PHD

Biochemical effects of polychlorinated biphenyls with reference to transfer in breast milk

Duncan, Ian W.

Award date:
1988

Awarding institution:
University of Bath

Link to publication

Alternative formats
If you require this document in an alternative format, please contact:
openaccess@bath.ac.uk

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 10. Jan. 2021
BIOCHEMICAL EFFECTS OF POLYCHLORINATED BIPHENYLS

WITH REFERENCE TO

TRANSFER IN BREAST MILK

Submitted by IAN W. DUNCAN B.Sc., M.Sc.

for the degree of Ph.D.

of the University of Bath

1988

Copyright

Attention is drawn to the fact that copyright of this thesis rests with its author. This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author.

This thesis may be made available for consultation within the University Library and may be photocopied or lent to other libraries for the purposes of consultation.

SIGNED: Ian W. Duncan
Dedication

To my mother and father, in appreciation of their
support and understanding
CONTENTS

Page No.

Acknowledgements I
Abbreviations II
Summary III

CHAPTER ONE: INTRODUCTION

1.1 Commercial Importance of PCBs, and the Beginning of the Environmental Problem 1

1.2 Composition of Commercial PCBs and the Complexity of the Problem 4

1.3 Occurrence of PCBs 7

1.4 Toxicity

1.4.1 Acute Toxicity 9
1.4.2 Chronic Toxicity 10

1.5 Environmental Effects of PCBs and Evaluation of the Hazards 14

1.6 Transfer of PCBs from Mother to Young 16

1.7 Disposition in Animals

1.7.1 Effects of Congener Structure 18
1.7.2 Effects of Pregnancy and Lactation 20

1.8 Enzyme Induction

1.8.1 The Hepatic Mixed-Function Oxidase System and Cytochrome P-450 22
1.8.2 Effects of Enzyme Inducers 23
1.8.3 Single PCB Congeners as Enzyme Inducers 26
1.8.4 Relationship between Enzyme Induction and Toxicity 27

1.9 Objectives of this Study 28

CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials 31

2.2 Synthesis and Purification of PCB Congeners

2.2.1 General Procedure 32
2.2.2 Instrumentation 35
| 2.2.3 | Purification of PCB 156 | 36 |
| 2.2.4 | Purification of PCBs 157 and 169 | 40 |
| 2.2.5 | Purification of PCB 189 | 44 |
| 2.2.6 | Purification of PCBs 97 and 118 | 46 |

### 2.3 Animals

### 2.4 Dosing

2.4.1 AROCLOR 1254 Maternal Dosing Regimens 50
2.4.2 Maternal Dosing of Rats with PCB Congeners 51
2.4.3 Co-administration of PCBs 97 and 169 to Rats 52
2.4.4 Dosing of Immature Rats with PCB Congeners (Organ Weight Study) 54
2.4.5 PCB 169 Administration to Mice (Neurotoxicity Study) 55
2.4.6 Topical Administration of PCB Congeners to Mice (Dermal Study) 55

### 2.5 Biological Measurements

2.5.1 AROCLOR 1254 Maternal Dosing Regimens 56
2.5.2 Maternal Dosing of Rats with PCB Congeners 57
2.5.3 Co-Administration of PCBs 97 and 169 to Rats 57
2.5.4 Dosing of Immature Rats with PCB Congeners (Organ Weight Study) 57
2.5.5 PCB 169 Administration to Mice (Neurotoxicity Study) 58
2.5.6 Topical Administration of PCB Congeners to Mice (Dermal Study) 58

### 2.6 Biochemical Assays

2.6.1 Preparation of Microsome Suspensions 58
2.6.2 Protein Determinations 60
2.6.3 Cytochrome P-450 Concentration 61
2.6.4 Aniline Hydroxylase Activity 63
2.6.5 Aminopyrine-N-Demethylase Activity 67

**CHAPTER THREE: IDENTITY, PURITY AND YIELDS OF PCB CONGENERS**

**RESULTS AND DISCUSSION**

3.1 PCB Yields and Purities 73
3.2 PCB 156 75
3.3 PCB 157 78
3.4 PCB 169 81
3.5 PCB 189 84
3.6 PCB 118 87
3.7 PCB 97 90
3.8 Discussion

CHAPTER FOUR: PCB EFFECTS IN PUPS AFTER DIFFERENT MATERNAL DOSING REGIMENS: RESULTS AND DISCUSSION

4.1 Litter Size
4.2 Litter Weight
4.3 Pup Weight
4.4 Pup Survival
4.5 Organ Weights
4.6 Liver Microsome Protein Concentration
4.7 Aniline Hydroxylase Activity
4.8 Aminopyrine-N-Demethylase Activity
4.9 Discussion

CHAPTER FIVE: PCB EFFECTS IN PUPS AFTER MATERNAL DOSING WITH DIFFERENT CONGENERS: RESULTS AND DISCUSSION

5.1 Litter Size
5.2 Litter Weight
5.3 Pup Weight
5.4 Pup Survival
5.5 Liver Weight
5.6 Liver Microsome Protein Concentration
5.7 Liver Cytochrome P-450 Concentration
5.8 Liver Cytochrome P-450 Absorption Maximum
5.9 Liver Aniline Hydroxylase Activity
5.10 Liver Aminopyrine-N-Demethylase Activity
5.11 Discussion

5.11.1 Litter Size and Pup Weight
5.11.2 Liver Parameters
5.11.3 PCB Effects in Second Litters
5.11.4 Sex Differences in Response to PCBs
5.11.5 Effect of Contaminants in the PCB Congeners
CHAPTER SIX: CO-ADMINISTRATION OF PCBs 97 AND 169
RESULTS AND DISCUSSION

6.1 Study 1: Oral Dosing
6.2 Study 2: Intraperitoneal Dosing
6.3 Discussion

CHAPTER SEVEN: PHARMACODYNAMIC STUDIES: RESULTS AND DISCUSSION

7.1 Organ Weight Study
7.2 Dermal Toxicity Study
7.3 Neurotoxicity Study
7.4 Discussion
  7.4.1 Organ Weight Study
  7.4.2 Dermal Toxicity Study
  7.4.3 Neurotoxicity Study

CHAPTER EIGHT: GENERAL DISCUSSION

8.1 Summary of Main Results
8.2 PCB Mode of Action: Receptor Binding
8.3 Structure-Activity Relationships
  8.3.1 Correlations between Receptor Binding, Toxicity and Enzyme Induction
  8.3.2 QSAR: Metabolism
  8.3.3 QSAR: Enzyme Induction
8.4 Relative Toxicities of the Congeners Used
8.5 Induction Patterns seen in this Study
8.6 Is Breast-Feeding Hazardous?
8.7 Criticisms
8.8 Conclusions
8.9 Recommendations for Future Work

REFERENCES

APPENDICES 1-9
ACKNOWLEDGEMENTS

I wish to express my thanks to my supervisors, Dr. L.J. Notarianni and Dr. T.M. Jefferies for their advice and encouragement throughout this project. I also gratefully acknowledge the financial support of the Science and Engineering Research Council and the use of facilities in the School of Pharmacy and Pharmacology.

The technical support provided by the Animal House staff and the technicians of the Pharmacology Department and the photographic expertise of Mr. R. Sadler were invaluable. Thanks are due to Dr. T. Macleod, Consultant Pathologist at the Royal United Hospital, Bath, for undertaking the processing of tissues for histological examination.

The interesting discussions provided by Dr. M.P. Seymour as a fellow postgraduate student were much appreciated.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHH</td>
<td>aryl hydrocarbon hydroxylase</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate</td>
</tr>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>MC</td>
<td>3-methylcholanthrene</td>
</tr>
<tr>
<td>mcg</td>
<td>microgram</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PB</td>
<td>phenobarbitone</td>
</tr>
<tr>
<td>PCB</td>
<td>polychlorinated biphenyl</td>
</tr>
<tr>
<td>PCDF</td>
<td>polychlorinated dibenzofuran</td>
</tr>
<tr>
<td>QSAR</td>
<td>quantitative structure-activity relationships</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TCDF</td>
<td>2,3,7,8-tetrachlorodibenzofuran</td>
</tr>
</tbody>
</table>
SUMMARY

Amongst the 209 possible PCB congeners a large number are environmental contaminants and are transferred to suckled offspring in milk. As congeners vary in potency the toxicological hazard posed to human breast-fed infants is difficult to assess. The aim of this study is to compare the toxicity of selected PCB congeners administered to female rats by measuring PCB effects in their suckled pups after transfer in milk.

Due to the difficulty and cost of obtaining single PCB congeners commercially, six congeners (PCBs 97, 118, 156, 157, 169 and 189) were synthesized by a reported method and purified by a novel process involving preparative HPLC. Their identities and purities were established using NMR, mass spectroscopy and gas chromatography.

The optimal timing of maternal dosing, in order to detect effects in 21-day old suckled pups, was investigated using AROCLOR 1254, a commercial PCB mixture. The magnitude of PCB effects on litter size, pup weight, liver:body ratio, liver microsomal protein concentration, and the activities of liver aniline hydroxylase and aminopyrine demethylase was unchanged when dams were exposed to PCBs either before mating, during gestation or during lactation. No sex differences were observed.

The synthesized PCB congeners were then administered to lactating rats. Their effects on the parameters listed above and on the
cytochrome P-450 concentration in liver microsomes were measured and compared in the 21-day old suckled pups. The decreasing order of potency was PCB 169 > PCB 157 > PCB 118 > PCB 189 > PCB 97. The effects of PCBs 169 and 157 were detected in second litters suckled by the same dams without further PCB exposure. No sex differences were observed, apart from a foetotoxic effect of PCB 169 in second litters. The results are discussed in the light of current PCB structure-activity theory.

Rats were also dosed directly with both PCBs 169 and 97. No synergism was detected in their combined effects on liver parameters at the doses used.

The rank order of potency indicated by the congener study was compared to that suggested by dermal toxicity tests carried out with PCBs 157, 189 and 97 applied directly to the skin of hairless mice and by the effects of the congeners on organ weights in weanling rats dosed directly. These tests confirmed the general order of potency already indicated.
CHAPTER ONE

INTRODUCTION

1.1 The Commercial Importance of Polychlorinated Biphenyls, and the Beginning of the Environmental Problem

Polychlorinated biphenyls (PCBs) have been manufactured in the United States since the 1920s by the Monsanto Chemical Co., St. Louis, Missouri and marketed under the trade name AROCLOR. This group of compounds has been of great industrial importance, being ideal for use in a variety of technically demanding situations. They are extremely stable both thermally and chemically, have a low flammability and water solubility, and a high electrical resistivity. Consequently they have been widely used in closed systems such as capacitors, transformers, and heat transfer fluids, and in open systems as plasticisers and adhesives. In post-war years they have been in demand for moisture proofing and sealing, and for use in synthetic resins, rubbers, paints and varnish (1).

Other companies have manufactured and marketed PCBs over the past few decades: in Japan under the trade name of KANECHLOR, in the USSR as SOVOL, in Italy as FENCLOR, in Germany as CLOHEN and in France as PHENOCLOR and PYRALENE. Over the sixty-odd years since PCB production first began on an industrial scale in the United States, many millions of pounds of these chemicals have been produced and distributed world-wide. The history of their production and use is
the subject of an interesting review by Brinkman and De Kok (2).

Mass environmental damage due to man's uncontrolled activities has become a feature of the twentieth century. An outstanding example of this is the problems created by the widespread use of pesticides, an issue which was brought sharply into focus in 1962 by Rachel Carson's book Silent Spring (3) in which she states:

"The most alarming of all man's assaults upon the environment is the contamination of air, earth, rivers, and sea with dangerous and even lethal materials. This pollution is for the most part irrecoverable."

The concern aroused by Carson's analysis of the pesticide problem was soon overshadowed by similar concern over PCBs, which in the opinion of Brinkman and De Kok (2) "attained the dubious honour of surpassing the chlorinated insecticides as the most talked-about organochlorine pollutants."

In the late 1960s two events occurred which were to introduce the era of concern over PCBs. The first was in 1966 when a Swedish scientist reported the presence of previously undetected substances in extracts of wildlife samples prepared for gas chromatographic analysis. These substances were identified as PCBs (4). The second was in 1968 in Japan when hundreds of people were poisoned by cooking oil which had been contaminated with KANECHLOR 400, a commercial PCB product. This catastrophe became known as the "Yusho" (rice oil) incident, and the victims experienced neurological, dermatological, reproductive, immunological and hepatic abnormalities which came to be known as the symptoms of "Yusho" (5). These two events aroused concern over the safety of PCBs in the environment, and since then the number of scientific reports on the
occurrence, chemical analysis and toxicology of the PCBs has proliferated enormously. A great deal of progress has been made towards understanding, but not necessarily solving, the problems they appear to pose. It is now clear that PCBs have spread to almost every corner of the globe, having been detected in the tissues of a multitude of animal and plant species as well as in soil and in the sediments of rivers, lakes and oceans. The distribution of PCBs has been reviewed by various authors (6,7,8).

Following the disturbing events of the late 1960s, Monsanto (the principal manufacturer) ceased PCB production for virtually all uses except in closed systems. As had been the case in the production of these chemicals, the United States took the lead in subsequent attempts to control their use. In 1976 Congress passed the Toxic Substances Control Act which controls the use of PCBs. In 1977 the manufacture and distribution of PCBs except in totally enclosed systems was banned. The American attempts to tackle the problem and the responses of various interests and pressure groups have been discussed by Miller (9).

Attention has also been attracted by the polybrominated biphenyls, the brominated analogues of the PCBs. These caused concern when they entered the food chain in contaminated cattle feed in the United States. Used mainly as fire retardants, they were less widely used than the PCBs but elicit many of the toxic effects typical of the halogenated hydrocarbons (10).
1.2 Composition of Commercial PCBs and the Complexity of the Problem

Figure 1.1 shows the general reaction used in the manufacture of PCBs, a process which is based on the catalytic chlorination of biphenyl (5).

