Draft

AN ANIMAL MODEL TO SIMULATE THE RESPONSE OF THE HUMAN FETUS AND NEONATAL TO POLYCHLORINATED BIPHENYL EXPOSURE

by

Edward J. Calabrese, Ph.D., Ed.D.
Assistant Professor - Occupational and
and Environmental Medicine
Assistant Director - Environmental
Health Resource Center
University of Illinois
School of Public Health

November, 1975

Note: This proposal is incomplete and has not been submitted to any funding agency.

MONS 090176

Research Plan

A. Introduction

1. Objective

The basic objective in the proposed research is to develop an animal model which will simulate the response of human fetuses and neonatals to PCB's in order to determine if this subgroup of the human population is at high risk to PCB exposure. The secondary objective includes determination of the importance of conjugate glucuronidation in the detoxification and excretion of PCB's.

2. Background

Since the late 1960's when polychlorinated biphenyls (PCB's) were first recognized as a potentially widespread environmental contaminant hundreds of research papers have been published concerning their structure, industrial uses, presence in the environment, toxicity to numerous animals from insects to man, tissue storage, metabolism and excretion.[1,2] As a result of such data accumulation, regulatory agencies are now trying to approach the problem of standard setting with respect to PCB's. Standards for air, water and food are necessary if the total human exposure is to be regulated and controlled. One important component in standard setting is a consideration of the individuals within the population who may be at high risk to the pollutant because of various genetic, physiological, psychological and behavioral traits. Individuals lacking the ability to detoxify and excrete PCB's represent such a high risk group since they will be unable to prevent undue accumulation of toxic levels of PCB's within the body.

Mammals, including rabbits, dogs, and humans have been reported to excrete PCB's in part by conjugate glucuronidation and/or sulfonation.[1,3] The urinary excretion of biphenyl and 4-chlorobiphenyl has been studied in rabbits. Biphenyl glucosiduronic acid and 4-hydroxybiphenyl were isolated from urine. Rabbits fed 4-chlorobiphenyl excreted 4-(p-chlorophenyl)-phenol and 4-chlorobiphenyl glucosiduronide. Twice as much 4-chlorobiphenyl as biphenyl was excreted as the glucosiduronic acid derivative. It was suggested that other low chlorinated biphenyls are excreted in a similar manner.[3]

In dogs injected with 2,4,4'-trichloro-2'hydroxydiphenyl ether, nearly 100% of the material excreted in their urine and feces over a 5-day period, appeared as the glucuronide or sulfate conjugate. Human adults similarly exposed excrete 65% in urine and 20% in feces after intravenous injection. It is excreted as a free compound or as a glucuronide.[1] Other evidence suggests that some chlorinated biphenyls are hydroxylated by species such as the rat and pigeon. No evidence of reductive dechlorination was observed in either the trout, rat or pigeon.[4]

During the excretory process foreign substances (PCB's) usually first undergo several metabolic transformations including oxidations, reductions, and hydrolysis. It often happens that following an oxidative, reductive or hydrolytic reaction conjugation of the foreign substance with either glucuronic acid, sulfate, glycine, cysteine, methyl or acetyl occurs. Of these reactions, glucuronic acid conjugation is probably the most important in the animal kingdom since it occurs extensively in man and all laboratory animals with the exception of the cat. In the cat, glucuronic acid conjugation is not entirely absent, but occurs at a low level compared with other mammalian species. Conjugations are generally regarded as detoxification mechanisms, for compounds which undergo these reactions are converted into products which are usually less toxic and more rapidly excreted than their precursors.[5-8]

