

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

**ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600**

UMI[®]



Université d'Ottawa • University of Ottawa

**The Effects of PCB 126 on Embryotoxicity and
 β -Adrenoceptors in Chicken (*Gallus domesticus*) Embryos**

By

Andrea L. Katynski

Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfillment of the requirements for the
Masters of Science in Biology

Ottawa-Carleton Institute of Biology

Thèse soumise à
l'École des études supérieures et de la recherche
Université d'Ottawa
en vue l'obtention de la maîtrise ès sciences

L'Institut de biologie d'Ottawa-Carleton



**National Library
of Canada**

**Acquisitions and
Bibliographic Services**

**395 Wellington Street
Ottawa ON K1A 0N4
Canada**

**Bibliothèque nationale
du Canada**

**Acquisitions et
services bibliographiques**

**395, rue Wellington
Ottawa ON K1A 0N4
Canada**

Your file Votre référence

Our file Notre référence

0-612-66060-5

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

Canada

ABSTRACT

Halogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs) comprise some of the most toxic and persistent environmental pollutants associated with various developmental and reproductive effects in mammals, birds and fish. It has been previously reported that PCBs induce oxidative stress through various mechanisms including increased production of reactive oxygen species, lipid peroxidation and altered antioxidant status; and oxidative stress can modify β -adrenoceptor (β -AR) function. This thesis examines the effects PCBs on β -AR function in an avian model, to test the hypothesis that if PCB toxicity in the liver is mediated in some part by oxidative damage, such as increased lipid peroxidation, then the function of a membrane-bound receptor, such as the β -AR, may be modified through oxidative damage to membrane lipids.

Injection of 1.6 μg PCB 126/kg egg into the air cell prior to incubation increased the % mortality and the incidence of abnormalities, including liver lesions and subcutaneous edema, in day 19 chicken (*Gallus domesticus*) embryos. Hepatic lipid peroxidation and EROD activity were significantly increased in PCB 126-treated embryos. These results indicate that the dose of PCB 126 used in this study induced a significant toxic response in day 19 embryos.

Pharmacological characterization of chick embryo hepatic β -ARs showed that they preferentially bind mammalian β_2 -agonists and antagonists. PCB 126 significantly decreased hepatic β -AR affinity (K_d) compared to vehicle-treated embryos. There were, however, no significant differences between control and PCB 126-treated embryos, or control and vehicle-treated embryos. This suggests there is an effect of the corn oil vehicle; it may protect the liver from increased lipid peroxidation and potential alterations

in membrane fluidity. No significant differences in hepatic β -AR numbers (B_{max}) were found between control, vehicle and PCB 126-treated embryos.

Future studies should focus on specific changes in membrane fluidity and membrane lipid composition to further examine the role of oxidative damage in mediating effects such as changes in membrane structure and function, and the implication of these changes to membrane-bound protein function.

RÉSUMÉ

Les hydrocarbures aromatiques halogénés, dont les biphényles polychlorés (BPCs), figurent parmi les polluants environnementaux les plus toxiques et persistants ayant des effets néfastes sur le développement et la reproduction des mammifères, oiseaux et poissons. Des études antérieures ont démontré que les BPCs créent un stress oxydatif par l'intermédiaire de plusieurs mécanismes tel la formation de radicaux libres et la peroxydation lipidique. Le stress oxydatif peut entraîner une modification du fonctionnement des récepteurs adrénérgiques de types β ou β -adrénocepteurs (β -AR). À l'aide d'un modèle avien, cette thèse examine l'effet des BPCs sur le fonctionnement des β -ARs. L'hypothèse avancée stipule que la toxicité des BPCs dans le foie provient en partie du dommage causé par les oxydants, plus précisément la peroxydation lipidique membranaire, qui pourrait donc modifier le fonctionnement des récepteurs membranaires tel les β -ARs.

Une injection de 1.6 μ g BPC 126/kg d'œuf, avant la période d'incubation, dans le réservoir d'air augmente le pourcentage de mortalité et la fréquence d'anormalités, dont des lésions au foie et des accumulations d'eau sous-cutanée, chez l'embryon de poulet (*Gallus domesticus*) de 19 jours. Les résultats démontrent une augmentation significative de la peroxydation des lipides hépatiques et de l'activité de l'enzyme 7-éthoxy résorufine-O-dééthylase chez les embryons traités au BPC 126, indiquant par le fait même un dosage adéquat afin de produire une réponse toxique chez les embryons de 19 jours.

La caractérisation pharmacologique des β -ARs hépatiques d'embryon de poulet démontre que ces derniers se lient préférentiellement aux agonistes et antagonistes

adrénergiques de type β_2 des mammifères. Les embryons traités aux BPC 126 ont une diminution significative de l'affinité (K_d) des β -ARs hépatiques comparé aux embryons traités au véhicule. Par contre, aucune différence significative n'a été observée entre le K_d des β -ARs hépatiques des embryons contrôles et ceux traités aux BPC 126, de même que pour le K_d des β -ARs hépatiques des embryons contrôles et ceux traités avec le véhicule. Ceci semble indiquer la présence d'un effet causé par le véhicule (l'huile de maïs). Il est possible que le véhicule protège le foie des embryons contre l'augmentation de la peroxydation des lipides pouvant affecter la fluidité des membranes et ainsi le K_d . On n'observe aucune différence significative dans le nombre de β -AR (B_{max}) entre les embryons contrôles et ceux traités avec le véhicule ou les BPCs.

Des études futures devraient porter sur les changements de fluidité membranaire et de composition lipidique des membranes afin d'examiner d'avantage le rôle que joue le dommage des oxydants sur la structure et le fonctionnement des membranes, sans oublier l'impact de ces changements par rapport au fonctionnement des protéines membranaires.

ACKNOWLEDGEMENTS

There are many people who deserve credit for helping me through this project and most importantly, seeing it come to an end. First of all, I would like to thank Tom Moon for his guidance and input. Working with him has been an outstanding experience and I feel privileged to have been a “Moonie” these last couple of years. No worries Tom... I’ll take the lava lamp and disco ball with me when I leave! I would also like to thank Sean Kennedy (Canadian Wildlife Service) for his input and advice with the toxicology aspects of this project. Many thanks to everyone in Sean’s lab, especially Stephanie Jones and Angela Lorenzen for introducing me to the wonders of the egg world. I would like to thank Xiaolei Jin for all her help; she’s been an invaluable source of information throughout the course of this project. My committee members, Jim Fenwick and Cam Wyndam also deserve thanks for contributing their ideas to this project. I would like to thank Jules Blais for allowing us to use his lab for our PCB analysis, and Linda Kempe for helping with the protocol development and validation. I thank Tamara DiMuccio, an honors student in our lab, for her work on the EROD assays.

My fellow Moonies, Michel Lortie and Stephen Dugan, deserve many thanks for seeing me through countless binding assays and broken beta-counters... thanks, Mike for bringing ice-cream to the potluck and Doogie, for eating peanut butter everyday! And of course Moonies past and present: Nicola, Nada, Martin, Marosh, Jamie, Christian, Steve, Carol and Caroline — thanks for all the snacks, good chats and especially the laughs! I would also like to thank everyone in Perry’s lab: Alex, John, Katherine, Colin, Pat and Stu, as well as some of those Trudeau followers across the hall: Doug and Katherine L. Many thanks also go to Emilie, Kerry and Teri. All of you made the last two years an unbelievably great time!

TABLE OF CONTENTS

	Page No.
ABSTRACT	i
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF ABBREVIATIONS	ix
LIST OF TABLES	x
LIST OF FIGURES	xi
INTRODUCTION	1
1.1 PCBs in the Environment	3
1.2 PCB Toxicity: Field and Laboratory Studies	4
1.3 HAH Mode of Action: The Aryl Hydrocarbon Receptor	6
1.4 Cytochrome P450s	7
1.5 Oxidative Stress: A Mediator of the Effects of PCBs	9
1.6 The Beta-Adrenergic Receptor (β-AR)	13
1.7 β-ARs and Xenobiotics	15
1.8 Oxidative Stress Effects and β-AR Function	17
1.9 Objective and Hypothesis of Study	18
MATERIAL AND METHODS	
2.1 Egg Injection Protocol	20
2.2 Liver Membrane Preparation	21
2.3 Hepatocyte Isolation	22
2.4 β-Adrenoceptor Binding Assays	23

2.5 Competition Assays	25
2.6 Lipid Peroxidation	26
2.7 EROD Activity in Liver Microsomes	27
2.8 Analysis of PCB Tissue Concentration	29
2.9 Statistics	30

RESULTS

3.1 Effects of PCB 126 Injection	32
3.1.1 Embryotoxicity	32
3.1.2 EROD Activity	32
3.1.3 PCB 126 Tissue Analysis	32
3.2 Characterization of the Avian Hepatic β-Adrenoceptor	41
3.2.1 Competition Assays	41
3.3 Effect of PCB 126 on Lipid Peroxidation and β-AR Binding Curves	46
3.3.1 Lipid Peroxidation	46
3.3.2 β-AR Saturation Curves	50

DISCUSSION

4.1 Mortality and Abnormalities	58
4.2 EROD Activity	60
4.3 PCB 126 Tissue Content	62
4.4 Pharmacological Characterization of the Chick β-AR	64
4.5 Lipid Peroxidation	66

4.6 β-AR Saturation Assays	69
4.7 Does Oxidative Stress Play a Role in PCB-Induced Alterations of β-AR Function?	73
4.8 Conclusions	74
REFERENCES	76
APPENDIX	90

ABBREVIATIONS

ACase	adenylyl cyclase
AHH	aryl hydrocarbon hydroxylase
AHR	aryl hydrocarbon receptor
AR	adrenergic receptor
ARNT	aryl hydrocarbon nuclear translocator protein
BAAM	bromoacetyl alprenolol menthane
CAT	catalase
CGP	(-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-benzimidazol-2-one])
EROD	ethoxyresorufin-<i>O</i>-deethylase
GPCR	G-protein coupled receptor
GPx	glutathione peroxidase
GR	glutathione reductase
GSSG	glutathione disulfide
H₂O₂	hydrogen peroxide
HAH	halogenated aromatic hydrocarbon
LPO	lipoygenase
MFO	mixed function oxidase
O₂⁻	superoxide anion
OH[•]	hydroxyl radical
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzodioxin
PCDF	polychlorinated dibenzofuran
PHS	prostaglandin-H-synthase
PROC	procaterol
ROS	reactive oxygen species
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TCDD	2,3,7,8-tetrachlorodibenzo-<i>p</i>-dioxin
XO	xanthine oxidase
XRE	xenobiotic responsive element

LIST OF TABLES

Table No.		Page No.
1-1.	Environmental Levels of PCBs in Various Bird Species	4
1-2.	Effects of PCB126 in Laboratory Studies	5
3-1.	Effects of PCB126 on Mortality Rate, Abnormalities, Liver and Embryo Weight	33
3-2.	Mean Inhibitory Constants (K_i) for β-AR Agonists and Antagonists	47
3-3.	β-AR Binding Affinities (K_d) and Number of Binding Sites (B_{max})	57

LIST OF FIGURES

Figure No.	Page No.
1-1. General Structures of HAHs	1
1-2. Working Model for Induction of <i>CYP1A</i>	7
1-3. Biochemical Pathways of ROS Formation, Cytoprotection and Macromolecular Damage	10
3-1. EROD activity in liver microsomes	35
3-2. Hepatic PCB126 content	38
3-3. Sample chromatograph	40
3-4. β -AR agonist competition assay	43
3-5. β -AR antagonist competition assay	45
3-6. Hepatic lipid peroxidation	49
3-7. A. β -AR saturation curve in control hepatocytes	52
B. Scatchard analysis	52
3-8. A. β -AR saturation curve in vehicle-treated hepatocytes	54
B. Scatchard analysis	54
3-9. A. β -AR saturation curve in PCB126-treated hepatocytes	56
B. Scatchard analysis	56
A-1. GC calibration curve for PCB126	91
A-2. GC calibration curve for Mirex	91
A-3. GC calibration curve for PCB204	93
A-4. GC calibration curve for PCB30	93

CHAPTER 1:
INTRODUCTION

Up to 10 positions on the biphenyl ring may be chlorine substituted, leading to 209 potential PCB congeners. Over a 50-year period, more than a billion kilograms of PCBs were produced and 135 individual congeners have been identified in environmental samples (Erickson 1986). PCB mixtures were commercially sold under a variety of trade names, such as Aroclor™, Chlophen™, Kaneclor™ and Fenclor™.

Concern over the toxic potential of PCBs to human health was sparked by the report of a mass accidental poisoning incident in 1968 in Kyushu, Japan. Over 1000 people were exposed to PCBs (among other HAHs) after using contaminated rice oil. Symptoms of their exposure included chloracne, and abnormalities in hepatic and nervous system function (Kuratsune *et al.* 1971). In addition, children born to exposed mothers were reported to be small, hyperpigmented and had inflamed conjunctival glands. A similar incident involving PCB-contaminated cooking oil occurred 10 years later in Taichung, Taiwan, where exposed individuals exhibited chloracne and skin hyperpigmentation. Children born to exposed Taiwanese mothers showed speech problems, developmental and psychomotor delays (Rogan and Gladen 2000), and scored lower on developmental/cognitive tests than non-exposed children of the same age (Hsu *et al.* 1988). These incidents highlighted the potential impact of PCBs on human health, leading to a ban on PCB use in the late 1970s. However, because of the high degree of chlorination in certain PCB congeners, they resist biodegradation and persist in various environmental niches, even reaching remote regions of the Arctic through atmospheric transport and deposition (Tanabe 1988).

1.1 PCBs in the Environment

Different classes of PCB congeners, depending on the position and number of chlorine substitutions, elicit a wide spectrum of biological responses. Toxicity is, in part, determined by the chlorine substitutions in the *meta*- and *para*-, as opposed to *ortho*-, positions (Fig. 1) on the biphenyl ring (Safe 1994). Non- or mono-*ortho* substituted congeners, like 3,3',4,4',5-Pentachlorobiphenyl (PCB 126) can assume a relatively planar conformation and induce toxic responses similar to those of the laterally chlorinated 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic of the dioxin congeners (Peterson *et al.* 1993). PCBs are known to bioaccumulate along food chains, due to their lipophilic nature and resistance to biotransformation (Kimbrough and Jensen 1989). Bioconcentration within food chains results in elevated levels of dietary contaminants in top level predators, such as carnivores (Macek and Korn 1970).

The colonial fish-eating birds of the Great Lakes are a valuable sentinel species for studying the effects of persistent organic chemicals for a number of reasons (Fox 1993): (1) they are top-level predators; (2) the Great Lakes food chains have a history of toxic chemical induced injury and impairment; (3) they are widely distributed and species such as the herring gull and bald eagle nest year round; and (4) their behavior is well-studied. Other birds, such as seabirds, are also especially vulnerable to PCB accumulation again due to their position in the food web and high concentration of PCB deposits along coastlines and the open sea (Safe 1994; Fox 1993).

Table 1-1 summarizes a number of recent studies examining PCB levels in various bird species and geographic locations.

Table 1-1. Environmental Levels of PCBs in Various Bird Species

Reference	Species	Chemical Source	[PCBs] µg/g (ppm)				Study Date and Location
Bellwood <i>et al.</i> , 1990	Great Blue Heron (<i>Ardea herodias</i>)	pulp and paper effluent	control site	0.636 ¹		1988 Coastal British Columbia, Canada	
			low site	1.630 ¹		Nicomald Vancouver	
			high site	0.880 ¹		Codfish	
Schwartz and Stalling, 1991	Foster's Tern (<i>Sterna fuscata</i>)	recycled paper, industrial and domestic waste	control site	4.79 ¹		1991 Wisconsin, USA	
			contaminated site	21.0 ¹		Lake Poygan Green Bay	
Mink <i>et al.</i> , 1994	Common Tern (<i>Sterna hirundo</i>)	industrial waste, atmospheric deposition	control site	6.22 ^{2,3}		1994	
			contaminated sites	24.45 ^{2,3}		Netherlands and Belgium, North Sea coast	
Rattner <i>et al.</i> , 1994	Black-Crowned Night Heron (<i>Nycticorax nycticorax</i>)	industrial, urban, agricultural waste	control site	1.12 ⁴		1989 USA	
			low site	1.54 ⁴		Chincoteague National Wildlife Reserve, VA	
			intermediate site	3.43 ⁴		West Marin Island, San Francisco Bay, CA	
			high site	9.32 ⁴		Bair Island, San Francisco Bay, CA Cut Island, Green Bay, WI	
Carter <i>et al.</i> , 1997	Great Blue Heron (<i>Ardea herodias</i>)	not specified	contaminated site			3.0 ⁵	1993 USA Upper Mississippi River (MN, WI, IA, IL)
Rattner <i>et al.</i> , 1997	Black-Crowned Night Heron (<i>Nycticorax nycticorax</i>)	urban and industrial contaminants	control site 1	0.29	0.15	1991 USA	
			site 2	2.66	1.29	Chincoteague National Wildlife Reserve, VA	
				chick	1.29	Baltimore Harbor, MD	
				adult	1.29	Rock Creek Park, Washington, DC	
Lorenson <i>et al.</i> , 1999	Herring Gull (<i>Larus argentatus</i>)	industrial, agricultural waste	control site	total PCBs ⁶		non-ortho PCBs ⁷	
			site 1	3.3	3.5	1997 Canada	
			site 2	19	28.5	Kent Island, NB	
			site 3	27.5	37	Papoose Island, Lake Superior	
			site 4	23	17	Snake Island, Eastern Lake Ontario	
			site 5	42.5	42.0	Hamilton Harbor, Western Lake Ontario Middle Island, Western Lake Erie	
Danabalan <i>et al.</i> , 1999	Bald Eagle (<i>Haliaeetus leucocephalus</i>)	industrial contaminants	site 1	0.1295 ⁷		Ontario, Canada	
			site 2	0.0919 ⁷		1990-1994, 1996 Lake Erie	
			site 3	0.0471 ⁷		1994 Lake Huron	
			site 4	0.0943 ⁷		1993 Lake Nipigon	
			site 5	0.1498 ⁷		1992, 1994 Lake Superior 1992 Lake of the Woods	
Danabalan <i>et al.</i> , 1999	Bald Eagle (<i>Haliaeetus leucocephalus</i>)	industrial contaminants	site 1	84 ¹		1974-1988 Lake Erie	
			site 2	26.4 ¹		1989-1994 Lake Erie	
			site 3	3.27 ¹		1993-1996 Lake of the Woods	
Hoffman <i>et al.</i> , 1999	Great Blue Heron (<i>Ardea herodias</i>)	plant operations, waste disposal	control site	0.14 ^{1,2}		1992-1994 Oak Ridge Reservation, TN, USA	
			contaminated site	0.16 ^{1,2}		Long Island, Lonsay Island KCS, Midway Hill	
Finowski <i>et al.</i> , 1999	House Sparrow (<i>Passer domesticus</i>)	not specified	urban site	carcass	0.234 ²	1994-1996 Warsaw, Poland	
			road site	0.252 ²	0.0035	Dziakow Lesny, Pol.	
					0.0015	Kopa Kierdzinska, KK and Kierdzin, KL	
Carter <i>et al.</i> , 2000	Town Sorelifer (<i>Tachycineta bicolor</i>)	not specified	control site	egg ¹	0.28	0.13	1998 Upper Mississippi River, IA, USA
			site 1	0.40	0.14	Figuon Creek	
			site 2	0.54	0.14	Lindsay Harbor Duck Creek	
Rattner <i>et al.</i> , 2000	Black-Crowned Night Heron (<i>Nycticorax nycticorax</i>)	agricultural, municipal point and non-point sources	control site	0.42 ²		1997 Delaware, FL, USA	
			contaminated site	1.22 ²		Middle Island, Rehoboth Bay Pee Patch Island, Delaware Bay	
Ganaga <i>et al.</i> , 2000	Common Coot (Phalaropus carbo)	industrial, agricultural waste	control site 1	egg ³	chick	5.6	1999 Japan
			site 2	8.5	2.7	40	Lake Biwa Shinobu Pond, Tokyo Bay Goshima Island, Tokyo Bay

1. [total egg homogenate]
2. µg/g lipid
3. [yolk sac]
4. [embryo (-liver) + yolk sac]
5. [embryo (-liver + blood) + yolk sac]
6. [carcass (-liver + digestive tract)]
7. [digesting plume]
8. Archer 1264TM

1.2 PCB Toxicity: Field and Laboratory Studies

Deleterious effects such as wasting syndrome, immunotoxicity, impaired reproduction, porphyria and related liver damage, skeletal malformities and endocrine dysfunction in mammals have long been associated with dioxins, PCBs and structurally related HAHs (Hoffman *et al.* 1996). The fish-eating birds of the Great Lakes also exhibit

teratogenic and reproductive dysfunction as a result of high PCB accumulation (Guruge *et al.* 2000). Although there are species-specific differences in sensitivity to PCBs, in all cases the embryonic stage is considered most sensitive (Cantrell *et al.* 1998).

Embryotoxic effects of PCBs and HAHs observed in both field and laboratory studies include decreased hatchability, congenital malformations, edema, reduction in organ and body weight, induction of hepatic mixed function oxidases and embryonic wasting (Murk *et al.* 1996; Elliot *et al.* 1996). Recent laboratory studies examining the effects of PCB126 on various bird species are summarized in Table 1-2.