Figure 1.1. Reaction used in the manufacture of PCBs by the catalytic chlorination of biphenyl.

The reaction gives rise to a mixture of PCB congeners, each differing in the number or position of chlorine atoms on the rings. The numbering system normally used for PCBs is shown in figure 1.2.

Figure 1.2. Numbering system commonly used to describe PCB congeners.

Numbers are written in ascending order, e.g. 2,3,3',4,4',5-
hexachlorobiphenyl. There are 209 possible PCB congeners. Their identification has been simplified by Ballschmiter and Zell (11) who proposed a numbering system in which each congener is identified by a number between 1 and 209, in increasing order of chlorination. For example, 2,3,3',4,4',5-hexachlorobiphenyl is PCB 156. By varying the manufacturing conditions the proportions of the different congeners present can be varied to yield mixtures with correspondingly different chemical properties. Consequently the total chlorine content also varies and this is reflected, in the case of the AROCLORS, in the product name. AROCLOR 1254 for example is based on the 12-carbon biphenyl nucleus and contains 54% by weight of chlorine. The composition of some of the different AROCLOR products is shown in table 1.1, the data in which should be regarded as approximate because conflicting reports have appeared in the literature (2).

Table 1.1. Composition and chlorine content of some commonly used AROCLOR products. Data from Hutzinger et al.(12).

<table>
<thead>
<tr>
<th>AROCLOR</th>
<th>Chlorine Composition %w/w of each Congener Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%w/w     mono  di  tri  tetra  penta  hexa  hepta  octa  nona</td>
</tr>
<tr>
<td>1242</td>
<td>42       3     13    28    30    22    4</td>
</tr>
<tr>
<td>1248</td>
<td>48       2     18    40    36    4</td>
</tr>
<tr>
<td>1254</td>
<td>54       11    49    34    6</td>
</tr>
<tr>
<td>1260</td>
<td>60       12    38    41    8    1</td>
</tr>
</tbody>
</table>
The complex composition of the commercial PCBs has far-reaching analytical and toxicological implications. There is considerable variation between the different congeners in toxic potency, and distinct structure-activity relationships have been described. These are discussed in chapter eight. The variation in toxicity between different congeners has meant that chemical analysis of samples for PCBs needs to be congener-specific in order to allow meaningful toxicological assessment. This contrasts with earlier analytical methods which tended to measure only total PCB levels. Recent advances in analytical methodology, and the development of synthetic methods to provide the pure congeners necessary as analytical standards, have contributed immensely to our present understanding of the PCB problem. The chemical properties of PCBs have been reviewed in detail by Hutzinger et al. (12).

In addition, it has now been established that many commercial PCB products contain other chlorinated hydrocarbons, e.g. polychlorinated dibenzo furans (PCDFs; 13,14) and polychlorinated naphthalenes (PCNs; 15,16). PCDFs are in general considerably more toxic than the PCBs (17) and the toxicity of the chlorinated naphthalenes has been demonstrated (18). PCDFs as well as polychlorinated quaterphenyls (PCQs) were found in the cooking oil implicated in the Yusho incident as well as in the tissues of some of the victims (19,20,21,14,22). PCDFs and PCQs have also been found in the cooking oil implicated in the Taiwan poisoning incident, and PCDFs have been found in the blood of some of the victims (23). The presence of
these compounds has complicated interpretation of the toxicological data obtained from these incidents.

1.3 Occurrence of PCBs

PCBs are eliminated only slowly from animals in natural ecosystems (24). The lipophilic nature and metabolic stability of these compounds favours their accumulation in fatty animal tissues, which form depots for these and other fat-soluble hydrocarbons such as organochlorine pesticides. As early as 1968 a pattern of PCB accumulation at the top of natural food chains was emerging, with birds of prey and fish-eating birds accumulating the highest levels of these pollutants (25). In 1969 several species of marine animals including fish, mussels and seals were observed to contain PCBs in their tissues (26). Since then the list has continued to grow, and now includes zooplankton, shrimp, crabs, sharks, whales, raptors, gulls, ducks, polar bears, hares, foxes and cows (6).

Given the ubiquitous nature of PCBs it is not surprising that they have been found in the food of man. Cows excrete PCBs in their milk (27) and consequently milk products such as butter are contaminated (28). Particular concern has arisen over fish (29). The situation was considered sufficiently serious for the United States Food and Drug Administration (FDA) in a 1979 ruling to reduce the tolerances for PCBs in milk and dairy products from 2.5 to 1.5 ppm (fat basis), in poultry from 5 to 3 ppm (fat basis), in eggs from 0.5 to 0.3 ppm and in fish and shellfish from 5 to 2 ppm (30). In a recent survey of food products in Canada it is estimated that the total annual
intake from fruit, root and leaf vegetables, milk, eggs and meat is about 32 mcg per person (31). The contamination of the Japanese environment with PCBs, and the levels in food, have been reviewed by Fujiwara (32).

The occurrence of PCBs in food products has inevitably led to their accumulation in man. Mes et al. (33) reported total PCB levels of 0.9 ppm (of wet tissue) in Canadian human adipose tissue samples collected in 1972. Various other organochlorine residues were also present, such as DDT, DDE and dieldrin. In 1985 Safe et al. (34) measured the concentrations of about 59 different PCB congeners in a human milk sample collected in the Great Lakes region of the United States. Jensen and Sundstrom (35) identified some 40 different PCB congeners in Swedish human adipose tissue samples collected sometime prior to 1974, while a total level of 1.1 ppm (of dry tissue) has recently been reported from Italy. Human blood and milk have been reported to contain trace levels of PCBs in countries as far apart as Canada (36), Norway (37), and Japan (38) with typical blood levels being 2-3 ppb of whole blood. Solorach and Vaz (39) recently reported data from a UNEP/WHO pilot project which measured OC compounds in human milk from 10 different countries. PCBs were detected in milk from 7 countries, typical levels being 1-2 ppm of milk fat. In the United Kingdom a similar picture has emerged (40). PCBs have also been detected in samples of human liver and kidney (41).
1.4 Toxicity

1.4.1 Acute Toxicity

The toxic effects of the PCBs in animals and man have been the subject of various reviews (10,42,43,5). The acute symptoms of PCB poisoning in man were demonstrated in the Yusho incident and again in a similar outbreak of PCB poisoning that occurred in Taiwan in 1979, and included swelling of the upper eyelids, increased discharge from the eyes, visual disturbances, acne, itching and a dark pigmentation of the skin. A feeling of weakness, numbness of the limbs and headaches are common, and fever, jaundice and diarrhoea may also occur (44,45). The acute toxicity in laboratory animals develops over one to three weeks (10) and is characterised in rats by diarrhoea, weight loss, ataxia and weakness (46). Other effects include increased liver weight and induction of hepatic microsomal enzyme activity. There is wide variation between the susceptibility of different animal species, mink for example being much more sensitive than rats (47).

The oral LD$_{50}$ of the AROCLORS varies from 1 to 10 gKg$^{-1}$ body weight in rats (48,46). Acute oral toxicity decreases with increasing chlorine content in rats (49), but this is not the case in quail (10). The pattern of administration appears to affect toxicity, as exposure over a period of weeks can be more toxic than the same total amount of PCB given as a single dose. This aspect has been discussed by Nelson et al.(50). This observation has important implications for human health, since the pattern of human exposure
to PCBs is one of low dose, chronic ingestion in food. Relatively short term animal feeding studies may not realistically reflect such conditions.

It has been stated in various reviews that young animals tend to be more sensitive to the acute effects of PCBs than adults, and females more sensitive than males (10,51,43). In fact the evidence from the original papers quoted in such reviews is not conclusive for PCBs (1,48,52), although chronic feeding studies have indicated that females are more sensitive to AROCLOR 1260 (1).

1.4.2 Chronic Toxicity

The effects of the occupational exposure of man to PCBs have been well studied, and include deaths due to cancer (53), liver enzyme induction (54), dermal toxicity (55) and neurotoxicity (56). The toxic effects of PCBs seen in such cases generally tend to be less than would be expected on the basis of plasma PCB levels, by comparison with those seen in the Yusho incident. This raises the question already mentioned of the role played by other compounds in Yusho (see section 1.2). The incidence of chloracne, for example, is much lower amongst occupationally exposed workers than amongst Yusho victims (57). Concern has also been expressed about possible reproductive effects due to occupational exposure, but conclusive studies assessing this in humans are lacking (58).

In laboratory rats, a myriad of toxic effects have been observed after chronic exposure to PCBs. Liver toxicity is a major effect.
Kimbrough et al. (1) reported increased liver weight with hypertrophy of liver cells, cytoplasmic inclusions, and accumulation of pigment and lipid in rats fed AROCLORS 1254 and 1260 for eight months. AROCLOR 1254 proved to be the more toxic of the two mixtures used. AROCLOR 1260 was more toxic to females than to males, while AROCLOR 1254 was not. These effects were common at a dietary PCB level of 100 ppm. Adenofibrosis occurred at 500 ppm.

Schaeffer et al. (59) reported liver tumours in rats fed CLOHEN A60 100 ppm in the diet for 700 days. In the same study, CLOHEN A30, a less chlorinated PCB mixture, was less toxic which contrasts with the study of Kimbrough et al. already mentioned in which the less chlorinated PCB mixture was more toxic. The study of Schaeffer et al. also showed that after 800 days the PCB-exposed rats experienced a lower mortality rate and a lower incidence of neoplastic lesions other than hepatocellular carcinoma. Young (60) concluded from this that in the long term, exposure to the PCB had actually been beneficial. How such an effect is caused by PCBs is not clear. The processes involved in the production or suppression of neoplasia by PCBs seem poorly understood.

Bruckner et al. (61) found that porphyria, detected as an increase in urinary coproporphyrin excretion, was a sensitive indicator of AROCLOR 1242 exposure. In another study, exposure of rats to AROCLOR 1260 100 ppm in their diet for 21 months produced hepatic tumours whereas in a range of other organs the incidence of tumours was no greater than controls (62). Thus the liver appears to be the main target for PCB-induced carcinoma in the rat.
AROCLORS are also reported to be toxic to the adrenals of rats, causing a reduction in adrenal weights and in circulating adrenocortical hormone levels. These effects were seen after several months of exposure to dietary concentrations as low as 1 ppm (63). Rats also experience thyrotoxicity after PCB exposure. This is thought to be due to pathological effects on the thyroid gland as well as to an increase in the metabolism of thyroxine (64,65).

Reproductive effects of PCBs have been observed in rats by various workers (66,48,67) and include reductions in uterine and ovarian protein, decreased foetal and pup survival, and a hypoandrogenic condition in males that leads to reproductive impairment. Increased metabolism of sex hormones has been suggested as a possible mechanism for such effects (68). However not all workers have reported foetotoxicity. Villeneuve et el. (69) administered AROCLOR 1254 orally to pregnant Wistar rats in doses of up to 100 mgKg\(^{-1}\)day\(^{-1}\) from day 6 to day 15 of gestation. They observed no decline in average litter size compared to controls. They did however observe foetotoxic effects in rabbits in the same study.

In contrast, Spencer (66) fed AROCLOR 1254 to Holtzman rats at concentrations of up to 900 ppm in their diet, again from day 6 to day 15 of gestation. They observed a significant (p<0.05) decrease in the foetal survival rate per litter at birth at dietary concentrations of 300 ppm and above. According to their figures for daily food intake, this dietary level is equivalent to 4.7 mg/rat/day which approximates to about 19 mgKg\(^{-1}\)day\(^{-1}\) for a 250 g
rat. Thus they observed foetotoxicity in their adult Holtzman rats at a much lower dose than Villeneuve et al. had used in their Wistars. Teratogenicity due to PCBs has been reported in mice (70).

Immunotoxicity is manifest in rats as a decline in spleen and thymus weights (71,19). The weights of other organs are also affected (71), some of which have been mentioned.

Generally, the toxic effects induced by PCBs in rats are seen in many other species and there is great variation in sensitivity between species. For example, mink are markedly more sensitive than rats (47). Hamsters are considered to be relatively resistant to the effects of the halogenated hydrocarbons in general (72) but hamster studies using PCBs in particular seem uncommon. There appears to be a tendency for young animals and females to be more sensitive to halogenated hydrocarbons in general, but as mentioned in section 1.4.1 this needs clarification as regards the PCBs. Rats and mink do not tend to show dermal toxicity with PCBs, whereas humans, monkeys, cows, hairless mice and the rabbit ear tend to show acneform or other skin lesions as major effects. Chickens develop subcutaneous oedema and hydropericardium, termed "chick oedema disease" (73), and this disorder is specific to chickens (74). The reasons for these species-specific effects are unknown.

In contrast the neurological, immunological, hepatic, reproductive, hormonal and enzyme induction effects of PCBs tend to appear in a wide variety of species (10,43). Most of the carcinogenic studies with PCBs seem to have been carried out in rats and mice. The
picture now emerging is that PCBs tend to be only weak tumour
initiators but are more active as tumour promoters (75,76).

1.5 Environmental Effects of PCBs and Evaluation of the Hazards

The widespread occurrence and the toxicity of the PCBs as discussed
in sections 1.3 and 1.4 suggest that these compounds may be exerting
a considerable effect on the stability of natural ecosystems and
consequently may present long-term problems for wildlife
conservation and human health. In a review paper in 1975 Peakall
(77) tried to evaluate the environmental hazards of PCBs. He
expressed concern over the release of large amounts of stable,
foreign materials into the environment but could reach no firm
conclusions as to the seriousness of the problems this might have
caused.

