exposure to DDT, Aroclor 1254 and 1260, biphenyl, and a series of pure chlorobiphenyls. Significant ($p<0.05$) increases in activity were observed with all agents tested. It should be noted that biphenyl caused a marked increase in this enzyme activity.

Figure 3 shows the influence of the DDT isomers, commercial Aroclors, biphenyl, and the series of isomerically pure chlorobiphenyls on pentobarbital-induced sleeping time. Highly significant ($p<0.05$) reductions in sleeping time were observed with the

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MONS 202595

commercial Aroclors and DDT isomers. Biphenyl and 4-chlorobiphenyl caused no significant ($p>0.05$) changes in sleeping time. Of the dichloro-isomers, only the 4,4'-isomer significantly reduced the duration of effect. Both tetrachloro-isomers caused significant reductions in sleeping time though the 2,5,2',5'-isomer was less effective. The hexa- and octa-chlorobiphenyls caused a marked reduction in sleeping times, being comparable to those observed with p,p'-DDT and the commercial Aroclors.

Figure 4 summarizes the influence of the various agents tested on the hepatic mixed function oxidases aniline hydroxylase (A), p-nitroanisole O-demethylase (B), and aminopyrine N-demethylase (C). As has been observed by others, we found that the most responsive enzymes were those closely associated with the smooth endoplasmic reticulum. The results in vitro reflected the observations of altered sleeping times in vivo. One can see that, even with this limited number of pure chlorobiphenyls, not all caused effects of the same magnitude. To summarize the results of this study, treatment with biphenyl caused slight induction while 4-chlorobiphenyl did not. The mixed function oxidases were markedly induced by pure hexa- and octa-chlorobiphenyls and also by di- and tetra-chlorobiphenyls with chlorines substituted at the 4-positions of the rings. Considering the dichloro-isomers, when the 4- and 4'-positions were occupied, there was a much greater inductive effect for all of the enzyme activities than was observed with the 2,2'- and 2,4'-isomers. The same positional phenomenon was observed for the two tetra-chlorobiphenyls studied, the induction caused by 2,4,2',4'-tetra-chlorobiphenyl being much greater than that observed with the 2,5,2',5'-isomer. The results obtained for the higher chlorinated analogs suggested that the positions of the chlorine atoms were not as important. Our results, with the exception of the marked effects obtained following treatment with 4,4'-dichlorobiphenyl, confirmed the observations of other investigators who have found that penta-, hexa- and octa-chlorobiphenyls had greater enzyme-inducing potential than did low chlorine-containing biphenyls (refs. 8-11).

On the basis of our initial studies, we came to the conclusion that the biphenyl nucleus could exert some effect on hepatic enzyme levels (fig. 4) though the only function markedly affected was the BSP-GSH conjugation (fig. 2). Much more important conclusions were that not all congeners possessed the same toxicologic properties and that, while greater inductive effects were observed with highly chlorinated biphenyls, marked ultrastructural and enzymatic changes were observed with specific di- and tetra-chlorobiphenyls, particularly those with chlorines substituted on the 4-position on the ring. We have extended the investigation to a broader series of mono-, di-, tri-, and tetra-chlorobiphenyls in an attempt to confirm the importance of position of the chlorine on the ring structure of low chlorine-containing congeners. Isomerically-pure chlorobiphenyls, synthesized by my colleagues or purchased from Analabs Inc. (North Haven, Conn.), were injected intraperitoneally using the same regimen (50 mg/kg/day for 3 consecutive days, killing the animals 96 hr after the third injection). A 12,000 g-20 min supernatant from 20% w/v homogenates of liver was used as the enzyme source. This study has been published recently (ref. 12). Some of the pertinent results are shown in the next figures.

It was essential, before studying the effects of chlorination and position, to determine what effects the biphenyl nucleus had on hepatic enzymes. Table 1 shows the results of the i.p. administration of vehicle (peanut oil), commercially available biphenyl (Eastman Organic Chemicals, Rochester, N.Y.), and purified biphenyl on the activities of hepatic O-demethylase (OD), aniline hydroxylase (AH), carboxylesterase (CE), and the BSP-GSH conjugating enzyme. Treatment with unpurified biphenyl resulted in significant ($p<0.05$) increases in activities of three of the enzymes. In contrast, biphenyl repurified by thin layer chromatography caused a significant increase only in BSP-GSH conjugating enzyme activity, suggesting the presence of an impurity in the commercial material. Since relatively pure chlorobiphenyls were to be used, repurified biphenyl was used for the control groups of animals.

The influence of monochlorobiphenyls on hepatic O-demethylase, aniline hydroxylase, carboxylesterase, and BSP-GSH conjugating enzyme activities are compared in table 2 with the effects following treatment with biphenyl. While changes in microsomal O-demethylase were not observed, all three monochloro-isomers significantly increased aniline hydroxylase and carboxylesterase levels. The BSP-GSH conjugating enzyme activity was not affected.

The influence of a series of di-, tri-, and tetrachlorobiphenyls on the selected enzymatic functions are shown in figures 5, 6, and 7, respectively. With each series of isomers, a chlorine atom on the 4-position caused a more marked induction of hepatic drug-metabolizing enzyme activities than did a chlorine atom at any other position. As one increased the degree of chlorination, subsequent substitution at the 2-position was next in importance followed by substitution at the 3-position. The results conclusively demonstrated that not all isomers of the mono-, di-, tri-, and tetrachloro-

MONS 202596

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	Activities (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]	8.8± 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 $p<0.05$.

MONS 202597

Table 2. 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).

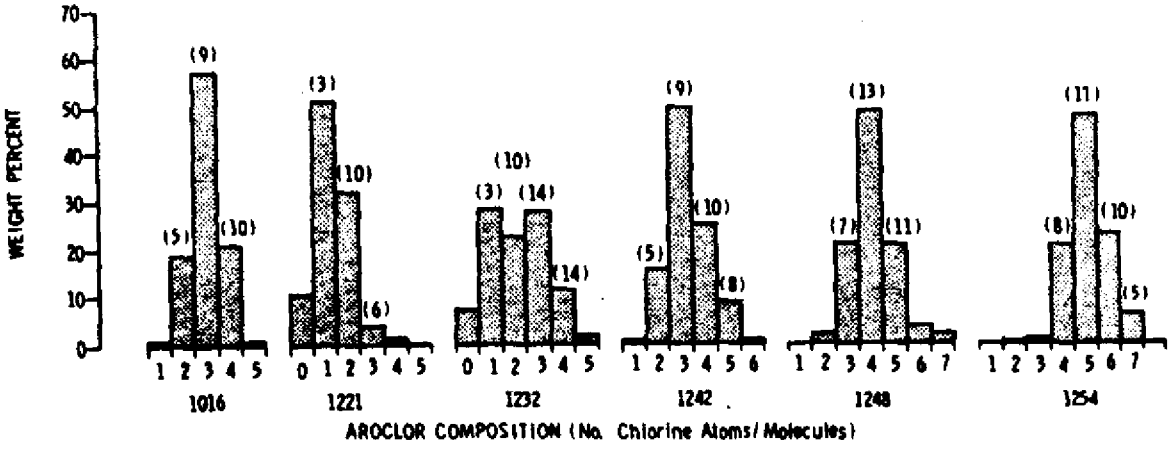

Figure 1. The congener composition of commercially available Aroclors based on the weight percent of biphenyls bearing different numbers of chlorine atoms/molecule. Data were obtained from reports by Webb and McCall (ref. 6), Sissons and Welti (ref. 5), and from information supplied by the Monsanto Industrial Chemicals Company.