Table 1-2. Effects of PCB126 in Laboratory Studies

Reference	Species and Age	Treatment	LD ₅₀	Observed Effects
Hedlman <i>et al.</i> , 1996	American herring (<i>Falco sparverius</i>) nestling	Oral dose daily for 10 days @ 50, 250, 1000µg/g	not determined	50µg/g: enlarged liver, mild congestive necrosis, >10fold increase in MFO ¹ induction, mild-moderate lymphoid depletion 250µg/g: multifocal congestive necrosis, decreased brain growth, spleen weight, lymphocyte depletion in spleen and bursa, degenerative thyroid lesions 1000µg/g: previous effects and decreased brain weight, decreased hepatic thiol content, increase in plasma ALT, AST, LDH-L activity ²
Powell <i>et al.</i> , 1996a	Chicken (<i>Gallus domesticus</i>) hatchlings	Injection into yolk on day 0 incubation @ 0.1-12.8 µg/g egg	2.3 µg/g	3.2µg/g: increased relative brain, heart and liver weight 3.2, 6.4, 12.8µg/g: increase in structural defects and edema, decreased hatchling weight
Powell <i>et al.</i> , 1996b	Chicken (<i>Gallus domesticus</i>) hatchlings	Injection into yolk on day 0 incubation @ 0.1-8.1 µg/g egg	0.6 µg/g	increased albuminemia, decreased body weights at 2-3weeks of age, decreased bursa and liver weight, increased heart weight
Elliot <i>et al.</i> , 1997	American herring (<i>Falco sparverius</i>) adult (1 yr)	single oral dose @ 25 µg/g, sacrificed after 5 days	not determined	increased hepatic and renal EROD ³ , detection of CYP1a protein in liver
	Japanese Quail (<i>Coturnix c. japonica</i>) 8 weeks			increased hepatic and renal EROD ³ , APND ⁴ induction, increased hepatic and renal porphyrin
Zhao <i>et al.</i> , 1997	Chicken (<i>Gallus domesticus</i>) embryo	Injection into yolk on day 4 incubation @ 0.5-12.0 µg/g egg, sacrificed after 14 days	3.0 µg/g	dose-dependent increase in lethality, malformations (eye, beak, head, wing, and feet), edema, liver lesions
Hedlman <i>et al.</i> , 1998	Chicken (<i>Gallus gallus</i>) hatchling	Injection into air cell on day 4 incubation @ 0.3-3.2µg/g	0.4 µg/g	malformations (beak, skull, feet, eye, head), severe edema, decreased embryo growth, EROD induced 80fold compared to tern, 1000fold compared to herring, altered hepatic thiol
	American herring (<i>Falco sparverius</i>) hatchling	Injection into air cell on day 4 incubation @ 0.23-233µg/g	65 µg/g	moderate edema, decreased embryo growth, decreased liver weight, malformations, maximum EROD induced 12fold, increased hepatic thiol
	Common tern (<i>Sterna hirundo</i>) hatchling	Injection into air cell on day 4 incubation @ 44-434µg/g	104 µg/g	mild edema, malformations, maximum EROD induced 196fold
Fair and Gorman, 1999	Chicken (<i>Gallus domesticus</i>) embryo	Injection into air cell on day 0 incubation @ 0.051-0.50µg/g, sacrificed after 20 days	1.01 µg/g	decreased thymus mass, decreased lymphoid cell number, decreased bursa mass, dose-dependent malformations (beak, head, eye, feet) and edema (abdominal, subcutaneous, neck)

1. MFO- mixed function oxidase
2. ALT- alanine aminotransferase, AST- aspartate aminotransferase, LDH-L- lactate dehydrogenase -L
3. EROD- ethoxycoumarin-O-deethylase
4. APND- aminopyrine-N-demethylase

A similar order of HAH toxicity is apparent across a diverse range of vertebrates, with TCDD being the most toxic, followed by coplanar compounds such as non- or mono-*ortho* PCBs (Cantrell *et al.* 1996). This suggests there is an evolutionary conserved mechanism mediating the potent developmental and reproductive toxicity of these compounds.

1.3 HAH Mode of Action: The Aryl Hydrocarbon Receptor

The discovery of a specific, high-affinity cytosolic receptor that binds PCBs and other toxic HAHs significantly improved our understanding of the mode of action of these compounds. Poland and Glover (1975) dubbed this receptor the “aryl hydrocarbon” receptor (AHR). The unliganded AHR exists as a complex, comprised of the receptor itself, two heat shock proteins (hsp90's), and other proteins (Poellinger *et al.* 1993). It has been postulated that two different overlapping binding sites, $3 \times 10 \text{ \AA}$ for HAHs (Poland and Knutson 1982) and $7 \times 14 \text{ \AA}$ for PAHs (Landers and Bunce 1991) exist on the AHR. Ligand binding initiates activation of the cytosolic AHR, the dissociation of the hsp's and subsequent translocation into the nucleus, where a heterodimer is formed between the AHR and a nuclear translocator protein (ARNT) (Hahn 1998; Reyes *et al.* 1992). The AHR-ARNT complex is unique in that it is the only member of the base-loop-helix-loop (bHLH/PAS) gene family whose activity is dependent on the binding of a ligand (Chang and Puga 1998). Gene transcription is activated by the AHR-ARNT complex binding to the DNA consensus sequence commonly referred to as the xenobiotic responsive element

(XRE) in the regulatory domains of the dioxin/PCB responsive genes (Jones *et al.* 1986; Neuhold *et al.* 1989).

It should be noted that by themselves, neither the AHR nor ARNT show any detectable affinity for the XRE sequence until the AHR-ARNT complex is formed (Poellinger *et al.* 1993).

Several of the genes which are transcriptionally regulated by the AHR-ARNT complex are Phase I and Phase II drug-metabolizing enzymes, including cytochrome P450s (Fig. 1-2), glucuronosyltransferases, glutathione transferases and aldehyde dehydrogenases (Nebert and Gonzalez 1987).

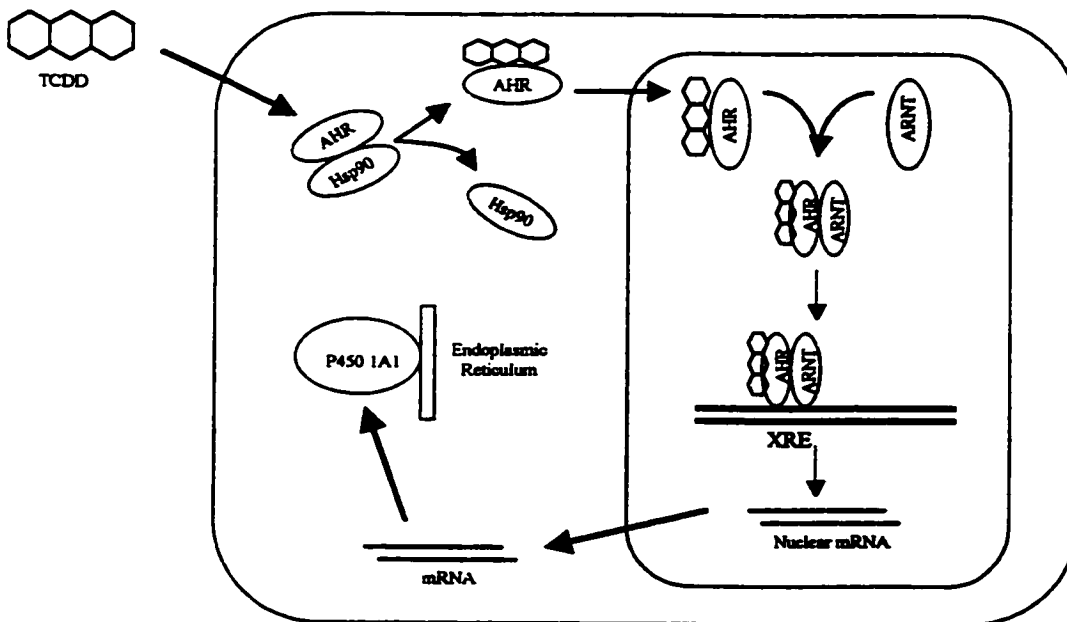


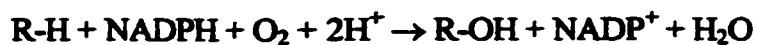
Figure 1-2. Working model for the induction of CYP1A transcription by AHR-ligands

1.4 Cytochrome P450s

The AHR-dependent induction of the *CYP1A1* gene leads to the expression of cytochrome P4501A1 (Whitlock 1989). Cytochrome P450s are mixed function oxidases

(MFOs) capable of converting a hydrophobic substrate into a water-soluble derivative (Whitlock *et al.* 1989); therefore, these enzymes play a key role in the elimination, detoxification, and in some cases, metabolic activation of the substrate. P450s metabolize both endogenous (cholesterol, steroids, liposoluble vitamins, prostaglandins) and exogenous (drugs, pesticides, organic pollutants) substrates (Riviere and Cabanne 1987). They are integral membrane proteins found primarily in the endoplasmic reticulum and mitochondria. The constitutive levels of cytochrome P450s varies considerably between tissues, however their activity can be induced several fold following exposure to TCDD, PCBs and other AHR ligands (Annas *et al.* 1998).

Cytochrome P450s catalyze phase I reactions involved in xenobiotic biotransformation through oxidation of a lipophilic substrate by the activation of molecular oxygen (Whitlock *et al.* 1989). P450s function by introducing one oxygen atom into the substrate while reducing a second oxygen atom to water (Riviere and Cabanne 1987):



Phase II biotransformation involves the conjugation of the oxygenated substrate with a polar moiety, such as glucuronic acid or sulfate, via a transferase reaction (Whitlock *et al.* 1989). This process increases the water-solubility of the exogenous substrate and facilitates its elimination.

To date, purification and classification of cytochrome P450s has led to the identification of 74 gene families including 481 P450 genes and 22 pseudogenes (Walker 1998). However, only a few forms have been properly characterized in birds, with most of this work done in domestic fowl (*Gallus domesticus*), a species very different from

many other avian species (Nelson *et al.* 1996). Rifkind *et al.* (1994) characterized two TCDD-induced cytochrome P450 isoforms in chicken embryo liver, one showing catalytic selectivity for arachidonic acid, and the second showing catalytic selectivity for aryl hydrocarbon hydroxylase (AHH) and 7-ethoxyresorufin deethylase (7-EROD). These two isoforms, TCDD_{AA} and TCDD_{AHH}, are coinduced in the liver in equal amounts and account for all the TCDD-induced P450.

EROD activity is commonly used as an “index of *CYP1A1* activity” because it is principally catalyzed by *CYP1A1* (Sinclair *et al.* 1997). Since induction of cytochrome *P4501A1* is reflective of xenobiotic binding to the AHR, EROD activity is considered a reliable biomarker of AHR agonist exposure (Fox 1993).

1.5 Oxidative Stress: A Mediator of the Effects of PCBs

Numerous studies have implicated oxidative damage as a contributor to the general toxicity syndrome of TCDD, PCBs and other P450 inducers (see reviews by Stohs 1990 and Wells *et al.* 1997). As a consequence of aerobic metabolism, various oxygen species are produced by a number of different enzymatic systems, however oxidative stress occurs when the production of reactive oxygen species (ROS) overwhelms the antioxidant capacity of a cell or multicellular system and subsequently leads to oxidative tissue injury. Xenobiotics such as TCDD and PCBs have been shown to induce oxidative stress in many model systems, including mammals (Albro *et al.* 1978, Al-Bayati *et al.* 1987; Wells *et al.* 1997, Alsharif *et al.* 1994; Wells *et al.* 1997; Stohs *et al.* 1986; Stohs 1990), fish (Cantrell *et al.* 1996, Otto and Moon 1996) and birds (Hoffman *et al.* 1996; Hoffman *et al.* 1998). Xenobiotic bioactivation through

cytochromes P450, prostaglandin H synthases (PHS) or lipoxygenases (LPOs) may lead to the formation of free radical reactive intermediates or quinone metabolites that undergo redox cycling (Wells *et al.* 1997). Superoxide anions ($O_2^{\cdot-}$), formed as a result of xenobiotic metabolism, are detoxified by superoxide dismutase (SOD), which catalyzes the reaction of $O_2^{\cdot-}$ and H_2 to hydrogen peroxide (H_2O_2) (Kelly *et al.* 1998). However, increased cellular concentrations of superoxide and hydrogen peroxide may lead to formation of highly toxic, although short-lived, hydroxyl radicals (OH^{\cdot}). These radicals are capable of irreversibly oxidizing cellular macromolecules such as lipids, proteins and DNA (Kelly *et al.* 1998). Other H_2O_2 detoxifying enzymes include catalase (CAT) which rapidly dismutates H_2O_2 to water and oxygen. Glutathione peroxidase (GPx) is another important scavenger of H_2O_2 , whose activity depends on the NADPH-dependent reduction of glutathione disulfide (GSSG) by glutathione reductase (GR). NADPH levels are maintained by glucose-6-phosphate dehydrogenase (G-6-PDH) (Wells *et al.* 1997) (Fig. 1-3).

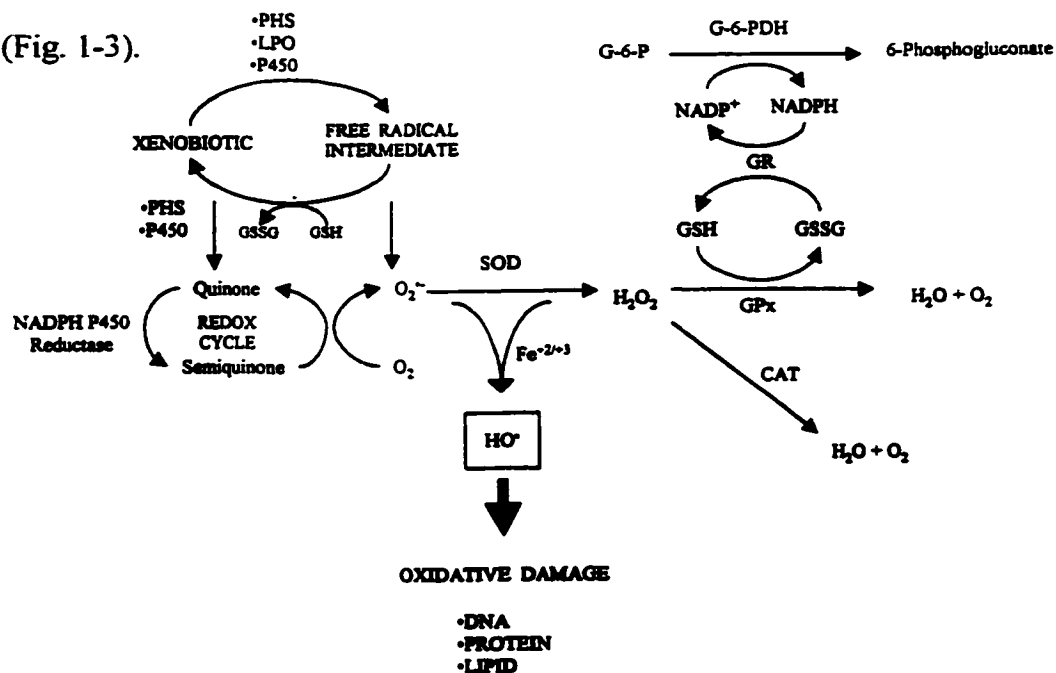


Figure 1-3. Postulated biochemical relationships among pathways of xenobiotic-initiated formation of ROS, associated pathways of cytoprotection and macromolecular damage. PHS-prostaglandin H synthase; LPO-lipoxygenase; P450-cytochrome P450.

Stohs *et al.* (1990) outlined various indicators of TCDD-induced oxidative cell injury which included lipid peroxidation, DNA damage, changes in membrane fluidity, disruption of Ca^{2+} homeostasis, and changes in non-protein sulfhydryl content and NADPH content of hepatic subcellular compartments. Changes in these parameters were measured over a 12-day period following a single injected dose of $100\mu\text{g}$ TCDD/kg body weight in female rats. The most pronounced effects were a 400-500% increase in hepatic mitochondrial, microsomal and nuclear membrane lipid peroxidation and a 600% increase in hepatic nuclear DNA strand breaks 3-5 days post treatment (Stohs *et al.* 1990). Mitochondrial, microsomal and nuclear membrane fluidity gradually decreased 30-40%, which further substantiates TCDD-induced alterations in membrane structure and function. Stohs *et al.* (1986) found that CAT significantly inhibited TBARS formation (an indicator of lipid peroxidation) in microsomes of TCDD-treated rats, suggesting a role for H_2O_2 production in response to TCDD. Microsomal membrane lipid peroxidation was also inhibited in a dose-dependent fashion by SOD, which additionally suggests a role for the superoxide anion in TCDD-induced oxidative injury. Albro *et al.* (1978) observed an accumulation of lipofuscin pigments (considered byproducts of lipid peroxidation) in the hearts of TCDD-injected rats. After treating rats with $0.3\text{-}0.55\mu\text{g}$ PCB126/kg, Hassoun *et al.* (2000) also observed a dose-dependent increase in hepatic lipid peroxidation. In addition to oxidative stress induced alterations in membrane fluidity as a result of membrane lipid peroxidation, PCBs have also been associated with alterations in the physical characteristics of membrane lipids. Borlakoglu *et al.* (1990) showed that a single intraperitoneal injection of 1.5mmol Aroclor™ 1254/kg significantly

increased the degree of unsaturation in pigeon hepatic microsomal membranes after 120 hours. Increased fatty acid desaturase activity, membrane proliferation and P450 activity were also observed.

TCDD and related Ah ligands have also been shown to inhibit the activity of antioxidant enzymes GPx in birds and SOD in rats, respectively (Stohs *et al.* 1986, Hermansky *et al.* 1988a). Hoffman *et al.* (1996) noted a significant relationship between hepatic PCB 126 concentrations and the increasing ratio of oxidized to reduced glutathione (GSSG:GSH) in nesting American kestrels, indicating that TCDD and other P450 activators may induce oxidative stress by inhibiting antioxidant enzyme activities, by inhibiting the removal of ROS, leading to the accumulation of ROS.

Possible sources of ROS in response to TCDD and PCBs include macrophages, microsomes and mitochondria (Stohs 1990). Olivero and Ganey (2000) showed that PCBs stimulate the activation of neutrophils and subsequent superoxide production through the activation of phospholipase A₂. Macrophages produce superoxide anion and hydrogen peroxide through a NADPH oxidase system, while microsomal production of hydrogen peroxide, superoxide and hydroxyl radicals are mediated by the induction of NADPH-dependent cytochrome P450. TCDD also activates xanthine oxidase (XO), an enzyme which converts xanthine to uric acid via an oxygenation reaction. In addition to being a source of reactive oxygen species, XO is activated in the presence of TCDD-induced decreases in glutathione and sulfhydryl content (Hassan *et al.* 1983).

If oxidative stress is a major mediator of the deleterious effects of TCDD and other P450 inducers, then treatment with exogenous antioxidants or free radical scavengers should decrease or eliminate TCDD-induced oxidative damage. Cantrell *et al.*

(1996) observed DNA degradation, lipid peroxidation and loss of functional integrity in cells of the embryonic vasculature of TCDD-treated medaka, while administration of the antioxidant N-acetylcysteine significantly protected the embryo against these effects. Treatment with piperonyl butoxide, a cytochrome P4501A1 inhibitor, also provided complete protection from the embryotoxicity of TCDD, suggesting that induction of P4501A1 contributes to increased cellular oxidative stress.

1.6 The Beta-Adrenergic Receptor (β -AR)

The catecholamines adrenaline and noradrenaline bind to adrenergic receptors (ARs). These receptors were originally classified into the α - and β -subtypes by Alquist (1948). All adrenoceptors are members of the superfamily of seven transmembrane-spanning domain G-protein coupled receptors (GPCR) which includes receptors for peptide hormones such as glucagon, vasopressin and dopamine and other bioactive amines (Fabbri *et al.* 1998). With advances in molecular biology and radioligand binding techniques, Alquist's original α - and β -receptors have been further subdivided into α_1 (A, B, C), α_2 (A, B, C, D) and β_1 , β_2 , β_3 (Fabbri *et al.* 1998).

The metabolic effects of the binding of adrenaline and noradrenaline to the β -AR include the stimulation of hepatic and muscular glycogenolysis, gluconeogenesis in the liver, and lipolysis in the adipose tissue, resulting in a net increase in blood glucose and fatty acid concentrations. The β -AR also mediates the cardio-stimulatory effects of catecholamines through increases in cardiac output, heart rate, and rate and strength of cardiac contractions (Rhoades and Pflanzner 1996).

The physiological and structural properties of the β_2 -AR have been thoroughly investigated. The first β -AR to be sequenced and cloned in birds was from the erythrocyte membrane of turkey. The turkey β -AR is a 54kDa receptor composed of 483 amino acids (Yarden *et al.* 1986). It is predominantly arranged in seven membrane-spanning sequences with a long cytoplasmic carboxy-terminus. The extra-cellular amino-terminal domain contains a consensus sequence for N-glycosylation.

The signal transduction pathway of the β -AR is dependent on the activation of membrane-bound adenylyl cyclase (ACase). ACase is activated by the binding of GTP to a regulatory G_s protein on the inner surface of the cell membrane (Yarden *et al.* 1986). Activated ACase increases intracellular concentrations of cyclic AMP (cAMP) that subsequently activates various intracellular protein kinases A. Because the β -AR, the G -protein and ACase are all embedded in the cell membrane, their activity may be influenced by the fluid state of the membrane. Alberts *et al.* (1989) reported that the fluidity of the membrane allows the AR and G -proteins to move laterally within the membrane. Additionally, the activation of ACase is dependent on the collisions between the molecular components of the signal transduction pathway, and changes in the bilayer fluidity may directly influence the activity of this pathway (Houslay 1986).

There is scant information on the pharmacological characterization of the avian α -AR or β -AR. Reithmann *et al.* (1987) characterized the adult chicken heart muscle ARs as mainly the β_1 -subtype, according to mammalian classification, while the non-muscle cells were mainly β_2 -AR. Port *et al.* (1992) reported chick embryo myocytes to possess a mixed population of β_1/β_2 -subtypes. Dickinson *et al.* (1981) concluded that the

chick erythrocyte had a mixed population of β -ARs, unlike either the mammalian β_1 - or β_2 -AR.

1.7 β -ARs and Xenobiotics

Despite the lack of studies addressing possible receptor-level compensation to polycyclic aromatic hydrocarbon (PAH) exposure, Steevens *et al.* (1996) hypothesized that xenobiotic exposure may result in receptor down- or up-regulation, as a compensatory response to chemical stress. To test this hypothesis, a field study was conducted on brown bullheads (*Ameiurus nebulosus*) from the Black River, Ohio, a site with a known history of PAH contamination, including benzo[a]pyrene and benz[a]anthracene, resulting from a coking plant effluent prior to 1983. A 1982 survey of the brown bullheads showed a 60% incidence of liver tumors and 39% incidence of cancers (Baumann *et al.* 1982). The bullhead survey was repeated in 1987, after the plant closure in 1983, and the incidence of liver tumors and cancers decreased to 32% and 10%, respectively (Baumann and Harshbarger 1995). However, following PAH-contaminated sediment removal by dredging in 1990, the incidence of tumors and the ambient PAH concentrations were increased compared to the values obtained from the 1987 survey (Jones 1996). Bullheads from the Black River and Old Woman Creek (a reference site), aged 3 years and older, were collected in May of 1993. β -AR affinity (K_d) and number (B_{max}) were measured in membranes from gill, heart and brain. The number of receptors was reduced by 2.4-fold in the gill membranes of Black River bullheads, with no change in binding affinity. No significant changes in K_d and B_{max} were detected in the heart or brain ARs of fish from either sampling locations.

A previous study using chick embryos also showed that treatment with PCBs decreased the contractile response of cardiac ventricular muscle to β -AR stimulation (Rifkind *et al.* 1984). Studies performed on isolated atrial and ventricular muscle strips following *in vivo* or *in ovo* treatment with TCDD showed that TCDD decreases β -AR mediated contraction in rat (Hermansky *et al.* 1988b), guinea pig (Brewster *et al.* 1987, Canga *et al.* 1988) and bird (Canga *et al.* 1993) hearts. Muscle contractility was measured as the ability of an isolated strip to generate tension (recorded using a myogram or force transducer) following the addition of isoproterenol, a β -AR agonist, to the tissue bath surrounding the strip. In all of the aforementioned studies, treatment with TCDD compromised the contractile strength of the heart, however, whether this was due to a direct effect on the muscle or due to a disruption of the β -AR pathway was not determined. TCDD may cause a down-regulation of β -ARs or reduce the sensitivity of the receptor for its agonist; however, neither receptor density nor affinity for isoproterenol were measured in this study. Canga *et al.* (1993) reported no significant difference in total cAMP production in control or TCDD-treated chick embryos; however, this result may not take into consideration subcellular changes in cAMP that may not reflect changes in the whole organ. The effects of TCDD on β -AR-mediated contraction are also consistent with the AhR-mediated action of TCDD, as treatment with phenobarbital (a P450 inducer which is not an AhR ligand) failed to affect the contractile ability of the heart (Canga *et al.* 1993).