Attempts have recently been made to assess the situation in specific
geographical regions such as the Great Lakes ecosystem in the United
States and in the Baltic, Mediterranean and Australian ecosystems
(78). These assessments highlight some alarming trends, such as
reproductive toxicity in pinnipeds (79) which has been proposed as
the cause of the marked decline in the common seal population in the
Dutch Wadden Sea (80). Recently the importance of the more toxic PCB
congeners present in commercial PCB mixtures has received attention,
as these have now been detected in environmental samples (208).

In spite of such analyses, the total long-term environmental impact
of PCBs is still a matter of conjecture. It is not clear what action
can usefully be taken other than minimising the use of PCBs and tackling the problems of their safe disposal and destruction. The general outlook has been discussed by Tanabe (8).

Assessment of the public health effects of PCBs is also difficult. Monitoring of foodstuffs, restriction of the use of PCBs and maintenance of normal standards of industrial safety ensure that human exposure consists of the ingestion of only trace amounts in the diet throughout life. The chronic effects of such exposure are unknown. Particular concern has arisen over the appearance of PCBs in human milk because of the effects they may have on breast-fed infants.
1.6 Transfer of PCBs from Mother to Young

PCBs have been shown to be transferred to offspring both in utero across the placenta and in milk in different animal species such as mice \((81,82)\), rats \((83,84)\), rhesus monkeys \((85)\) and man \((86,87)\) with the milk route accounting for a much greater proportion of the total PCB transferred. Most of these studies involved the direct measurement of total PCB in body fluids or tissues. Gallenberg and Vodicnik \((88)\) reported that female mice treated with a \(^{14}C\)-labelled hexachlorobiphenyl 50 mgKg\(^{-1}\) as a single i.p. dose two weeks before mating had eliminated over 98% of the dose by day 20 of lactation and that minimal placental transfer had occurred.

The kinetics of the transfer of PCB from mother to offspring has been studied in rats by Takagi et al. \((89)\) who administered an oral tracer dose of a PCB mixture uniformly labelled with \(^{14}C\). Their results indicate the pattern of PCB movement. Less than 0.28% of the \(^{14}C\) dose actually absorbed by the dams during gestation was found in the foetus at day 18 of gestation. Suckled pups whose mothers had received PCB during gestation and lactation showed tissue PCB levels that continued to increase from birth for 11 days and then declined slowly over the following weeks. The highest levels in weanling (i.e. three-week old) pups were found in subcutaneous fat. The PCB levels in the tissues of the dams indicated that substantial amounts of PCB had been removed by lactation and very little by placental movement, compared to virgin controls. Ando \((83)\) investigated the transfer of a single i.p. dose of 2,2',4,4',5,5'-hexachlorobiphenyl labelled with \(^{14}C\) administered to rats after mating. Placental
transfer accounted for 2.7% of the dose, while 39.2% was transferred in milk by the sixteenth day postpartum.

In man, exposure in utero to PCBs has been reported to cause low birth weight and smaller head circumference in the case of mothers whose diet normally included contaminated lake fish and who had therefore experienced a greater than usual level of exposure (90). Placental PCB transfer was also considered to be the cause of a syndrome seen in newborn babies of Yusho victims, and characterised by skin pigmentation, gingival hyperplasia and abnormal calcification of the skull (91). Rogan et al. (92) concluded that hypotonicity and hyporeflexia in neonates were associated with high PCB levels. An increase in PCB blood levels with duration of breast-feeding has been demonstrated (93).

Toxicity observed in offspring suckled by PCB-exposed mothers has been attributed to PCB transfer in several animal species. The effects include induction of hepatic microsomal enzymes in mice (94) and rats (95), liver enlargement with morphological changes in the liver in rats (84) and immunocompetence (measured as a reduction in T cell helper activity) in mice (96). Acne, swelling of the eyelids, loss of facial hair, hyperpigmentation of the skin and decreased survival of infants have been reported in rhesus monkeys (85). The appearance of the Yusho syndrome in a human infant breast fed for several months has been reported (5). The mother had consumed PCB-contaminated rice oil, but not until after delivery. However the case is not well documented.
An interesting recent development is the indication that synergism may occur amongst the congeners present in a PCB mixture with respect to their toxic effects. This was suggested by Leece et al. (97) who observed that the degree of enzyme induction caused by two PCBs administered to rats within a few days of each other was greater than the sum of the effects of either congener given alone. As human milk is characterised by a large number of different PCB congeners, the implications for breast-fed babies could be important.

1.7 Disposition in Animals

1.7.1 Effects of Congener Structure

Variation between PCB congeners in their rate of elimination from virgin rats has been reported in several different species. Grant et al. (98) in 1971 found that the different components in AROCLOR 1254 were not all eliminated at the same rate from rats. Hutzinger et al. (99) reported that the rate of metabolism of PCBs in trout, pigeons and rats became slower as the number of chlorine atoms in the molecule increased. Burse et al. (100) fed AROCLOR 1242 or AROCLOR 1016 to rats at a level of 100 ppm in their diet for six months and then measured total PCBs in different organs over several months, after the animals had been returned to a PCB-free diet. Their results show that AROCLOR 1016, which differs from AROCLOR 1242 by containing fewer of the more highly chlorinated PCB congeners, was eliminated more rapidly from tissues than AROCLOR 1242. PCB congener structure has been shown to affect retention in
tissues in various species, with the substitution pattern as well as the total number of chlorine atoms present in the molecule being important (101,102). This aspect is discussed further in chapter eight.

It has been observed that the PCB congener composition in biological samples, including human adipose tissue, does not closely resemble the composition of the commercial mixtures from which they originated (35,38). This phenomenon is attributed to kinetic differences between congeners. In general, the more highly chlorinated congeners tend to be eliminated more slowly from animal tissues than the less chlorinated PCBs (98). However, the position of the chlorine atoms on the rings is important in determining elimination rate (see section 8.3.2).

The relationship between congener structure and retention in tissues has implications for the environmental toxicity of PCBs. Parkinson et al. (103) prepared a mixture of PCB congeners that resembled the PCB composition of a Japanese sample of human breast milk. They compared the enzyme inducing effects of this mixture with those of a commercial mixture, Kanechlor 500, which was a widely used product in Japan and a likely source of PCB pollution in that country. They found that the prepared mixture was seven times more potent than Kanechlor 500 and concluded that this was due to the higher proportion of certain penta- and hexachlorobiphenyls in the prepared mixture compared to the commercial product. Thus selective bioconcentration of PCBs in the human body increases the toxicity of the PCB burden in breast milk.
1.7.2 Effects of Pregnancy and Lactation

The kinetics of PCBs in pregnancy and lactation has been studied by Vodicnik (104) who administered a $[^{14}\text{C}]$-labelled tetrachlorobiphenyl 150 mgKg$^{-1}$ i.p. to virgin, 15-day pregnant and lactating (1 day postpartum) mice. Doses were all calculated as a function of non-pregnant body weight. By four days post-dose virgin mice had eliminated about 20% of the radioactivity given, pregnant mice 10% and lactating mice 90%. Most of the radioactivity eliminated by the lactating mice could be accounted for in the suckled pups, which were examined at the same time as their mothers. The concentrations of radioactivity in the liver and adipose tissue of pregnant mice did not decline over the four day period following dosing, while the levels in these tissues generally declined in virgin and lactating mice.

A similar pattern of elimination was observed by Vodicnik and Lech (105) who injected mice with a $[^{14}\text{C}]$-labelled hexachlorobiphenyl 100 mgKg$^{-1}$ two weeks before mating. The virgin controls showed a steady decline in tissue levels from blood, muscle, kidney, adipose tissue and liver. Pregnant mice showed a slower decline with higher levels in all these tissues. Mammary gland levels however increased during pregnancy. A marked increase in the levels in all tissues except muscle was seen at parturition followed by a rapid decline during lactation.

The plasma events associated with these changes in PCB distribution were investigated by Spindler-Vomachka and Vodicnik (106) who...
administered a [\textsuperscript{14}C]-labelled hexachlorobiphenyl to rats by intravenous injection. They found that in virgin rats, 50% of the total dose in the plasma was associated with the low density lipoprotein (LDL) fraction. The hyperlipidemia which is a feature of late pregnancy was characterised by an increase in very low density lipoproteins (VLDL) and an increase in the proportion of the radioactivity associated with the VLDL fraction, compared to virgin controls. By midlactation the levels of VLDL had begun to decrease again so that plasma radioactivity was more evenly distributed amongst the different lipoprotein fractions in the plasma.

These studies indicate that the profound physiological changes that occur during pregnancy and lactation affect the disposition of PCBs in the maternal body. Consequently PCB elimination slows down during pregnancy, thereby favouring PCB accumulation, and speeds up markedly during lactation, compared to the non-pregnant, non-lactating state. Thus the fate of a given PCB dose within the body is influenced by the physiological state of the animal and by the structure of the congeners present (see section 1.7.1). The time that has elapsed since administering a PCB mixture will influence the congener composition in tissues, and hence the congener composition of PCBs transferred in milk. The tendency for the toxicity of a given PCB dose to be greater if it is given over a period of time rather than as a single dose was mentioned in section 1.4.1. These considerations suggest that the timing of PCB administration, with respect to the duration of dosing, the interval between dosing and transfer in milk, and the changing physiological state of the animal may influence the toxicity experienced by the
1.8 Enzyme Induction

1.8.1 The Hepatic Mixed-Function Oxidase System and Cytochrome P-450

In recent years, attention has focussed on the importance of hepatic enzyme systems in the metabolism of environmental chemicals. These systems increase the water solubility of their substrates by processes such as oxidation and reduction (Phase 1 metabolism) followed in some cases by conjugation (Phase 2 metabolism) thereby increasing excretion of the substrate in body fluids, particularly urine. The majority of the mammalian liver enzymes concerned in these processes are located in the endoplasmic reticulum and can be studied in vitro using isolated microsomal fractions. A major group of these enzymes is the mixed-function oxidases. Based on the hemoprotein cytochrome P-450, these oxidases form complex electron transport systems which are responsible for a variety of oxidative reactions such as hydroxylation and O- and N-dealkylation. The functioning of this system has been discussed by Parke (107).

Studies on the nature of the liver enzyme systems have focussed on cytochrome P-450, which can be inhibited by some drugs (such as SKF-525A) and induced by others (such as phenobarbitone). Early work by Omura and Sato (108) in 1964 identified a pigment in liver microsomes and indicated its hemoprotein nature. This pigment, termed cytochrome P-450, forms a complex with carbon monoxide (CO). This property forms the basis of a method developed by the same
workers for quantifying the levels of the pigment present in a sample, by utilising the intense absorption band shown in the 450 nm region by the CO complex of the reduced pigment.

Recent studies have shown that there are a number of different cytochromes similar in nature and properties to the pigment described by Omura and Sato, and the number seems to increase steadily (109,110). Cytochrome P-450 appears to exist as many different isozymes, each differing in certain characteristics such as electrophoretic mobility, amino acid composition, immunological properties and in the wavelength of the absorption maximum shown by the reduced cytochrome P-450:CO complex. These are all referred to under the collective name of "cytochrome P-450". Variation between animal species, and between the sexes in the same species, in the nature of the P-450 cytochromes present have been reported (111,112). In 1982 Guengerich et al. (109) announced the purification of eight distinct forms of cytochrome P-450 from rat liver.

1.8.2 Effects of Enzyme Inducers

The ability of a wide range of compounds including drugs, carcinogens, pesticides and steroid hormones to increase (i.e. induce) the activity of hepatic microsomal enzymes has been reviewed by Conney (113). The inducers generally tend to be lipid soluble at physiological pH values, become localised in the endoplasmic reticulum of the liver and to bind to microsomal enzymes (107). A major effect of enzyme inducers is to cause an increase in the
synthesis of new enzyme protein which can be measured in isolated microsome fractions.

Inducers are characterised by the relative increases they cause in the levels of the different liver cytochromes and consequently by the shift in the absorption maximum. Administration of phenobarbitone (PB) to animals causes an increase in absorption at 450 nm, resulting in no shift in the wavelength of the maximum, while 3-methylcholanthrene (MC) induction causes a shift in the maximum to about 447-448 nm (114). The effects of these compounds have been used to classify different inducers as "PB-type" or "MC-type" depending on the wavelength of the resulting absorption maximum. Compounds have also been identified as "mixed-type" inducers as their administration to animals results in an absorption maximum somewhere between 450 and 447 nm.

Ryan et al. (115) isolated and characterised cytochrome P-450 isozymes from PB-treated rats designating them as cytochromes P-450\textsubscript{a} and P-450\textsubscript{b}, and from MC-treated rats designating them as cytochromes P-450\textsubscript{a} and P-450\textsubscript{c}. They also recorded the absorption maximum of the reduced P-450:CO complex of each of these isozymes, and found them to be 452, 450 and 447 nm for isozymes P-450\textsubscript{a}, P-450\textsubscript{b} and P-450\textsubscript{c} respectively. More recently, Ryan et al. (110) added cytochromes P-450\textsubscript{d} and P-450\textsubscript{e} to the list. The present situation as summarised by Safe (10) is that PB causes induction of cytochrome P-450 isozymes a, b and e while MC induces a, c and d. AROCLOR 1254 induces a, b, c, d and e which suggests a mixed-type of induction.
Associated with cytochrome P-450 induction are increases in the activities of the various dependent microsomal enzymes. FB administration produces marked increases in the activities of N-demethylase enzymes such as ethylmorphine-N-demethylase (116), aminopyrine-N-demethylase (117) and benzphetamine-N-demethylase (153) as well as increasing the activity of aniline hydroxylase (119). The activity of benzo[a]pyrene hydroxylase (also known as aryl hydrocarbon hydroxylase or AHH) is also induced by FB, but to a much lesser extent than by MC (116). FB induction is characterised by proliferation of the endoplasmic reticulum with a consequent increase in microsomal protein in the liver, and an increase in liver weight (117).