MONS 202598

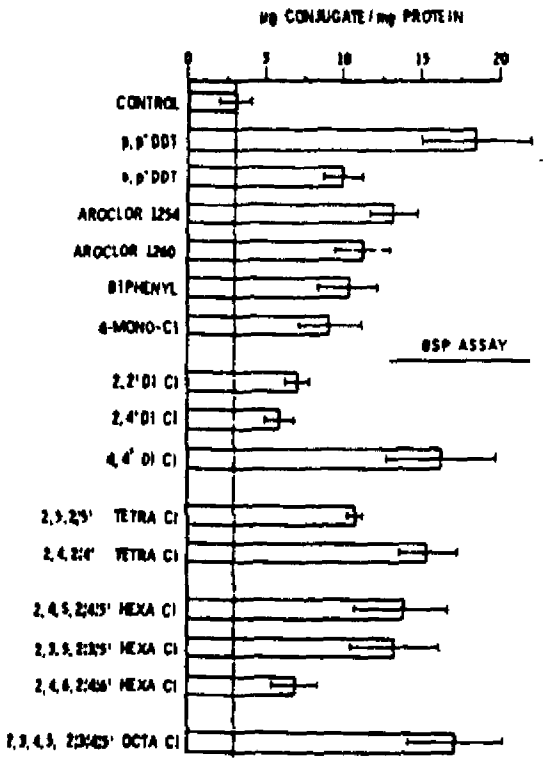

Figure 2. The effect of pretreatment of young male rats with DDT (o,p', and p,p'-isomers), Aroclors 1254 and 1260, biphenyl, and a series of isomerically pure chlorobiphenyls on the hepatic cytoplasmic enzyme which conjugates sulfobromophthalein (BSP) with reduced gluthathione. Activities are expressed as μg of conjugate formed mg^{-1} protein min^{-1}. Animals were treated by i.p. injection for 3 consecutive days, enzyme activity being determined 96 hr after the last injection. The values (bars) represent mean activities ±S.D. of the means of 19 control animals and 7 animals per treated group.

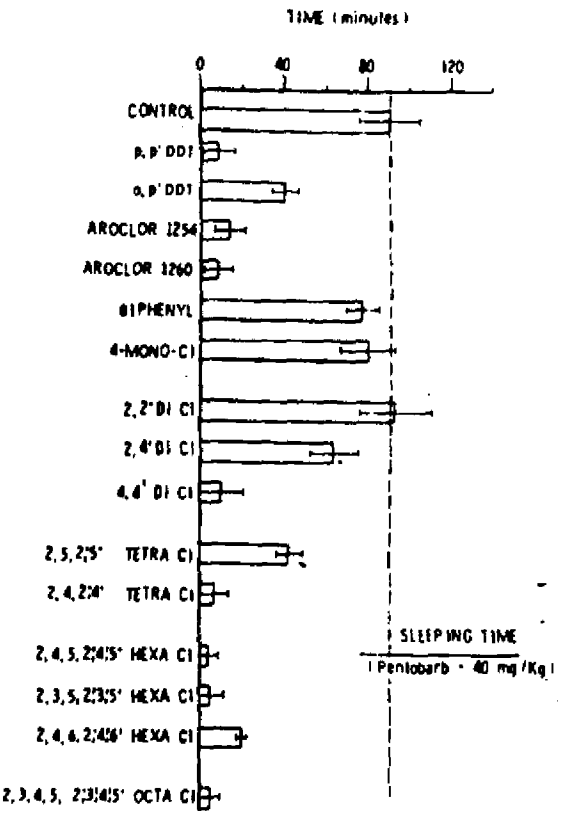

Figure 3. The effect of pretreatment of young male rats with DDT (o,p' and p,p'-isomers), Aroclor 1254 and 1260, biphanyl, and a series of isomerically pure chlorobiphenyls on the sleeping time produced by an injection of 40 mg/kg sodium pentobarbital. For other details, see figure 2.

NONS 202599

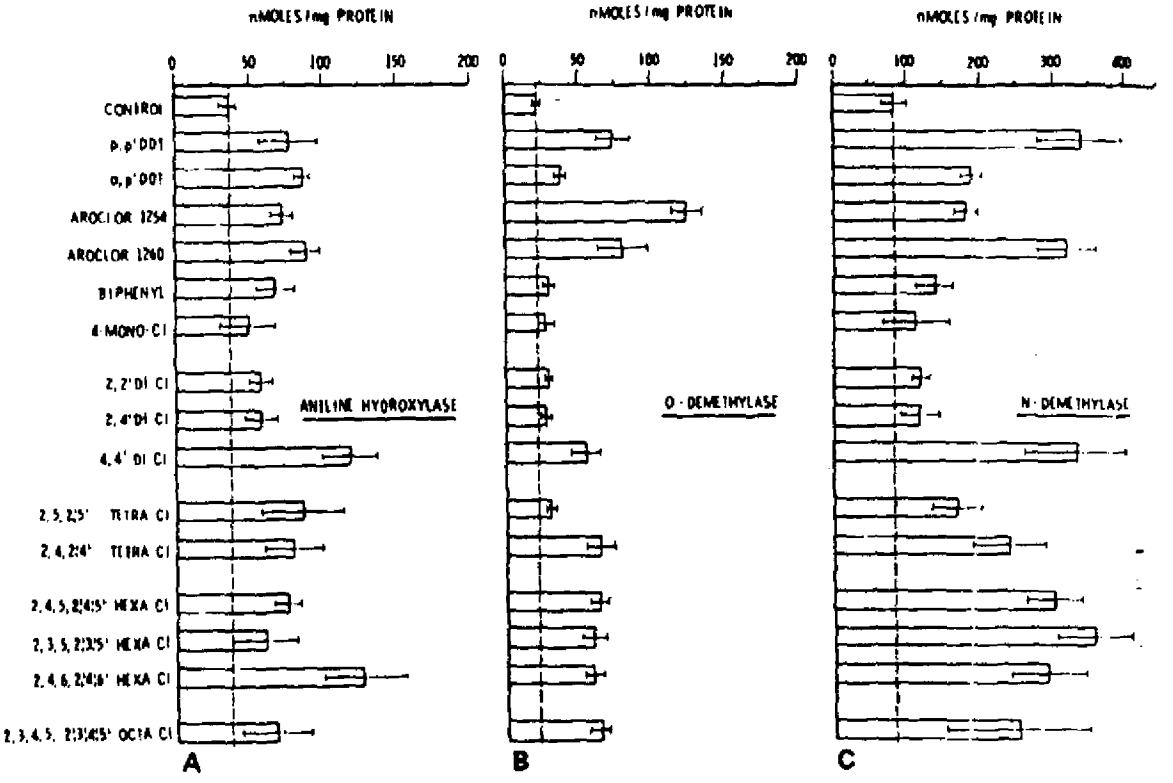

Figure 4. The effect of pretreatment of young male rats with DDT (o,p'- and p,p'-isomers), Aroclor 1254 and 1260, biphenyl, and a series of isomerically pure chlorobiphenyls on hepatic microsomal aniline hydroxylase (A), p-nitroanisole O-demethylase (B), and aminopyrine N-demethylase (C), activities. Activities are expressed as nmoles of product formed/mg microsomal protein/30min incubation. For other details, see figure 2.