1.8 Oxidative Stress Effects and β -AR Function

A number of studies using mammalian models have suggested that oxidative stress can alter β -AR function. Haenen *et al.* (1990) exposed ventricular membranes, and atrial and ventricular muscle strips to 0.1 mM cumene hydrogen peroxide to induce oxidative stress. The formation of cAMP, a critical second messenger in β -AR signal transduction, was reduced in both atrial and ventricular membrane preparations. However, oxidative stress did not alter cAMP production in membranes incubated with forskolin, which directly stimulates ACCase circumventing binding to the β -AR. This finding suggests that reduced cAMP formation during oxidative stress is most likely caused by an uncoupling between the receptor and ACCase or the associated G-protein. Haenan *et al.* (1990) also observed an increase in the number of β -ARs on ventricular membranes after treatment with cumene hydroperoxide, and the maximal inotropic response of the muscle strips to isoproterenol (a β -agonist) was reduced by 50%. Persad *et al.* (1998) showed that hydrogen peroxide reduced ACCase activity in rat heart membrane preparations, although direct effects on β -AR density and agonist affinity varied with the receptor subtype (β_1 vs β_2).

Reduction in membrane fluidity can depress β -AR signal transduction (Peters 1988). Oxidative stress-induced lipid peroxidation is known to decrease membrane fluidity, therefore the reduced cAMP formation after oxidative stress may possibly be attributed to the effects of lipid peroxidation on the physical state of the membrane. Persad *et al.* (1998) have suggested that increased lipid peroxidation decreases the fluidity of myocardial plasma membranes; this can lead to the uncoupling of the β -AR-G protein-ACCase complex, hence hindering cAMP production and overall β -adrenergic

stimulation of the heart. Since TCDD similarly induces lipid peroxidation in the heart, the decreased β -adrenergic responsiveness of hearts following treatment with TCDD may be mediated by lipid peroxidation and subsequent impairment of β -AR signal transduction.

1.9 Objective and Hypothesis of Study

Despite the ever-increasing body of knowledge on the toxic effects of PCBs, the molecular mechanisms by which these compounds exert their effects after AHR-induced gene transcription remain unclear. Two conclusions drawn from the aforementioned studies include: (1) PCBs induce oxidative stress through various mechanisms including increased production of ROS, lipid peroxidation and altered antioxidant status; and, (2) oxidative stress (whether xenobiotically induced or not) can modify β -AR function and signal transduction by altering receptor density, reducing ACase activity and/or cAMP production. To date there have been no studies examining the effects of AHR-ligands, such as PCBs, on β -ARs in an avian model, therefore the objectives of this study are to (1) pharmacologically characterize the β -ARs in the chick embryo liver; (2) determine if a parameter of oxidative stress, lipid peroxidation, is induced by the *in ovo* treatment of PCB126; and, (3) determine the effects of PCB126 injection *in ovo* on the liver β -AR density (B_{max}) and affinity (K_d).

The hypothesis is that if PCB toxicity in the liver is mediated in some part by oxidative damage, such as increased lipid peroxidation, then the function of a membrane-bound receptor, such as the β -AR, may be modified through oxidative damage to membrane lipids. If decreased adrenergic responsiveness is due to some effect at the

receptor level, then *in ovo* injection of PCB126 may result in (i) decreased receptor affinity for an endogenous or synthetic agonist, or (ii) decreased receptor density.

In addition, general toxicity of PCB126 will be evaluated in day 19 chicken embryos in terms of observable abnormalities and mortalities, EROD induction, and the resulting hepatic concentration of PCB126 after injection into the air cell of the egg.

CHAPTER 2:
MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Egg Injection Protocol

White Leghorn (*Gallus domesticus*) chicken eggs were obtained from Agriculture Canada (Ottawa, ON, Canada). Prior to incubation, eggs were weighed and candled to determine the location of the air cell. The injection surface was sterilized with 70% ethanol and injections of PCB 126 or vehicle were performed in a sterile transfer hood. Two small holes, approximately 1mm diameter, were drilled on the egg surface using a Multi-Tool drill (RYOBI America Corporation, Anderson, SC, USA). Using sterilized pipette tips, PCB126 or a corn oil vehicle were injected into the air cell of each egg. A PCB126 stock solution (8 μ g/mL) was prepared by dissolving 5mg of >99% PCB126 (Ultra Scientific, Kinstown, RI, USA) in 2mL dichloromethane; this stock was further diluted (2:3 v/v) into sterile-filtered corn oil (100% Pure Mazola, Best Foods Canada Inc., Etobicoke, ON, Canada). The stock solution was placed in a sterile transfer hood for at least 6 hours following preparation to allow the dichloromethane to evaporate prior to injecting the eggs. A standard injection volume of 10 μ L/50g egg was adjusted for individual eggs to give a final concentration 1.6 μ g PCB 126/kg. This dose was chosen based on the results of a dose response study using 0.4-2 μ g PCB 126/kg, which yielded a LD50 of approximately 1.5 μ g/kg (Jin *et al.*, 2001). After injection, both holes were sealed with melted paraffin wax and a small square of scotch tape was placed over the wax and injection site. Eggs were placed on their side in plastic trays and incubated for 19 days at 37°C and 65-75% humidity in an egg incubator (Ocatagon 250, Brinsea

Products Ltd., England, UK) with automatic rotation. Eggs were candled during incubation and non-fertile eggs and dead embryos were removed. On day 19, embryos were rapidly removed from the eggs, sacrificed by decapitation and weighed after removing the yolk sac. Livers used for TBARS, EROD and tissue analysis were rapidly removed, rinsed with 0.9% saline, weighed and quickly frozen in liquid nitrogen. Samples were stored at -80°C until analyses were undertaken.

2.2 Liver Membrane Preparation

Crude membrane fractions were isolated based on methods previously described by Daveloose *et al.* (1993) following dissection of approximately 30–40 livers from day 19 embryos. The livers were quickly removed and placed in a petri dish on ice containing Hepes-Earle buffer (115mM NaCl, 1.8mM CaCl_2 , 0.8mM MgCl_2 , 5mM glucose, 2.5mM Hepes, pH 7.4). The livers were rinsed several times with buffer at 4°C , then minced and rinsed again in Hepes buffer (150mM NaCl, 0.8mM MgCl_2 , 10mM Hepes, 10^{-4} Phenyl methylsulfonyl fluoride (PMSF), pH 7.4), and homogenized (1:9 volumes of buffer) using a Potter-Elvehjem Teflon-glass homogenizer attached to a hand-held Black & Decker drill running at medium speed for 10-20 sec. The homogenate was centrifuged at $1900 \times g$ for 10 min in a Sorvall RC 5B Plus (SS 34 rotor) centrifuge (Dupont Co., Newton, CT, USA) at 4°C . The supernatant was further centrifuged at $40000 \times g$ for 20 min at 4°C . The resulting pellet was resuspended in Tris buffer (50mM TrisHCl, 10mM MgCl_2 , adjusted to pH 7.4) and centrifuged at $40000 \times g$ for 15 min at 4°C for a total of three washes. The final pellet was resuspended in Tris buffer (50mM TrisHCl, 10mM

MgCl₂, adjusted to pH 7.4), aliquotted into 1.5mL plastic microcentrifuge tubes, frozen in liquid nitrogen and stored at -80°C until used.

2.3 Hepatocyte Isolation

Hepatocytes were isolated from livers of day 19 embryos using methods previously described for White Leghorn chicken embryos (Fischer and Marks 1976; Kennedy *et al.* 1993). Approximately 35-45 embryos were decapitated, the livers were removed and rinsed in Krebs-Ringer buffer (KRB) (48mM NaCl, 120mM KCl, 1.0mM CaCl₂•H₂O, 1.2mM MgSO₄•7H₂O, 1.2mM KH₂PO₄, 25mM NaHCO₃, pH ~7.2-7.4). Livers were pooled, minced with a razor blade and digested in 75mL KRB containing 400U/mL Type IV hepatocyte collagenase (Sigma, St.Louis, MO, USA) for 45-50 min with gentle shaking in a 37°C water bath. Following tissue disassociation, the collagenase/liver solution was filtered through four CellMicroSieves™ (200, 100, 50, 25 μm, BioDesign Inc., Carmel, NY) and 100mL of 2% bovine serum albumin (essential fatty-acid free BSA, 2g dissolved in 100mL KRB) (ICN Biomedicals Inc., OH, USA) was added to the filtrate. The filtrate was centrifuged for 5 min at 300 x g using a Beckman GS-6 centrifuge (Beckman Instruments Inc., Fullerton, CA, USA) at room temperature (21°C), in four 50mL polystyrene centrifuge tubes. The resulting pellets were combined and resuspended in 25mL of Waymouth Medium (Gibco, Burlington, ON, Canada) and 24mL of Percoll (Pharmacia Biotech, Sigma, St.Louis, MO, USA) and sucrose to remove the blood cells. The percoll-sucrose solution was prepared by combining 90mL of Percoll with 10mL of 2.5M sucrose; 24mL aliquots of this medium were placed in four sterile polystyrene tubes and refrigerated until used. The hepatocyte

suspension was centrifuged at 50 x g for 10 min as above. The upper cell layer was carefully removed from the medium with a glass Pasteur pipette and transferred to a pre-weighed 15mL polystyrene centrifuge tube and resuspended in 10mL of DNase (1mg of 4000U/mg DNase I, Sigma, in 100mL KRB). The tube was gently rocked by hand for 5-8 min until the clumps disappeared, and again centrifuged at 50 x g for 5 min. The resulting supernatant was discarded and the pellet was rinsed with Waymouth medium and centrifuged at 50 x g for 5 min two additional times. After the final centrifugation, the medium was discarded and the pellet was weighed and resuspended in Waymouth medium at a concentration of 100mg cells per mL. The cells were placed in an incubator (Forma Scientific, Caltec Scientific, Mississauga, ON, Canada) at 37°C, 3.9% CO₂ for approximately 1h before being used.

Cell viability was measured in a live/dead-staining assay using a Zeiss Axiophot fluorescent microscope (Carl Zeiss Inc., West Germany). Fluorescein diacetate (5mg/mL) (Molecular Probes Inc., Eugene, OR, USA) dissolved in acetone is stored frozen (-30 °C) until use. An ethidium bromide (Molecular Probes Inc.) stock solution (1mg/mL) is prepared in 1x Hanks' buffer, and diluted further to a 100µg/mL working solution by diluting 1mL ethidium bromide in 9mL of 1x Hanks' buffer. The stain was made for daily use by combining 7.5µL fluorescein diacetate, 50µL ethidium bromide and 1.2mL 1x Hanks' buffer in a foil-wrapped 15mL disposable centrifuge tube, capped and vortexed for 5-10 sec. A 1:1 volume of stain:cells dilution is used to count live and dead cells; dead cells are red and live cells are green under a fluorescent microscope.

2.4 β -Adrenoceptor Binding Assays

Binding assays were performed according to methods previously published by our laboratory (Dugan and Moon 1998). Hepatocytes prepared from separate pools of livers from control, vehicle (corn oil) and dosed (1.6 μ g PCB126/kg) eggs were assayed following a 1h rest period following isolation (see above). The tritium-labeled hydrophilic mixed β -AR antagonist (-)-³H-CGP 12177 ((-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-³H]benzimidazol-2-one) (Amersham Canada Ltd., Oakville, ON; specific activity 50Ci/mmol) was used in saturation experiments to characterize β -AR binding sites. Fifty μ L of hepatocytes (suspended at 100mg cell per mL in Weymouth medium) were incubated in 5mL Falcon polystyrene round-bottom clear tubes with 90 μ L of Tris buffer (50mM Tris-HCl, 10mM MgCl₂, pH 7.4) and 10 μ L of (-)-³H-CGP 12177 (at concentrations of 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05 nM) to determine the total β -AR binding on the isolated hepatocytes. Non-specific binding was determined in the presence of 50 μ L of cells, 50 μ L of 10 μ M (-)-alprenolol (Sigma, St.Louis, MO, USA), 40 μ L of Tris buffer, and 10 μ L of various (-)-³H-CGP 12177 concentrations. At each ³H-CGP concentration, total (T; only ³H-CGP) and non-specific (NS; ³H-CGP + alprenolol) samples were run in duplicate. The tubes were incubated at room temperature (21°C) for 2h. This incubation time was determined to be optimal by a preliminary time course assay. Prior to termination of the reaction, the cell harvester (Brandel 24R, Gaithersburg, MD, USA) equipped with a vacuum pump (VacuTrol, Spectrum Medical Industries Inc., Los Angeles, CA, USA) was rinsed with ice cold 0.9% w/v NaCl to remove residual radioactive contamination. Borosilicate glass filters (No. 32, Mandel Scientific, Guelph, ON, Canada) were pre-rinsed with ice cold 0.9% NaCl. The binding assay is terminated

by aspirating the incubation medium + cells from each tube through the cell harvester, followed by repeated washing (3X) with ice cold 0.9% NaCl. The borosilicate filters are placed in polyethylene scintillation vials with 4mL of Safety-Solve Liquid Scintillation cocktail (Research Products International Corp., Mount Prospect, IL, USA) and left in the dark for approximately 24h. The radioactivity in each vial was counted using a Packard 2500 TR liquid scintillation counter. The specific disintegrations per minute (dpm) for each ^3H -CGP concentration was calculated by subtracting the NS dpm from the T dpm. Binding assay data were further analyzed using EBDA software (Biosoft-Elsevier) (Munson and Rodbard 1980).

2.5 Competition Assays

Displacement assays were performed to pharmacologically characterize the β -AR subtype. Frozen crude liver membrane preparations (prepared as stated above) were thawed and resuspended in Tris buffer (50mM Tris-HCl, 10mM MgCl_2 , pH 7.4). The protein concentration of these aliquots was determined using the method of Lowry *et al.* (1951) using BSA dissolved in Tris buffer as a standard. As previously outlined, 50 μL of membranes (containing ~250 μg protein) are incubated in the presence of 10 μL of 1nM ^3H -CGP and 90 μL of Tris buffer (see above). Total and non-specific binding were determined in the absence and presence, respectively of 50 μL of five concentrations (10, 1, 0.1, 0.01, 0.001 μM) of the β -AR agonists (-)-norepinephrine, (-)-epinephrine, procaterol, or (\pm)-dobutamine and antagonists (\pm)-ICI 118551, bromoacetyl alprenolol menthane (BAAM), atenolol, or (\pm)-CGP 12177A (all from Sigma, St. Louis, MO, USA). Adrenaline, noradrenaline, procaterol and 12177 CGP stock solutions (3mM) were

prepared in Tris buffer (50mM Tris-HCl, 10mM MgCl₂, pH 7.4). ICI 118551 and dobutamine stock solutions (3mM) were prepared in ddH₂O. Atenolol and BAAM stock solutions (3mM) were prepared in 10% EtOH and dimethylsulfoxide (DMSO), respectively. Following vigorous vortexing (Maxi Mix I, Type 16700 Mixer, Thermolyne, IO, USA), the stock solutions were diluted 100-fold in Tris buffer (see above) to obtain the 10µM working solution, and subsequent 10-fold dilutions were performed to obtain the 1, 0.1, 0.01 and 0.001µM working solutions. The agonist/antagonists were prepared with the lights off and stored in foil-wrapped microcentrifuge tubes to protect them from photodegradation. Incubations were carried out for 2h at room temperature (21°C) and terminated by filtration as previously stated above. Final results are expressed as % maximum binding of each displacing agonist/antagonist compared with the total binding of 5nM ³H-CGP.

2.6 Lipid Peroxidation

Lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS) (Uchiyama and Mihara 1978) in frozen livers from day 19 control, vehicle and dosed (1.6µg PCB126/kg) embryos. Frozen tissues were ground into powder in liquid nitrogen using a mortar and pestle. A 10% homogenate containing (1) 10 volumes of 50mM Tris-1.15% KCl (pH 7.4) and (2) 10mM butylated hydroxytoluene (BHT, dissolved in toluene, final concentration of 500µM) per liver sample was prepared. The homogenate was sonicated (Krontes Microultrasonic Cell Disruptor, Mandel Scientific, Guelph, ON, Canada) in a 1.5mL plastic microcentrifuge tube for 20-30 sec on ice. A sample volume of 250µL tissue homogenate or Tris-KCl (blank) was added to a screw

top 15mL polypropylene centrifuge tube containing 1.5mL of 1% (v/v) phosphoric acid, 0.5mL of 0.6% thiobarbituric acid (TBA), and 500 μ M BHT (volume adjusted for each liver sample). After vortexing, the pH of this mixture was checked using color-fixed pH indicator strips (Sigma, St. Louis, MO), adjusted to 1.5-2 using 10N NaOH and the tubes were boiled in a water bath for 45min with caps loosely screwed on. After cooling on ice, 2mL of butanol-1 were added and the tubes were vigorously shaken by hand to extract the reaction product. The tube was then centrifuged (Beckman GS-6) for 20-30 min at 3000 rpm at room temperature (21°C). One mL of the upper layer was transferred to a cuvette and scanned at 400-600 nm (Beckman DU640 Spectrophotometer, USA). A TBARS standard curve was generated by the same procedure using malonaldehyde (1,1,3,3-tetraethoxypropane; Sigma). Sample TBARS concentrations were calculated from the differences in peak heights at 533 and 510nm.

2.7 EROD Activity in Liver Microsomes

Ethoxyresorufin-*O*-deethylase (EROD) activity was measured in microsomes prepared from frozen livers of day 19 control, vehicle and dosed (1.6 μ g PCB126/kg) embryos. Individual livers were weighed, thawed on ice for 10 min, minced with a cold razor blade or scissors, and transferred to a 2mL Potter-Elvehjem homogenizing tube on ice. Potassium phosphate buffer (0.1M, pH 7.4) was added to the tube, at a volume of 800 μ L per 0.5g liver. Samples were homogenized with 12 up and down strokes using a Wheaton Overhead Stirrer (Wheaton Instruments, Millville, NJ, USA). The homogenates were then centrifuged (Sorvall RC 5B) at 9000 x g for 15 min at 4°C. The supernatants were transferred to ultracentrifuge tubes and further centrifuged at 100 000 x g for 1 h at

4°C using a Beckman L8-55M ultracentrifuge (Beckman Instruments Ltd., Fullerton, CA). The supernatants were discarded and any fat adhering to the side of the tubes was removed with a Q-tip. The final pellets were resuspended in 1 mL phosphate buffer (0.1M, pH 7.4) and 250µL aliquots were transferred to cryogenic vials which were frozen in liquid nitrogen and stored at -80°C until used.

EROD activity was assayed according to methods by Kennedy *et al.* (1993; 1995) using 48 well Falcon plates. Microsomes were thawed on ice. A resorufin (Sigma, St.Louis, MO) stock solution (130µM in MeOH) and a BSA (Pierce, Rockford, IL) stock solution (2mg/mL in 0.05M sodium phosphate buffer) were prepared prior to running the assay. Varying amounts (160-95µL) of sodium phosphate buffer (0.05M, pH 8.0), resorufin (7µM working solution in phosphate buffer) and BSA (1.25-25µL) were added to wells prior to the addition of the samples. In sample wells, 10 µL of thawed microsomes and 50µL of 35µM 7-ethoxyresorufin (7-ER) (Sigma) were added. The plate was placed on a dry block heater (VWR Heat Block, VWR Scientific, Mississauga, ON) at 37°C for 10 min, followed by the addition of 25µL of freshly prepared NADPH (2.4mM in phosphate buffer) to each well and another 10 min incubation at 37°C. Finally, 100µL of 2.16mM fluorescamine (Sigma, St.Louis, MO) in acetonitrile was added to each well and the plate was incubated for a further 15 min at room temperature. The plate was scanned for resorufin at 530 nm (excitation) and 590 nm (emission), and for protein at 400 nm (excitation) and 460 nm (emission) using a fluorescence plate reader (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA). EROD results were reported as pmol resorufin/min/mg protein. Resorufin production by EROD was

quantified against a resorufin standard curve, and total protein was quantified against a BSA standard curve.

2.8 PCB Tissue Analysis

Hepatic PCB126 concentration was measured in individual livers from day 19 embryos treated with 1.6µg PCB126/kg. Individual liver samples were prepared for extraction by grinding each liver in 5g of anhydrous sodium sulphate (BDH, Toronto, ON) using a pre-chilled mortar and pestle on dry ice. The powder was then transferred to a 1.1 x 30cm glass column, containing a 1cm glass wool plug. The column was eluted with 50mL of 1:1 dichloromethane/hexane (EM Science, Merck KGA, Gibbstown, NJ) at a flow rate of approximately 1 mL/min. The extract was concentrated to 2.5 mL by rotary evaporation with a Büchi Rotavapor (Switzerland) and vacuum pump (Gast, Model DAA-V175-EB, MFG Corp., Beuton Harbor, MI) while gradually increasing the pressure from 400 to 650 psi. After transfer to a graduated centrifuge tube, 1/10th of the extract volume was removed to a pre-weighed aluminum dish and dried in an oven at 60°C overnight. The dish was weighed again the next day to determine the lipid volume of the extract. The remaining extract was evaporated in the centrifuge tube to 1.9mL under N₂ gas. The extract plus 40µL of PCB 204/30 internal standard (55/51 ng/mL) (Canadian Center for Inland Waters, Environment Canada, Burlington, ON) was added to a 1.1 x 30cm glass column containing a 1cm glass wool plug, 8g of activated Florisil (Supelco, Bellafonte, PA) and 1cm layer of anhydrous sodium sulphate. The column was pre-rinsed with hexane and then eluted with a further 40–45mL of hexane. The extract was

concentrated to 1 mL by rotary evaporation and 1 mL of isooctane (EM Science, Merck KGA, Gibbstown, NJ) was added. The final extract was concentrate to 200 μ L under N₂ gas. The extract was transferred by pasteur pipette to a glass vial, 5 μ L of Mirex (200pg/ μ L) (Canadian Center for Inland Waters, Environment Canada, Burlington, ON) was added and the vial was stored at -30°C until analysis. Chemical recoveries were routinely determined to be >85%.

Gas chromatographic analysis was performed on a Hewlett-Packard 6890GC/uECD equipped with a HP7683 Injector and a HP 5973 Mass Selective Detector. The analytical column was a HP-5 19091J-433 (cross-linked with 5% phenyl methyl siloxane), with dimensions of 30m and 0.25 μ m ID. A 1 μ L splitless sample injection was used. The GC conditions were as follows: injector carrier gas was helium (250°C) at a flow rate of 3.6mL/min, and the detector carrier gas was ultrahigh purity nitrogen (350°C) at a flow rate of 58.7mL/min. The column program was as follows: 1.3mL/min constant pressure, 80°C for 2min, 10°C ramp to 110°C, 3°C/min to 280°C and a 5min hold for a total run time of 68min.