In contrast MC greatly increases the activities of AHH, ethoxyresorufin-O-deethylase and to a lesser extent aniline hydroxylase, while having no noticeable effect on the N-demethylase enzymes (120,117,121). MC induction is not characterised by proliferation of the endoplasmic reticulum or by large increases in microsomal protein or liver weight, compared to the increases in these parameters caused by FB (117).

Alvares et al. (116) administered AROCLOR 1254 i.p. to rats at a dose of 25 mgKg\(^{-1}\)day\(^{-1}\) for six days and prepared liver microsomes from the dosed animals 24 hours after the last dose. They reported a significant increase (p<0.05) in the activities of AHH and ethylmorphine-N-demethylase and in the level of cytochrome P-450. The absorption maximum of the P-450:CO complex was shifted from 450 to 448 nm. In the same study, FB produced similar changes except
that the extent of induction of AHH was less than that due to PCB and the absorption maximum remained at 450 nm. MC also induced AHH but failed to induce the N-demethylase. The inductive effects of AROCLOR 1254 were therefore somewhere between those of PB and MC, and so it is classified as a mixed-type inducer.

1.8.3 Single PCB Congeners as Enzyme Inducers

Following early reports of mixed-type induction caused by commercial PCB mixtures such as AROCLOR 1254, Goldstein et al. (122) measured the effects of a range of single PCB congeners on drug metabolising enzymes in rat liver. On the basis of their results they classified each congener as either a pure MC- or a pure PB-type inducer. The former group were found to consist of those congeners which lacked chlorine atoms in any of the ortho (2 and 6) positions relative to the phenyl-phenyl bridge, i.e. congeners in which the substitution pattern favours coplanarity of the two phenyl rings in the molecule. The PB-type inducers were those congeners possessing one or more ortho chlorine atoms, the presence of which reduces coplanarity of the rings due to steric interactions. However, examination of their data reveals that in fact some of their congeners behaved more like mixed-type inducers. Mixed-type induction was subsequently reported in some ortho substituted congeners by Parkinson et al. (123,124).

The use of enzyme activity assays to indicate the induction properties of a compound was used by Yoshimura et al. (120). They administered inducing doses of PB or MC or one of a selection of
purified PCB congeners to rats on two consecutive days and prepared liver microsomes 24 hours after the last dose. MC caused a significant (p<0.05) increase in the activity of aniline hydroxylase and in the concentration of cytochrome P-450, but did not increase the activity of aminopyrine-N-demethylase or NADPH-cytochrome c reductase or the level of cytochrome b5. PB on the other hand increased all these parameters as did a combination of MC and PB given together. Each of the coplanar PC congeners used showed an induction pattern similar to MC, while the remaining congeners showed a pattern similar to PB or PB with MC. They were therefore able to reliably identify the MC-type inducers with this combination of measurements. The inducing characteristics of the coplanar PCBs have been confirmed by other workers (121,122).

1.8.4 Relationship between Enzyme Induction and Toxicity

Substantial progress has been made towards explaining the biological mode of action of PCBs and in defining their structure-activity relationships. These are reviewed by Safe et al.(70). The PCBs are thought to exert their toxic and inductive effects by binding to a cytosolic receptor, known as the Ah ("Aromatic hydrocarbon") receptor. The receptor binding affinity is thought to increase with the degree of coplanarity of the PCB molecule (see section 1.8.3). A correlation has been established between the receptor binding affinities of different PCB congeners and their capacity to induce AHH. These effects have been broadly correlated with toxic potency. The coplanar PCBs (i.e. the MC-type inducers) such as 3,3',4,4',5,5'-hexachlorobiphenyl are more active biologically than
the mono-ortho substituted congeners, which in turn are generally regarded as more active than the di-ortho substituted congeners. This is discussed further in chapter eight.

These observations have led to the theory that hepatic enzyme induction and receptor binding studies can be used to predict the toxic potency of a given PCB congener (10). In other words, measurements of enzyme induction can perhaps be used as toxicity indicators. Considering the high costs involved in full-scale routine toxicity testing and the fact that there are 209 possible PCB congeners, it would be useful to develop rapid, cheap and reliable methods of comparing and broadly classifying each one according to expected potency. This need has been emphasized by Safe (10). To be practical such methods should require minimal quantities of the pure congeners, since these are not readily obtained commercially and must often be synthesized in-house. Those that are available commercially are generally very expensive.

1.9 Objectives of this Study

The primary area of interest in this study is the transfer of PCB toxicity in human breast milk. The preceding discussion has highlighted concern for the health of breast-fed infants who, according to the available information, are now consuming PCBs through their mothers' milk. The mass of toxicity data on PCBs encourages this concern. Many different PCB congeners occur in milk and show differing toxic potencies. Assessing the total toxicity burden due to PCBs in milk is therefore complicated. Some important
questions need answering: At what point does PCB-induced toxicity in
the infant become unacceptable, and when should breast-feeding be
avoided? To answer these questions, information is needed not only
about the levels and identity of the congeners present in a given
milk sample, but also about their relative toxicity. This study is
concerned with the assessment of the relative toxicity of different
congeners.

Differences between PCB congeners with respect to their transfer in
milk have been discussed in section 1.7.1. The toxicity experienced
by a breast-fed infant, due to a given congener ingested by its
mother, must be influenced by the pharmacokinetic as well as the
pharmacodynamic characteristics of that congener. This suggests that
comparative toxicity testing using different congeners must involve
milk transfer if the results are to be used to relate maternal
dietary intake to PCB-induced effects in breast-fed infants.

The objectives adopted were therefore as follows:

1. To investigate the importance of the timing of maternal PCB
   exposure when measuring the effects of PCBs transferred by
   lactation (section 1.7);

2. Using the results of 1. above, to develop a relatively rapid
   method (based on enzyme induction) of comparing the toxic
   potencies of different PCB congeners transferred to rat pups
   by lactation (section 1.8);
3. To then demonstrate the use of this method by comparing the effects of selected PCB congeners;

4. To investigate the possibility that synergistic effects may occur between PCB congeners (section 1.6);

5. To assess the extent to which the comparison of the toxic potencies of different congeners obtained in 3. above agrees with comparisons made using other (non-enzyme) effects.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

Acetylacetone, 4-aminophenol hydrochloride, aminopyrine, aniline, CHAPS (3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate), D-glucose 6-phosphate (monosodium salt), glucose-6-phosphate dehydrogenase (type XI), and NADP (monosodium salt) were obtained from Sigma Chemical Company, Poole, Dorset.

AROCLO 1254 was kindly supplied by Dr. M. Cooke, Department of Inorganic Chemistry, University of Bristol, Bristol.

Carbon monoxide gas and formaldehyde solution (37-41%) were obtained from BIH Chemicals Ltd., Poole, Dorset.

Olive oil (BP grade) was bottled by Crosse & Blackwell, Croydon, Surrey.

The reactants used in the syntheses of the PCB congeners were obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset.

Solvents used were HPLC grade and supplied by Fisons plc, Loughborough, Leics.
Laboratory reagents used were analytical grade.

2.2 Synthesis and Purification of PCB Congeners

2.2.1 General Procedure

Synthetic Method. The PCB congeners were synthesized by the Cadogan Coupling of a chlorinated aniline in excess of a chlorinated benzene (125) as shown in figure 2.1.

Figure 2.1. General reaction used in the synthesis of PCB congeners.

Depending on the reactants used, one or more PCB congeners are obtained, together with numerous by-products which vary in colour from pale yellow to a dark brownish-red. To synthesize PCBs 97, 118, 156, 157, 169 and 189 it was necessary to carry out four different reactions which are detailed in Table 2.1.

For each reaction, the chlorinated aniline and the chlorinated benzene were weighed and placed in a three-necked 100ml round-bottomed flask. A mercury-in-glass thermometer was placed in
Table 2.1. Reactants used and congeners obtained in PCB syntheses.

<table>
<thead>
<tr>
<th>Reactants</th>
<th>PCB Products *</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4,5-trichloroaniline 25mmol=4.91g</td>
<td><img src="2,4,5-trichloroaniline" alt="Image" /> 2,3',4,4',5-Pentachlorobiphenyl PCB 118 2,2',3',4,5-Pentachlorobiphenyl PCB 97</td>
</tr>
<tr>
<td>1,2-dichlorobenzene 250mmol=36.75g</td>
<td><img src="1,2-dichlorobenzene" alt="Image" /></td>
</tr>
<tr>
<td>iso-pentyl nitrite 40mmol=5.6ml</td>
<td>2,3',4,4',5-Pentachlorobiphenyl PCB 118 2,2',3',4,5-Pentachlorobiphenyl PCB 97</td>
</tr>
<tr>
<td>3,4-dichloroaniline 12mmol=1.94g</td>
<td><img src="3,4-dichloroaniline" alt="Image" /></td>
</tr>
<tr>
<td>1,2,3,4-tetrachlorobenzene 130mmol=28.01g</td>
<td>2,3',4,4',5-Hexachlorobiphenyl PCB 156</td>
</tr>
<tr>
<td>iso-pentyl nitrite 20mmol=2.8ml</td>
<td>2,3',4,4',5-Hexachlorobiphenyl PCB 156</td>
</tr>
<tr>
<td>3,4,5-trichloroaniline 30mmol=5.89g</td>
<td><img src="3,4,5-trichloroaniline" alt="Image" /> 2,3',4,4',5-Hexachlorobiphenyl PCB 157 3,3',4,4',5,5'-Hexachlorobiphenyl PCB 169</td>
</tr>
<tr>
<td>1,2,3-trichlorobenzene 400mmol=72.58g</td>
<td><img src="1,2,3-trichlorobenzene" alt="Image" /></td>
</tr>
<tr>
<td>iso-pentyl nitrite 40mmol=5.6ml</td>
<td>2,3',4,4',5-Hexachlorobiphenyl PCB 157 3,3',4,4',5,5'-Hexachlorobiphenyl PCB 169</td>
</tr>
<tr>
<td>3,4,5-trichloroaniline 25mmol=4.91g</td>
<td><img src="3,4,5-trichloroaniline" alt="Image" /> 2,3',4,4',5-Hexachlorobiphenyl PCB 157 3,3',4,4',5,5'-Hexachlorobiphenyl PCB 169</td>
</tr>
<tr>
<td>1,2,3,4-tetrachlorobenzene 266mmol=57.37g</td>
<td><img src="1,2,3,4-tetrachlorobenzene" alt="Image" /></td>
</tr>
<tr>
<td>iso-pentyl nitrite 40mmol=5.6ml</td>
<td>2,3',4,4',5,5'-Heptachlorobiphenyl PCB 189</td>
</tr>
</tbody>
</table>

* PCB identity numbers according to Ballschmiter and Zell (11).
one of the side necks and a water condenser in the central neck. The third neck was stoppered and used for the addition of the iso-pentyl nitrite. The flask was heated in a thermostatically controlled oil bath to maintain the contents at a temperature of 120-130°C, with stirring. This resulted in a liquid mixture of reactants to which the iso-pentyl nitrite was added dropwise over a period of one hour. Following this the reaction mixture was held refluxing at 120-130°C for about a further 20 hours. The flask was then allowed to cool yielding, in the case of all four reactions carried out, a solid dark red-brown mass which was subjected to one of the clean-up procedures described below. Each PCB congener clean-up is described in the order in which it was carried out to indicate how the procedures were developed.

Clean-up Procedures. In every case vacuum distillation was used to remove excess chlorobenzene as the first stage of the clean-up procedure followed by liquid column chromatography. TLC was then used to select suitable systems for the purification of the PCB reaction products by preparative scale HPLC. The spots on the TLC plates were visualised by viewing under UV light (254 nm).

Identity and Purity Tests. The identity of each of the PCB congeners was confirmed by proton nuclear magnetic resonance spectrometry, 70 eV mass spectrometry and comparison of melting points with published values where available. These techniques, along with capillary gas chromatography, also allowed assessment of purity.
2.2.2 **Instrumentation**

For all the PCB congeners made the HILC clean-up was achieved using a ZORBAX ODS 250 x 21.2 mm column, with a solid phase particle size of 5-6 μm (Du Pont (UK) Ltd., Stevenage, Herts.). A 2.0 ml injection loop was used. The pump was part of a Du Pont 830 preparative HILC unit linked to a Du Pont UV detector set at 2.56 AUFS, with a preparative scale flow cell. The unit was fitted with an oven to allow control of the column temperature. The optimum mobile phase, flow rate and oven temperature were selected for each PCB. The detector was set at 254 nm except for the PCB 157/169 separation.

The proton NMR spectra were recorded using a JEOL GX 270 FT-NMR. The PCB samples were dissolved in CDCl₃ with TMS as internal reference standard. The spectra were recorded at 20°C using 64 scans with a resolution of 0.2 Hz.

The 70 eV mass spectra were recorded using a VG 707E double focussing, extended geometry instrument with a PDP 8A based operating system. The ionisation mode used was electron impact (EI).

The capillary GC was carried out under the following conditions:
**Instrument:** Perkin-Elmer Sigma 3 gas chromatograph.
**Column:** 39m, 0.25mm id, fused silica, polymethylsiloxane (OV-1) 0.2μm film (Alltech England, Carnforth, Lancs.).
**Carrier gas:** Hydrogen at 17 psi.
**Oven programme:** 100°C for one minute, then 4°C min⁻¹ to 250°C. Hold.
Detector: Flame ionisation.

Inj./Det. temp.: 275°C.

Injection mode: 2 to 4 μl in heptane/hexane solution, split (low ratio). Heptane was used for solutions of PCBs 156, 157 & 169 and hexane for PCBs 189, 118 & 97.

2.2.3 Purification of PCB 156

PCB 156 was synthesized as indicated in section 2.2.1.

Vacuum Distillation. The first stage in the clean-up procedure was the removal of excess chlorobenzene by vacuum distillation.