MONS 202600

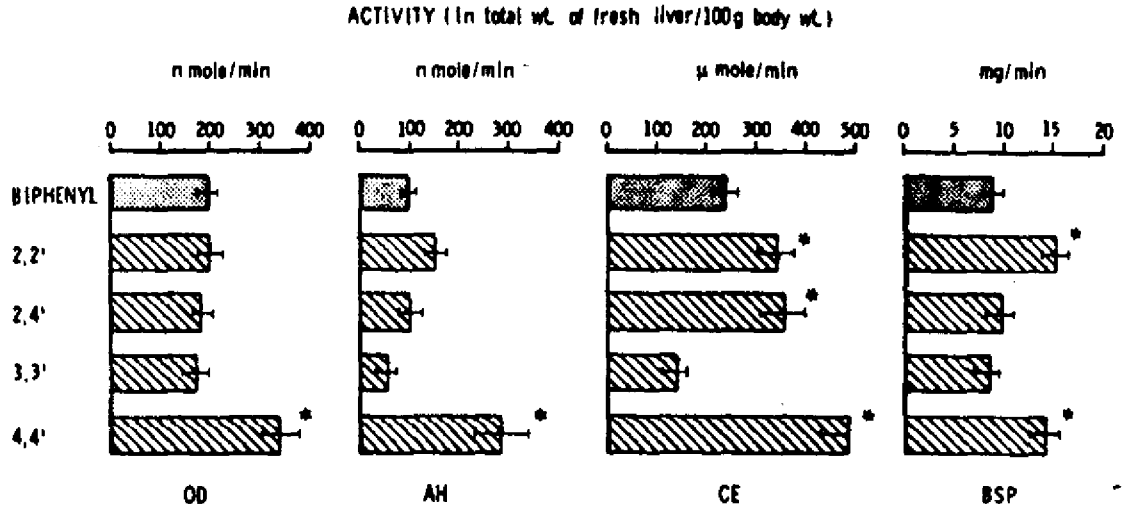

Figure 5. The effect of pretreatment of young male rats with biphenyl and series of isomerically pure dichlorobiphenyls on hepatic p-nitroanisole O-demethylase (OD), aniline hydroxylase (AH), carboxylesterase (CE), and solfobromophthalein-glutathione conjugating enzyme (BSP) activities. Animals were treated by i.p. injection for 3 consecutive days and were killed and assayed 96 hr after the last injection. The values (bars) represent the mean enzymatic activities ± S.D. (lines) of 18 control, biphenyl-treated rats and 6 animals per treated group. The asterisk (*) indicates values statistically different ($p < 0.55$) from biphenyl-treated controls.

MONS 202601

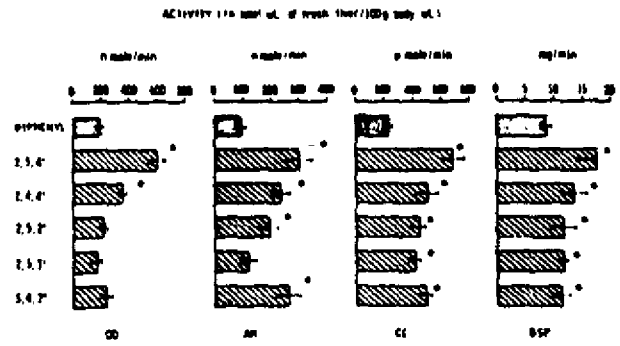

Figure 6. The effects of pretreatment of young male rats with biphenyl and a series of isomerically pure trichlorobiphenyls on hepatic p-nitroanisole O-demethylase (OD), aniline hydroxylase (AH), carboxylesterase (CE), and sulfobromophathalein-gluthathione conjugating enzyme (BSP) activities. For other details, see figure 5.

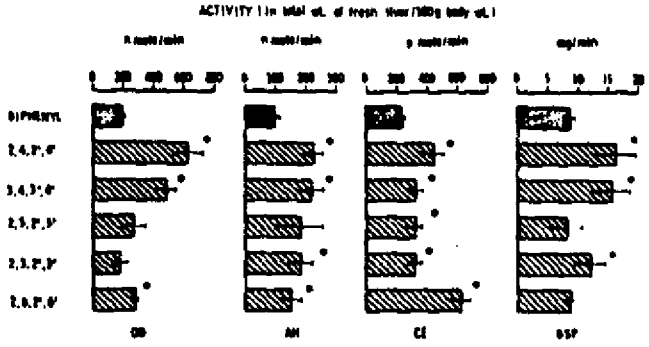

Figure 7. The effects of pretreatment of young male rats with biphenyl and a series of isomerically pure tetrachlorobiphenyls on hepatic p-nitroanisole O-demethylase (OD), aniline hydroxylase (AH), carboxylesterase (CE), and sulfobromophthalein-glutathione conjugating enzyme (BSP) activities. For other details, see figure 5.

MONS 202602

biphenyls possess the same toxicologic properties, the position of chlorination being as important as degree of chlorination.

The porphyrogenic nature of chlorinated biphenyls is well known (refs. 2,13-15). Goldstein et al. and Grote et al. demonstrated marked increases in δ-aminolevulinic acid synthetase following treatment with commercial chlorobiphenyls (refs. 15,16). This enzyme, located in hepatic mitochondria, is the rate-limiting catalyst in the synthesis of heme and prophyrins. Recently, Goldstein et al. completed a study of the effects on chick liver, feeding five different, isomerically pure hexachlorobiphenyls at concentrations of 400 ppm or 3 weeks (ref. 17). All agents caused uroporphyrin accumulation, increased hepatic cytochrome $P\text{-}_{450}^{450}$, and microsomal drug-metabolizing enzymes but only 3,4,5,3',4',5'-, 2,3,4,2',3',4'-, and 2,4,5,2',4',5'-hexachlorobiphenyl caused gross accumulation of hepatic porphyrins. These isomers were also the most toxic. It is notable that these three isomers had both the *m*- and *p*-positions occupied by chlorines, while the other two isomers (2,3,6,2',3',6'- and 2,4,6,2',4',6'-) had either the *m*- or the *p*- positions unoccupied.

Several investigators have demonstrated that the low chlorine-containing congeners in commercial mixtures disappeared more rapidly from tissues than did the highly chlorinated biphenyls (refs. 18-22). In our earliest paper, we suggested that the magnitude of hepatic enzyme induction might be related to the rate of degradation and elimination of the congeners from the body (3). There is ample evidence of hydroxylated derivatives of chlorobiphenyls being eliminated from mammalian systems and, no doubt, we shall hear more about them at this meeting (refs. 23-27). Evidence in the literature suggests two possible mechanisms of biotransformation. The first and most rapid mechanism involves the formation of an arene oxide intermediate and requires the presence of unsubstituted adjacent (vicinal) carbon atoms in the nucleus (ref. 24,27,28). The second and much slower mechanism uses a different hydroxylating system for isolated unsubstituted positions as are found in highly chlorinated biphenyls (ref. 26). The position of chlorination in low chlorine-containing biphenyls could have considerable directing influence over the pathway of biotransformation. Certain low chlorine biphenyls would be less effective (and less toxic) due to rapid biotransformation via arene oxide intermediates. Others, substituted at a position (i.e., 4-position) which would disrupt the vicinal carbon arrangement, would be unable to undergo rapid biotransformation and would persist in vivo. The toxicity of these agents may be closely correlated with lipid solubility, high concentration in the liver, and along duration of action, but it is evident that the position of the substituent chlorines on the biphenyl nucleus may be the key factor governing these other properties.

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DISCUSSION

DR. JOHN V. MOORE (National Institute of Environmental Health Sciences, Research Triangle Park, North Caroline): Have you done any investigatory work to see what the name of the impurity might be?