2.9 Statistics

Graphs were constructed using Sigma Plot 2000 software (Jandel Scientific). Data are expressed as means \pm SEM of (n) independent experiments. Statistical analyses were undertaken using Sigma Stat 2.01 software (Jandel Scientific).

One way ANOVA and/or ANOVA on ranks were used to test for significant differences in EROD activities and extent of lipid peroxidation between control, vehicle- and PCB126-treated embryos. When a significant difference was found by one way

ANOVA , Tukey's test and/or Dunn's pairwise multiple comparison were used to isolate which groups differed.

K_i , K_d and B_{max} values for β -AR competition and saturation assays were calculated using EBDA software. One way ANOVA followed by Dunn's pairwise multiple comparison were used to test for significant differences between K_d and B_{max} values for control, vehicle- and PCB126-treated embryos.

GC calibration curves for PCB126, PCB204, PCB30 and Mirex were analyzed by linear regression using HP Chemstation Plus software (Hewlett Packard). Chromatograph peak areas were integrated and analyzed using HP Chemstation Plus software (Hewlett Packard).

CHAPTER 3:

RESULTS

3. RESULTS

3.1. Effects of PCB 126 Injection

3.1.1 Embryotoxicity

The effects of PCB126 on chick embryo mortality rate, incidence of abnormalities, and liver and embryo weight are shown (Table 3-1). Eggs were injected in the air sac with a corn-oil vehicle or PCB 126 (1.6 µg/kg egg) prior to incubation and embryos were dissected and weighed on day 19 of incubation, approximately 2 days prior to hatching. PCB126-treatment increased the mortality rate and the incidence of liver abnormalities and edema. Embryo and liver weights did not change significantly between control, vehicle- or PCB126-treatments.

3.1.2 EROD Activity

PCB126 (1.6 µg/kg egg) induced EROD activity approximately 12-fold in liver microsomes prepared from day 19 chick embryos (Fig. 3-1). Mean EROD activities were 342.9 ± 59.3 pmol resorufin/min/mg protein in the PCB126-treated microsomes, compared with 30.2 ± 3.6 and 26.7 ± 5.0 pmol resorufin/min/mg protein in the vehicle-treated and control microsomes, respectively. There was no significant difference in basal EROD activity in the control or vehicle-treated embryos.

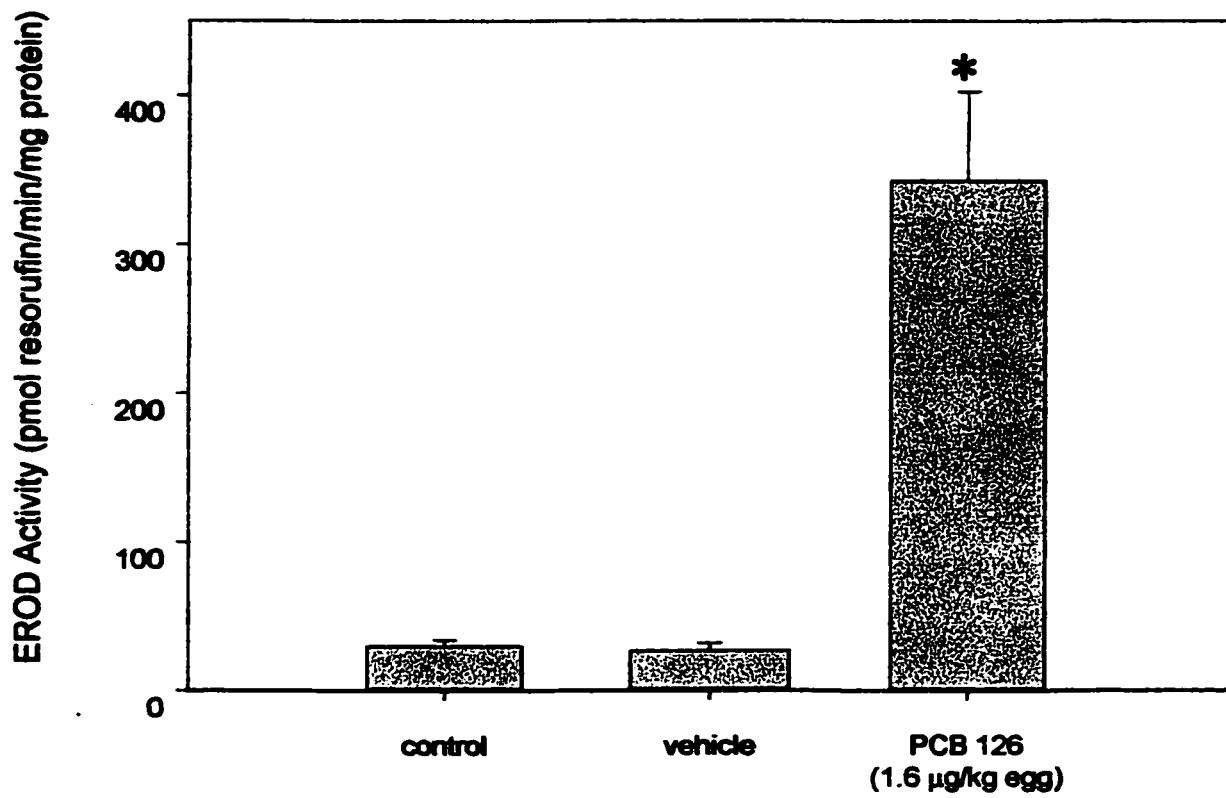
3.1.3 PCB126 Tissue Analysis

PCB126 content in the livers of day 19 chick embryos was measured by GC-ECD, after injecting 1.6 µg/kg egg into the air cell of the eggs prior to incubation. The

Table 3-1. Effects of PCB 126 on chick embryo mortality rate, incidence of abnormalities, liver and embryo weight. Eggs were injected in the air sac with a corn-oil vehicle or PCB 126 (1.6 µg/kg egg) prior to incubation. Embryos were dissected and weighed on day 19 of incubation, approximately 2 days prior to hatching. All values are means ± SEM.

	Control	Vehicle Injected	1.6µg/kg PCB 126
% Dead by embryonic day19	5.26	2.70	22.33
% Abnormalities (Edema, liver discoloration, liver lesions)	5.26	5.41	10.45
Embryo Weight (g)	23.67 ± 0.37	23.39 ± 0.39	23.50 ± 0.38
Liver Weight (g)	0.43 ± 0.01	0.42 ± 0.01	0.47 ± 0.01
Sample size	38	37	67

Figure 3-1. EROD activity (pmol resorufin/min/mg protein) in liver microsomes from control, vehicle and PCB126-treated day 19 chick embryos. Eggs were injected in the air sac with a corn-oil vehicle or PCB 126 (1.6 µg/kg egg) prior to incubation. EROD activity and protein concentrations in microsomal fractions were obtained using a fluorescence plate reader. Bars are means ± SEM (n= 5 or 6) for each treatment group. Statistical analyses were performed using Kruskal Wallis one-way ANOVA on ranks, followed by Dunn's pairwise multiple comparison. The “ * ” indicates a significant difference at P<0.05 compared to the control and vehicle-treatment.



average PCB126 content in the six livers analyzed was 0.67 ± 0.17 ng/g liver; the average lipid-based content for the same samples was 10.2 ± 1.9 ng/g lipid (Fig. 3-2). Two livers from vehicle (corn-oil)-injected and untreated control eggs were also analyzed and PCB126 levels in those livers were not detected.

A sample chromatograph with peaks for PCB126, and the internal standards (PCBs 204 and 30) and Mirex is shown (Fig. 3-3). The PCB126 sample was extracted from an individual liver from a day 19 chick embryo injected with $1.6 \mu\text{g/kg}$ egg prior to incubation. Retention time for PCB126 is approximately 45 min.

PCB126 sample concentrations were quantified using chromatograph peak areas compared with the PCB126 standard peak areas. A PCB126 standard curve was generated using concentrations of 0.8, 1.0, 1.5, 2.5 and 3.0 ng/mL PCB126 in iso-octane (Fig. A-1).

Mirex is added to all samples and standards prior to GC analysis to measure variations between injections and evaporative losses prior to injection. A concentration of 5ng/mL was added to all samples and the resulting Mirex concentrations varied minimally in all samples analyzed (Fig. A-2). Retention time for Mirex was approximately 50min.

Internal standards PCBs 204 and 30 (55 and 51 ng/mL) were added to the samples during the extraction process, prior to elution from the florasil column. The resulting sample concentrations of PCB 204/30 are quantified against a standard curve to give a measure of chemical recovery during the extraction process. The PCB 204 standard curve (Fig. A-3) was generated using concentrations of 5.1, 30.6, 56.1, 86.1 and 107.1 ng/mL, while the PCB30 standard curve (Fig. A-4) concentrations were 5.5, 33.0, 60.5, 88.0 and

Figure 3-2. PCB 126 content (ng/g) in the liver of day 19 chick embryos, injected with 1.6 µg/kg egg prior to incubation. Bars are means ± SEM (n=6) expressed as PCB126 content per gram liver and per gram lipid.

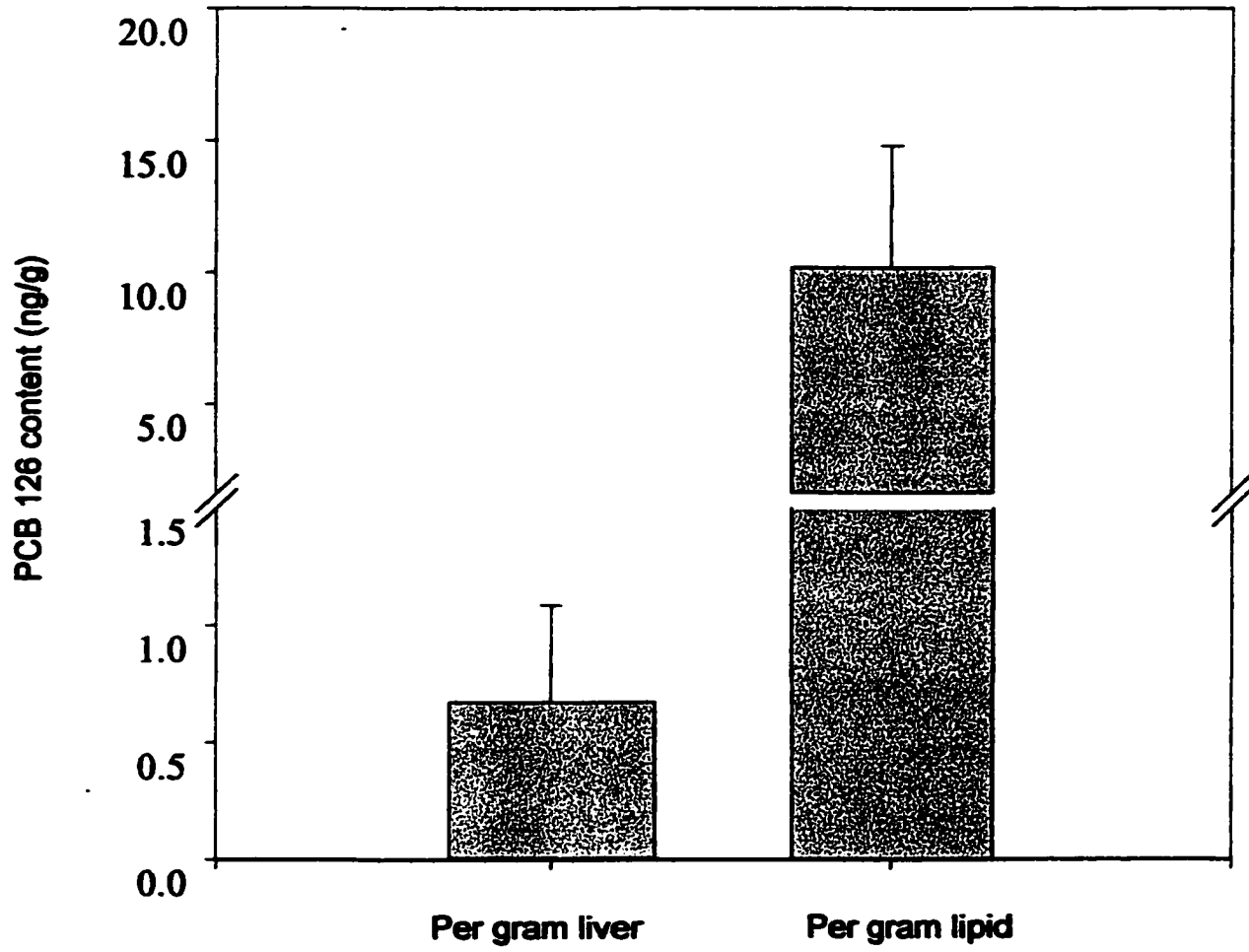
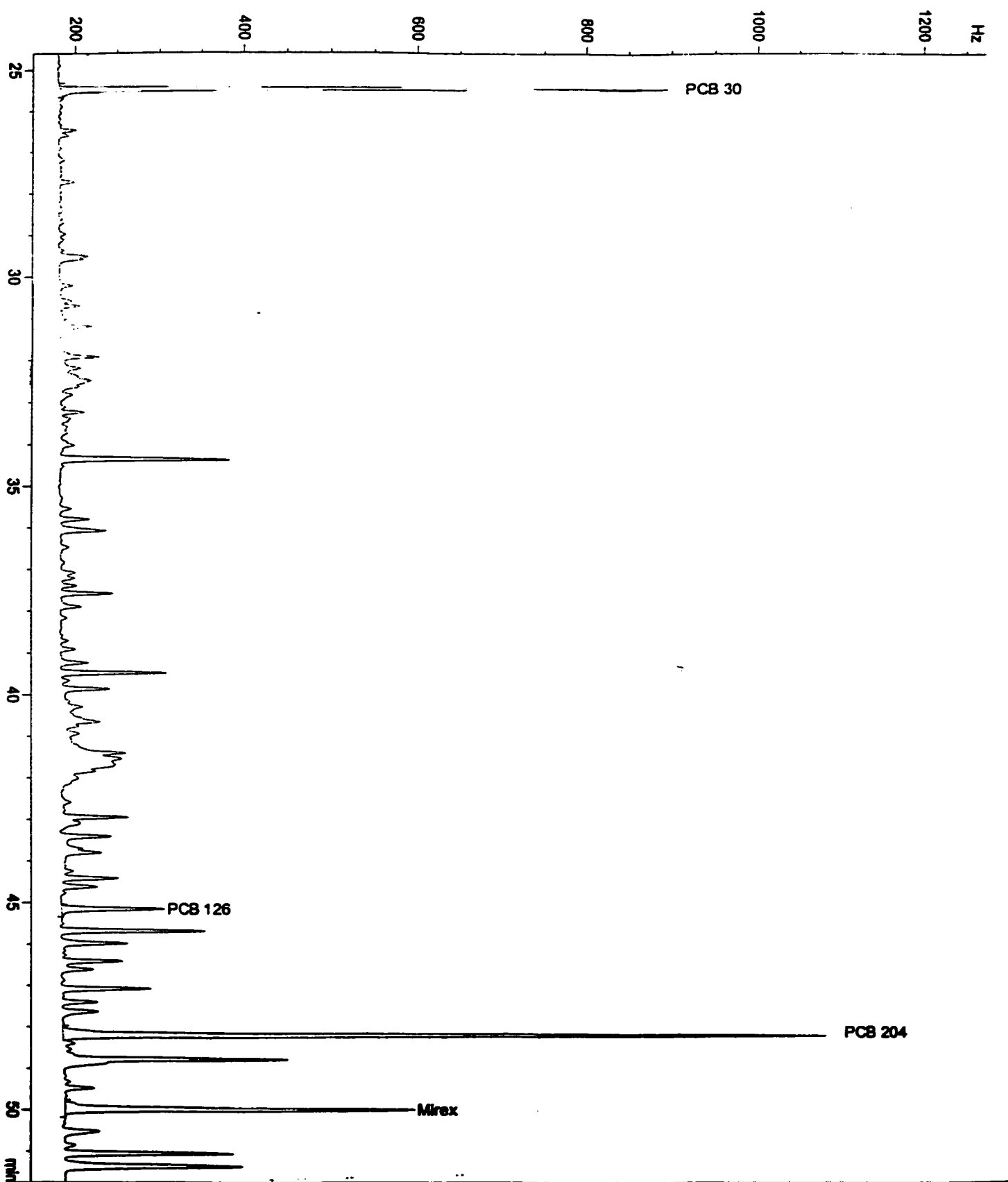


Figure 3-3: Sample chromatograph showing a sample peak (PCB126) and internal standard peaks (PCB30, PCB204 and Mirex). Injection volume is 1 μ L. The sample is an individual liver from a day 19 chick embryo injected with 1.6 μ g/kg egg PCB126 prior to incubation.



115.5 ng/mL. All recoveries were >85%. Retention times for PCBs 204 and 30 were 48 and 25 min respectively.

3.2 Characterization of Avian Hepatic β -Adrenoceptor

3.2.1 Competition Assays

Competition assays were performed on crude membranes prepared from pooled day 19 chick embryo livers using mammalian β -AR agonists (Fig. 3-4). Membranes (250 μ g protein/50 μ L) were incubated for 120 min with 1nM 3 H-CGP 12177 in the absence (total binding) and in the presence of increasing concentrations of various β -agonists. Neither noradrenaline (β_1 -AR agonist) nor dobutamine (β_1 -AR agonist) achieved 50% displacement of 3 H-CGP 12177 at the highest concentration of 10^{-5} M. The β_2 -agonists were much more effective at displacing 3 H-CGP 12177. Adrenaline, at the highest concentration used, displaced at least 50% of the 3 H-CGP 12177, while procaterol displaced 80% of the 3 H-CGP 12177. These results suggest that the day 19 chick embryo liver β -ARs preferentially bind mammalian β_2 -agonists.

Competition assays were also performed on crude membranes prepared from pooled day 19 chick embryo livers using mammalian β -AR antagonists (Fig. 3-5). Membranes (250 μ g protein/50 μ L) were incubated for 120 min with 1nM 3 H-CGP 12177 in the absence (total binding) and in the presence of increasing concentrations of various β -antagonists. Atenolol (selective β_1 -antagonist) was ineffective in displacing 3 H-CGP 12177, whereas bromoacetyl alprenolol menthane (BAAM) (β_2 -antagonist) displaced 50% of the 3 H-CGP 12177 at the highest concentration used. ICI 118551 (selective β_2 -

Figure 3-4. Agonist competition for β -AR binding sites on day 19 chick embryo liver membranes. Membranes (250 μ g protein/50 μ L) were incubated for 120min with 1nM 3 H-CGP 12177 in the absence (total binding) and in the presence of increasing concentrations of various β -agonists (noradrenaline, adrenaline, procaterol, dobutamine). All values are means \pm SEM of four independent experiments using pools of ~30 eggs per membrane preparation.

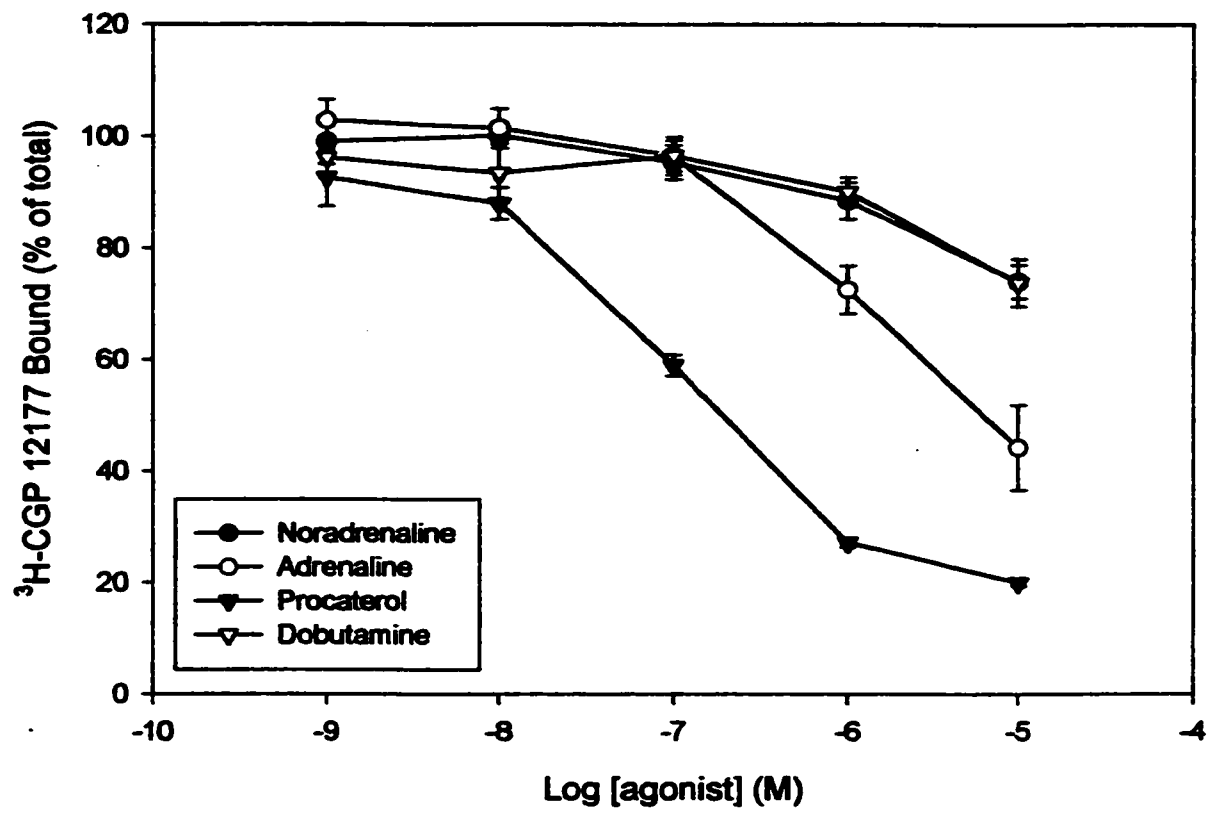
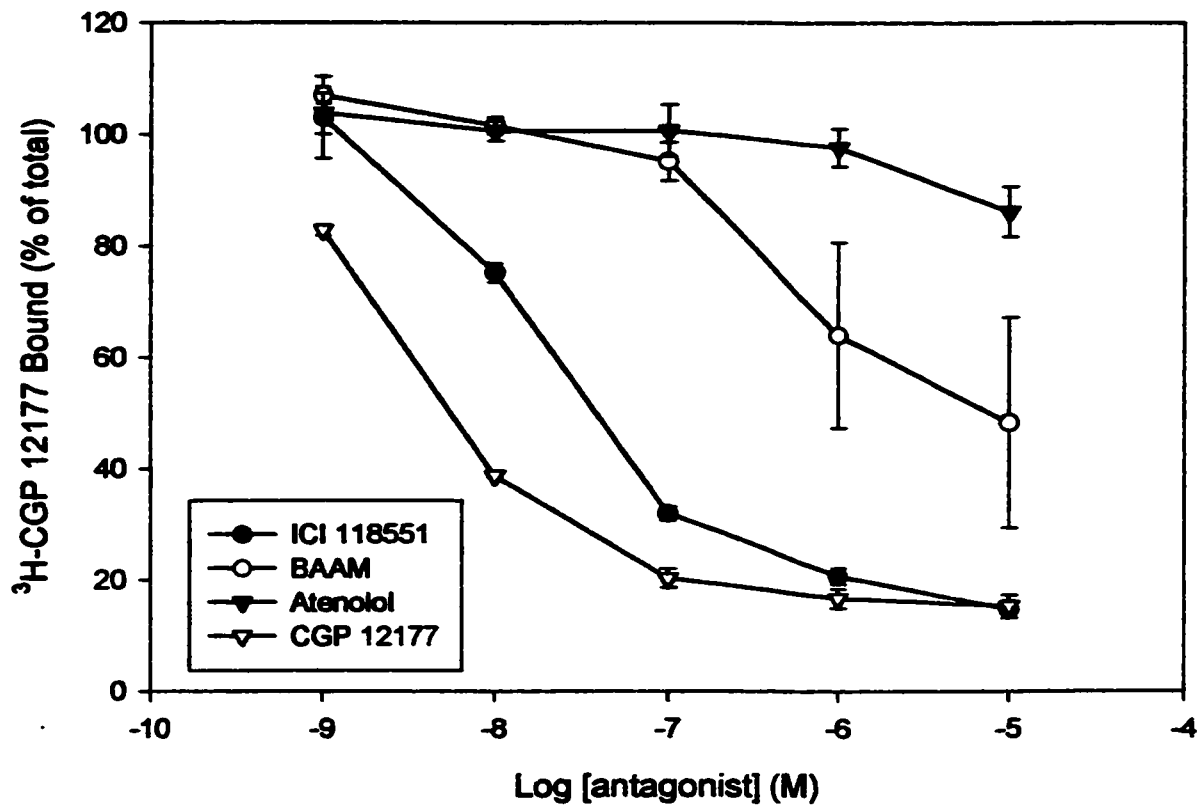


Figure 3-5. Antagonist competition for β -AR binding sites on day 19 chick embryo liver membranes. Membranes (250 μ g protein/50 μ L) were incubated for 120min with 1nM 3 H-CGP 12177 in the absence (total binding) and in the presence of increasing concentrations of various β -antagonists (ICI 118551, BAAM, atenolol, CGP 12177). All values are means \pm SEM of four independent experiments using pools of ~30 eggs per membrane preparation.



antagonist) and CGP 12177 (non-selective β -agonist/antagonist) effectively displaced ^3H -CGP 12177 at all concentrations used. These results support that chick embryo liver β -ARs are of the β_2 -subtype and preferentially bind mammalian β_2 -agonists and antagonists.