Liquid Column Chromatography. TLC was used to investigate the movement of the reaction products over silica. A system using a silica plate (Silica GF<sub>254</sub>, Merck, BDH Chemicals Ltd., Poole, Dorset) with hexane as the mobile phase indicated that the PCB was the most mobile component with the mobility of the components decreasing as their colour darkened. The separation of the PCB from the majority of the coloured by-products was then achieved using a glass column (52 x 300mm) wet packed in hexane to a depth of about 250mm with silica gel (Kieselgel 60, particle size 0.063-0.200 mm, Merck) through which the reaction products, dissolved in hexane, were passed using hexane as eluant. The first fraction was collected until just before the first coloured (pale yellow) pigments eluted. Four further fractions were collected, their colour becoming gradually darker with successive fractions. The hexane was removed from each fraction using a rotary evaporator. The
earlier fractions smelled slightly of chlorobenzene, indicating further purification was necessary.

**Reversed-Phase HPTLC.** It has been shown that PCBs can be separated by reversed-phase TLC using paraffin oil coated kieselguhr plates and an acetonitrile/methanol/water mobile phase (126), indicating the suitability of reversed-phase systems for separating PCBs. It was therefore decided to attempt purification of PCB 156 by preparative scale reversed-phase HPTLC. A suitable mobile phase was determined by TLC using RP-HPTLC plates (RP-18, F254, Merck) to compare the movement of various solutes including 1,2,3,4-tetrachlorobenzene, 3,4-dichloroaniline and the different fractions eluted from the column. The results of the RP-HPTLC are shown in Figure 2.2.

**Figure 2.2.** Separation of components from the synthesis of PCB 156 by RP-HPTLC using 100% acetonitrile as mobile phase.
It was concluded that a mobile phase consisting of 100% acetonitrile was capable of separating PCB 156 from any residual chloroaniline or chlorobenzene. TLC indicated also that probably only the first and second column fractions contained significant amounts of PCB 156. Column eluate should therefore be collected until about 100ml after the first appearance of colour. Collection beyond this point increases the amount of coloured impurities without significantly increasing PCB recovery.

Preparative HPLC. Following the results of the TLC studies, preparative reversed-phase HPLC was used to isolate PCB 156 from the various components in the first and second fractions obtained from the silica/hexane column clean-up. Residues from these two fractions were dissolved in a minimum amount of acetonitrile and subjected to preparative HPLC separation using repeated injections. The mobile phase used was 100% acetonitrile at a flow rate of 14 ml min⁻¹ and an oven temperature of 35°C. The HPLC equipment used is detailed in section 2.2.1. The column and injection port were maintained at 35°C to increase the solubility of the residue in the mobile phase, as well as to sharpen the peak shapes and speed up elution. The RP-HPTLC results were used to identify the peaks. The PCB 156 fraction was collected in a round-bottomed flask and the acetonitrile was removed by rotary evaporation. A typical chromatogram is shown in Figure 2.3.

Final Purification. The PCB residue obtained from the HPLC clean-up was recrystallised from 95% aqueous ethanol. As the crystals obtained seemed to have a very slight orange tinge, they
were dissolved in hexane and passed through a SEP-PAK silica cartridge (Waters Associates Inc., Northwich, Cheshire). This retained the pigment. The solvent was then evaporated and the residue dried in a vacuum oven at 78°C for four hours, yielding a white crystalline powder.

Figure 2.3. Chromatogram obtained during the clean-up of PCB 156 by preparative HPLC.
2.2.4 Purification of PCBs 157 and 169

PCBs 157 and 169 were synthesized as indicated in section 2.2.1.

As well as removal of impurities from the PCBs, it was also necessary to separate the two PCBs formed in this reaction. As in the case of PCB 156 (2.2.3) the first stage in the clean-up was the removal of excess chlorobenzene from the crude reaction products by vacuum distillation followed by the removal of most of the coloured impurities by silica/hexane column chromatography, collecting eluate from the column until about 100ml after the appearance of yellow colour.

 Investigations using Reversed-Phase HPTLC. To achieve separation of PCBs 157 and 169, the suitability of the same HPLC conditions as were used to purify PCB 156 was investigated using reversed-phase HPTLC. The movement of the PCB residue obtained from the silica/hexane column eluate was examined on reversed-phase HPTLC plates using 100% acetonitrile as mobile phase. This indicated that the system could not achieve adequate separation of the two PCBs although removal of trichlorobenzene and some of the coloured impurities was possible. This result was not surprising as the two PCB molecules are very similar in structure, varying only perhaps in dipole moment and molecular shape. However, to facilitate the study of this problem the PCB residue was subjected to the HPLC clean-up used for PCB 156, and this removed the chlorobenzene (traces of which remained after the vacuum distillation step) and some coloured pigments. A typical chromatogram is shown in Figure 2.4.
Figure 2.4. Chromatogram obtained during the preliminary clean-up of PCBs 157 and 169 by preparative HPLC.

HPLC/RP-HPTLC Studies. HPLC investigations were carried out using a Hypersil phenyl column of 25cm x 5mm id and 5μm particle size (Shandon Southern, Runcorn, Cheshire) with mobile phases based on acetonitrile and later trying mobile phases based on tetrahydrofuran (THF). THF was investigated because in addition to having a marked dipole moment like acetonitrile, it is also a cyclic molecule and may thus be able to exploit differences in the molecular shape of the two PCBs to achieve separation. Separation was in fact achieved with 55% v/v THF in water. Following this, RP-HPTLC was used to assess the suitability of THF-based mobile phases for use on a C18
stationary phase rather than on the phenyl stationary phase (because
the only preparative HILC column available to hand was the C_{18}
column used for PCB 156). Mobile phases of both 70% v/v THF in water
and 80% v/v THF in water achieved good separation of PCBs 157 and
169 on RP-HPTLC plates. The results of the RP-HPTLC are shown in
Figure 2.5.

Figure 2.5. Separation of PCBs 157 and 169 by RP-HPTLC using
75% v/v THF in water as the mobile phase.

Preparative HILC. Following the HPTLC studies, separation of PCBs
157 and 169 was carried out by preparative reversed-phase HILC,
using repeated injections the PCB 157/169 residue obtained from the
preliminary HILC clean-up dissolved in mobile phase. A mobile phase
of 75% v/v THF in water was used at a flow rate of 7.0 mlmin^{-1} and
an oven temperature of 35°C. The HPLC equipment used is detailed in section 2.2.1. A typical chromatogram is shown in Figure 2.6.

Figure 2.6. Chromatogram obtained during the separation of PCBs 157 and 169 by preparative HPLC.

The identity of the peaks was assigned on the basis that PCB 157, having a dipole moment, would be expected to elute before PCB 169 which has no dipole moment. This had been borne out by the previous RP-HPTLC studies and was later confirmed in the identity determinations. The detector was set at a wavelength (297nm) which was off the absorption maximum in order to prevent the absorbance exceeding the dynamic range of the detector. The two PCBs were collected in separate flasks after emerging from the detector cell. In order to avoid cross contamination, collection of PCB 157 was stopped before the bottom of the valley between the peaks was
reached, and collection of PCB 169 was started just after the valley point (Figure 2.6). After removal of the solvent by rotary evaporation, PCB 169 had a slight yellow colouration.

**Final Purification.** The last stage in the purification procedure was the recrystallisation of each PCB from absolute ethanol followed by drying in a vacuum oven at 80°C for four hours. This yielded both PCBs as white crystalline materials, although PCB 169 appeared to have a waxy consistency.

2.2.5 *Purification of PCB 189*

PCB 189 was synthesized as indicated in section 2.2.1.

The crude, red-brown reaction residue containing PCB 189 was subjected to the same clean-up by vacuum distillation (to remove the majority of any excess chlorobenzene) followed by elution with hexane down a silica column (to remove coloured impurities) as was used with the PCBs discussed previously. After removal of the hexane solvent the (slightly yellow) crystalline residue was dissolved in a minimum amount of acetonitrile. RP-HPTLC was then used to assess the likely suitability of the same HPLC conditions as were used for PCB 156, for the remaining clean-up of PCB 189. The RP-HPTLC results are shown in Figure 2.7 and indicated that separation would be possible.
The solution of PCB 189 in acetonitrile was then subjected to HILC clean-up by repeated injections using 100% acetonitrile as the mobile phase at a flow rate of 11.2 mL min\(^{-1}\) and an oven temperature of 55°C. The HILC equipment used is detailed in section 2.2.1. A typical chromatogram is shown in Figure 2.8. Again the HPTLC results indicated the identities of the peaks. The final purification stage was the removal of the acetonitrile solvent by rotary evaporation followed by recrystallisation of the PCB from methanol and drying in a vacuum oven at 80°C for four hours.
Figure 2.8. Chromatogram obtained during the clean-up of PCB 189 by preparative HPLC.

2.2.6 Purification of PCBs 97 and 118

PCBs 118 and 97 were synthesized as indicated in section 2.2.1.

As described in previous sections, vacuum distillation to remove excess chlorobenzene followed by silica column clean-up were used as the initial clean-up stages for the crude residue containing PCBs 97
and 118. Heptane was used in the column elution which was again continued for about 100 ml after the yellow band had started to elute. The heptane was then removed by rotary evaporation. Studies with reversed-phase HPTLC indicated that 70% v/v THF in water, i.e. the same system as was used to separate PCBs 157 and 169, could also separate PCBs 97 and 118. The HPTLC results are shown in Figure 2.9.

Figure 2.9. Separation of PCBs 97 and 118 by RP-HPTLC using 70% v/v THF in water as the mobile phase.

<table>
<thead>
<tr>
<th>Solvent Front</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB 97+</td>
<td>1,2</td>
</tr>
<tr>
<td>156</td>
<td>118</td>
</tr>
<tr>
<td>DCB</td>
<td></td>
</tr>
</tbody>
</table>

In order to increase the HPLC separation a slightly lower proportion of THF (65% v/v in water) was used, at a flow rate of 11.6 ml min⁻¹ and an oven temperature of 50°C. The HPLC equipment used is detailed in section 2.2.1. A saturated solution of the PCB residue in mobile phase was used for the HPLC and repeated injections were made. A
A typical chromatogram is shown in Figure 2.10.

**Figure 2.10.** Chromatogram obtained during the separation of PCBs 97 and 118 by preparative HPLC.

As before, the HPTLC results and dipole moment considerations were used to determine the identities of each peak. After evaporation of the mobile phase, both PCBs 97 and 118 appeared off-white in colour, suggesting some impurities were still present. This was reflected in the presence of impurity peaks in the HPLC chromatogram (Figure 48).
2.10) in contrast to the cleaner appearance of PCBs 157 and 169 after HPLC clean-up and the presence of fewer impurity peaks in their chromatogram (Figure 2.6). This indicates that a preliminary HPLC clean-up, as was performed with PCBs 157 and 169 using acetonitrile as mobile phase, would have been desirable for PCBs 97 and 118. As this rather time-consuming stage had been omitted, PCB 118 was subjected to a second heptane/silica column clean-up after the HPLC stage, stopping the elution before any colour appeared. After removal of the heptane by rotary evaporation PCB 118 was recrystallised from methanol, yielding white crystals.

PCB 97 was recrystallised three times from 50% v/v acetonitrile in methanol, yielding slightly off-white crystals. Both PCBs were finally dried in a vacuum oven at 78°C for four hours.
2.3 Animals

All rats used were virgin female Wistar albino rats, Bath University strain. In the neurotoxicity study, virgin female C57BL mice (30-43g) obtained from stock bred in-house were used. In the dermal toxicity study male hairless mice of the MFl-hr strain were used. All animals were housed under laboratory conditions (12 hour/12 hour light cycle) and fed commercial rat chow (CRM, Labsure, Manea, Cambs.) and water ad libitum.

2.4 Dosing

A variety of dosing schedules were used, which are outlined below.

2.4.1 AROCLOR 1254 Maternal Dosing Regimens

Each rat was assigned to one of three different dosing regimens. In each regimen, five rats were each dosed with a total dose of 180 mgKg$^{-1}$ of AROCLOR 1254 in olive oil, administered in six separate doses of 30 mgKg$^{-1}$ per dose by oral dosing tube. Each dose was calculated individually on the day of administration using the body weight on that day. A further five rats received olive oil only, using the same dosing pattern, and served as controls for that dosing regimen. The three dosing regimens used are outlined below:

Regimen 1: Mature rats (250 to 300g at mating) were dosed once weekly for six weeks and mated ten days after the last dose.
Regimen 2: Mature rats (200 to 250g at mating) were mated and then dosed twice weekly for three weeks during gestation, starting on the day of conception.

Regimen 3: Mature rats (200 to 250g at mating) were mated and dosed twice weekly for three weeks starting on the day of parturition.

Litters were weighed on the day of birth and offspring were allowed to suckle normally from their mothers. At age 21 days the litters were weighed again and two males and two females from each litter were each weighed separately and killed by concussion followed by cervical dislocation. Their livers were immediately removed and placed in ice-cold phosphate buffer (buffer no.1, Appendix 1) and then used for the preparation of microsome suspensions by the method described in section 2.6.

2.4.2 Maternal Dosing of Rats with PCB Congeners

Mature rats (200 to 250g) were mated and litters were born as normal and weighed on the day of birth. Each dam (with her litter) was then assigned to one of 6 different treatment groups with 5 dams per group. Dams were dosed by oral dosing tube twice weekly for three weeks starting on the day of parturition, during which time the pups were allowed to suckle normally from their mothers. Each group received a different PCB congener dissolved in olive oil, with the exception of one (control) group which received olive oil only. The congeners used were PCBs 97, 118, 157, 169 and 189. The total dose of each PCB administered was 0.5 mmolKg\(^{-1}\) with the exception of
PCB 169 for which a total dose of $0.05 \text{ mmol Kg}^{-1}$ was used. As already indicated, this total dose was divided into 6 single doses given over the 3 week period between parturition and weaning. Each dose was calculated individually on the day of administration using the body weight on that day.