DR. ECOBICHON: Not that I know of. I don't even belive Eastmen Kodak is aware of it.

MONS 202604

TOXICOLOGY OF SELECTED SYMMETRICAL HEXACHLOROBIPHENYL ISOMERS: I. BIOLOGICAL RESPONSES IN CHICKS AND MICE

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Abstract

One-day-old cockerels were fed: (I) 3,10,30,100, and 300 ppm of 3,4,5,3',4',5'-hexachlorobiphenyl (HCB); (II) 400 ppm of 2,3,4,2',3',4'-HCB; (III) 400 ppm of 2,4,5,2',4',5'-HCB; (IV) 400 ppm of 2,3,6,2',3',6'-HCB; and (V) 400 ppm of 2,4,6,2',4',6'-HCB. Surviving chicks were sacrificed at 21 days. Male mice were fed 10, 30, 100 and 300 ppm of three of the above isomers (I, III, V), and survivors were sacrificed at 28 days. HCB's levels in adipose tissue and liver were determined. There were variations among the isomers as to dose and pathologic effects. Isomer (I) showed the greatest effect of those studied on mortality, body weight gain, liver, thymus, and spleen; it also attained the highest tissue concentration. It was the only isomer which produced porphyrin accumulation; and, in the chicks, produced hydropericardium, ascites, and edema. The decreasing order of overall toxicity was I >> V > II, III, IV. A general similarity of response was observed in both chicks and mice.

INTRODUCTION

A number of publications have described various toxicologic effects of commercial polychlorinated biphenyls. The presence of toxic impurities, variable chlorine content, or chlorine substitution patterns make interpretation and comparison of the results of these studies quite difficult.

By comparison, little work has been done on assessing the general toxic effects of single polychlorinated biphenyls. The objective of this report is to describe the first results obtained from a comperetive, systematic study of the general biological effects of some hexachlorobiphenyl (HCB) isomers in chicks and mice.

The HCB's were selected because of (1) their predominant presence in higher chlorineted formulations (ref. 1); (2) their relative stability in the environment (particularly their persistance in human tissue) (ref. 1); and (3) their strong inductive effect on several biological parameters (ref. 2). The specific isomers studied were selected as biphenyl models representing different physicochemical properties. Chicks were used in this experiment because of their high sensitivity to polychlorinated hydrocarbons; mice were selected to confirm results in a mammelian species.

MATERIALS AND METHODS

The experimental protocol is summarized in table 1. The table illustretes the chemicals and dose levels used, the number of animals per dose level, and the duration of the experiment. The HCB's were mixed in a chick edame bioessay diet or a standard powdered mouse diet. The animals were housed in temperature- and humidity-controlled rooms. The chicks were kept in wire-floored cages, the mice in separate plastic cages. Food and water were provided *ad libitum* for the entire experimental period. The animals were observed daily; body weight was recorded twice a week; food consumption was measured once a week for the chicks and three times a week for the mice. Control groups were observed under identical experimental conditions.

Complete necropsies were performed on dead or moribund animals and on the surviving animals at the end of the experimental period. Blood samples were obtained at this time and analyzed for packed cell volume, total serum protein, and protein fractionation. Liver, spleen, and heart weights were recorded for the chicks; in mice, kidneys, right testicle, and adrenals were also weighed. All organs and tissues were examined under UV light for the presence of red fluorescence as an indication of porphyrin accumulation. After fixation, tissues were prepared for histopathologic examination using standard methods.

All animals were assigned to a given dose according to a table of random numbers. Diet, adipose tissue, and liver were collected for HCB's residue analysis.

RESULTS

The complete results of this study are described in a series of papers either in press or in preparation (refs. 3-6). This presentation is a summary comparison of major biological responses observed in chicks and mice. Table 2, which summarizes the chick effects, shows that 3,4,5,3',4',5'-HCB is the most toxic isomer studied, causing death in all the chicks, even at the lowest dose used. You will note that this dose is less than 1/100 of the diet concentrations of the other HCB's. Only one out of ten

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MONS 202605

chicks died in the 2,3,6,2',3',6'-HCB group; no animals of the other groups died during the experiment. Reduction in body weight gain was observed in all treatment groups.

At the dose levels studied, significant differences in liver weights and histopathologic changes were observed with all HCB isomers. The most toxic was 3,4,5,3',4',5'-HCB, even at the lowest dose. It was the only isomer in which UV fluorescence (porphyrin accumulation) was observed. This finding was quite pronounced, especially in the lining layer of the gizzard, liver, and bones. The slight pathologic effect of the liver observed at 10 ppm is probably due to the very early death of the chicks. The thymus of these chicks was extremely involuted while only slight effect was caused by the other isomers.

Table 3 summarizes the major pathological changes observed: Marked edema of subcutaneous tissue, denoted by gelatinous appearance of subcutaneous fat; marked depletion of lymphocytes in the spleen; diarrhea and soiling of the cloacal area; turbid ascitic fluid; hydropericardium; and loss of visceral fat were findings seen only with the 3,4,5,3',4',5'-HCB. Fatty metamorphosis and single-cell or focal necrosis of the liver, were present, albeit to a variable extent in all animals. In addition, characteristic large black spots, 1 to 5 mm in diameter, were observed under the capsule and on cut surfaces of the liver of birds which received 2,4,6,2',4',6'-HCB. This change was caused by marked dilatation of sinusoids with a result of accumulation of blood. In some cases, the epicardium of chicks fed 2,3,4,2',3',4'-HCB was markedly edematous.

Table 4 summarizes the effects in mice. Again, 3,4,5,3',4',5'-HCB was the most toxic, causing one death at 30 ppm and decreasing body weight gain at the 10-ppm level. Although there were no deaths at 10-ppm level in this experiment, which lasted to the 28th day, in one subsequent experiment, mice fed a diet with 10 ppm died an average of 39.4 days (range 36-47). Only one mouse fed the highest dose (300 ppm) of 2,4,5,2',4',5'-HCB died before the end of the experimental period; in contrast, all mice fed 2,4,6,2',4',6'-HCB at the same level died.

The body weight gain of the mice fed 100 ppm of 2,4,5,2',4',5'-HCB or 2,4,6,2',4',6'-HCB were not significantly reduced. The dose-related increase in liver weight was caused by all three isomers, with the greatest effect being in the 3,4,5,3',4',5'-HCB group. The absence of liver pathology in the mice fed with 300 ppm 3,4,5,3',4',5'-HCB is, again, considered to be related to the early time of death. Only 3,4,5,3',4',5'-HCB caused porphyrin accumulation and a dramatic atrophy of the lymphatic organs.

The major pathological changes observed in the mice are summarized in table 5. Gelatinous appearance of subcutaneous fat, presence of blood in the gastrointestinal tract, and hemmorrhage in the retrobulbar area of the eye were characteristic of the mice that died due to 3,4,5,3',4',5'-HCB toxicity. Accentuation of the hepatic lobules, swelling and hyalinization of the hepatocytes, fatty metamorphosis, and single or focal necrosis were not related to a particular chemical since these changes were observed in all HCB groups to various degrees. Cardiomyopathy and passive congestion of the lung were caused only by 2,4,6,2',4',6'-HCB at the 300-ppm dose.