The mean inhibitory constants (K_i 's) for the β -agonists (adrenaline and procaterol) and β -antagonists (ICI 118551 and CGP 12177) were calculated using EBDA software (Table 3-2). The K_i represents the concentration (M) of the agonist/antagonist that displaces 50% of ^3H -CGP 12177 binding to β -ARs. K_i 's could only be calculated for compounds that achieved at least 50% displacement of the ^3H -CGP 12177. The order of potency with respect to the ability of these compounds to displace ^3H -CGP 12177 was CGP 12177 > ICI 118551 > procaterol > adrenaline.

3.3 Effect of PCB 126 on Lipid Peroxidation and β -AR Binding Curves

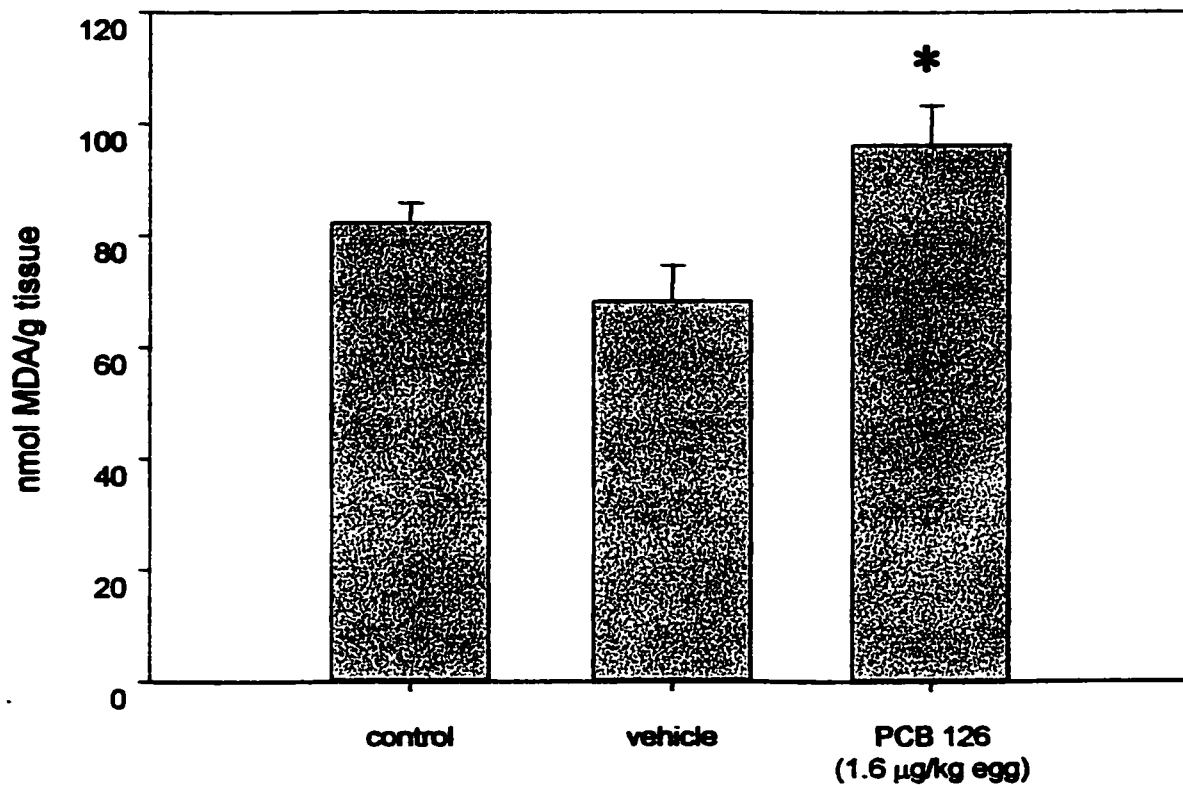
3.3.1 Lipid Peroxidation

TBARS were significantly increased in the livers of PCB126-treated (1.6 $\mu\text{g}/\text{kg}$ egg) embryos (Fig. 3-6) compared with control and vehicle-injected eggs. Mean TBARS levels were 78.3 ± 3.5 , 65.0 ± 5.1 and 103.9 ± 6.7 nmol MDA/g tissue in control, vehicle- and PCB126-treated embryos, respectively. There were no statistically significant differences in lipid peroxidation between the control and vehicle-treated embryos, although there was a trend to decreased TBARS in the vehicle group.

Table 3-2. Mean inhibitory constants (K_i) for β -agonists (adrenaline and procaterol) and β -antagonists (ICI 118551 and CGP 12177). The K_i represents the concentration (M) of the agonist/antagonist that displaces 50% of ^3H -CGP 12177 binding to β -ARs in day 19 chick embryo liver membranes. All values are means \pm SEM of four independent experiments.

		K_i (M)
Agonist	Adrenaline	$3.75 \times 10^{-6} \pm 2.00 \times 10^{-7}$
	Procaterol	$1.07 \times 10^{-7} \pm 9.19 \times 10^{-9}$
Antagonist	ICI 118551	$2.06 \times 10^{-8} \pm 1.83 \times 10^{-9}$
	CGP 12177	$3.24 \times 10^{-9} \pm 4.12 \times 10^{-10}$

Figure 3-6. Lipid peroxidation (TBARS) (nmol/g tissue) in livers from control, vehicle and PCB126-treated day 19 chick embryos. Eggs were injected in the air sac with a corn-oil vehicle or PCB 126 (1.6 µg/kg egg) prior to incubation. Bars are means ± SEM (n=9) for each treatment group. Statistical analyses were performed using one-way ANOVA followed by Tukey's post-hoc test. The “ * ” indicates a significant difference at P<0.05 compared to the control and vehicle-treatment.



3.3.2 β -AR Saturation Curves

β -AR binding kinetics for hepatocytes isolated from control, vehicle- and PCB126-treated embryos are shown in Figures 3-7A, 3-8A and 3-9A, respectively. Eggs were injected in the air sac with a corn-oil vehicle or PCB 126 (1.6 $\mu\text{g}/\text{kg}$ egg) prior to incubation and embryos were dissected on day 19 on of incubation, approximately 2 days prior to hatching. For all treatments, specific binding of ^3H -CGP 12177 increased and saturated as the concentration of ^3H -CGP 12177 increases. Scatchard analysis (using EBDA software) of data from control (Fig. 3-7B), vehicle (Fig. 3-8B) and PCB126-treated (Fig. 3-9B) binding curves was linear, indicating a single binding site for ^3H -CGP 12177.

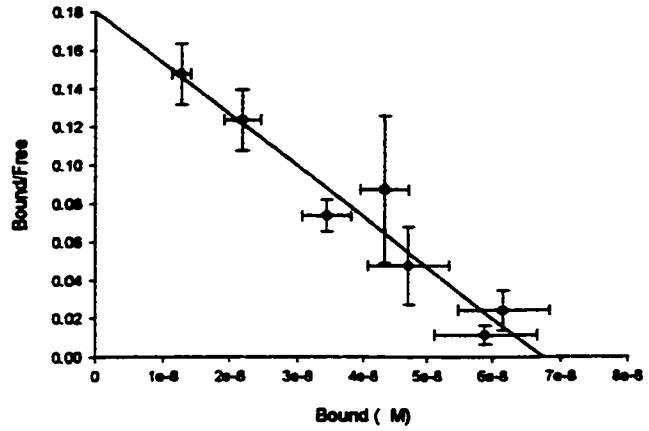
The affinity of the chick embryo liver β -AR for ^3H -CGP 12177 is significantly lower (indicated by the increased K_d) in the PCB-treated hepatocytes compared to vehicle ($p < 0.05$); however, there is no significant difference in K_d 's between the PCB126-treatment and control, or between the vehicle and control (Table 3-3).

There is no statistically significant difference amongst the maximum number of binding sites (B_{max} 's) in the control, vehicle- and PCB126-treated hepatocytes (Table 3-3).

Figure 3-7A. Saturation curve of β -ARs on isolated hepatocytes from control day 19 chick embryo livers. Hepatocytes (5mg/50 μ L) were incubated for 120 min with increasing concentrations of 3 H-CGP 12177 (0.05-10nM) in either the absence (total binding) or presence of 10 μ M alprenolol (non-specific binding). Specific binding was determined by subtracting the non-specific binding from the total binding. Data points are means \pm SEM of six independent experiments done in duplicate, consisting of pooled livers from 35-45 eggs per experiment.

B. Scatchard plot of data from A, transformed using EBDA software. Data points are means \pm SEM of six independent experiments done in duplicate, consisting of pooled livers from 35-45 eggs per experiment.

B.



A.

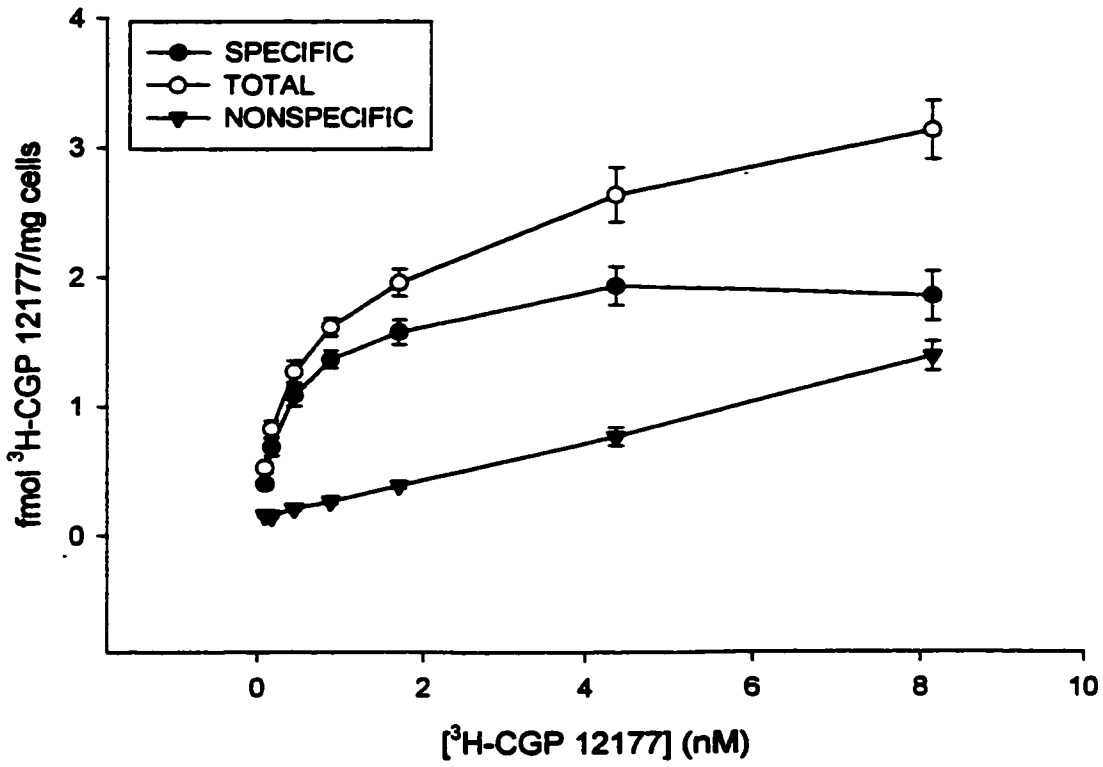
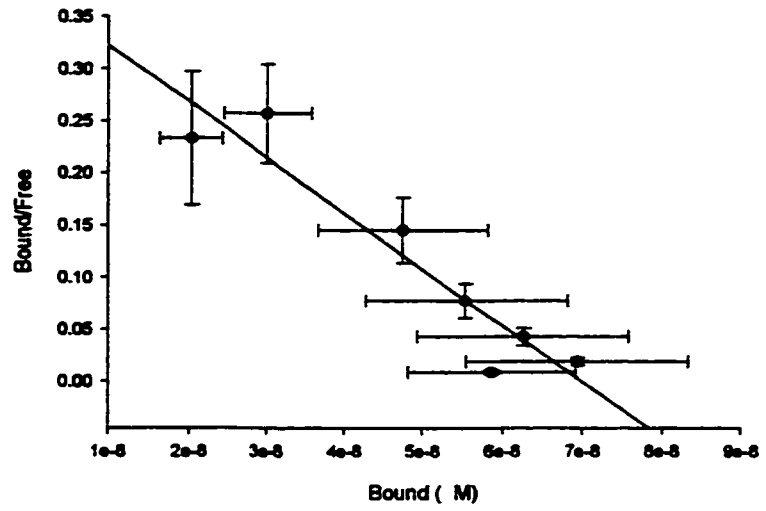


Figure 3-8A. Saturation curve of β -ARs on isolated hepatocytes from vehicle-treated day 19 chick embryo livers. Eggs were injected in the air sac with a corn-oil vehicle (10 μ L /50g egg) prior to incubation. Hepatocytes were incubated as in Fig.3.7A. Data points are means \pm SEM of six independent experiments done in duplicate, consisting of pooled livers from 35-45 eggs per experiment.

B. Scatchard plot of data from A, transformed using EBDA software. Data points are means \pm SEM of six independent experiments done in duplicate, consisting of pooled livers from 35-45 eggs per experiment.

B.



A.

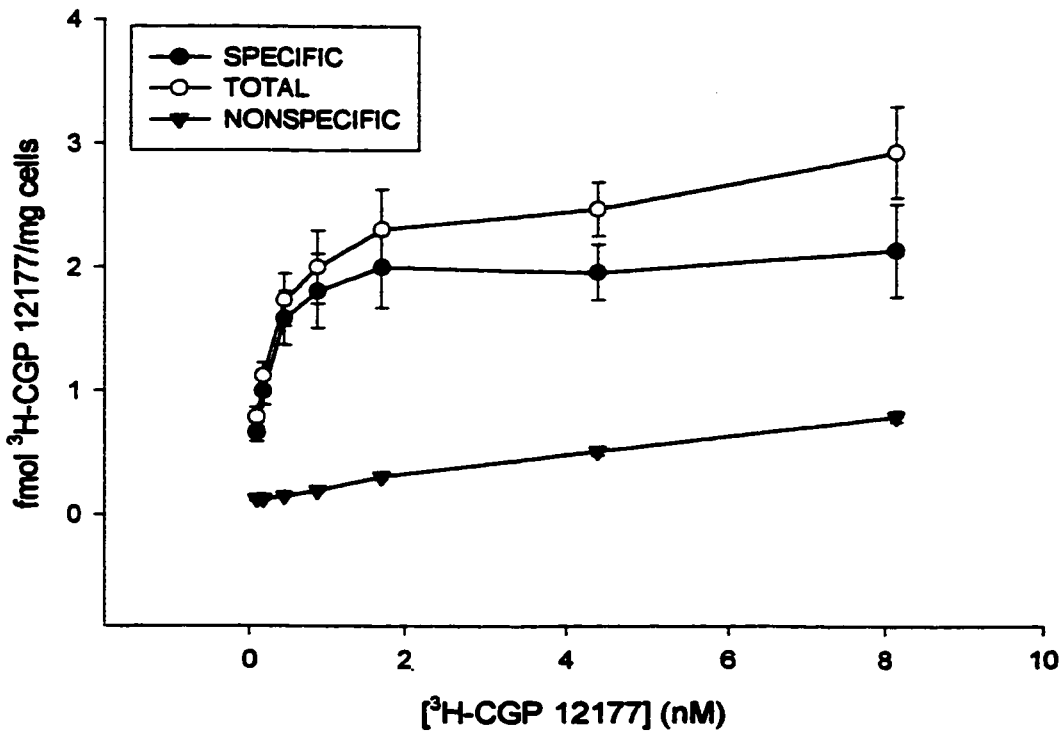


Figure 3-9A. Saturation curve of β -ARs on isolated hepatocytes from PCB126-treated day 19 chick embryo livers. Eggs were injected in the air sac with PCB126 (1.6 μ g/kg egg) prior to incubation. Hepatocytes were incubated as in Fig.3.7A. Data points are means \pm SEM of nine independent experiments done in duplicate, consisting of pooled livers from 35-45 eggs per experiment.

B. Scatchard plot of data from A, transformed using EBDA software. Data points are means \pm SEM of six independent experiments done in duplicate, consisting of pooled livers from 35-45 eggs per experiment.

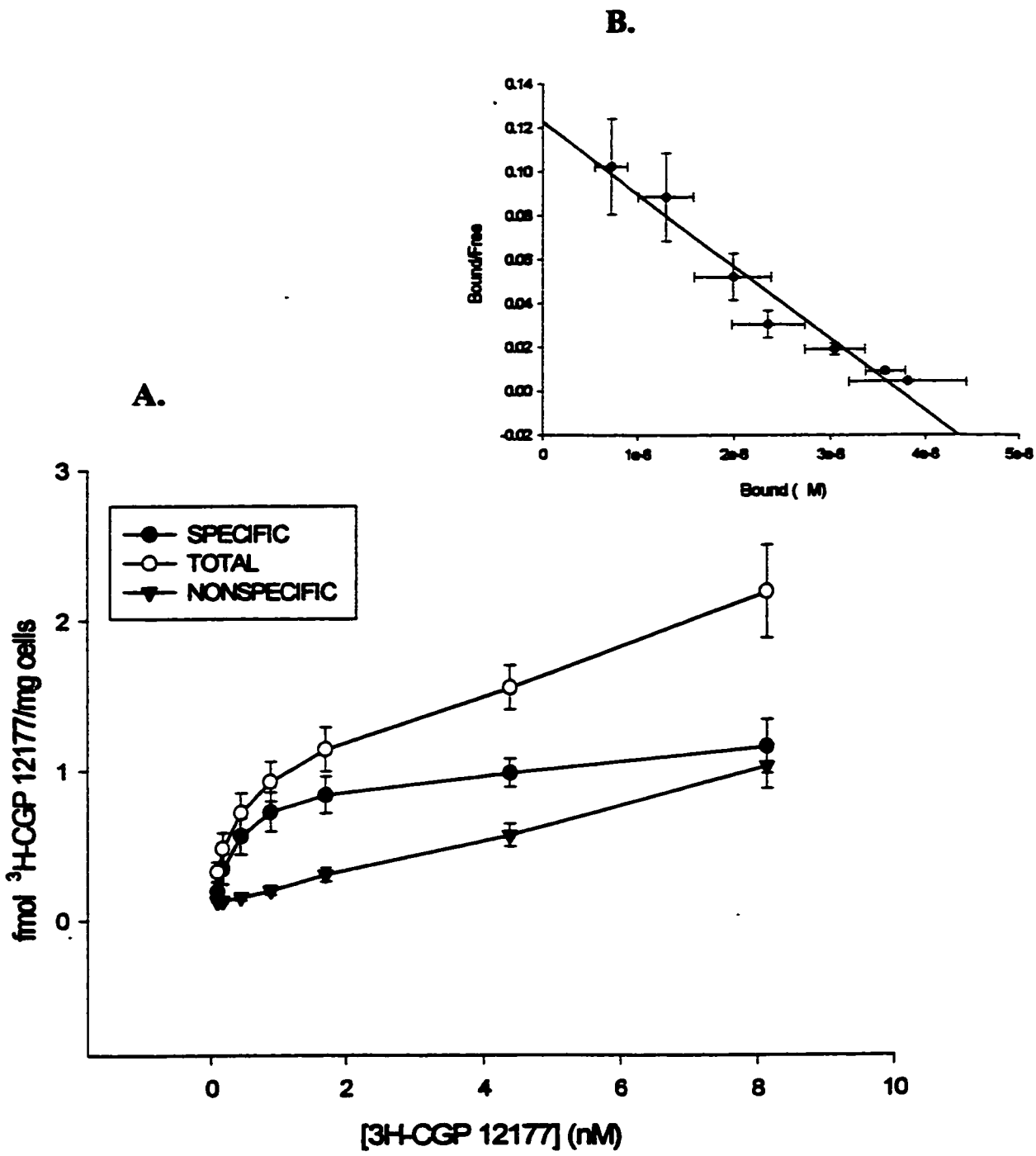


Table 3-3. Binding affinities (K_d) (nM) and maximum number of binding sites (B_{max}) (fmol/mg cells) for β -ARs on isolated hepatocytes from control, vehicle- and PCB126-treated day 19 chick embryo livers. Binding parameters were determined from Scatchard analysis as noted in Fig.3.7B, 3.8B and 3.9B. All values are means \pm SEM of six to nine independent experiments done in duplicate, consisting of pooled livers from 35-45 eggs per experiment. Statistical analyses were performed using one-way ANOVA on ranks, followed by Dunn's pairwise multiple comparison. The “ * ” indicates a significant difference at $P < 0.05$ compared to the vehicle.