The widespread occurrence of glucuronic acid conjugation may be due to the facility with which glucuronic acid can be produced in the body from carbohydrate sources and the variety of chemical groups to which glucuronic acid can be transferred enzymatically. The other conjugation mechanisms are more restricted in their occurrence and this is probably because of the limited availability of the conjugating agents such as glycine and cystaine (via glutathione) and to the small number of chemical groupings to which conjugating agents such as sulfate, glycine, cystaine, methyl, and acetyl can be transferred. The activity and amount of the transferring enzymes concerned in these conjugations are probably also limiting factors. Glycine conjugation is confined mainly to aromatic carboxyl groups, sulfate to phenolic hydroxyl groups and occasional aromatic amino groups, cysteine or glutathione conjugation to some aromatic hydrocarbons and halogenated hydrocarbons, and methylation

to certain hydroxyl and amino groups, and acetylation to some amino and hydrazine groups. However, glucuronic acid conjugation can occur with several types of hydroxyl, amino, and carboxyl groups and with sulfhydryl groups.[5-8]

The effect of conjugation of a substance with glucuronic acid is to produce a strongly acidic compound which is more water-soluble at physiological pH values than the precursor. The majority of foreign substances are ultimately cleared from the body via their excretion in the urine and bile, most often in the form of polar conjugates such as glucuronides with the avenue of excretion of the glucuronide probably varying with the species of animal considered.[5,6]

It is apparent that individuals lacking the ability to form either conjugate glucuronides or sulfates or both will be at high risk with respect to toxic compounds of a phenolic nature. The recognition of low levels of glucuronide formation in the cat have been suggested as an explanation to the widely observed phenomenon in veterinary literature[9-12] that phenolic compounds should not be used on or around cats because of increased incidence of toxicity in this species. Furthermore, intravenous injections of phenol in cats, pigs, dogs and goats revealed that cats were at least 2 times more sensitive with respect to fatality caused by the phenol.[13] Toxicity in this study was related to the partial deficiency in the cat to conjugate phenol with glucuronic acid. The author concluded that the cat is more likely to be poisoned by acute doses of phenol and is more likely to become chronically affected with phenol toxicity due to the inability to rapidly form and excrete glucuronides. Biochemical studies with the cat have also indicated that conjugate glucuronidation is the major pathway for the detoxification and excretion of phenol (e.g., more than twice as effective as sulfonation).[13] Because cats are hypersusceptible to phenolic-like compounds as a result of their diminished capacity to form glucuronides it is strongly suspected that human embryos, fetuses, and neonatals (2 to 3 months old) which also are deficient in a functional conjugate glucuronidation system will also be predisposed to the toxic effects of phenolic compounds including PCB's.[8,14,15]

Usually by the age of 2 to 3 months adult levels of most enzyme systems are achieved.[8,14,15] Unfortunatly, this "developmental immaturity" in the

unborn and the very young may predispose them to the toxic effects of certain substances since they may be unable to detoxify and excrete them as quickly as necessary.[16] It has already been pointed out that human adults do excrete, in part, PCB's via conjugate glucuronidation. Clinical experience has shown that infants may respond differently to doses of drugs which are easily tolerated by older children and adults. Presumably this is because older children and adults have fully functioning enzyme detoxification systems while the neonatal lacks such development. Inefficient glucuronidative mechanisms in the very young have broad significance since the absence of such mechanisms prevent the prompt elimination of toxic substances from the body. A further problem for the very young with regard to PCB's is that in addition to having a difficult time excreting PCB's they may also consume more PCB's per unit of body weight than at any other time during their life span. For example, samples of human milk from two cities in California contained average PCB levels of 60 ppb while average levels in human milk in Sweden and Germany were 16 ppb and 100 ppb, respectively. Based on a daily milk intake of 150 µg/kg, breast fed infants in California would ingest about 9 µg/kg/day of PCB's. A range of 1-3 µg/kg/day has been suggested as a "reasonable" level for an Acceptable Daily Intake.[2,16] One µg/kg/day has been reported as being 100 times less than the lowest "no effect" level in animal studies.[17] Thus, in children the margin is reduced to about a factor of 10 if excretory activity is equivalent with adults. However, as a result of the immature enzyme systems the safety factor of 10 may be considerably reduced. How much - is presently unknown.