Retention indices of the mixed liquid phase used for gas chromatography may serve as an index of lipophilicity (table 6). Except for 2,4,6,2',4',6'-HCB, decreasing indices for this isomeric series correlated exactly with decreasing adipose tissue accumulation in chicks. The highest values were shown by 3,4,5,3',4',5'-HCB and 2,3,6,2',3',6'-HCB the lowest. A similar trend in tissue accumulation and retention indices is evident in mice (table 7), but not at all dose levels. A greater concentration of 3,4,5,3',4',5'-HCB was observed in all cases, particularly in the liver.

CONCLUSIONS

There are definite differences in the toxicity of the HCB isomers tested. Comparing toxicity using such biological parameters as mortality, body weight gain, liver effects, porphyrin accumulation, involution of the lymphatic organs, and fluid accumulation in chicks, the decreasing order of overall toxicity would be 3,4,5,3',4',5'-HCB >> 2,4,6,2',4',6'-HCB > 2,4,5,2',4',5'-, 2,3,4,2',3',4'-, 2,3,6,2',3',6'-HCB.

The differences in pathologic effects observed during these experiments were quantitative and, to some degree, qualitative. It is important to note that the nature of the toxicity of 3,4,5,3',4',5'-HCB mimics the effects caused by the dibenzofurans.

Although certain toxicologic characteristics differentiate the biological responses in each species tested, the major toxicopathologic effects were common to both chicks and mice.

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MONS 202606

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Table 1. Protocol for hexachlorobiphenyl isomers toxicity experiments in mice and chicks[a]

Chemical	Dose level (ppm)		No. of animals/ dose level		Experimental period (day)	
	Mice	Chicks	Mice	Chicks	Mice	Chicks
(>99 percent purity)						
3,4,5,3',4',5'-HCB	3 10 30 100 300	3 10 30 100 300	5	10	28	21
2,3,4,2',3',4'-HCB	-	400	-	10	-	21
2,4,5,2',4',5'-HCB	10 30 100 300	400	5	10	28	21
2,3,6,2',3',6'-HCB	-	400	-	10	-	21
2,4,6,2',4',6'-HCB	10 30 100 300	400	5	10	28	21

[a]Five-week old C57BL/6 male mice; 1-day-old white leghorn cockerels.

NONS 202607

Table 2. Summary of the biological effects of hexachlorobiphenyl isomers in chicks

Chemical	Dose level (ppm)	Mortality	Body weight gain (% of control)	Liver effect	Porphyrin Accumulation	Thymus effect
3,4,5,3',4',5'-HCB	3	10/10	-	+++	-	+++
	10	10/10	-	++	+++	+++
2,3,4,2',3',4'-HCB	400	0/8	68	++	-	+
2,4,5,2',4',5'-HCB	400	0/10	66	++	-	+
2,3,6,2',3',6'-HCB	400	1/10	80	++	-	+
2,4,6,2',4',6'-HCB	400	0/10	83	+++	-	+

Table 3. Summary of major pathological changes in chicks given different hexachlorobiphenyl isomers for 21 days

Chemical	Dose level (ppm)	Liver	Thymus	Spleen	Fluid Accumulation
3,4,5,3',4',5'-HCB	3	Marked; fatty metamorphosis, focal necrosis.	Marked; involution.	Marked; depletion of lymphocytes.	Subcutaneous edema, ascites, hydropericardium.
2,3,4,2',3',4'-HCB	400	Moderate; hyalinization, single-cell necrosis.	Slight; involution.	NS[a]	Epicardial edema.
2,4,5,2',4',5'-HCB	400	Moderate; single-cell necrosis.	Slight; involution.	NS[a]	NS[a]
2,3,6,2',3',6'-HCB	400	Moderate; fatty metamorphosis, focal necrosis, giant cell formation.	Slight; involution.	NS[a]	NS[a]
2,4,6,2',4',6'-HCB	400	Marked; fatty metamorphosis, focal necrosis, giant cell formation, marked focal dilatation of sinusoids.	Slight; involution.	NS[a]	NS[a]

[a]NS = not significant.

MONS 202608

Table 4. Summary of the biological effects of hexachlorobiphenyl isomers in mice

Chemical	Dose level (ppm)	Mortality	Body weight gain (% of control)	Liver effect	Porphyrin Accumulation	Thymus effect
3,4,5,3',4',5'-HCB	10	0/5	12	++	+	++
	100	3/5	-	+++	+++	+++
	300	5/5	-	-	-	+++
2,4,5,2',4',5'-HCB	100	0/5	91	+	-	-
	300	1/5	58	++	-	+ -
2,4,6,2',4',6'-HCB	100	0/5	85	+	-	-
	300	5/5	-	++	-	++

Table 5. Summary of major pathological changes in mice given different hexachlorobiphenyl isomers for 28 days

Chemical	Dose level (ppm)	Liver	Thymus	Spleen	Heart
3,4,5,3',4',5'-HCB	30	Marked; fatty metamorphosis, single-cell necrosis.	Marked; involution.	Moderate; depletion of lymphocytes.	NS[a]
2,4,5,2',4',5'-HCB	300	Slight; swelling of hepatocytes.	Slight; involution.	NS[a]	NS[a]
2,4,6,2',4',6'-HCB	300	Marked; fatty metamorphosis, single-cell necrosis.	Marked; involution.	Moderate; depletion of lymphocytes.	Moderate; cardiomyopathy.

[a]NS = not significant.

MONS 202609

Table 6. Gas chromatographic retention indices and tissue hexachlorobiphenyl levels in chicks

Chemical	Dose level (ppm)	GC retention index value	Concentration (ppm) Adipose T.	Liver
3,4,5,3',4',5'-HCB	100	2,820	1,210	-
	300		4,912	
2,3,4,2',3',4'-HCB	400	2,678	3,998	44
2,4,5,2',4',5'-HCB	400	2,542	3,921	59
2,3,6,2',3',6'-HCB	400	2,478	2,893	9
2,4,6,2',4',6'-HCB	400	2,346	4,172	24

Table 7. Gas chromatographic retention indices and tissue hexachlorobiphenyl levels in mice

Chemical	Dose level (ppm)	GC retention index value	Concentration (ppm) Adipose T.	Liver
3,4,5,3',4',5'-HCB	10	2820	508	95
	30		1,383	498
	100		2,278	887
	300		6,912	1,344
2,4,5,2',4',5'-HCB	10	2542	296	7
	30		416	30
	100		1,864	83
	300		3,923	637
2,4,6,2',4',6'-HCB	10	2346	99	6
	30		582	15
	100		1,402	122
	300		4,329	1,022

MONS 202610

TOXICOLOGY OF SELECTED SYMMETRICAL HEXACHLOROBIPHENYL ISOMERS: CORRELATING BIOLOGICAL EFFECTS WITH CHEMICAL STRUCTURE

James D. McKinney, Ph.D.*

Abstract

The symmetrical hexachlorobiphenyls (HCB's) represent model PCB's with high and constant chlorine content permitting unequivocal study of a given substitution pattern. These isomers show separate and distinct biological responses which can be related to chemical structure via effects of varying chlorine substitution on compound lipophilicity and metabolism. Compound purity must also be assured. Relative molecular polarizability can be correlated with lipophilicity and biological activity and is measurable by chromatographic and spectroscopic techniques. These parameters were highest in HCB's with planar symmetry and 4,4' substitution. The rates of metabolism of these isomers are considered of lesser importance than the potentially highly toxic nature of some of the intermediary and terminal metabolites.