At age 21 days the litters were removed from their mothers and weighed again. At the same time two males and two females from each litter were weighed individually and killed by concussion followed by cervical dislocation. Their livers were immediately removed and placed in ice-cold phosphate buffer (buffer no.1, Appendix 1) and then used for the preparation of microsome suspensions by the method described in section 2.6. After removal of the litter each dam was mated again during the following week and allowed to deliver and rear her second litter as before. No further dosing was carried out. The pups were treated in the same manner as the first litters and microsome suspensions prepared from the livers of two males and two females from each litter.

2.4.3 Co-Administration of PCBs 97 and 169 to Rats

Study 1: Oral Dosing. Three week old rats (40-57g at first dosing) were each assigned to one of four different treatment groups with four rats per group. Dosing was then carried out on days 1 and 8, using either a solution of a PCB dissolved in olive oil or olive oil alone. This protocol is summarized in Table 2.3.
Table 2.3. Dosing protocol used to study the combined effects of two PCB congeners in the first combination study.

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>Treatment</th>
<th>Received</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 8</td>
</tr>
<tr>
<td>1</td>
<td>olive oil</td>
<td>PCB 169</td>
</tr>
<tr>
<td>2</td>
<td>PCB 97</td>
<td>olive oil</td>
</tr>
<tr>
<td>3</td>
<td>PCB 97</td>
<td>PCB 169</td>
</tr>
<tr>
<td>4</td>
<td>olive oil</td>
<td>olive oil</td>
</tr>
</tbody>
</table>

As can be seen, the dosing protocol involved the administration of PCB 97 seven days prior to the administration of PCB 169 (treatment group 3). Each of these PCBs were in addition administered separately (groups 1 and 2) with group 4 serving as a control. The doses given were 0.3 mmol Kg$^{-1}$ for PCB 97 and 0.025 mmol Kg$^{-1}$ for PCB 169, administered as single doses by oral dosing tube. On day 13 rats were weighed and killed by concussion followed by cervical dislocation and their livers were removed and used to prepare microsome suspensions according to the method described in section 2.6.

Study 2: i.p. Dosing. Sixteen-day old rats (37-46g at first dosing) were each assigned to one of four different treatment groups with four rats per group. Dosing was then carried out on days 1 and 7, using either a solution of a PCB dissolved in olive oil or olive oil alone. The dosing protocol used is summarised in table 2.4 and was
based on that used in the first combination study with some modifications in timing and dose.

Table 2.4. Dosing protocol used to study the combined effects of two PCB congeners in the second combination study.

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>Treatment</th>
<th>Received</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>olive oil</td>
<td>PCB 169</td>
</tr>
<tr>
<td>2</td>
<td>PCB 97</td>
<td>olive oil</td>
</tr>
<tr>
<td>3</td>
<td>PCB 97</td>
<td>PCB 169</td>
</tr>
<tr>
<td>4</td>
<td>olive oil</td>
<td>olive oil</td>
</tr>
</tbody>
</table>

PCB 97 was administered 6 days prior to the administration of PCB 169, the doses were increased to 0.5 mmolKg⁻¹ for PCB 97 and 0.05 mmolKg⁻¹ for PCB 169, and doses were administered by i.p. injection. On day 10, rats were weighed, killed by concussion followed by cervical dislocation and their livers removed and used to prepare microsome suspensions by the method described in section 2.6.

2.4.4 Dosing of Immature Rats with PCB Congeners (Organ Wt. Study)

Sixteen-day old female Wistar rats (32-43g) were each allocated to one of five treatment groups, with four rats per group. Each rat received a single i.p. dose of 0.5 mmolKg⁻¹ PCB in olive oil.
Controls (7 rats) received olive oil only. All rats were killed by CO₂ gassing 14 days after dosing and the liver, thymus gland, heart, spleen and kidneys were immediately removed.

2.4.5 **PCB 169 Administration to Mice (Neurotoxicity Study)**

Five female mice (30-43g) received 30 mgKg⁻¹/day⁻¹ PCB 169 dissolved in olive oil on days 10-16 of gestation. The total dose administered to each mouse was therefore 210 mgKg⁻¹. In addition, five mice received an equivalent dose of olive oil only and served as controls. All doses were administered by oral dosing tube.

2.4.6 **Topical Administration of PCB Congeners to Mice (Dermal Study)**

On each dosing day, 0.2 ml of a 5 mgml⁻¹ solution of a PCB congener dissolved in acetone was slowly applied by automatic pipette to the skin of the back, upper flanks and rump of each animal. Care was taken to ensure complete coverage of the whole area with PCB as the acetone evaporated. Dosing was carried out on each of five days per week for six consecutive weeks. This provided a total dose of 30mg of PCB per animal over the six weeks of the study. In addition, five mice each received acetone only instead of PCB solution, applied in the same manner, and served as controls. The study was carried out in two stages:

1. Six mice (35-46g, Bath University stock) were treated with either PCB 157 (four mice) or acetone only (two mice);

2. Eleven mice (23-33g) obtained from an external supplier
(Olac Ltd., Shaw's Farm, Bicester, Oxon.) were treated either with PCB 189 (four mice) or PCB 97 (four mice) or acetone only (three mice).

Mice were weighed at weekly intervals during the study. On completion of the six-week dosing period mice were killed by concussion followed by cervical dislocation. The skin of the back was removed as one complete sheet, keeping the epidermis and dermis intact and undamaged. The sheet of skin was spread over a microscope slide to maintain it in a flat position and prevent it from curling up. The slide and skin were then immersed in a solution of 40% formaldehyde. Skin sections were later prepared for histological examination by the standard procedure of processing to paraffin blocks, cutting a section of thickness 5μm and staining with haematoxylin and eosin.

2.5 Biological Measurements

PCB-induced changes in the dosed animals or their pups were monitored by measuring the parameters detailed below.

2.5.1 AROCLOR 1254 Maternal Dosing Regimens

For each litter, the litter size and litter weight at birth and at age 21 days were recorded, differentiating between male and female pups. For each microsome suspension prepared, measurements were made of protein concentration, aniline hydroxylase activity and aminopyrine-N-demethylase activity (see section 2.6).
In addition, for each 21 day old rat killed the organ weight:body weight ratio was recorded for thymus gland, heart, spleen and kidneys.

2.5.2 Maternal Dosing of Rats with PCB Congeners

For each litter, the litter size and litter weight at birth and at age 21 days were recorded, differentiating between male and female pups. For each microsome suspension prepared, measurements were made of protein concentration, cytochrome P-450 concentration, aniline hydroxylase activity and aminopyrine-N-demethylase activity (see section 2.6).

2.5.3 Co-Administration of PCBs 97 and 169 to Rats

For each microsome suspension prepared, measurements were made of the protein concentration, cytochrome P-450 concentration, aniline hydroxylase activity and aminopyrine-N-demethylase activity (see section 2.6).

2.5.4 Dosing of Immature Rats with PCB Congeners (Organ Wt. Study)

After removal of the organs from each animal and patting dry on filter paper, the weights of the liver, thymus gland, heart, spleen and kidneys were recorded.
2.5.5 PCB 169 Administration to Mice (Neurotoxicity Study)

Following the birth of each litter, offspring were allowed to suckle normally from their mothers until weaning at age 3 weeks. During this period, and for a further 3 weeks following weaning, pups were examined at five-day intervals for signs of abnormal behaviour (particularly circular movements).

2.5.6 Topical Administration of PCB Congeners to Mice (Dermal Study)

Histological examination of the mouse skin samples was carried out using a Weiss light microscope under x250 magnification.

2.6 Biochemical Assays

2.6.1 Preparation of Microsome Suspensions

Rat livers were weighed and then homogenised in ice-cold M/15 phosphate buffer pH 7.35 containing 1mM EDTA and 20% glycerol (buffer no.1, Appendix 1), using a Potter-Elvehjem type glass homogeniser equipped with a teflon pestle. Six vertical strokes of the homogeniser were used at a homogeniser speed of 2,000 rpm. The homogenate was then centrifuged at 10,000 xg for 20 minutes in a MSE High Speed 18 centrifuge. The resultant supernatant fraction was recentrifuged at 100,000 xg for one hour in a Beckman L8-70M ultracentrifuge. The microsome pellet obtained was suspended in M/15 phosphate buffer pH 7.35 (buffer no.2, Appendix 1) using a
hand-driven ground-glass homogeniser to yield a suspension with a protein concentration of between 5 and 10 mg ml\(^{-1}\). All operations were carried out at 4°C. EDTA was included in the homogenisation buffer because it is reported to prevent aggregation of microsome particles and their subsequent appearance in the 10,000 xg pellet, and to decrease the degree of contamination of the 100,000 xg (microsomal) pellet with ribosomes (127). Glycerol was included in both buffers no.1 and no.2 to inhibit the conversion of cytochrome P-450 to P-420 (128,129).

Microsome suspensions were stored in small containers (each holding about 0.5ml suspension) which were frozen (-20°C) immediately after preparation until being thawed at room temperature immediately before use. By storing the suspensions in small aliquots, each sample was subjected to the freeze/thaw process only once before use, thereby minimising any damage to the proteins that the freezing/defrosting process may cause. The stability of deep frozen microsome samples has been reviewed by Hubbard et al. (130) who concluded that enzyme activities are not generally adversely affected by storage at -80°C or below for periods of up to about 7 months. In order to check the stability of samples stored in this project, microsome suspensions were prepared according to the method described above, using livers from rats dosed with AROCLOR 1254. The samples were stored at -20°C, thawed at room temperature and the activity of aminopyrine-N-demethylase determined in each sample at intervals of 0, 1, 5 and 6 months. The results are shown in Table 2.5 and indicate that enzyme activity was generally not significantly affected by storage for 6 months. In practice, all
enzyme activity determinations were carried out within three months of sample preparation.

Table 2.5. Effect of storage time on the activity of aminopyrine-N-demethylase in liver microsome suspensions. All samples were stored at -20°C and thawed once before use.

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>0 months</th>
<th>1 month</th>
<th>5 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>117</td>
<td>93</td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>98</td>
<td>90</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>101</td>
<td>85</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>108</td>
<td>99</td>
<td>103</td>
</tr>
</tbody>
</table>

*Expressed as a percentage of the activity at zero months (i.e. assayed on the day following sample preparation) for the same animal.

2.6.2 Protein Determinations

Microsomal protein concentrations were determined by the method of Lowry et al. (131) using bovine serum albumin as standard. Albumin standard solutions were made using a x40 dilution of buffer no.2 (Appendix 1) in saline. The assay showed a linear relationship between absorbance of the final solutions and the protein
concentration of the standards, up to a limit of 0.4 mgml\(^{-1}\). Dilutions (x40) of the microsomal suspensions were made using 0.9% saline, to give a protein concentration in the range 0.1-0.3 mgml\(^{-1}\) before being used in the assay.

2.6.3 Cytochrome P-450 Concentration

Cytochrome P-450 concentrations in liver microsome suspensions were determined by the method of Omura and Sato (108) using microsome suspensions solubilised with the non-denaturing detergent (CHAPS) developed by Hjelmeiland (132). Microsome suspensions were diluted with phosphate buffer containing glycerol and CHAPS (buffer no.4, Appendix 1) to give a clear solution with a protein concentration of 1.0 mgml\(^{-1}\). This solution was transferred to sample and reference cuvettes and the baseline absorption spectrum recorded between 400 and 500 nm. The instrument used was a Perkin Elmer Lambda 3 dual beam spectrophotometer, scanning at 60 nmmin\(^{-1}\). The sample cuvette was then gassed with carbon monoxide by bubbling the gas through the solution in the cuvette at a rate of about 1 bubble sec\(^{-1}\) for 90 seconds. The spectrum was recorded (spectrum 1) giving a single maximum around 420 nm which represented the absorbance due to the haemoglobin:CO complex.

Approximately three milligrams of sodium dithionite were then added to each cuvette, the sample cuvette was gassed a second time with CO in the same manner as before, and the spectrum recorded again (spectrum 2). This also showed a minor maximum around 420 nm, this time representing the absorbance due to both the reduced cytochrome
P-420:CO and the haemoglobin-CO complexes. Spectrum 2 also showed a maximum around 450 nm, representing the absorbance due to the reduced cytochrome P-450:CO complex, the peak height of which was obtained by subtracting the absorbance at 490 nm from that at the maximum. Using this peak height, the concentration of cytochrome P-450 was calculated in nmol/mg protein assuming an extinction coefficient of 91 M⁻¹ cm⁻¹. The results obtained ranged from 0.3 to 2.5 nmol/mg protein. The wavelength of this absorbance maximum was also recorded. A typical set of spectra is shown in Appendix 2.

The tendency of cytochrome P-450 to convert to P-420 (108) during sample preparation may lead to an underestimation of the amount of the former originally present in vivo. The approximate magnitude of this conversion is indicated by the relative peak heights of the maxima at 420 and 450 nm seen in spectrum 2. However, because the minor (420 nm) peak occurs on the shoulder of the main (450 nm) peak, an accurate estimate of the concentration of the components giving rise to the minor peak cannot be obtained. In addition, the size of the minor peak is also affected by the haemoglobin content of the solution (133) since the haemoglobin:CO complex also shows a maximum in this region (spectrum 1). A comparison of spectra 1 and 2 for each sample indicated that generally the absorbance maximum at 420 nm was due mainly to the presence of haemoglobin in the samples, in which case little conversion of P-450 to P-420 had occurred. The calculation of P-450 concentration was based on this assumption.