A tragic example of toxicity arising from the inability to form glucuronides has been reported. The drug chloramphenicol, which is known to be metabolized in humans by the action of the glucuronic pathway, caused the death of more than 30 premature babies who had been treated with the antibiotic for infectious diseases. Theoretically, the premature babies were not able to conjugate the drug with glucuronic acid and thus the drug could only be slowly excreted and so tended to accumulate in the body to toxic proportions eventually leading to death.[15]

An additional problem encountered by many neonatals is that approximately five percent of the mothers of normal infants secrete milk which inhibits the activity of glucuronyl transferase (and thus the glucuronidation process) by more than 20 percent via the action of a steriod present in the breast milk. Inhibition of this glucuronyl transferase has been reported for up to 49 days after birth. Clinically these children have been reported to develop unusually severe neonatal jaundice. This condition develops because glucuronide formation which assists in the elimination of bilirubin (breakdown product of hemoglobin) is partially inhibited. Cow's milk does not contain sufficient amounts of this steroid to affect a noticeable inhibition of the glucuronidation process. Consequently about 5 percent of the neonatals breast fed would be expected to have their ability to excrete PCB's impaired.[18] Because the benefits of breast feeding have been widely popularized in newspapers, on television and in popular magazine articles since the late 1960's in the U.S., it is expected that any problems associated with the inhibition of the glucuronidation process by the steroid is more widespread than in previous decades. Administration of the antibiotic novobiocin has also been associated with unconjugated hyperbilirubinemia in infants.[19] Novobiocin is a noncompetitive inhibitor of glucuronyl transferase activity in vitro.[20] Thus children receiving concomitant exposure of novobiocin and PCB's would be expected to have their ability to detoxify and excrete PCB's impaired.

After the neonatal period, a broad range of conditions is associated with the improper or incomplete development of the glucuronide conjugation system. The usual physiological problem associated with these conditions is the inadequate detoxification and excretion of bilirubin. This spectrum extends from the frequently occurring "mild" condition known as Gilbert's syndrome to the very rare, but severe and often fatal Crigler-Najjar syndrome.[21]

Since the clinical effects associated with these syndromes are considered to be caused by metabolic disturbances of the glucuronide conjugation scheme, it is expected that PCB elimination in these individuals would be impeded. Although Gilbert's syndrome has been mentioned as being "encountered relatively often" in a clinical sense, no estimates of the gene frequency of such a trait have been reported.

Concomitant exposure to PCB's and other drug-like chemicals may potentiate the toxic effects of PCB's. For example, rats and monkeys given SKF525A during the initial 24 hours of exposure to PCB succumbed rapidly as compared to the control group.[21] Of possible significance is the fact that SKF525A inhibits the proper functioning of the glucuronic pathway.[15]

Individuals with liver infections may also be at high risk with respect to PCB's. For example, depression of glucuronide synthesis has been observed in humans with infectious hepatitis.[15]

After considerable review of the PCB literature it has become apparent that any studies concerning the rate of accumulation, storage and elimination of PCB's in cats are entirely lacking. It is suggested that cats may represent an animal model simulating the way a human fetus, neonatal and adults with disturbances in the glucuronidation conjugation pathway may respond to PCB exposure.

Rationale

In attempting to establish standards for environmental stressors it is important to consider the health effects of such stressors on high risk populations.

A crucial component in this process is the development of an animal modal system which will allow predictions and extrapolations to human populations. Considerable evidence indicates that phenolic and biphenolic compounds are detoxified and excreted primarily via the glucuronidation process in mammals. We believe that the adult cat, the only known adult mammal having extremely low activity of the conjugate glucuronidation process, represents a model simulating humans with immature and non-functional glucuronidation enzyme systems, that is, fetuses, neonatals and enzyme deficient adults with the Gilbert's and Crigler-Najjar syndromes.