INTRODUCTION

A previous paper (ref. 1) has shown the importance of the degree of chlorination of PCB's in terms of their distribution and excretion in the rat. Of particular concern is the implication that not more than 20 percent of the hexachlorobiphenyl isomer studied would ever be excreted. Therefore, hexachlorobiphenyls may represent PCB's with both high chlorine content and biological half life. Our studies with symmetrical hexachlorobiphenyl isomers (HCB's) sought to determine the varying biological effects of HCB's as a function of their varying substitution patterns (figure 1). Two of the isomers studied are found in about 3 to 4 percent (area percent by flame ionization gas chromatography) in Aroclors 1254 and 1260. Several major components of these same Aroclors contain various combinations of the substitution patterns studied. Since the effects of chlorine substitution in biphenyls on spectral and chromatographic properties may be approximately predicted (refs. 2,3) using additive parameters obtained from chlorobenzenes, it may be possible to predict the biological behavior of the various combinations through study of the appropriate individual symmetrical isomers.

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TOXICOLOGICALLY SIGNIFICANT PROPERTIES

As previously described (ref. 4), our studies with both chickens and mice have shown separate and distinct biological effects at submaximal doses for the different isomers even though all of these isomers might be considered potent (relative to lower chlorinated isomers). In explaining these differences, we feel that the three major considerations are compound purity, lipophilicity, and metabolism. Intestinal adsorption was not considered since earlier work (ref. 5) failed to show appreciable differences for various PCB's.

Compound purity can be very important since both the individual synthetic isomers (ref. 6) and the Aroclor mixtures are known (ref. 7) to be contaminated with small amounts of chlorinated dibenzofurans which, in some species at least, are orders of magnitude more toxic than the PCB's. Since separate and distinct differences in the biological effects of 2,3,7,8-tetrachlorodibenzofuran and the HCB's were found in our work (refs. 8,9), one may be in a position to assess the involvement of dibenzofurans as contaminants and, as will be described later, as potential metabolites.

Relatively little work (ref. 10) has been done associating the effects of varying PCB structure with varying degrees of tissue accumulation. Our work (ref. 8) does appear to show a trend in adipose tissue accumulation of HCB's which correlated with molecular polarizability as measured by chromatographic and spectroscopic techniques. The HCB gas-chromatographic (GC) retention indices (increasing) generally correlated with their overall biological response and were highest in isomers with 3,4-substitution. The amount of HCB in the adipose tissue does not determine activity, but its distribution in this tissue and activity in the liver may both reflect lipophilicity. Since the GC retention index correlation is consistent with a number and variety of GC columns, it is possible that it may have some value in predicting lipophilicity. The lipophilicity in turn relates to biological activity through effective tissue concentration of a given HCB. The differences in adipose tissue accumulation do not appear to be a function of metabolic removal since the least accumulated isomer (2,3,6,2',3',6') was one of the least metabolized (ref. 11).

Considerations of metabolism must take into account the differences in rates of degradation and excretion as well as the nature of intermediary and terminal metabolites. Higher degradation rates (shorter biologi-

MONS 202611

cal half-life) are generally associated with those PCB's having vicinal (two-adjacent carbon atoms) positions unoccupied. These PCB's are also the only ones for which there is clear evidence to support arene oxide intermediates (refs. 1,12). The toxic properties of these PCB's may have more to do with the reactive nature of such arene oxide intermediates, with the main quantitative difference being associated with varying degrees of lipophilicity.

The more highly chlorinated PCB's such as the HCB's would show similar differences due to lipophilicity, but would be expected to be metabolized more slowly and by other mechanisms as well since vicinal unoccupied positions occur more infrequently. The probable metabolic pathways in chicks (based on metabolites identified in excreta) for the 2,4,5,2',4',5'-, 2,4,6,2',4',6'- and 3,4,5,3',4',5'-HCB's suggest that several reaction types, including isomerization, reductive dechlorination and oxidation with and without the loss of chlorine, are operating. The detailed methodology for identifying these metabolites will be described in a separate report (ref. 11). Microbial metabolism in the gut cannot be ruled out.

The metabolites of 2,4,5,2',4',5'-HCB include the meta-hydroxy compound, which appears to undergo further oxidation to the para-quinol, suggesting that a direct insertion hydroxylation reaction may be operating as reported (ref. 12) for further oxidation of 4'-chloro-4-biphenylol to 4'-chloro-3,4-biphenyldiol. However, arene oxide intermediate formation may be possible at 1,2-carbons with one unoccupied and the other occupied with either a phenyl or chlorine substituent probably resulting in concomitant chlorine (or phenyl) migration or dechlorination. Dechlorination does occur since a trace of pentachlorobiphenyl and pentachlorotrihydroxybiphenyl and pentachlorophenylbenzoquinone metabolites are found. These highly oxidized products including the *p*-quinone, which accompanies the *p*-quinol, are more chemically and perhaps biochemically reactive. Such products could be more toxic as a result of their increased reactivity unless they are rapidly excreted. The quinone form may not be rapidly excreted since it cannot undergo conjugation.

The metabolites identified in the excreta of chickens fed 2,4,6,2',4',6'-HCB contaminated food include 2,4,6,2',3',4'-HCB and a trace of 2,4,6,2',3'-PCB, which are accompanied by two compounds whose low-resolution mass spectral and chromatographic properties suggested hexachlorodibenzofuran and pentachlorodibenzofuran structures. High-resolution mass spectral analysis of the suspected hexachlorodibenzofuran metabolite showed that its molecular formula was $C_{12}H_6Cl_6$ and, therefore, was a methylhexachlorobiphenyl or the mass equivalent chloromethylpentachlorobiphenyl. Methyl ethers and esters are conceivable metabolites of PCB's, but this type of metabolite is unexpected and suggests a direct methylation (or chloromethylation) of an aromatic ring in the biphenyl molecule.

High-resolution mass spectral analysis of the suspected pentachlorodibenzofuran is in progress. However, preliminary low-resolution gas chromatography-mass spectral data obtained from perchlorination of the metabolite mixture indicated that some octachlorodibenzofuran was present. In the absence of the high-resolution data, it is conceivable that the suspected pentachlorodibenzofuran is the source of the octachlorodibenzofuran.

Data obtained from spiking of control chick feces with the 2,4,6,2',4',6'-HCB and performing the same extraction and cleanup procedure used for the chick metabolites cast doubt on the authenticity of the pentachlorobiphenyl metabolite and on an additional metabolite corresponding in molecular formula (by high-resolution mass spectral analysis) to $C_{12}H_4Cl_4O_2$ found in liver tissue and later in the excreta of the chicks fed the 2,4,6,2',4',6'-HCB.

It is clear from the structures of some of these metabolites that chlorine removal and migration have occurred. Therefore, these products may be derived from enzymatic one electron reduction of this system followed by loss of chloride ion to generate a reactive radical which can react directly with nucleophilic constituents or undergo a second electron reduction to an anion which can react directly with electrophilic constituents. Both electrophilic and nucleophilic radicals of hydrogen (dechlorination), chlorine (isomerization), and oxygen (oxidation) are among the possible recombinants which could account for the metabolites found. The methylhexachlorobiphenyl could also be the result of interaction with an electrophilic methyl donor. Perhaps of equal or greater toxicological significance would be the nature of nonexcretable metabolites derived from covalent bonding of these reactive radicals.

The propensity to lose chloride ion (ref. 13) stems from the favorable stereoelectronic properties associated with a high degree of ortho substitution. The possibility that any oxygenated products including dibenzofurans are the result of direct superoxide anion displacement of chlorine cannot be ruled out. Recent work (ref. 14) has shown that chlorine displacement by superoxide can take place in the mass spectrometer under negative chemical ionization conditions. An analogy to this and possibly an environmental source of dibenzofurans from PCB's would be photonucleophilic displacement of chlorine by water (ref. 15).