	Control	Vehicle	1.6 μ g/kg PCB126
K_d (nM)	0.399 \pm 0.055	0.183 \pm 0.031	0.921 \pm 0.171 *
B_{max} (fmol 3 H-CGP/mg cells)	0.644 \pm 0.069	0.780 \pm 0.126	0.540 \pm 0.082

CHAPTER 4:
DISCUSSION

4. DISCUSSION

It was hypothesized that if PCB toxicity in the liver is mediated in some part by oxidative damage, such as increased lipid peroxidation, then the function of a membrane-bound receptor, such as the β -AR, may be modified through oxidative damage to membrane lipids. If decreased adrenergic responsiveness is due to some effect at the receptor level, then *in ovo* injection of PCB126 may result in (i) decreased receptor affinity for an endogenous or synthetic agonist, or (ii) decreased receptor density. In testing this hypothesis, I have (1) pharmacologically characterized the β -ARs in the chick embryo liver; (2) determined if a parameter of oxidative stress, lipid peroxidation, is induced by the *in ovo* treatment of PCB126; and (3), determined the effects of PCB126 injection *in ovo* on the liver β -AR affinity (K_d) and density (B_{max}).

In addition, PCB126 embryotoxicity was evaluated in terms of observable mortalities and abnormalities, EROD activity, and the hepatic concentration of PCB126 following air cell injection prior to incubating the eggs.

4.1 Mortality and Abnormalities

The PCB 126 treatment in this study (1.6 μ g/kg egg injected into the air cell prior to incubation for an exposure period of 19 days) produced an array of toxic effects similar to those observed in previous studies with respect to embryo mortality and the incidence of abnormalities (Powell *et al.* 1996a; Powell *et al.* 1996b; Hoffman *et al.* 1996; Brunstrom and Lund 1988; Hoffman *et al.* 1998). The percentage of dead embryos in the PCB 126 treated-eggs, after 19 days of incubation, was approximately 4-fold

higher with respect to control eggs and 8-fold higher with respect to vehicle-treated eggs (Table 3-1). A concurrent study in our lab (Jin *et al.*, 2001) determined the approximate 50% lethal dose (LD50) for PCB 126 in chickens was 1.5µg/kg egg. This value was slightly lower than those reported by Powell *et al.* (1996a) (2.3µg/kg egg based on hatching) and Zhao *et al.* (1997) (3.6µg/kg egg based on surviving day 18 embryos), but higher than the LD50's reported by Powell *et al.* (1996b) (0.6µg/kg egg based on surviving day 18 embryos) and Hoffman *et al.* (1998) (0.4µg/kg egg based on hatching). Although the LD50s are similar in magnitude, differences in vehicle, site and time of injection and developmental stage of the embryo undoubtedly contribute to the variation of LD50 values.

The percentage of liver abnormalities (discoloration and lesions) and of tissue edema (subcutaneous and pericardial) was 2-fold higher in the PCB 126-treated embryos compared with control and vehicle-treated embryos (Table 3-1). The teratogenic potential of PCB126 in chicken embryos has been well established in the literature. Brunstrom and Andersson (1988) injected 2µg/kg egg into the air cell on day 4 of incubation and observed subcutaneous edema, microphthalmia and beak deformities in day 18 embryos. Powell *et al.* (1996a) and Hoffman *et al.* (1998) reported similar abnormalities in chicken hatchlings following dosing regimes of 3.2µg/kg egg injected into the yolk sac prior to incubation, and 0.3µg/kg egg injected into the air cell on day 4 of incubation, respectively. The percentage of abnormal embryos in Hoffman *et al.* (1998) ranged from 4- to 7-fold higher in all dosed groups (0.3-3.2 µg/kg) compared to vehicle-treated eggs.

No significant differences in body weight or liver weight were observed between the PCB-, vehicle-treated or control embryos (Table 3-1), in contrast to the results

reported by Powell *et al.* (1996a) where 3.2µg PCB 126/kg egg significantly lowered hatchling body weights and there was a trend of increasing liver weight at the same dose that lowered hatchling body weight. However in an additional study by Powell *et al.* (1996b), there were no significant differences in body weight between the vehicle and 0.9µg/kg egg PCB 126-treated hatchlings until 2 weeks post-hatch.

No histological analyses were undertaken during the course of this study to examine cellular changes in the liver or any other tissues. Zhao *et al.* (1997) reported PCB 126-induced changes in hepatic histopathology in day 18 chicken embryos following the injection of 2.0µg/kg egg into the yolk on day 4 of incubation. Liver sections exhibited large necrotic zones and mineralization following PCB 126-treatment, however the mechanism of PCB-induced necrosis remains unknown as there were no apparent vascular lesions in the liver and the mineral depositions were nonspecific.

4.2 EROD Activity

In *in vitro* studies, PCB 126 was shown to be the most potent inducer of EROD activity among 10 PCB congeners tested, with a concentration of 0.003nM causing EROD induction and an EC50 of 0.052nM in hepatocyte cultures prepared from day 19 chicken embryos (Kennedy *et al.* 1996). Brunstrom *et al.* (1995) exposed hepatocyte cultures from day 10 chicken embryo livers to PCB 126 for 48h and reported an EC50 of 0.044nM. Additional studies have shown PCB 126 to be a potent *CYP1A* inducer *in ovo* as well. Four- to 40-fold increases in hepatic EROD activity were observed in liver microsomes prepared from day 10 chicken embryos dosed with 0.092-7 nmol PCB 126/kg egg for 3 days, with an EC50 of 0.3nmol/kg egg (Brunstrom and Andersson

1988). More recently, Brunstrom and Haldin (1998) detected a 3- to 4-fold increase in hepatic EROD activity in day 18 chicken embryos dosed with 0.2 μ g PCB 126/kg egg on day 4 of incubation. Using a similar dosing protocol, Hoffman *et al.* (1998) measured a 17-fold increase in hepatic EROD activity in hatchlings dosed with 0.3 μ g PCB 126/kg egg on day 4 of incubation.

In this study, EROD activity was measured in microsomes prepared from livers of day 19 control, vehicle- and PCB 126-treated embryos. PCB 126 (1.6 μ g/kg egg) induced EROD activity approximately 12-fold in the liver (Fig. 3-1), indicative of PCB 126-induction of *CYP1A*, *in ovo*, in chicken embryos.

It is interesting to compare the EC50s for hepatic EROD activity *in vitro* (52pM from Kennedy *et al.* 1996; 44pM from Brunstrom *et al.* 1995) with hepatic EROD activity *in ovo* (300pM from Brunstrom and Anderson 1988). The *in ovo* EC50 is approximately 6- to 7-fold higher than the *in vitro* EC50s, suggesting that higher concentrations are required to induce EROD *in ovo*, possibly due to the fact that the proportion of PCB 126 actually transported to the liver of the developing embryo may be less than the proportion the cultured cells receive during *in vitro* dosing. Differences in methodology between these kinds of studies may also, at least in part, account for the differences in magnitude of these EC50 values.

Compared to other bird species, the chicken appears to be one of the most sensitive species with respect to PCB toxicity. Hoffman *et al.* (1998) compared EROD responses in hatchlings from three species (air cell injections on day 4 of incubation): chicken (*Gallus gallus*) (0.3-3.2 μ g/kg), American kestrel (*Falco sparverius*) (0.23-233 μ g/kg) and common tern (*Sterna hirundo*) (44-434 μ g/kg). The LD50s for these

species were 0.4µg/kg, 65µg/kg and 104µg/kg, respectively. Maximum EROD induction was 12-fold in the kestral and 19-fold in the tern, however in the chicken, EROD was induced 800-fold compared to the activity in the tern and 1000-fold compared to EROD activity in the kestral. Basal EROD activities were not significantly different between the three species (10.5, 5.9, 11.4 pmol resorufin/min/mg microsomal protein in chicken, kestral and tern, respectively) suggesting a much more sensitive mixed function oxidase (MFO) response is present in the chicken. Brunstrom and Halldin (1998) showed in order to produce an equivalent 3- to 4-fold increase in hepatic EROD activity in domestic duck, they needed to use a PCB 126 dose 50 times higher than that used in chickens. The EC50 for EROD induction in Pekin duck has been shown to be 250 times higher than the EC50 in chicken (Kennedy *et al.* 1996). Jin *et al.* (2001) found that the basal EROD activity was higher in day 26 duck embryos than in day 19 chickens, which suggests that there are species specific differences in basal EROD activity as well as induction. This is important to note when one attempts to extrapolate ecological risk to wild species based on results using a model species of high sensitivity, such as the chicken, in laboratory studies.

4.3 PCB 126 Tissue Content

Environmental levels of PCB 126 measured in a variety of bird species were summarized in Table 1-1 (see Introduction, p. 4). Concentrations of PCB 126 range from 0.14-84 µg/g in total egg homogenate (including yolk), 3.3-42.5 µg/g in the yolk sac, 0.29-9.32 µg/g in the embryo (including yolk) and 0.05-0.15 µg/g in fledgling plasma. Russel *et al.* (1999) proposed a model for estimating maternal transfer of hydrophobic

organic chemicals to the egg, based on the theory that passive transport processes during ovogenesis result in a chemical equilibrium between the chemical concentration in maternal tissues and in the egg. The assumptions of this model include: (1) distribution of the chemical within the maternal tissues is relatively rapid and homogeneous; (2) transfer of maternal lipoproteins during ovogenesis results in chemical levels in the egg which reflect those in maternal lipoproteins; and, (3) low metabolic transformation of contaminants in eggs due to underdeveloped enzyme systems in embryonic tissues. By analyzing contaminant (PCBs, TCB, HCB, DDE and Mirex) content in egg and maternal tissues of a few oviparous species of fish and turtles, and using literature data for birds, Russel *et al.* (1999) found that the lipid normalized egg/maternal tissue concentration ratios for individual chemicals were not significantly different from 1.0. They concluded that the maternal and embryonic organisms are exposed to the same effective internal contaminant concentration.

In my study, as with other laboratory studies, the delivery of the contaminant is not dependent on deposition during ovogenesis. Injection into the air cell of an unincubated egg raises an interesting question of whether or not the tissue distribution of the injected chemical will vary with different injection locations (air cell, yolk sac, the embryo) and times (prior, during or post-incubation)? These variables may have important implications when assessing the potential toxicity of a given chemical, as the mode and time of delivery will undoubtedly influence the response of the target organism. Although I did not examine the effects of these variables during the course of my study, I did measure the concentration of PCB 126 in the livers of day 19 chick embryos following air cell injection of 1.6 $\mu\text{g}/\text{kg}$ egg prior to incubation. Changes in liver

biochemistry and morphology are most commonly used to assess PCB toxicity, however the actual amount of PCB deposited in the liver per se is rarely reported in the literature.

The mean hepatic PCB 126 content was 0.67 ng/g liver or 10.2 ng/g on a lipid basis (Fig. 3-2), following air cell injection of 1.6 µg/kg egg prior to incubation. A higher percentage of PCB 126 content would undoubtedly be found in the yolk and tissues with high lipid content such as the adipose tissue. Hoffman *et al.* (1996) reported hepatic PCB 126 accumulation following oral dosing for 10 days in nestling American kestrels (*Falco sparverius*). Dosing with 50, 250 and 1000 µg PCB 126/kg resulted in hepatic concentrations of 156, 380 and 1098 µg PCB 126/kg, respectively. However, dosing with 1000 µg PCB 77/kg and 4000 µg PCB 105/kg in nestling American kestrels resulted in hepatic concentrations of 892 and 1677 µg/kg, respectively (Hoffman *et al.* 1993). From these studies, it is apparent that differences in dosing protocols, chemical congeners and the developmental stage of the target organism all affect the partitioning of PCBs into the liver.

4.4 Pharmacological Characterization of the Chick β-AR

The characterization of avian β-ARs has been limited very few tissues (heart, erythrocyte, muscle) in species such as chicken and turkey. Using classic mammalian β-agonists and -antagonists, Reithmann *et al.* (1987) characterized the adult chicken heart muscle ARs as mainly of the β₁-subtype, while the non-muscle cells were mainly β₂, and Port *et al.* (1992) reported chick embryo myocytes to be a mixed population of β₁/β₂-subtypes. Dickinson *et al.* (1981) concluded that the chick erythrocyte had a mixed population of β-ARs, distinct from the mammalian β₁- or β₂-ARs.

Competition assays were performed using a number of mammalian β -AR agonists: noradrenaline, which preferentially binds to β_1 -ARs, adrenaline, which preferentially binds to β_2 -ARs, procaterol, a selective β_2 agonist and dobutamine, a selective β_1 agonist. Low (<20%) to no displacement of ^3H -CGP 12177 occurred in the presence of the β_1 -agonists, noradrenaline and dobutamine (Fig. 3-4). The β_2 -agonists (adrenaline and procaterol) were much more effective at displacing ^3H -CGP 12177. Adrenaline, at the highest concentration used, displaced at least 50% of the ^3H -CGP 12177, while procaterol significantly displaced 80% of the ^3H -CGP 12177 (Fig. 3-4). The order of the various agonists' relative ability to displace ^3H -CGP 12177 was procaterol > adrenaline > noradrenaline = dobutamine. These results imply that the day 19 chick embryo liver β -ARs preferentially bind mammalian β_2 -agonists.

In addition, competition assays using mammalian β -AR antagonists further suggest the chick embryo liver β -AR is of the β_2 -subtype. In this case, the classic mammalian β -antagonists were used: (\pm)-ICI 118551, a selective β_2 antagonist, bromoacetyl alprenolol menthane (BAAM), which preferentially binds to β_2 -ARs, atenolol, a selective β_1 antagonist, and CGP 12177, a non-selective mixed β -agonist/antagonist. Atenolol was ineffective in displacing ^3H -CGP 12177, BAAM displaced 50% of the ^3H -CGP 12177 at the highest concentration used, while ICI 118551 and CGP 12177 most effectively displaced ^3H -CGP 12177 at all concentrations used (Fig. 3-5). The order of the various antagonists relative abilities to displace ^3H -CGP 12177 binding was CGP 12177 > ICI 118551 > BAAM > atenolol. These data support the predominant β -AR in the chick hepatocyte being of the β_2 -subtype.

4.5 Lipid Peroxidation

Lipid damage has been studied extensively in toxicity studies, primarily because of the critical role of lipids in maintaining membrane structure and function, and cellular viability. The presence of polyunsaturated fatty acids (PUFAs) and the increased solubility of oxygen in lipid (versus aqueous solution) make membranes particularly sensitive to oxidative attack (Scott and Eaton 1997). The process of lipid peroxidation is initiated by the abstraction of a hydrogen from the hydrocarbon chain, typically occurring at CH₂ groups adjacent to C-C double bonds (Scott and Eaton 1997). The resulting product is a resonance-stabilized conjugated diene that may further react with molecular oxygen to form peroxy radicals. Peroxy radicals are also capable of abstracting hydrogen from additional lipid molecules to form lipid hydroperoxides (LOOH) in a chain-reaction fashion. One of the most commonly used assays to measure oxidative lipid degradation is the TBARS (thiobarbituric acid reactive substances) assay, which measures the reaction of lipid peroxidation products and thiobarbituric acid (TBA) (Kelly *et al.* 1998). Although widely used, the results of the TBARS assay must be interpreted with some caution, as there are a few methodological flaws with this test. For example, this test is most frequently used as a measure of malondialdehyde (MDA), a secondary product of lipid peroxidation. However TBA reacts with a number of other oxidation products (such as 4-hydroxynoneal, unsaturated aldehydes and endoperoxides from enzymatic routes) in addition to MDA (Kelly *et al.* 1998). Also, a portion of TBA-reactive material may form during the heating of the sample (by a metal-catalyzed reactions) rather than being present before sample manipulation (Scott and Eaton 1997). Therefore, lipid peroxidation measured by the TBARS assay provides only a crude and

rather non-specific estimate of oxidative lipid damage. It is also important to consider that membrane lipids may be much more susceptible to peroxidation following cell injury or death (xenobiotic or otherwise induced) (Kehrer 1993), therefore lipid peroxidation may only serve as a marker of cell injury or death, as opposed to a defining mechanism.

The toxicity of PCBs and other related compounds has been extensively associated with parameters of oxidative stress including lipid peroxidation in a number of studies (Alsharif *et al.* 1994; Hassan *et al.* 1983; Stohs *et al.* 1986; Stohs *et al.* 1990; Stohs *et al.* 2000). Stohs *et al.* (1990) observed 400-500% increases in hepatic mitochondrial, microsomal and nuclear membrane lipid peroxidation 3-5 days after a single injection of 100µg TCDD/kg in female rats, with mitochondrial, microsomal and nuclear membrane fluidity gradually decreasing 30-40%, suggesting TCDD-induced alterations in membrane structure and function. Hassoun *et al.* (2000) showed that PCB 126 (0.3-0.55µg/kg) increased hepatic lipid peroxidation in a dose-dependent fashion in rats following a subchronic exposure. PCB 126, at doses of 6.3 and 25µg/kg, significantly increased hepatic lipid peroxidation in lake trout (*Salvelinus namaycush*) 6 weeks post intraperitoneal injection (Palace *et al.* 1996).

In my study, lipid peroxidation was significantly increased ($P < 0.001$) in the livers of PCB 126 (1.6µg/kg)-treated embryos after 19 days of *in ovo* exposure, compared to control and vehicle-treated chicken embryos (Fig. 3-6). These findings are in agreement with those of Jin *et al.* (2001) who detected significant increases in lipid peroxidation in the livers and adipose tissue of PCB 126 (1.6µg/kg)-dosed chicken embryos. My results are in contrast to those of Hoffman *et al.* (1998) who did not detect any significant increase in hepatic lipid peroxidation in PCB 126-exposed (0.3-3.2 µg/kg) common tern

hatchlings, even at doses that caused 50% mortality. Additionally, Hoffman *et al.* (1998) reported no significant increase in hepatic lipid peroxidation in day 19 chicken embryos dosed with 0.3 μ g PCB 126/kg. Differences in dosing protocols (injection site and stage of development) as well as differences in methodology may contribute to the variable results with respect to PCB 126-induced lipid peroxidation. However, the increased degree of lipid peroxidation measured in this study further reinforces the hypothesis that oxidative stress, at least in part, mediates PCB 126 toxicity in the chicken embryo.

Studies by Mohammadpour *et al.* (1988) and Alsharif *et al.* (1994) have shown a correlation between lipid peroxidation and the AHR, the receptor that mediates the effects of PCBs and other structurally related HAHs. Using responsive and non-responsive mice strains, Mohammadpour *et al.* (1988) observed a significant increase in hepatic lipid peroxidation in responsive mice treated with a single dose of TCDD of 0.5 μ g/kg; doses 5-14 times higher were required to induce the same increase in lipid peroxidation in non-responsive strains. The AHR levels in the livers of the non-responsive mice strains were found at lower concentrations and in a lower affinity form than the AHRs in the responsive strain (Poland and Glover 1990; Okey *et al.* 1989). With respect to differences in species sensitivity in birds, the chicken embryo AHR has been shown to have much higher affinity for TCDD than the AHRs of other bird species (Sanderson and Bellward 1995), providing another possible explanation for the increased sensitivity of the chicken to the toxic effects of AHR-ligands.

A study by Toborek *et al.* (1995) further corroborates the relationship between P450 1A induction, oxidative stress and PCB toxicity. They exposed porcine pulmonary-artery derived endothelial cells to PCBs 77, 114 and 153 at concentrations of 0.85 to

3.4 μ M for 24h and observed a dose-dependent disruption in endothelial barrier function resulting in increased albumin transfer across the endothelial monolayer in those cells treated with PCBs 77 and 114. These cells additionally showed marked increases in cellular oxidative stress, lipid hydroperoxide production and intracellular Ca²⁺ concentration. Increased content and activity of cytochrome P4501A was additionally observed in endothelial cells exposed to PCBs 77 and 114. In contrast, PCB 153 had no effect on endothelial barrier function or cellular oxidative stress. Interestingly, PCBs 77 and 114 bind to the AHR and induce cytochrome P450 1A, whereas PCB 153 is not an AHR ligand and induces cytochrome P450 2B (Farin *et al.* 1994).

From these studies it can be concluded that the interaction of the AHR and the induction of *P4501A1* play a critical role in mediating PCB-induced oxidative lipid damage.

4.6 β -AR Saturation Assays

The main objective of my study was to test the hypothesis that if PCB toxicity in the liver is mediated in some part by oxidative damage, such as increased lipid peroxidation, then the function of a membrane-bound receptor, such as the β -AR, may be modified through oxidative damage to membrane lipids. The K_d was significantly higher in hepatocytes from the PCB126-treated group ($p < 0.05$) compared to the vehicle group, suggesting that the affinity of the β -AR decreased with PCB 126-treatment (Table 3-3). However, there were no significant differences in K_d 's between the PCB126-treated and control groups, or between the vehicle and control groups. Although I would have to measure changes in membrane fluidity to determine the direct effects of PCB126 on

membrane lipid composition and membrane-receptor conformation; the results suggest that perhaps the corn-oil vehicle is having some effect that may be masking the full magnitude of the PCB effect. The corn-oil vehicle may in fact protect the liver to some extent, perhaps acting as an antioxidant due to its high vitamin E content. Vitamin E (α -tocopherol), a major cellular lipid-soluble antioxidant, can suppress fatty acid oxidation by contributing a hydrogen atom from its phenolic group to lipid-derived radicals (Kelley *et al.* 1995). This action prevents the peroxy radical from attacking the unsaturated fatty acids of adjacent membrane lipids, thereby inhibiting the propagation of lipid peroxidation (Kelley *et al.* 1995). Surai *et al.* (1999a) found that vitamin E concentration in the liver of turkey embryos remained constant between embryonic days 16-22, and peaked significantly just prior to hatching. In addition, Surai *et al.* (1999b) observed that after feeding hens a high vitamin E diet (365 μ g VE/g feed), chick embryo tissues including liver and brain exhibited significantly reduced susceptibility to lipid peroxidation. It is interesting to note that there was a trend towards a decrease in the extent of lipid peroxidation (Fig. 3-6) in the vehicle-group as well, however there was no statistically significant difference between the control and vehicle groups. This trend towards decreased lipid peroxidation and increased β -AR affinity in the vehicle-treated embryos may be indicative of a protective effect of the vehicle

The B_{max} (receptor density) values were not significantly different amongst hepatocytes from control, vehicle- or PCB 126-treated embryos (Table 3-3), suggesting that the PCB 126-treatment did not alter the number of β -ARs in day 19 chick embryo hepatocytes.

This thesis is the first report of the effects of PCB 126 on hepatic β -AR function in an avian model. As previously stated, Steevens *et al.* (1996) hypothesized that exposure to PAHs in brown bullhead may result in β -AR down- or up-regulation, as a compensatory response to chemical stress. A decreased number of β -ARs was found only in the gill of exposed fish, with no change in receptor affinity. A possible explanation for this decrease in receptor number may be that xenobiotic stress increased levels of circulating adrenaline, an endogenous β -AR ligand, and caused vasodilation and increased blood flow through the gills (Mazeaud and Mazeaud 1981; Aota *et al.* 1990). β -AR numbers may be reduced, or down-regulated, in order to compensate for increased blood flow and the subsequent increase in the uptake of hydrophobic xenobiotic compounds at the gill. I did not measure catecholamine levels during the course of my study; however it would be interesting to see if a 19-day exposure to PCB 126 would alter circulating catecholamine levels in chicken embryos. Lorenzen *et al.* (1999) found a significant negative correlation between basal plasma cortisosterone levels and yolk sac PCDD/PCDF/PCB concentrations in Great Lakes herring gull embryos. These data suggest that the hypothalamo-pituitary-adrenal axis is in fact a potential target of organochlorine toxicity.