In order to test the hypothesis that the glucuronidation process is of principle importance in detoxifying and excreting a specific PCB (Aroclor 1016) it will be necessary to compare a group of cats exposed only to the Aroclor with other treatment groups which have the indentical PCB exposure but have had their conjugate glucuronidation processes either significantly inhibited or stimulated. It is predicted that the inhibition treatment will make the

cats more sensitive to the toxic effects of the PCB's if glucuronidation is a major route of detoxification and excretion. In the case of the stimulation of conjugate glucuronidation, it is predicted that the cats will be less sensitive to the PCB intoxication because of increased ability of the glucuronidation pathway to detoxify and excrete PCB's.

Experimental Design

In order to test the hypothesis, cats will be used as the experimental animal in both the acute and chronic phases of the study.

1. Acute Phase I - For Determination of LD_{50}
   a. Intraperitoneal Injection of Aroclor 1016
      (1) Experimental group - Five groups of cats with 10 males (randomly chosen) in each group will be established. Each group of cats will be injected once with a different concentration of Aroclor 1016. Concentrations will range between 500-2000 mg/kg of body weight.
      (2) Sham control - A sham control will be established consisting of 5 groups of cats with 10 males in each group. Each cat will be injected once with the same amount of vehicle (Emulfor/ethanol/water - 1:1:8) similar to that used in a corresponding "experimental group."
      (3) Control group - A group of 10 male cats will not receive any injections.
   b. Oral Administration of Aroclor 1016
      An "experimental," "sham control" and "control group" will be established as in part 1a, "Intraperitoneal Injection of Aroclor 1016."
      (1) Experimental group - will be fed Aroclor 1016 once in concentrations of 1000-3000 mg/kg.
      (2) Sham control - Each cat will receive a single dose of the same amount of vehicle (Emulfor/ethanol/water - 1:1:8) used in a corresponding feeding in the "Experimental group," part 1b1.
      (3) Control group - This group will receive neither the Aroclor 1016 nor the vehicle.
2. Chronic Phase
   a. Intraperitoneal Injection of Aroclor 1016
      (1) Experimental group - Five groups of cats with 10 males in each group will be established randomly. Each group of cats will be injected every other day with a different concentration of Aroclor 1016. Injections will be carried on for a

period of 3 months. The concentrations of Aroclor 1016 will be 1/100-50/100 of the LD_{50} value determined in Acute Phase I (1a).

(2) Sham control - The sham controls will contain the same numbers and groups of cats as the "Experimental group," [2a-(1)]. Injections of the vehicle will be given every other day for a period of 3 months. Each cat in the "sham control" will be injected with the same amount of vehicle used in a corresponding injection in the "Experimental group."

(3) Control group - A group of 10 male cats will not receive any injections.

b. Oral Administration of Aroclor 1016

An "experimental," "sham control" and "control group" will be established as in part 2a, "Intraperitoneal Injection of Aroclor 1016."

(1) Experimental group - Each cat will be fed every other day for a period of 3 months, twice the amount of Aroclor 1016 that was injected into cats in the "Experimental group" [2a-(1)].

(2) Sham control - Each cat will be fed every other day, for a period of 3 months, the same amount of the vehicle that was fed cats in the "Experimental group" [2b-(1)].

(3) Control group - This group will receive neither the Aroclor 1016 nor the vehicle.

3. Acute Phase II

Experimental design is exactly the same as in "Acute Phase I" except that all cats will be injected with 500 mg/kg of Novobiocin for a three day period prior to injection of the Aroclor 1016.

4. Acute Phase III

Experimental design is exactly the same as in "Acute Phase I" except that all cats will be injected with 50 mg/kg of body weight in corn oil, 10 ml/kg of 3 methylcholanthrene for a 3-day period prior to injection of the Aroclor 1016.