MONS 202612

In contrast, the 2,3,6,2′,3′,6′-HCB was metabolized to a trace amount of a monohydroxy metabolite, but no other PCB's or other oxygenated products were found. Therefore, four ortho chlorines alone are not enough; it may be necessary to have high lipophilicity (4,4′-substitution) as well as vicinal unoccupied positions available for appreciable metabolism.

No metabolites were detected in the excreta of chickens fed food contaminated with the 3,4,5,3′,4′,5′-HCB. However, this isomer has separate and distinct biological effects indicative of a chlorinated dibenzofuran. Since this isomer has a high lipophilicity and accumulation in tissue, its slow metabolism to an elusive, but potentially highly toxic, dibenzofuran may be significant. Enzymatic oxidation at any one of the equivalent unoccupied ortho positions could lead via rearrangement to a "transannular epoxide" intermediate which would undergo a rather facile oxidation to the symmetrical 2,3,4,6,7,8-hexachlorodibenzofuran.

Examination of chicken tissue and mouse excreta and tissue for metabolites is in progress. Preliminary work with tissue from 2,4,5,2′, 4′,5′- and 2,4,6,2′,4′,6′-HCB treated chicks has confirmed the presence of tissue metabolites. Metabolites found in tissue are also found in excreta, but not all the metabolites found in excreta are in tissue. At least one metabolite found in the liver and adipose tissue of the 2,4,5,2′,4′,5′-HCB and in the liver and excreta of the 2,4,6,2′,4′,6′-HCB treated chicks was of particular interest, since its mass spectral properties (low- and high-resolution measurements) corresponded to a tetrachlorodibenzodioxin (TCDD), a hydroxytetrachlorodibenzofuran, a tetrachlorophenylbenzoquinone or a methoxytetrachlorobiphenyl. The exact structure assignment cannot be made at this time, but failure to methylate this metabolite under two sets of reaction conditions would cast doubt on the hydroxytetrachlorodibenzofuran possibility. As mentioned earlier, this compound may not be a metabolite, but it remains a potential problem as an artifact of the extraction and cleanup procedure for certain PCB's, which could interfere in analysis for TCDD in environmental samples.

SUMMARY

High compound lipophilicity is clearly a contributing factor to HCB toxicity in both chickens and mice. The potentially highly toxic chlorinated dibenzofurans are a problem since they are known to be contaminants of certain Aroclor mixtures and at least one synthetic isomer and are potential environmental transformation products including metabolites of certain PCB's. The exact chlorine substitution patterns found in these compounds may be of considerable importance toxicologically (ref. 16), as has been the case for the structurally similar chlorinated dibenzodioxins. The requisite substitution patterns for dibenzofuran formation found in components of Aroclors most likely would include a combination of those providing high chlorine content, in particular ortho chlorine, high lipophilicity and tissue accumulation, and unavailability of unoccupied vicinal positions. Other PCB's having all ortho positions occupied might also favor ortho oxygenation. Other PCB's may be metabolized to a high degree of ortho-substituents, one of which is hydroxy and another chlorine, which through further metabolism could lead to dibenzofurans, again provided that other requirements are met. Further study is needed with the appropriate purified isomers to clearly establish the formation of dibenzofuran metabolites and determine if microsomal enzymes are responsible.

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DISCUSSION

DR. STEVEN SAFE (Department of Chemistry, University of Guelph, Ontario, Canada): You mentioned the requirement for 1,2-carbons unsubstituted for arene oxide formation and hydroxylation. We have observed both chlorine migration (NIH shift) and dechlorination. So you do not need unsubstituted positions but you can have a shift.

DR. McKINNEY: I do not believe I said it that way. I believe I said that the only clear evidence that I have seen for oxides has been in cases where adjacent carbon atoms were present.

DR. SAFE: With migration of the chlorine?

DR. McKINNEY: Yes, that is an unusual situation.

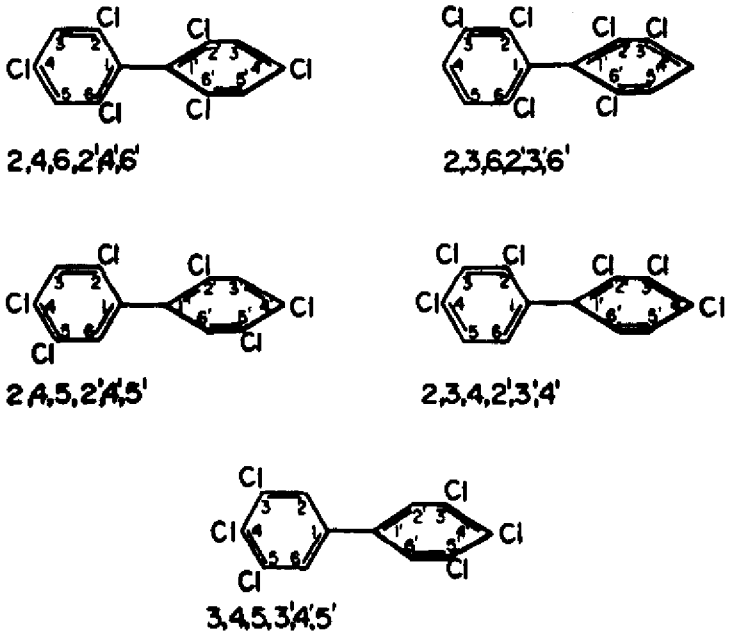

Figure 1. Hexachlorobiphenyl structures.

MONS 202614

TOXICITY OF 2,3,7,8-TETRACHLORODIBENZOFURAN—PRELIMINARY RESULTS

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Abstract

There are at present no reports on the toxicity of pure chlorinated dibenzofurans. The paper below describes ongoing research on 2,3,7,8-tetrachlorodibenzofuran (TCDF), the most toxic isomer of the group. The work is based on the premise that due to the similar structures of chlorinated dibenzofurans and chlorinated dibenzodioxins, the nature of response and the level of exposure needed to induce that response may be similar for the two compounds.

Although no conclusions can be drawn yet, the following observations have been made: (1) Hegharn chicks given 5 μg/kg TCDF showed fluid accumulation, enlarged heart, reduced size of thymus and spleen, then death; (2) Hartley guinea pigs showed weight loss, depression, and diminution of thymus and spleen prior to death; (3) Rats and mice had only mild toxicological reactions to a 6,000 μg/kg dose of TCDF.

Trends at this point indicate that TCDF is highly toxic at low doses to chicks and guinea pigs, as is tetrachlorodibenzodioxin (TCDD). For rats and mice, however, the correlation is not strong. Before accurate assessments can be made of the health significance of chlorinated dibenzofurans, the specific structures of their isomers must be elucidated.

The toxicological significance of exposure to chlorinated dibenzofurans is an issue of great interest. Interest has been generated by the identification of chlorinated dibenzofurans as impurities of two European PCB's (refs. 1,2,3); American PCB's (ref. 4); the presumptive findings of dibenzofurans in the urine of rats fed Aroclor 1254 (ref. 5) as well as the identification of chlorinated dibenzofurans in PCB-contaminated rice oil (ref. 6). There are no reports which describe the toxicologic effects of pure chlorinated dibenzofurans.