To test the reliability of the assay, a series of dilutions of the same microsome suspension was prepared using buffer no.4 (appendix
1). This provided solutions (in duplicate) containing 0.5, 1.0, 1.5 and 2.0 mgml⁻¹ protein. The absorbance spectrum of the reduced P-450:CO complex was recorded for each solution using the method already described. The results obtained were used to construct a graph of absorbance (at the maximum) against protein concentration (Appendix 3). The graph shows that these two parameters are positively correlated, which forms the basis for the assay, and shows that a protein concentration of 1.0 mgml⁻¹ is suitable.

2.6.4 Aniline Hydroxylase Activity

Aniline hydroxylase activity in the microsomal suspensions was assayed by the colorimetric determination of p-aminophenol (PAP) produced in reaction mixtures incubated at 37°C for 25 minutes. The composition of the incubations was based on that used by Yoshimura et al. (120) and is shown in table 2.6. The measurement of PAP was based on the method of Brodie and Axelrod (134) as adapted by Imai et al. (135). Known amounts of PAP carried through the incubation and assay procedures served as standards. Before the modified assay could be adopted for use, some validation work was carried out to test the reliability of the method.

Incubations were carried out in 10ml stoppered tubes to which solutions of the various components of the incubation had been added by automatic pipette. Each batch of incubations consisted of standards (containing known amounts of PAP) and tests (containing a microsomal suspension being assayed). The enzyme in each standard was inactivated before incubation by the addition of trichloroacetic
acid (TCA). Details of the solutions added to these incubations are given in Appendix 4. After incubation with gentle shaking in a water-bath at 37°C for 25 minutes, the enzyme reaction was terminated in test incubations by the addition of 0.4ml 25% w/v aqueous TCA solution. All tubes were then centrifuged at 3000 rpm for ten minutes, after which 1.0ml of the supernatant was added to 0.3ml of 15% w/v aqueous sodium carbonate solution followed by 0.5ml 4% w/v phenol in 0.4M sodium hydroxide solution (freshly prepared). After mixing, the solutions were incubated at 20°C for one hour to allow colour formation and their absorbances read at 630 nm against the standard incubation containing 0 mcg PAP. The instrument used was a Pye Unicam 8620 single beam, fixed wavelength spectrophotometer.

Table 2.6. Composition of incubations used in the assay of aniline hydroxylase activity.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline</td>
<td>1 μmole</td>
</tr>
<tr>
<td>NADP</td>
<td>0.33 μmoles</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>8 μmoles</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>6 μmoles</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>1 unit</td>
</tr>
<tr>
<td>Microsomal suspension</td>
<td>equiv. to 1mg protein</td>
</tr>
<tr>
<td>Buffer No. 3, appendix l</td>
<td>to 1.0 ml</td>
</tr>
</tbody>
</table>

64
From a calibration curve of absorbance against PAP concentration in the standard incubations, (see below) the amount of PAP formed in each test incubation was determined and used to calculate the enzyme activity in units of mmol PAP formed/mg protein/minute. Initially incubations were carried out in triplicate, but once the precision of the assay had been shown in use only duplicates were used.

**Standard Incubations.** The assay requires a linear relationship between the absorbance of the coloured solutions measured at the end of the assay and the amount of PAP present. An example of the absorbances obtained from the standard incubations is presented in Table 2.7 which shows a linear relationship \((r=0.998)\) over the range 0-6 mcg PAP/incubation.

**Table 2.7.** Absorbance of final solutions obtained with the assay of aniline hydroxylase activity, using triplicate standard incubations of inactivated enzyme.

<table>
<thead>
<tr>
<th>PAP, mcg/incubation</th>
<th>Absorbances</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0, 0.016, 0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.027, 0.026, 0.025</td>
</tr>
<tr>
<td>1.0</td>
<td>0.060, 0.066, 0.060</td>
</tr>
<tr>
<td>2.0</td>
<td>0.145, 0.137, 0.134</td>
</tr>
<tr>
<td>4.0</td>
<td>0.297, 0.308, 0.298</td>
</tr>
<tr>
<td>6.0</td>
<td>0.438, 0.432, 0.464</td>
</tr>
</tbody>
</table>
**Assay Validation using Active Enzyme.** Measurements were made of the change in reaction rate with time and with the amount of protein present, using microsomes prepared from the livers of immature rats dosed with AROCLOR 1254. The effect of time on reaction rate was assessed by carrying out the assay using a constant amount of the same microsomal suspension (equivalent to 1mg protein) incubated at various times (0, 5, 10, 15, 20, 25 and 30 minutes) at 37°C. Each incubation was performed in triplicate and the results are shown graphically in Appendix 5. A linear relationship was found between the absorbance (and therefore the amount of PAP produced) and time up to 30 minutes. From these results a standard incubation time of 25 minutes was adopted for use in this assay, being within the region of linearity. The effect of varying the amount of protein present in the incubations was assessed by carrying out the assay using varying amounts of the same microsomal suspension, to provide a range of up to 1.5mg protein per incubation. Each incubation was performed in triplicate and the results are shown graphically in Appendix 6. A linear relationship was found between absorbance and the amount of protein present. This indicated the suitability of using 1.0mg protein per incubation in this assay.

The precision of the assay was assessed from the variation in the absorbances obtained from triplicate incubations. Table 2.8 shows the data used to construct the graph in Appendix 6, showing the change in absorbance against the amount of protein present. The variation between similar incubations is generally acceptable. This precision is also reflected in the data in Table 2.7, which was obtained using standard incubations.
Table 2.8. Absorbance of final solutions in the assay of aniline hydroxylase activity, using triplicate incubations of different amounts of the same microsomal suspension incubated at 37°C for 25 minutes.

<table>
<thead>
<tr>
<th>Volume of suspension incubated, ml</th>
<th>Absorbances</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0, 0.001, 0.002</td>
</tr>
<tr>
<td>0.05</td>
<td>0.042, 0.042, 0.041</td>
</tr>
<tr>
<td>0.10</td>
<td>0.081, 0.085, 0.087</td>
</tr>
<tr>
<td>0.15</td>
<td>0.121, 0.127, 0.122</td>
</tr>
<tr>
<td>0.20</td>
<td>0.159, 0.161, 0.162</td>
</tr>
<tr>
<td>0.25</td>
<td>0.191, 0.192, 0.186</td>
</tr>
<tr>
<td>0.30</td>
<td>0.214, 0.215, 0.211</td>
</tr>
</tbody>
</table>

2.6.5 Aminopyrine-N-Demethylase Activity

Aminopyrine-N-demethylase activity in the microsomal suspensions was assayed by the colorimetric determination of formaldehyde produced in reaction mixtures incubated at 37°C for 25 minutes. The composition of the incubations was based on that used by Yoshimura et al. (120) and is shown in table 2.9. The measurement of formaldehyde was based on the method of Nash (136) as adapted by Cochin and Axelrod (137). Known amounts of formaldehyde carried
through the incubation and assay procedures served as standards. Before the modified assay could be adopted for use, some validation work was carried out to test the reliability of the method.

Table 2.9. Composition of incubations used in the assay of aminopyrine-N-demethylase activity.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopyrine</td>
<td>1 µmole</td>
</tr>
<tr>
<td>Semicarbazide</td>
<td>11 µmoles</td>
</tr>
<tr>
<td>NADP</td>
<td>0.33 µmoles</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>8 µmoles</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>6 µmoles</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>1 unit</td>
</tr>
<tr>
<td>Microsomal suspension</td>
<td>equiv. to 1 mg protein</td>
</tr>
<tr>
<td>Buffer No. 3, appendix 1</td>
<td>to 1.0 ml</td>
</tr>
</tbody>
</table>

Incubations were carried out in 10ml stoppered tubes to which solutions of the various components of the incubation had been added by automatic pipette. Each batch of incubations consisted of standards (containing known amounts of formaldehyde) and tests (containing a microsomal suspension being assayed). The enzyme in each standard was inactivated before incubation by the addition of zinc sulphate solution. Details of the solutions added to these incubations are given in Appendix 7. After incubation with gentle shaking in a water-bath at 37°C for 25 minutes, the enzyme reaction
was terminated in test incubations by the addition of 0.4ml 15% w/v aqueous zinc sulphate solution followed after five minutes by 0.4ml saturated barium hydroxide solution. At this stage the standard incubations also received 0.4ml saturated barium hydroxide solution. All incubation tubes were then centrifuged at 3000 rpm for 10 minutes, after which 1.0ml of each supernatant was added to 0.4ml double-strength Nash reagent (freshly prepared, containing ammonium acetate 7.5g and acetyl acetone 0.1ml in water to 25.0ml). After mixing, the solutions were incubated at 60°C for 30 minutes to allow colour formation and their absorbances measured at 415nm against the standard incubation containing 0 mcg formaldehyde. The instrument used was a Pye Unicam 8620 single beam, fixed wavelength spectrophotometer.

From a calibration curve of absorbance against formaldehyde concentration in the standard incubations (see below) the amount of formaldehyde formed in each test incubation was determined and used to calculate the enzyme activity in units of nmol HCHO formed/mg protein/minute. Initially incubations were carried out in triplicate, but once the precision of the assay had been demonstrated in use only duplicate assays were considered necessary.

**Standard Incubations.** The assay requires a linear relationship between the absorbance of the coloured solutions measured at the end of the assay and the amount of formaldehyde present. An example of the absorbances obtained from the standard incubations is presented in Table 2.10 which shows a linear relationship (r=0.998) over the range 0-6 mcg HCHO/incubation.
Table 2.10. Absorbance of final solutions in the assay of aminopyrine demethylase activity, using triplicate standard incubations of inactivated enzyme.

<table>
<thead>
<tr>
<th>HCHO, mcg/incubation</th>
<th>Absorbances</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0, 0.026, 0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.070, 0.065, 0.057</td>
</tr>
<tr>
<td>1.0</td>
<td>0.104, 0.106, 0.122</td>
</tr>
<tr>
<td>2.0</td>
<td>0.192, 0.214, 0.218</td>
</tr>
<tr>
<td>4.0</td>
<td>0.362, 0.403, 0.388</td>
</tr>
<tr>
<td>6.0</td>
<td>0.601, 0.604, 0.599</td>
</tr>
</tbody>
</table>

Assay Validation using Active Enzyme. Measurements were made of the change of reaction rate with time and the amount of protein present, using microsomes prepared from the livers of immature rats induced with AROCLOR 1254. The effect of time on reaction rate was assessed by carrying out the assay using a constant amount of microsomal suspension (equivalent to 1.0 mg protein) incubated at various times (0, 5, 10, 15, 20, 25 and 30 minutes) at 37°C. Each incubation was performed in triplicate and the results are shown graphically in Appendix 8. A linear relationship was found between the absorbance (and therefore the amount of formaldehyde produced) and time up to 30 minutes. From these results a standard incubation time of 25 minutes was adopted for use in this assay, being within the region of linearity. The effect of varying the amount of protein present in
the incubations was assessed by carrying out the assay using varying amounts of the same microsomal suspension, to provide a range of up to 1.5mg protein per incubation. Each incubation was performed in triplicate and the results are shown graphically in Appendix 9. A linear relationship was found between the absorbance and the amount of protein present. This indicated the suitability of using 1.0mg protein per incubation in this assay.

The precision of the assay was assessed from the variation in the absorbances obtained from triplicate incubations. Table 2.11 shows the data used to construct the graph in Appendix 9, showing the change in absorbance against the amount of protein present. The variation between similar incubations is generally acceptable. This precision is also reflected in Table 2.10, which was obtained using standard incubations.
Table 2.11. Absorbance of final solutions obtained in the assay of aminopyrine-N-demethylase activity, using triplicate incubations of different amounts of the same microsome suspension incubated at 37°C for 25 minutes.

<table>
<thead>
<tr>
<th>Volume of suspension incubated, ml</th>
<th>Absorbances</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.021, 0, 0.030</td>
</tr>
<tr>
<td>0.05</td>
<td>0.122, 0.142, 0.173</td>
</tr>
<tr>
<td>0.10</td>
<td>0.227, 0.240, 0.275</td>
</tr>
<tr>
<td>0.15</td>
<td>0.336, 0.343, 0.367</td>
</tr>
<tr>
<td>0.20</td>
<td>0.430, 0.440, 0.417</td>
</tr>
<tr>
<td>0.25</td>
<td>0.465, 0.462, 0.502</td>
</tr>
<tr>
<td>0.30</td>
<td>0.524, 0.530, 0.556</td>
</tr>
</tbody>
</table>
CHAPTER THREE

IDENTITY, PURITY AND YIELDS OF PCB CONGENERS

RESULTS AND DISCUSSION

The identity of each of the PCB congeners synthesized was confirmed by proton nuclear magnetic resonance spectra, mass spectrometry and the comparison of melting points with published values where available. These techniques along with capillary gas chromatography also allowed an assessment of purity. The results are presented and discussed below.

3.1 PCB Yields and Purities

The yields and purities of each of the purified PCB congeners are shown in Table 3.1.

The yield of PCB 118 was thought to be low due to some being left behind on the second silica column clean-up. The relatively low yield of PCB 169 compared to that of PCB 157 (a product of the same reaction) may be at least partly due to the fact that the reaction favours the latter, so that a greater yield of PCB 157 would be expected.

Table 3.1 also shows the yields expressed as a percentage of the theoretical maximum. This indicates the efficiency of the entire synthesis and clean-up procedure in the production of PCB product. The low percentage yield of PCB 156 compared to the others was
possibly due to the smaller amounts of reactants used (see section 2.2.1) resulting in proportionally greater losses during clean-up. Apart from the low value for PCB 156, the percentage yields from the different reactions are fairly consistent.