EXPERIMENTAL DESIGN - POLYCHLORINATED BIPHENYLS - AROCLOR 1016

EXPERIMENT / TREATMENTS	ACUTE						CHRONIC	
	NO PRETREATMENT I		PRETREATMENT					
			NOVOBIOCIN II		3-METHYL-CHOLENTHRENE III			
	IP	ORAL	IP	ORAL	IP	ORAL	IP	ORAL
EXPERIMENTAL * +	SINGLE DOSE 500-2000 mg/kg of AROCLOR	SINGLE DOSE 1000-3000 mg/kg of AROCLOR	IP INJECTION OF 500 mg/kg EVERY DAY FOR 3 DAYS		IP INJECTION OF 50 mg/kg EVERY DAY FOR 3 DAYS		1/100-50/100 OF LD_{50} (ACUTE I) EVERY OTHER DAY FOR 3 MONTHS	1/50-1 OF THE LD_{50} DOSE (ACUTE I) EVERY OTHER DAY FOR 3 MONTHS
	SAME AMOUNT OF AROCLOR 1016 ADMINISTERED AS IN "ACUTE I-NO PRETREATMENT"							
SHAM CONTROL * +	VEHICLE ONLY - ADMINISTERED ONCE						VEHICLE ADMINISTERED EVERY OTHER DAY FOR 3 MONTHS	
CONTROL ** +	ONE GROUP OF 10 MALE CATS IN EACH OF THE 8 EXPERIMENTS							
STATISTICAL ANALYSIS	PROBIT ANALYSIS & DOSE-LOG PLOT						MULTIPLE REGRESSION ANALYSIS ANOVA SIGNED RANK TEST	

* 5 GROUPS OF 10 MALE CATS EACH
** NO CHEMICALS ADMINISTERED
\+ TISSUE BIOPSY & GROSS PATHOLOGY

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Methods and Materials

All animals used in these experiments will be adult male conditioned cats. The cats will be kept in cages at the Biological Resource Laboratory at the University of Illinois at the Medical Center under the same environmental conditions. Food and water will be given ad libertum. Aroclor 1016 will be dissolved in a mixture of Emulfor/ethanol/water (1:1:8) and the mixture injected intraperitoneally (ip).[26] This same preparation, but in a different concentration so that the Aroclor 1016 and vehicle will be able to be administered in a small gelatin capsule, will be administered in studies on the effect of oral administration. The animals will be anesthetized with ether with a retractor placed in its mouth. The capsule will then be placed into the esophagus. The trachea will be massaged until the capsule is swallowed.

In Acute Phase III, cats will be pretreated with an ip injection of 3-methylcholanthrene (50 mg/kg of body weight in corn oil, 10 ml/kg)[23] 3 days prior to the administration of Aroclor 1016 in order to increase the rate of synthesis of the glucuronide. In another acute study (Acute Phase II), cats will be pretreated (ip injection) with Novobiocin[20] at 500 mg/kg of body weight for 3 days prior to the administration of Aroclor 1016. Novobiocin is given in order to decrease the rate of synthesis of the glucuronide.

In all acute studies, blood will be drawn 1 day prior to and at 6 days after administration of the Aroclor 1016. Blood will also be drawn at death or sacrifice. Tissue biopsy and gross pathology will be performed on a random sample of cats from each acute and chronic study.

Levels of free Aroclor 1016 in tissues will be determined by the following method. One to two ml of plasma or 100-200 mg of liver, kidney, brain or adipose tissue will be extracted with hexane. Urine, 10-20 ml, will be extracted at approximately pH 7 with hexane. Each sample in a volume of 0.5 ml will be eluted from a microsilica gel column using a 1:1 benzene-hexane mixture. Samples will then be analyzed with a tritium source electron capture detector operated in the direct current mode at 12v.[24]

Cats in the acute studies will be monitored at 12, 24, 36 and 48 hours after administration of the Aroclor 1016 and at daily intervals thereafter.

The acute studies will be terminated after a 3 month period.