Several authors have speculated that due to similar chemical structure, the nature of the response and the level of exposure to induce that response could be similar for chlorinated dibenzofurans and chlorinated dibenzodioxins. To determine the role PCB's per se or a chlorinated dibenzofuran contaminant may play in the toxicity of PCB's, and to simultaneously correlate chlorinated dibenzofuran response to chlorinated dibenzodioxins, initial experiments were undertaken using 2,3,7,8-tetrachlorodibenzofuran. The dibenzofuran with chlorines at the 2,3,7, and 8 positions was felt to be the isomer of initial interest given that it is the isomeric configuration that is the most toxic chlorinated dibenzodioxin.

In all of the studies which I am to report upon today, the 2,3,7,8- tetrachlorodibenzofuran (TCDF) used†† was of 88 percent purity with the remaining components primarily a pentachlorodibenzofuran. The TCDF was dissolved in acetone, which was then further mixed with corn oil. All doses of TCDF were administered by gavage. We have conducted general toxicity studies with TCDF in chicks, mice, guinea pigs, and rats.

In most instances, the data to be presented represent current status of ongoing research; as such, it does not allow for a clear concise conclusion at this time. However, I do feel the data to be sufficient for one to perceive trend, and I present it with this in mind.

In one experiment (ref. 7), TCDF was administered daily by gavage utilizing white leghorn chicks that were 1 day of age when the study commenced. Birds which received 5 μg/kg per day TCDF died in an average of 11.5 days. General unthriftiness and apparent depression preceded death. One of six chicks receiving 1 μg/kg per day TCDF died on day 19. Body weight and food consumption decreased in this group during the 14th to 21st day of the study. The experiment was designed to terminate on the 21st day. It was our impression that had the study endured an additional week, most, if not all, chicks at the 1 μg/kg dose would have died. The most striking gross pathologic change observed was the accumulation of clear fluid, as evidenced by marked subcutaneous edema, ascites, and hydropericardium, with the severity of the fluid accumulation greatest at the 5 μg/kg dose. The hearts of these birds also appeared enlarged and flabby (rounded). The second striking change was a marked reduction in size of the thymus and spleen. Histologically, size reduction could be

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MONS 202615

accounted for by the marked depletion of lymphocytic cell types in the spleen and cortex of the thymus. Similar pathologic changes were found to occur at the 1 μg/kg dose, albeit to a milder extent. There were no significant effects observed on liver weight; mild liver pathology was found to occur only at the 5 μg/kg dose. Total serum protein levels were reduced with a marked reduction in serum albumin.

The toxic response described is similar to that observed in "chick edema disease," a syndrome later shown to be produced by chlorinated dibenzodioxins. This TCDF response is in general agreement with the response that Vos observed in birds that received PCB's that contained low levels of tetrachloro- and pentachlorodibenzofurans.

Toxicity studies with TCDF in Hartley guinea pigs are summarized in table 1. This table shows that the single oral LD_{50} dose lies between 5 and 10 μg/kg. It is also evident that there is an inverse relationship between increased dose and decreased mean time to death. The bottom of the table shows, for comparative purposes, that the LD_{50-30} (that dose which causes 50 percent mortality in a 30-day period) of 2,3,7,8-tetrachlorodibenzodioxin (2 μg/kg) compares quite closely with the TCDF value. TCDF toxicity in guinea pigs is one of a progressive weight loss followed by depression at least 24 to 36 hours preceding death. As was observed in the chicken toxicity studies, there was severe diminution in size of thymus and spleen. Similarly, this finding was found to correlate with a lymphocyte depletion in the periarterialor lymphocytic sheaths and follicles of the spleen; or severe atrophy of the thymus cortex. Although the numbers of animals evaluated were small, the trend of changes present in the bladder, adrenal, kidney, and liver were similar to that previously associated with tetrachlorodibenzodioxin toxicity (ref. 8).

A single experiment in which TCDD was administered to rats at doses up to 1,000 μg/kg failed to show any toxic effects. These rats were terminated 4 weeks after dosing. Histopathologic evaluation of tissues from the rats failed to reveal any changes that could be associated with tetrachlorodibenzofuran toxicity.

Table 2 summarizes the TCDF toxicity findings in male C57B1/6 mice. Per os dose levels up to and including 6,000 μg/kg in a single dose failed to produce any observable effects during the ensuing 28 to 30 days. Histopathologic examinations of these animals failed to reveal any effects on thymus, spleen, or other organs, save for the liver. Here, a mild effect with the suggestions of a mild toxicity was observed in mice that received 6,000 μg/kg per os. A definite toxic response was observed in mice that received a 6,000 μg/kg dose administered subcutaneously. Although mortality was not observed, there was a depression in body weight gain during the second to fifth day following dosing. Organ weights recorded at time of necropsy 30 days after the dose had been administered showed an increase in the liver weight as well as liver to body weight ratio.

Table 1. 2, 3, 7, 8-tetrachlorodibenzofuran toxicity in Hartley guinea pigs

Per os dose (μg/kg)	Mortality	Mean time to death
80	5/5	10.8 days
40	5/5	11.6
20	5/5	12.2
15	6/6	12.0
10	9/11	15.5
5	3/11	18.0
0	0/11	-0-

LD_{50} 2,3,7,8-TCDD = 2.0 μg/kg; MTD = 20.6 days.

MONS 202616

Table 2. 2,3,7,8-tetrachlorodibenzofuran toxicity in male C57 BL/6 mice[a]

Dose:	0 µg/kg	6,000 µg/kg
Mortality:	None	None
Body weight:	Normal gain	Sl. depression 2nd-5th day
Liver/body weight:	1.513 + 0.05	1.728 + 0.04
Ratio:	5.76 + 0.05	6.86 + 0.06
Thymus/body weight:	0.045 + 0.002	0.017 + 0.001
Ratio:	0.19 + 0.01	0.06 + 0.007

[a]Administered subcutaneously to 6-week-old mice. Animals sacrificed 30 days post-administration. "No effects" per, os, at 6,000, 4,000, 2,500, 1,500, 1,200, 1,000, 800, 600, and 400 µg/kg. LD_{50} 2,3,7,8-TCDO = 200-250 µg/kg.

A more striking finding was the reduction in thymus weight with a concurrent reduction in thymus to body weight ratio. Histopathologic evaluations of tissues from these animals confirmed that the reduction in thymus weight was due to loss of lymphocytic elements. Evaluation of the liver evidenced a clear, but moderate, toxic response characterized by focal areas of single- cell necrosis and pleomorphism. The mouse studies show that there is at least a 30-fold diminution in TCDF toxicity when compared to TCDD. It further indicates, however, that the pattern of the toxicity likely will mimic that which is observed with tetrachlorodibenzodioxin (ref. 9).

The health significance of dibenzofurans in PCB toxicity can only be speculated upon at this time. It is clearly toxic at very low dose levels in the chick and guinea pig. However, studies to date with the mouse and, to a lesser extent, with the rat suggest that toxicity in these species may not be as closely correlatable to the corresponding dibenzodioxin analog. Furthermore, I believe the specific structure of chlorinated dibenzofuran isomers must be elucidated before their health significance can be projected with any reasonable degree of confidence. This opinion is based on work with chlorinated dibenzodioxins where marked variability in toxicity was seen dependent on the specific chlorinated isomer tested (ref. 10).

A final point addresses the tendency to speculate that the 3,4,5,3',4',5'-HCB toxicities as seen in mice and chickens are due to TCDF. Though the pattern of pathologic response is similar in both instances, the response which is observed is not pathognomonic for a specific chemical.

These dibenzofuran studies are continuing and are being extended to the rhesus monkey as soon as sufficient 2,3,7,8-TCDF is synthesized. A systematic evaluation of other chlorodibenzofurans is planned.

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