A few other studies on xenobiotic-induced alteration of β -adrenergic function have been reported for cardiac muscle preparations. Rifkind *et al.* (1984) have shown that cytochrome P448-inducing-PCB congeners decreased the contractile response of cardiac ventricular muscle to β -adrenergic stimulation in day 19 chick embryos following 9 day exposure to 500 nmoles of 3,4,3',4'-TCB or 3,4,5,3',4',5'-HCB per egg. Canga *et al.* (1993; 1988) obtained similar results using cardiac muscle strips from chick embryos

treated with 6.4-32.0 μg TCDD/kg and adult guinea pigs treated with 10 μg TCDD/kg body weight. In these studies, the authors proposed that decreased β -adrenergic responsiveness may have been due to (1) decreased β -AR number or affinity, or (2) the uncoupling of β -ARs from ACase. TCDD has previously been shown to reduce β -AR number and coupling in keratinocytes (Choi *et al.* 1987). Another possible explanation for decreased β -adrenergic responsiveness is that TCDD and PCBs may affect cAMP production, potentially altering cAMP-regulated processes and β -AR signal transduction. Weber *et al.* (1991) reported that TCDD inhibited rat liver phosphoenolpyruvate carboxykinase (PEPCK), a cAMP-dependent enzyme that contributes to the control of hepatic gluconeogenesis, thus implicating TCDD (and presumably other related compounds like PCBs) in the disruption of glucose metabolism. Future studies should examine glucose and glycogen production in hepatocytes isolated from PCB-treated embryos to determine whether the effects of PCB 126 on β -AR function may significantly impact glucose metabolism in isolated hepatocytes, thus leading to studies on physiological implications of PCB 126-induced disturbances of glucose metabolism *in vivo*.

4.7 Does oxidative stress play a role in PCB-induced alterations of β -AR function?

From my results I can conclude that PCB 126 induced hepatic lipid peroxidation in day 19 chick embryos following *in ovo* injection. However, the assay I used gives a non-specific measure of lipid peroxidation, therefore I cannot conclude that membrane lipids were a specific target of PCB 126-induced oxidative damage. A future study examining lipid peroxidation in isolated membrane preparations as well as a direct

measurement of membrane fluidity would prove useful in predicting the effects of PCB-mediated oxidative damage and/or alterations of membrane-bound receptors, such as the β -AR. Because the β -AR, the G-protein and ACase are all embedded in the cell membrane, their activity is undoubtedly influenced by the fluid state of the membrane.

Oxidative stress, whether induced by xenobiotic or other oxidants, has been shown to impact β -AR function by altering cAMP production (Haenen *et al.* 1990), ACase activity (Persad *et al.* 1998) and receptor-G-protein-ACase coupling through reductions in membrane fluidity (Peters 1988) (also see Introduction). Another confounding factor in the multitude of effects of PCB/TCDD-induced oxidative damage is the potential role of arachidonic acid (AA) metabolism. AA is a polyunsaturated membrane fatty acid, subject to metabolism by cytochrome P450 (Quilley and Rifkind 1986). Using chick embryos, Rifkind *et al.* (1990) showed that a dose of 6.3 pmol TCDD/egg caused a significant increase in P450 metabolism of AA, increasing the production of AA reactive metabolites, such as prostaglandins and leukotrienes. PCBs also increased prostaglandin production in chick heart and lung (Rifkind *et al.* 1984). Leukotrienes have been shown to increase vascular permeability and stimulate vasoconstriction, which may contribute to the prevalence of edema seen in TCDD- and PCB-treated animals (Burke *et al.* 1982; Dahlin 1981). With respect to the potential effects of AA metabolism on β -AR function, prostaglandins have also been shown to alter cAMP levels in cardiac tissue (Cantieri *et al.* 1980) and the autooxidation or co-oxidation of AA by endogenous iron can induce lipid peroxidation and lead to impaired membrane integrity (Karmazyn and Moffat 1985).

4.8 Conclusions

This thesis has examined the effect of PCB 126 on embryotoxicity, hepatic lipid peroxidation and hepatic β -adrenoceptors in White Leghorn chicken embryos. A few conclusions can be drawn from this work.

First, injection of 1.6 μ g PCB 126/kg egg into the air cell prior to incubation increased the % mortality and the incidence of abnormalities, including liver lesions and subcutaneous edema, in day 19 embryos.

Second, PCB 126 significantly increased in hepatic EROD activity in day 19 embryos, indicative of *CYP1A* induction *in ovo*.

Third, mean hepatic PCB 126 content following air cell injection of 1.6 μ g/kg egg, prior to incubation, was approximately 0.67ng/g liver, or 10.2 ng/g lipid.

Fourth, chick embryo hepatic β -ARs preferentially bind mammalian β_2 -agonists and antagonists.

Fifth, PCB 126 significantly increased lipid peroxidation, indicative of oxidative damage, in the liver of day 19 embryos.

Sixth, PCB 126 significantly decreased hepatic β -AR affinity compared to vehicle-treated embryos. There were, however, no significant differences between control and PCB 126-treated embryos, or control and vehicle-treated embryos. This suggests there is an effect of the corn oil vehicle; it may protect the liver from increased lipid peroxidation and potential alterations in membrane fluidity. There were no significant differences in hepatic β -AR numbers between control, vehicle and PCB 126-treated embryos.

There are numerous complex and potentially interactive pathways mediating PCB toxicity in the chick embryo. Clearly, future studies are required to elucidate the role of oxidative damage, such as lipid peroxidation, in mediating effects such as changes in membrane structure and function, and the implication of these changes to membrane-bound protein function. Due to the presence of a multitude of contaminants in our environment, as toxicologists we are obliged to continue exploring the molecular mechanisms of PCBs, and toxicants in general, to improve our ability to assess the risk of exposure to humans and wildlife alike.

REFERENCES

- Al-Bayati, Z. A. F., W. Murray, and S. J. Stohs. TCDD-Induced Lipid Peroxidation in Hepatic and Extrahepatic Tissues of Male and Female Rats. *Archives of Environmental Contamination and Toxicology* 16: 259-266, 1987.
- Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. *Molecular Biology of the Cell*. New York, Garland Publishing Inc. 1989, 681-726.
- Albro, P., J. Corbett, M. Harris, and L. Lawson. Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on Lipid Profiles in Tissues of Fisher Rat. *Chemical and Biological Interactions* 23: 315-330, 1978.
- Alquist, R. P. Study of Adrenotropic Receptors. *American Journal of Physiology* 153: 586-600, 1948.
- Alsharif, N. Z., T. Lawson, and S. J. Stohs. Oxidative Stress Induced by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin is Mediated by the Aryl Hydrocarbon (Ah) Complex. *Toxicology* 92: 39-51, 1994.
- Annas, A., B. Brunstrom, I. Brandt, and E. Brittebo. Induction of Ethoxyresorufin-*o*-deethylase (EROD) and Endothelial Activation of the Heterocyclic Amine Trp-P-1 in Bird Embryo Hearts. *Archives of Toxicology* 72(7):402-410, 1998.
- Aota, S., K. D. Holmgren, P. Gallagher, and D. J. Randall. A Possible Role for Catecholamines in the Ventilatory Responses Associated With Internal Acidosis or External Hypoxia in Rainbow Trout *Oncorhynchus mykiss*. *Journal of Experimental Biology* 151: 57-70, 1990.
- Baumann, P. C. and J. C. Harshbarger. Decline in Liver Neoplasms in Wild Brown Bullhead Catfish After Coking Plant Closes and Environmental PAHs Plummet. *Environmental Health Perspectives* 103: 168-170, 1995.
- Baumann, P. C., W. D. Smith, and M. Ribick. Polynuclear Aromatic Hydrocarbon (PAH) Residue and Hepatic Tumor Incidence in Two Populations of Brown Bullhead (*Ictalurus nebulosus*). In Dennis, A. J. and G. Fisher, eds. *Polynuclear Aromatic Hydrocarbons: Physical and Biological Chemistry*. Columbus, Battelle. 1982, 93-102.
- Bellward, G. D., R. J. Norstrom, P. E. Whitehead, J. E. Elliot, S. M. Bandiera, C. Dworschak, C. Chang, S. Forbes, B. Cadario, L. E. Hart, and K. M. Cheng. Comparison

of Polychlorinated Dibenzodioxin Levels with Hepatic Mixed-Function Oxidase Induction in Great Blue Herons. *Journal of Toxicology and Environmental Health* 30: 33-52, 1990.

Borlakoglu, J. T., J. D. Edwards-Webb, and R. R. Dils. Evidence for the Induction of Fatty Acid Desaturation in Proliferating Hepatic Endoplasmic Reticulum. *International Journal of Biochemistry* 23: 925-931, 1991.

Brewster, D. W., F. Matsumura, and T. Akera. Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on Guinea Pig Heart Muscle. *Toxicology and Applied Pharmacology* 89: 408-417, 1987.

Brunstrom, B. and P. L. Andersson. Toxicity and 7-Ethoxyresorufin-*O*-Deethylase-Inducing Potency of Coplanar Polychlorinated Biphenyls (PCBs) in Chick Embryos. *Archives of Toxicology* 62: 263-266, 1988.

Brunstrom, B., M. Engwall, and K. Hjelm. EROD Induction in Cultured Embryo Liver: A Sensitive Bioassay for Dioxin-Like Environmental Pollutants. *Environmental Toxicology and Chemistry* 14: 837-842, 1995.

Brunstrom, B. and K. Haldin. EROD Induction by Environmental Contaminants in Avian Embryo Livers. *Comparative Biochemistry and Physiology Part C*: 213-219, 1998.

Brunstrom, B. and J. Lund. Differences Between Chick and Turkey Embryos in Sensitivity to 3,3',4,4'-Tetrachlorobiphenyl and in Concentration/Affinity of the Hepatic Receptor for 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin. *Comparative Biochemistry and Physiology* 91C: 507-512, 1988.

Burke, J., R. Levi, Z. Guo, and E. Corey. Leukotrienes C4, D4 and E4: Effects on Human and Guinea Pig Cardiac Preparations *in vitro*. *Journal of Pharmacology and Experimental Therapeutics* 221: 235, 1982.

Canga, L., R. Levi, and A. B. Rifkind. Heart as a Target Organ in 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Toxicity: Decreased β -Adrenergic Responsiveness and Evidence of Increased Intracellular Calcium. *Proceedings of the National Academy of Science, USA* 85: 905-909, 1988.

Canga, L., L. Paroli, T. J. J. Blanck, R. Silver, and A. B. Rifkind. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Increases Cardiac Myocyte Intracellular Calcium and

- Progressively Impairs Ventricular Contractile Responses to Isoproterenol and to Calcium in Chick Embryo Hearts. *Molecular Pharmacology* 44: 1151, 1993.
- Cantieri, J., G. Graff, and N. Goldberg. Cyclic GMP Metabolism in Psoriasis: Activation of Soluble Guanylate Cyclase by Arachidonic Acid and 12-Hydroxy-5-, 8,10,14-Eicosatetraenoic Acid. *Journal of Investigative Dermatology* 74: 234, 1980.
- Cantrell, S. M., J. Joy-Schlezinger, J. J. Stegeman, D. E. Tillitt, and M. Hannink. Correlation of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-Induced Apoptotic Cell Death in the Embryonic Vasculature with Embryotoxicity. *Toxicology and Pharmacology* 148: 24-34, 1998.
- Cantrell, S. M., L. H. Lutz, D. E. Tillitt, and M. Hannink. Embryotoxicity of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD): The Embryonic Vasculature is a Physiological Target for TCDD-Induced DNA Damage and Apoptotic Cell Death in Medaka (*Orizias latipes*). *Toxicology and Applied Pharmacology* 141: 23-34, 1996.
- Chang, C. and A. Puga. Constitutive Activation of the Aromatic Hydrocarbon Receptor. *Molecular and Cellular Biology* 18: 525-535, 1998.
- Choi, E. J., M. J. Young, D. L. Toscano, W. F. Greenlee, and W. A. Toscano. *FASEB* 46: 2250, 1987.
- Custer, C. M., C. M. Custer, and M. Coffey. Organochlorine Chemicals in Tree Swallows Nesting in Pool 15 of the Upper Mississippi River. *Bulletin of Environmental Contamination and Toxicology* 64: 341-346, 2000.
- Custer, C. M., R. K. Hines, M. J. Melancon, D. J. Hoffman, J. K. Wickliffe, J. W. Bickham, J. W. Martin, and D. S. Henshel. Contaminant Concentrations and Biomarker Response in Great Blue Heron Eggs from 10 Colonies on the Upper Mississippi River, USA. *Environmental Toxicology and Chemistry* 16: 260-271, 1997.
- Dahlin, S. e. al. Leukotrienes Promote Plasma Leakage and Leukocyte Adhesion in Postcapillary Venules: *In vivo* Effects with Relevance to the Acute Inflammatory Response. *Proceedings of the National Academy of Science, USA* 78: 3887, 1981.
- Daveloose, D., H. Vezin, F. Basse, and J. Viret. Fluidity of Chicken Ventricular Plasma Membranes During Development *In ovo* and After Birth: Spin Labelling and Fluorescence Studies. *Journal of Molecular and Cellular Cardiology* 25: 1439-1444, 1993.

- De Voogt, P. and U. A. Brinkman. Production, Properties and Usage of Polychlorinated Biphenyls. In R.D.Kimbrough and A.A.Jensen, eds. Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins, and Related Products. Amsterdam, Elsevier. 1989, 3-45.
- Dickinson, K. E. and S. R. Nahorski. Atypical Characteristics of Frog and Chick Erythrocyte Beta-Adrenoceptors. *European Journal of Pharmacology* 74: 43-52, 1981.
- Donaldson, G. M., J. L. Shutt, and P. Hunter. Organochlorine Contamination in Bald Eagle Eggs and Nestlings from the Canadian Great Lakes. *Archives of Environmental Contamination and Toxicology* 36: 70-80, 1999.
- Dugan, S. G. and T. W. Moon. Cortisol Does Not Affect Hepatic α - and β -Adrenoceptor Properties in Rainbow Trout (*Oncorhynchus mykiss*). *Fish Physiology and Biochemistry* 18: 343-352, 1998.
- Elliot, J. E., S. W. Kennedy, and A. Lorenzen. Comparative Toxicity of Polychlorinated Biphenyls to Japanese Quail (*Coturnix c. japonica*) and American Kestrels (*Falco sparverius*). *Journal of Toxicology and Environmental Health* 51: 57-75, 1997.
- Elliot, J. E., R. J. Norstrom, A. Lorenzen, L. E. Hart, H. Philibert, S. W. Kennedy, J. Stegeman, G. D. Bellward, and K. M. Cheng. Biological Effects of Polychlorinated Dibenzo-*p*-dioxins, Dibenzofurans and Biphenyls in Bald Eagle (*Haliaeetus leucocephalus*) Chicks. *Environmental Toxicology and Chemistry* 15: 782-793, 1996.
- Erickson, M. D. Analytical Chemistry of PCBs. Boston, Butterworth. 1986.
- Fabbri, E., A. Capuzzo, and T. W. Moon. The Role of Circulating Catecholamines in the Regulation of Fish Metabolism: An Overview. *Comparative Biochemistry and Physiology Part C* 120: 177-192, 1998.
- Farin, E. M., T. H. Pohlman, and C. J. Omiecinski. Expression of Cytochrome P450s and Microsomal Epoxide Hydrolase in Primary Cultures of Human Umbilical Vein Endothelial Cells. *Toxicology and Applied Pharmacology* 124: 1-9, 1994.
- Fischer, P. W. and G. S. Marks. Chick Embryo Liver Cells Maintained in Serum Free Waymouth MD 705/1 Medium. *Tissue Culture Association Manual* 2: 449-452, 1976.

- Fox, G. A. What Have Biomarkers Told Us About the Effects of Contaminants on the Health of Fish-Eating Birds in the Great Lakes? The Theory and a Literature Review. *Journal of Great Lakes Research* 19: 722-736, 1993.
- Fox, L. and K. A. Grasman. Effects of PCB 126 on Primary Immune Organ Development in Chicken Embryos. *Journal of Toxicology and Environmental Health* 58: 233-244, 1999.
- Guruge, K. S., S. Tanabe, and M. Fukuda. Toxic Assessment of PCBs by the 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Equivalent in Common Cormorant (*Phalacrocorax carbo*) from Japan. *Archive of Environmental Contamination and Toxicology* 38: 209-521, 2000.
- Haenen, G., M. Veerman, and A. Bast. Reduction of β -Adrenoceptor Function by Oxidative Stress in the Heart. *Annual Reviews of Pharmacology and Toxicology* 35: 307-340, 1990.
- Hahn, M. E. The Aryl Hydrocarbon Receptor: A Comparative Perspective. *Comparative Biochemistry and Physiology Part C* 121(1-3): 23-53, 1998.
- Halbrook, R. S., R. L. Brewer, and D. A. Buehler. Ecological Risk Assessment in a Large River-Reservoir: Environmental Contaminant Accumulation and Effects in Great Blue Heron. *Environmental Toxicology and Chemistry* 18: 641-648, 1999.
- Hassan, M. Q., S. J. Stohs, and W. Murray. Comparative Ability of TCDD to Induce Lipid Peroxidation in Rats, Guinea Pigs and Syrian Golden Hamsters. *Bulletin of Environmental Contamination and Toxicology* 31: 649-657, 1983.
- Hassoun, E. A., A. C. Walter, A. Z. Alsharif, and S. J. Stohs. The Relative Abilities of TCDD and its Congeners to Induce Oxidative Stress in the Hepatic and Brain Tissues of Rats After Subchronic Exposure. *Toxicology* 145: 103-113, 2000.
- Hermansky, H., T. Holcslaw, W. J. Murray, R. Markin, and S. J. Stohs. Biochemical and Functional Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) on the Heart of Female Rats. *Toxicology and Applied Pharmacology* 95: 175-184, 1988.
- Hoffman, D. J., M. J. Melancon, J. Klein, J. D. Eisemann, and J. W. Spann. Comparative Developmental Toxicity of Planar Polychlorinated Biphenyl Congeners in Chickens, American Kestrels and Common Terns. *Environmental Toxicology and Chemistry* 17: 747-757, 1998.

- Hoffman, D. J., M. J. Melancon, J. Klein, C. P. Rice, J. D. Eisemann, R. K. Hines, J. W. Spann, and J. C. Meadow. Developmental Toxicity of PCB126 (3,3',4,4',5-Pentachlorobiphenyl) in Nestling American Kestrels (*Falco sparverius*). *Fundamental and Applied Toxicology* 34: 188-200, 1996.
- Hoffman, D. J., Rice, C. P., Melancon, M. J., Klein, P. N., Eisemann, J. D., and Hines, R. K. Developmental Toxicity of Planar PCB Congeners in Nestling American Kestrels. Society of Environmental and Toxicology and Chemistry . Abstract 1993.
- Houslay, M. D. Insulin, Glucagon and the Receptor-Mediated Control of Cyclic AMP Concentrations in Liver. *Biochem.Soc.Trans.* 14: 183-193, 1986.
- Hsu, C. C., T.-C. Chen, W. T. Soong, C.-C. Tsung, S.-J. Sue, C.-Y. Lui, and et al. A Six-Year Follow Up Study of Intellectual and Behavioural Development of Yu-Cheng Children: Cross-Sectional Findings of the First Field Work. *Chinese Psychology* 2: 40, 1988.
- Jin, X., Kennedy, S. W., Di Muccio, T., and Moon, T. W. Role of Oxidative Stress and Antioxidant Defense in 3,3',4,4',5-Pentachlorobiphenyl-Induced Toxicity and Species-Differential Sensitivity in Chicken and Duck Embryos. In press. 2001.
- Jones, J. M. Indicators of Exposure to Polycyclic Aromatic Hydrocarbons (PAH) in Populations of Brown Bullhead Catfish (*Ameiurus nebulosus*). 1996. Thesis. Ohio State University, Columbus, OH, USA.
- Jones, P. B., L. Durren, D. R. Galeazzi, and J. P. Jr. Whitlock. Control of Cytochrome P₁-450 Gene Expression: Analysis of a Dioxin-Responsive Enhancer System. *Proceedings of the National Academy of Science, USA* 83: 2802-2806, 1986.
- Karmazyn, M. and M. Moffat. Toxic Properties of Arachidonic Acid on Normal, Ischemic and Reperfused Hearts: Indirect Evidence for Free Radical Involvement. *Prost.Leuk.Med.* 17: 251-264, 1985.
- Kehrer, J. P. Free Radicals as Mediators of Tissue Injury and Disease. *Critical Reviews in Toxicology* 23: 21-48, 1993.

- Kelley, E. E., G. R. Buettner, and C. P. Burns. Relative Alpha-Tocopherol Deficiency in Cultured Cells: Free Radical-Mediated Lipid Peroxidation, Lipid Oxidizability, and Cellular Polyunsaturated Fatty Acid Content. *Archives of Biochemistry and Biophysics* 319: 102-109, 1995.
- Kelly, S. A., C. M. Havrilla, T. C. Brady, K. H. Abramo, and E. D. Levin. Oxidative Stress in Toxicology: Established Mammalian and Emerging Piscine Model Systems. *Environmental Health Perspectives* 106: 375-384, 1998.
- Kennedy, S. W., S. P. Jones, and L. Bastien. Efficient Analysis of Cytochrome P4501A Catalytic Activity, Porphyrins, and Total Proteins in Chicken Embryo Hepatocyte Cultures with a Fluorescence Plate Reader. *Analytical Biochemistry* 226: 362-370, 1995.
- Kennedy, S. W., A. Lorenzen, C. A. James, and B. T. Collins. Ethoxyresorufin-O-deethylase and Porphyrin Analysis in Chicken Embryo Hepatocyte Cultures with a Fluorescence Multiwell Plate Reader. *Analytical Biochemistry* 211: 102-112, 1993.
- Kennedy, S. W., A. Lorenzen, S. P. Jones, M. E. Hahn, and J. J. Stegeman. Cytochrome P4501A Induction in Avian Hepatocyte Cultures: A Promising Approach for Predicting the Sensitivity of Avian Species to Toxic Effects of Halogenated Aromatic Hydrocarbons. *Toxicology and Applied Pharmacology* 141: 214-230, 1996.
- Kimbrough, R. D. and A. A. Jensen. Halogenated Biphenyls, Terphenyls, Napthalenes, Dibenzodioxins and Related Products. Amsterdam, Elsevier. 1989.
- Kuratsune, M., T. Yoshimara, J. Matsuzaka, and A. Yamaguchi. Yusho, A Poisoning Caused by Rice Oil Contaminated with Polychlorinated Biphenyls. *HSMHA Health Report* 86: 1083-1091, 1971.
- Landers, P. L. and N. J. Bunce. The Ah Receptor and the Mechanism of Dioxin Toxicity. *Journal of Biochemical Toxicology* 276: 273-287, 1991.
- Lorenzen, A., T. W. Moon, S. W. Kennedy, and G. A. Fox. Relationships Between Environmental Organochlorine Contaminant Residues, Plasma Corticosterone Concentrations and Intermediary Metabolic Enzyme Activities in Great Lakes Herring Gull Embryos. *Environmental Health Perspectives* 107: 179-186, 1999.
- Lowry, O. H., N. J. Rosebrough, A. J. Farr, and R. J. Randall. Protein Measurement With the Folin-Phenol Reagent. *Journal of Biological Chemistry* 193: 265-275, 1951.