All cats in the chronic study will be administered Aroclor 1016 using the same methods employed in the acute studies. Aroclor 1016 will be administered every other day for a period of 3 months. Aroclor 1016 levels in urine, feces, blood, liver, kidney, brain and adipose tissue will be determined using the same methods employed in the acute studies. Additionally, in the chronic studies, levels of glucuronides and sulfonides will be determined from urine, feces, blood, liver, kidney, brain and adipose tissue using the following method:

Employing the method of Hanson et al,[25] the level of glucuronic acid will be determined for urine, feces, blood, liver, kidney, brain and adipose tissue. All glucuronic acid is assumed to be conjugated with Aroclor 1016 or one of its metabolites. With the level of total Aroclor 1016 and the level of glucuronic acid determined, subtraction of the glucuronic acid level from that of the free Aroclor 1016 level will give us the level of the sulfonation fraction.

Amyl alcohol will be used as the solvent and color intensity will be measured with a photoelectric absorptiometer. Extraction of the pigment with amyl alcohol will have a blue color with a sharp maximum absorption at 615-620 mμ. Tissues and solvent will be boiled in a water-bath to insure optimum color production.

Prodedure. 2 ml of solution containing 10-80 ug glucuronic acid, 2 ml 0·25% naphthoresorcinol solution and 3 ml conc. HCl in 6 X 5/8 in. pyrex tubes, are mixed by rotation and placed in a boiling water-bath for 2 hours. The tubes are cooled in ice for 10 minutes, 5 ml amyl alcohol added, and the tubes stoppered and shaken vigorously for 15 seconds. The contents are poured into 50 ml separating funnels and washed out with a further 5 ml amyl alcohol added to the separating funnels, the tubes being allowed to drain for 1 minute. The aqueous layer is separated, the amyl alcohol solution run into graduated tubes, and the funnels allowed to drain for 1 minute. The solution is made up to 11 ml with ethanol (to dissolve any fine droplets of water suspended in the amyl alcohol); the contents of the tubes are mixed and the color estimated by means of a photoelectric colorimeter.[27,28]

Maximum color production is obtained when 3 vol. conc. HCl in a total of 7 vol. solution are used. There is no sharp maximum for color production on varying the naphthoresorcinol concentration. The color intensity increases rapidly with rising concentrations to 0·25% solution and then only slowly for concentrations above this. Naphthoresorcinol 0·25 g dissolved in 100 ml distilled water will be used as the blank.

Tissue biopsy and gross pathology will be performed on a number of randomly chosen cats from each of the acute and chronic studies. Tissues will be fixed in a buffered 4% formaldehyde solution and stained with hematoxylin-eosin stain for light microscopic examination. Gross pathology will also be conducted to determine the presence of any tumors.

Statistical Analysis

Statistical analysis will be performed on all studies. The acute studies will be analyzed using probit analysis and dose-log plots. The chronic study will be analyzed using the Wilcoxin-Cox signed rank test, ANOVA, Scheffé multiple regression analysis and Duncan's Multiple range.

Supplies

1000 cats at $35 @		$35,000
Care of cats $85/day		30,000
1 full-time laboratory technician	14,000	28,000
½ time laboratory technician	7,000	14,000
Statistician (½ time)	7,000	7,000
Secretary	8,000	16,000
Computer time		700
Key punching		650
Books		500
Office supplies		300
Telephone		500
Xerox		500
Travel to conferences to present results of the study		1,500
Publication cost		3,000
Chemical reagents		5,000
Laboratory glassware and other supplies		4,500
Aroclor 1016 100 lbs.		5,000
Varian aerograph gas chromatograph 210040-01		6,800
Columns and packing		1,500
The Pharmacia Gel Electrophoresis System Electrophoresis Apparatus GE-4 91011		885
Gel and Eluant Reservoirs 90826		56
Sephadex University Dye Kit		20
Graphic illustrations and photographs		500
	TOTAL	$161,911

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