- Macek, K. M. and S. Korn. Significance of the Food Chain in DDT Accumulation by Fish. *Journal of Fisheries Research Board of Canada* 27: 1496-1498, 1970.
- Mazeaud, M. and F. Mazeaud. Adrenergic Responses to Stress in Fish. In Pickering, A. D., ed. *Stress and Fish*. London, UK, Academic. 1981, 49-75.
- Mohammadpour, H., W. Murray, and S. J. Stohs. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-Induced Lipid Peroxidation in Genetically Responsive and Non-Responsive Mice. *Archives of Environmental Contamination and Toxicology* 17: 645-650, 1988.
- Munson, P. F. and D. Rodbard. LIGAND: A Versatile Computerized Approach for Characterization of Ligand-Binding Systems. *Analytical Biochemistry* 107: 220-239, 1980.
- Murk, A. J., A. T. Bosveld, M. Van den Berg, and A. Brouwer. Effects of Polyhalogenated Aromatic Hydrocarbons (PHAHs) on Biochemical Parameters in Chicks of the Common Tern (*Sterna hirundo*). *Aquatic Toxicology* 30: 91-115, 1994.
- Murk, A. J., T. J. Boudewijn, P. L. Meininger, A. T. Bosveld, G. Rossaert, T. Ysebaert, P. Meire, and S. Dirksen. Effects of Polyhalogenated Aromatic Hydrocarbons and Related Contaminants on Common Tern Reproduction: Integration of Biological, Biochemical and Chemical Data. *Archives of Environmental Contamination and Toxicology* 31: 128-140, 1996.
- Nebert, D. W. and F. H. Gonzalez. P450 Genes: Structure, Evolution, and Regulation. *Annual Reviews of Biochemistry* 56: 945-993, 1987.
- Nelson, D. R., L. Koyman, T. Kamataki, J. J. Stegeman, R. Feyereisen, D. J. Waxman, N. R. Waterman, O. Gotoh, M. J. Coon, R. W. Estabrook, I. C. Gunsalus, and D. W. Nebert. P450 Superfamily: Update on New Sequences, Gene Mapping, Accession Numbers and Nomenclature. *Pharmacogenetics* 6: 1-42, 1996.
- Neuhold, L. A., Y. Shirayoshi, K. Ozato, J. E. Jones, and D. W. Nebert. Regulation of Mouse CYP1A-1 Gene Expression by Dioxin: Requirement of Two *Cis*-Acting Elements During Induction. *Molecular and Cellular Biology* 9: 2378-2386, 1989.
- Okey, A. B., L. M. Vella, and P. A. Harper. Detection and Characterization of Low Affinity Forms of Cytosolic AH Receptors in the Livers of Non-responsive Mice to Induction of Cytochrome P450 by 3-Methylcholanthrene. *Molecular Pharmacology* 35: 823-830, 1989.

- Olivero, J. and P. E. Ganey. Role of Protein Phosphorylation in Activation of Phospholipase A₂ by the Polychlorinated Biphenyl Mixture Aroclor 1242. *Toxicology and Applied Pharmacology* 163: 9-16, 2000.
- Otto, D. M. E. and T. W. Moon. Phase I and II Enzymes and Antioxidant Responses in Different Tissues of Brown Bullheads From Relatively Polluted and Non-Polluted Systems. *Archives of Environmental Contamination and Toxicology* 31: 147, 1996.
- Palace, V. P., J. F. Klaverkamp, W. L. Lockhart, D. A. Metner, D. Muir, and S. B. Brown. Mixed-Function Oxidase Enzyme Activity and Oxidative Stress in Lake Trout (*Salvelinus namaycush*) Exposed to 3,3',4,4',5-Pentachlorobiphenyl (PCB 126). *Environmental Toxicology and Chemistry* 15: 955-960, 1996.
- Persad, S., H. Rupp, R. Jindal, J. Arneja, and N. Dhalla. Modification of Cardiac Beta-Adrenoceptor Mechanisms by H₂O₂. *American Journal of Physiology* 274: H416-H423, 1998.
- Peters, R. Lateral Mobility of Proteins and Lipids in the Red Cell Membrane and the Activation of Adenylate Cyclase by β -Adrenergic Receptors. *FEBS Letters* 243: 1-7, 1988.
- Peterson, R., H. Theobald, and G. Kimmel. Developmental and Reproductive Toxicity of Dioxins and Related Compounds: Cross-Species Comparisons. *Critical Reviews in Toxicology* 23: 283-335, 1993.
- Pinowski, J., A. Niedwiejadowska, Z. Juricova, I. Literak, and J. Romanowski. Chlorinated Aromatic Hydrocarbons in the Brains and Lipids of Sparrows (*Passer domesticus* and *Passer montanus*) from Rural and Suburban Areas Near Warsaw. *Bulletin of Environmental Contamination and Toxicology* 63: 736-743, 1999.
- Poellinger, L., M. Whitlaw, I. Pongratz, A. Wilhelmsson, K. Gradin, and A. Berghard. The Dioxin Receptor: A Ligand-Activated Gene Regulatory Protein. *Pharmacol.Skin.Basel.Karger* 5: 103-111, 1993.
- Poland, A. and E. Glover. Genetic Expression of Aryl Hydrocarbon Hydroxylase by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: Evidence for a Receptor Mutation in Genetically Non-Responsive Mice. *Molecular Pharmacology* 11: 389-398, 1975.

- Poland, A. and E. Glover. Characterization and Strain Distribution Pattern of the Murine AH Receptor Specified by the AHd and AHb-3 Alleles. *Molecular Pharmacology* 38: 3306-3312, 1990.
- Poland, A. and J. C. Knutson. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and Related Halogenated Aromatic Hydrocarbons: Examination of the Mechanism of Toxicity. *Annual Reviews of Pharmacology and Toxicology* 22: 517-554, 1982.
- Port, J. D., C. C. Debellis, J. Klein, G. A. Peeters, W. H. Barry, and M. R. Bristow. Pharmacological Characterization of Chick and Frog *Beta* Adrenergic Receptors in Primary Cultures of Myocardial Cells. *Journal of Pharmacology and Experimental Therapeutics* 262: 217-224, 1992.
- Powell, D. C., R. J. Aulerich, J. C. Meadow, D. E. Powell, J. P. Giesy, K. L. Stromberg, and S. J. Bursian. Effects of 3,3',4,4',5-Pentachlorobiphenyl (PCB126) and TCDD Injected into the yolks of chicken eggs prior to incubation. *Archive of Environmental Contamination and Toxicology* 31: 404-409, 1996.
- Powell, D. C., R. J. Aulerich, K. L. Stromberg, and S. J. Bursian. Effects of 3,3',4,4'-Tetrachlorobiphenyl, 2,3,3',4,4'-Pentachlorobiphenyl, and 3,3',4,4',5-Pentachlorobiphenyl on the developing chicken embryo when injected prior to incubation. *Journal of Toxicology and Environmental Health* 49: 319-338, 1996.
- Quilley, C. P. and A. B. Rifkind. Prostaglandin Release by the Chick Embryo Heart is Increased by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and by Other Cytochrome P448 Inducers. *Biochemical and Biophysical Research Communications* 136: 582-589, 1986.
- Rattner, B. A., J. S. Hatfield, M. J. Melancon, C. M. Custer, and D. E. Tillitt. Relation Among Cytochrome P450, AH-Active PCB Congeners and Dioxin Equivalents in Pipping Black-Crowned Night-Heron Embryos. *Environmental Toxicology and Chemistry* 13: 1805-1812, 1994.
- Rattner, B. A., D. J. Hoffman, M. J. Melancon, G. H. Olsen, S. R. Schmidt, and R. J. Aulerich. Organochlorine and Metal Contaminant Exposure and Effects in Hatching Black-Crowned Night Herons (*Nycticorax nycticorax*) in Delaware Bay. *Archive of Environmental Contamination and Toxicology* 39: 38-45, 2000.
- Rattner, B. A., M. J. Melancon, C. P. Rice, W. Riley Jr., J. D. Eisemann, and R. K. Hines. Cytochrome P450 and Organochlorine Contaminants in Black-Crowned Night-Herons from the Chesapeake Bay Region, USA. *Environmental Toxicology and Chemistry* 16: 2315-2322, 1997.

- Reithmann, C., A. Thomschke, and K. Werdan. The Role of Endogenous Noradrenaline in the Beta-Blocker Withdrawal Phenomenon—Studies with Cultured Heart Cells. *Klin Wochenschr* 65: 308-316, 1987.
- Reyes, H., S. Reiz-Porszasz, and O. Hankinson. Identification of the Ah Receptor Nuclear Translocator Protein (Arnt) as a Component of the DNA Binding Form of the Ah Receptor. *Science* 256: 1193-1195, 1992.
- Rhoades, R. and R. Pflanzer. Human Physiology. Orlando, Saunders College Publishing. 1996, 436-439.
- Rifkind, A. B., M. Gannon, and S. S. Gross. Arachidonic Acid Metabolism by Dioxin-Induced Cytochrome P-450: A New Hypothesis on the Role of P-450 in Dioxin Toxicity. *Biochemical and Biophysical Research Communications* 172: 1180-1188, 1990.
- Rifkind, A. B., Y. Hattori, R. Levi, M. J. Hughes, C. P. Quilley, and D. R. Alsonso. The Chick Embryo as a Model for PCB and Dioxin Toxicity: Evidence of Cardiotoxicity and Increased Prostaglandin Synthesis. In A. Poland and R.D. Kimbrough, eds. Biological Mechanisms of Dioxin Action. Cold Spring Harbor Laboratory. 1984, 255-266.
- Rifkind, A. B., A. Kanetoshi, J. Orlinick, J. Capdevilla, and C. A. Lee. Purification and Biochemical Characterization of Two Major Cytochrome P-450 Isoforms Induced by 2,3,7,8-Tetrachlorodibenzo-p-dioxin in Chick Embryo Liver. *Journal of Biological Chemistry* 5: 3387-3396, 1994.
- Riviere, J. and F. Cabanne. Animal and Plant Cytochrome P-450 Systems. *Biochimie* 69: 743-752, 1987.
- Rogan, W. J. and B. C. Gladen. Neurotoxicology of PCBs and Related Compounds. *Neurotoxicology* 13: 27-36, 2000.
- Russel, R. W., F. A. Gobas, and G. D. Haffner. Maternal Transfer and *in ovo* Exposure of Organochlorines in Oviparous Organisms: A Model and Field Verification. *Environmental Science and Technology* 33: 416-420, 1999.
- Safe, S. H. Polychlorinated Biphenyls (PCBs): Environmental Impact, Biochemical and Toxic Responses and Implications for Risk Assessment. *Critical Reviews in Toxicology* 24: 87-149, 1994.

- Sanderson, J. T. and G. D. Bellward. Hepatic Microsomal Ethoxyresorufin-*O*-Deethylase-Inducing Potency *in ovo* and Cytosolic Ah Receptor Binding Affinity of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin: Comparison of 4 Avian Species. *Toxicology and Applied Pharmacology* 132: 131-145, 1995.
- Schwartz, T. R. and D. L. Stalling. Chemometric Comparison of Polychlorinated Biphenyl Residues and Toxicologically Active Polychlorinated Biphenyl Congeners in the Eggs of Forster's Terns (*Sterna forsteri*). *Archives of Environmental Contamination and Toxicology* 20: 183-199, 1991.
- Scott, M. D. and J. W. Eaton. Markers of Free-Radical-Mediated Tissue Injury: Tales of Caution and Woe. In Wallace, K. B., ed. *Free Radical Toxicology*. Taylor & Francis. 1997, 401-420.
- Sinclair, P. R., N. Gorman, H. S. Walton, J. F. Sinclair, C. A. Lee, and A. B. Rifkind. Identification of CYP1A5 as the CYP1A Enzyme Mainly Responsible for Uroporphyrinogen Oxidation Induced by AH Receptor Ligands in Chicken Liver and Kidney. *Drug Metabolism and Disposition* 25: 779-783, 1997.
- Steevens, J. A., P. C. Baumann, and S. B. Jones. A Comparison of β -Adrenoceptors and Muscarinic Cholinergic Receptors in Tissues of Brown Bullhead Catfish (*Ameiurus nebulosus*) From the Black River and Old Woman Creek, Ohio. *Environmental Toxicology and Chemistry* 15: 1551-1554, 1996.
- Stevens, L. *Avian Biochemistry and Molecular Biology*. Cambridge, Great Britain, University Press. 1996, 111-113.
- Stohs, S. J. Oxidative Stress Induced by 2,3,7,8-Tetrachlorobibenzo-*p*-dioxin (TCDD). *Free Radical Biology and Medicine* 9: 79-90, 1990.
- Stohs, S. J., Z. A. F. Al-Bayati, M. Hassan, W. Murray, and H. Mohammadpour. Glutathione Peroxidase and Reactive Oxygen Species in TCDD-Induced Lipid Peroxidation. *Advances in Experimental Biology* 197: 357-365, 1986.
- Stohs, S. J., M. Q. Hassan, and W. J. Murray. Induction of Lipid Peroxidation and Inhibition of Glutathione Peroxidase by TCDD. 241-252, 2000.
- Stohs, S. J., M. A. Shara, N. Z. Alsharif, Z. Z. Wahba, and Z. A. F. Al-Bayati. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-Induced Oxidative Stress in Female Rats. *Toxicology and Applied Pharmacology* 1990: 126-135, 1990.

- Surai, P. F., R. C. Noble, and B. K. Speake. Relationship Between Vitamin E Content and Susceptibility to Lipid Peroxidation in Tissues of the Newly Hatched Chick. *British Poultry Science* 40: 406-410, 1999.
- Surai, P. F., N. H. Sparks, and R. C. Noble. Antioxidant Systems of the Avian Embryo: Tissue-Specific Accumulation and Distribution of Vitamin E in the Turkey Embryo During Development. *British Poultry Science* 40: 458-466, 1999.
- Tanabe, S. PCB Problems in the Future: Foresight from Current Knowledge. *Environmental Pollution* 50: 5-28, 1988.
- Toborek, M., S. W. Barger, M. P. Mattson, P. Espandiari, L. W. Robertson, and B. Hennig. Exposure to Polychlorinated Biphenyls Causes Endothelial Cell Dysfunction. *Journal of Biochemical Toxicology* 10: 219-226, 1995.
- Uchiyama, M. and M. Mihara. Determination of Malonaldehyde Precursor in Tissues by Thiobarbituric Acid Test. *Analytical Biochemistry* 86: 271-278, 1978.
- Walker, C. H. Avian Forms of Cytochrome P450. *Comparative Biochemistry and Physiology Part C* 121: 65-72, 1998.
- Weber, L. W., M. Lebofsky, B. U. Stahl, J. R. Gorski, G. Muzi, and K. Rozman. Reduced Activities of Key Enzymes of Gluconeogenesis as Possible Cause of Acute Toxicity of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) in Rats. *Toxicology* 66: 133-144, 1991.
- Wells, P. G., P. M. Kim, R. R. Laposa, C. J. Nicol, T. Parman, and L. M. Winn. Oxidative Damage in Chemical Teratogenesis. *Mutation Research* 396: 65-78, 1997.
- Whitlock, J. P. Jr. The Control of Cytochrome P450 Gene Expression by Dioxin. *TIPS* 10: 285-288, 1989.
- Whitlock, J. P. Jr., M. S. Denison, J. M. Fisher, and E. S. Shen. Induction of Hepatic Cytochrome P450 Gene Expression by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin. *Molecular Biology in Medicine* 6: 169-178, 1989.
- Yarden, Y., H. Rodriguez, S. K. F. Wong, D. R. Brandt, D. C. May, J. Burnier, R. N. Harkins, E. Y. Chen, J. Ramachandran, A. Ullrich, and E. M. Ross. The Avian β -Adrenergic Receptor: Primary Structure and Membrane Topology. *Proceedings of the National Academy of Science, USA* 83: 6795-6799, 1986.

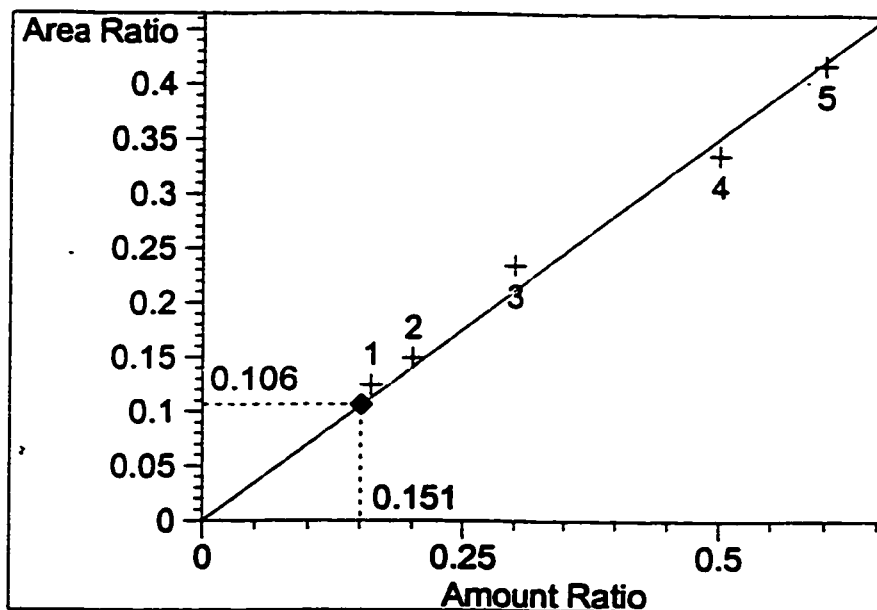
Zhao, F., K. Mayura, N. Kocurek, J. F. Edwards, L. F. Kubena, S. H. Safe, and T. D. Phillips. Inhibition of 3,3',4,4',5-Pentachlorobiphenyl-Induced Chicken Embryotoxicity by 2,2',4,4',5,5'-Hexachlorobiphenyl. *Fundamental and Applied Toxicology* 35: 1-8, 1997.

APPENDIX

Figure A-1. Sample GC calibration curve for PCB126. Standard concentrations were 0.8, 1.0, 1.5, 2.5 and 3.0ng/mL. A sample injection volume of 1 μ L was used. $R^2=0.99866$.

Figure A-2. Sample GC calibration curve for internal standard Mirex. Mirex (5ng/mL) is added to all samples prior to GC analysis to measure variations between injections and evaporative losses prior to injection. A sample injection volume of 1 μ L was used.

A-1.



A-2.

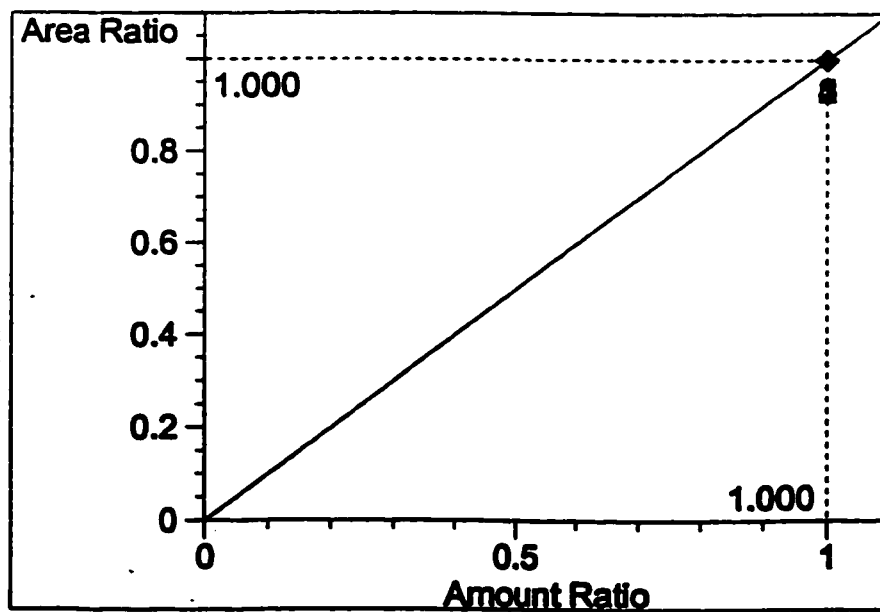


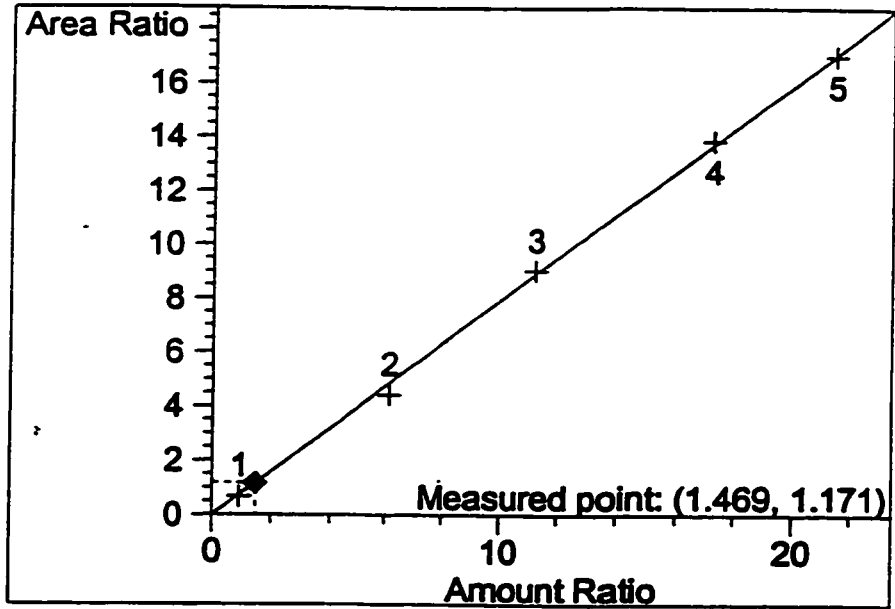
Figure A-3. Sample GC calibration curve for internal standard PCB204.

Standard concentrations were 5.1, 30.6, 56.1, 86.1 and 107.1ng/mL. An injection volume of 1 μ L was used. $R^2=0.99928$.

Figure A-4. Sample GC calibration curve for internal standard PCB30.

Standard concentrations were 5.5, 33.0, 60.5, 88.0, 115.5ng/mL. An injection volume of 1 μ L was used. $R^2=0.99980$.

A-3.



A-4.