<table>
<thead>
<tr>
<th>PCB No.</th>
<th>Yield, mg</th>
<th>Theoretical, g</th>
<th>% Yield</th>
<th>% Purity +</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
<td>720</td>
<td>8.163</td>
<td>13</td>
<td>99.0</td>
</tr>
<tr>
<td>118</td>
<td>360</td>
<td>&gt;99.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>156</td>
<td>240</td>
<td>4.332</td>
<td>6</td>
<td>&gt;98.0</td>
</tr>
<tr>
<td>157</td>
<td>935</td>
<td>10.830</td>
<td>11</td>
<td>99.4</td>
</tr>
<tr>
<td>169</td>
<td>214</td>
<td></td>
<td>98.0</td>
<td></td>
</tr>
<tr>
<td>189</td>
<td>749</td>
<td>9.888</td>
<td>8</td>
<td>&gt;98.0</td>
</tr>
</tbody>
</table>

* assumes 100% conversion of chlorinated aniline to PCB  
+ calculated from the gas chromatograms, assuming equal response factors for all peaks.

74
3.2 PCB 156

Melting Point. 124.1 to 124.9°C at 0.3°C min\(^{-1}\). No published value was available for comparison.

Proton NMR. There is good agreement between the proton NMR results for PCB 156 (Figure 3.1) and published data (125,138). Some very weak impurity signals were present in the region between \(\delta = 0.94\) and \(\delta = 3.70\) ppm. These are likely to be due to residual ethanol and possibly a hydrocarbon-like material which could have originated in the silica column or the HPLC column.

Mass Spectrometry. The mass spectrum obtained for PCB 156 (Figure 3.2) shows the expected m/z values, isotope clustering and fragmentation patterns that indicate a hexachlorobiphenyl (139). The few very weak impurity peaks present suggest slight traces of a hydrocarbon-like compound, ethanol, and possibly some heptachlorobiphenyl. The presence of a phthalate plasticiser was suggested by a peak at m/z = 149.

Capillary GC. The chromatogram obtained for PCB 156 shows a major peak at \(t = 34.1\) minutes (peak height 107.5 mm). Some small impurities were eluted up to \(t = 3.0\) minutes but none thereafter, suggesting that no long chain hydrocarbon type of impurities were present.
Figure 3.1. Proton NMR Spectra of PCB 156
Figure 3.2. 70 eV EI Mass Spectrum of PCB 156
3.3 PCB 157

Melting Point. 150.5 to 151.0°C at 0.5°C min⁻¹. No published value was available for comparison.

Proton NMR. There is good agreement between the proton NMR results for PCB 157 (Figure 3.3) and published data (125,138). Some very weak impurity signals were present which may be due to ethanol or a hydrocarbon chain although the indications were less clear than in the case of PCB 156. An extremely weak signal at $\delta = 7.54$ was due to the presence of very low levels of PCB 169.

Mass Spectrometry. The mass spectrum obtained for PCB 157 (Figure 3.4) shows the expected m/z values, isotope clustering and fragmentation patterns that indicate a hexachlorobiphenyl (139). No significant impurity peaks were seen.

Capillary GC. The chromatogram obtained for PCB 157 shows a major peak at $t = 35.4$ minutes (peak height 153.5mm). Some small impurities were eluted up to $t = 3.0$ minutes after which one minor contaminant appeared at $t = 4.7$ minutes (peak height 1.5mm) and one trace contaminant (PCB 169) at $t = 37.2$ minutes (peak height 1.0mm). Assuming equal response factors and peak shapes for the two isomers, PCB 169 represented about 0.6% of the two PCBs present.
Figure 3.3. Proton NMR Spectra of PCB 157
Figure 3.4. 70 eV EI Mass Spectrum of PCB 157
3.4 PCB 169

Melting Point. 192.0 to 193.0°C at 0.4°C min⁻¹. Published value 201 to 202°C (140).

Proton NMR. There is good agreement between the proton NMR results for PCB 169 (Figure 3.5) and published data (125,138). Some impurity signals were present but interpretation was difficult and indicated the possible presence of a hydrocarbon chain. Low levels of PCB 157 were indicated.

Mass Spectrometry. The mass spectrum obtained for PCB 169 (Figure 3.6) shows the expected m/z values, isotope clustering and fragmentation patterns that indicate a hexachlorobiphenyl (139). Several weak impurity peaks were seen indicating the presence of phthalates and hydrocarbon chains.

Capillary GC. The chromatogram obtained for PCB 169 shows a major peak at t = 37.2 minutes (peak height 145.5mm). Minor impurity peaks appeared up to t = 3.0 minutes and a small impurity peak at 20.7 minutes (peak height 1.5mm). Some PCB 157 was eluted at 35.4 minutes (peak height 2.0mm).
Figure 3.5. Proton NMR Spectrum of PCB 169
Figure 3.6. 70 eV EI Mass Spectrum of PCB 169
3.5 PCB 189

Melting Point. 151.0 to 153.0°C at 0.5°C min⁻¹. Published value 162 to 163°C (141).

Proton NMR. There is good agreement between the proton NMR results for PCB 189 (Figure 3.7) and published data (125,138). Some very weak impurity signals were present in the region between δ =1.05 and δ=2.18 ppm. A moderate signal appeared at δ =3.49 due to residual methanol. As with the other PCB congeners, these weak impurity signals may have been due to some hydrocarbon-like compound.

Mass Spectrometry. The mass spectrum obtained for PCB 189 (Figure 3.8) shows the expected m/z values, isotope clustering and fragmentation patterns that indicate a heptachlorobiphenyl (139). The few very weak impurity peaks present suggest slight traces of hydrocarbon chains and phthalates.

Capillary GC. The chromatogram obtained for PCB 189 shows a major peak at t = 38.6 minutes, the height of which exceeded the scale of the recorder. Rapidly eluted impurities were absent. A series of minor impurity peaks appeared between t = 21.5 and 36.9 minutes. The absence of the rapidly (up to 3.0 minutes) eluted contaminants, present in all the three PCBs already discussed, may be due to the use of methanol instead of ethanol as the recrystallisation solvent. The identities of the later appearing impurities were unknown.
Figure 3.7. Proton NMR Spectra of PCB 189
Figure 3.8. 70 eV EI Mass Spectrum of PCB 189
3.6 PCB 118

Melting Point. 106.0 to 106.5°C at 1.0°C min⁻¹. Published value 105 to 107°C (141) and 112 to 113°C (142).

Proton NMR. There is good agreement between the proton NMR results for PCB 118 (Figure 3.9) and published data (125,138). Some weak impurity signals appeared in the region between δ = 0.87 and δ = 3.50 ppm suggesting the presence of residual methanol and low levels of a hydrocarbon-like material.

Mass Spectrometry. The mass spectrum obtained for PCB 118 (Figure 3.10) shows the expected m/z values, isotope clustering and fragmentation patterns that indicate a pentachlorobiphenyl (139). Some weak peaks were seen indicating phthalate and hydrocarbon chain impurities. A cluster of peaks appeared between m/z = 392 and 398 and a pair of peaks at m/z = 360 and 362. These may have been due to traces of heptachlorobiphenyls and hexachlorobiphenyls, which may have originated from higher chlorinated impurities in the original reactants.

Capillary GC. The chromatogram obtained for PCB 118 shows a major peak at t = 32.0 minutes (peak height 149.5mm). There were no rapidly eluted contaminants visible. A few very small peaks (none > 1mm) appeared between t = 21.6 and 26.0 minutes. The only significant impurity peak appeared at 44.8 minutes (peak height 5mm) which may have been the heptachlorobiphenyl seen in the mass spectrum.
Figure 3.9. Proton NMR Spectra of PCB 118
Figure 3.10. 70 eV EI Mass Spectrum of PCB 118
3.7 PCB 97

Melting Point. 78.0 to 79.0°C at 0.5°C min⁻¹. Published value 81 to 82°C (141) and 81.5 to 82.5°C (142).

Proton NMR. There is good agreement between the proton NMR results for PCB 97 (Figure 3.11) and published data (138). A few weak impurity peaks were present, indicating a hydrocarbon chain type of compound, and a trace of residual methanol.

Mass Spectrometry. The mass spectrum obtained for PCB 97 (Figure 3.12) shows the expected m/z values, isotope clustering and fragmentation patterns that indicate a pentachlorobiphenyl (143) with very weak hydrocarbon and phthalate impurity peaks.

Capillary GC. The chromatogram obtained for PCB 97 shows a major peak at t = 28.3 minutes, the peak height of which exceeded the scale of the recorder. None of the very early peaks seen in some of the other PCBs were present. A series of very minor impurity peaks appeared between t = 18.8 and 26.8 minutes. The largest impurity was eluted at t = 36.8 minutes but was also very minor.

3.8 Discussion

The synthetic methods used, together with the proton NMR and mass spectroscopy results, confirmed the identity of each of the six PCB congeners made. These results and those of the capillary GC work indicated a degree of purity generally satisfactory for animal
Figure 3.11. Proton NMR Spectra of PCB 97
Figure 3.12. 70 eV EI Mass Spectrum of PCB 97
studies, with the contaminants seen being present at very low levels. A hydrocarbon-like impurity was detected in all congeners and could have originated in the solvents used or in the HPLC column. The phthalate impurity frequently seen may also have originated from the solvents. Traces of the recrystallisation solvent were commonly seen. Where ethanol had been used as the recrystallisation solvent, some very small GC peaks appeared early. These early peaks must have originated in the ethanol, since they were absent in PCBs 189, 118 and 97 for each of which methanol was used as the recrystallisation solvent. Although the off-white final colour of PCB 97 suggested that some coloured pigment may have still remained, the largest impurity peak seen on the gas chromatogram was very small compared to that of PCB 97 itself.

PCB 157 was contaminated with a very small amount (about 0.6% of total PCB 157+169 present) of PCB 169, which is reported to be considerably more potent biologically on a weight for weight basis than PCB 157. The possible toxicological significance of this is discussed in chapter 5.

PCB 118 contained traces of a heptachlorobiphenyl and possibly a hexachlorobiphenyl. Although they were present at low levels (the largest peak being about 3.3% of the peak height of PCB 118 itself) their structures were unknown and their likely biological effects can only be surmised. This point is also discussed further in chapter 5.

The presence of these low levels of impurities was reflected in the
general trend for the melting points to be depressed when compared to published values. This depression was not thought to be due to residual solvent, since further drying did not alter the melting points.

Further assurance about the identities of the PCB congeners can be obtained by considering their GC retention times which are shown in table 3.2. The results show that the order in which the congeners were eluted from the column was related to their level of chlorination, with the two pentachlorobiphenyls (PCBs 97 and 118) eluting first and the heptachlorobiphenyl (PCB 189) last. This trend has been observed by other workers (125).

Table 3.2. GC retention times of the PCB congeners

<table>
<thead>
<tr>
<th>PCB No.</th>
<th>Cl atoms</th>
<th>RT, mins</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
<td>5</td>
<td>28.3</td>
</tr>
<tr>
<td>118</td>
<td>5</td>
<td>32.0</td>
</tr>
<tr>
<td>156</td>
<td>6</td>
<td>34.1</td>
</tr>
<tr>
<td>157</td>
<td>6</td>
<td>35.4</td>
</tr>
<tr>
<td>169</td>
<td>6</td>
<td>37.2</td>
</tr>
<tr>
<td>189</td>
<td>7</td>
<td>38.6</td>
</tr>
</tbody>
</table>

The procedure developed in this study for the purification of the PCB congeners departs from previously reported methods (125,144) by
using preparative HPLC instead of preparative TLC. The latter has been the method commonly used for PCB congener purification prior to animal studies (103,145,146). Purification by HPLC was found to be convenient and quick and as such represents an improvement in the clean-up process. A report of this work was published in the Journal of Chromatography, 368, 174-179 (1986).
CHAPTER FOUR

PCB EFFECTS IN PUPS AFTER DIFFERENT MATERNAL DOSING REGIMENS: RESULTS AND DISCUSSION

This study examined the effects of varying the timing of administration of PCBs (before mating, during gestation or during lactation) to dams on the subsequent appearance of PCB related effects in their offspring. Measurements were made of litter size and weight, pup weight and pup survival. In 21-day old pups measurements were made of liver weight, liver microsomal protein concentration, and the activities of aniline hydroxylase and aminopyrine-N-demethylase in liver microsomes. For each parameter measured the unpaired student's t-test was used at the 5% confidence level to detect any differences due to PCB treatment compared to the controls used in the same dosing regimen. The results are presented and discussed below.

4.1 Litter Size

Table 4.1 shows the effect of AROCLOR 1254 on litter size at birth. No effect on this parameter was observed in any of the dosing regimens used. Dosing during lactation could not of course affect litters at birth.

96
Table 4.1. The effect of AROCLOR 1254 on litter size at birth

<table>
<thead>
<tr>
<th>Dosing Regimen</th>
<th>Treatment</th>
<th>Numbers of Pups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Males</td>
</tr>
<tr>
<td>Before Controls</td>
<td></td>
<td>7.3±2.5</td>
</tr>
<tr>
<td>mating AROCLOR</td>
<td></td>
<td>7.8±2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5)</td>
</tr>
<tr>
<td>During Controls</td>
<td></td>
<td>6.0±1.6</td>
</tr>
<tr>
<td>gestation AROCLOR</td>
<td></td>
<td>7.5±1.3</td>
</tr>
<tr>
<td>During Controls</td>
<td></td>
<td>6.0±2.4</td>
</tr>
<tr>
<td>lactation AROCLOR</td>
<td></td>
<td>5.8±2.2</td>
</tr>
</tbody>
</table>

Values represent mean of 4 litters±sd, except where shown otherwise in brackets.

4.2 Litter Weight

Total litter weight results are shown in table 4.2. Generally, AROCLOR 1254 had no significant effect on total litter weight at birth or at 21 days, and no effect on the litter weight increase over this period. The exception to this was a significantly heavier (p<0.05) litter weight at birth when dams were dosed before mating. This difference had disappeared by 21 days postpartum.
### Table 4.2. The effect of AROCLOR 1254 on total litter weights

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Treatment</th>
<th>Total Litter Wt. (g)</th>
<th>Birth</th>
<th>21 Days</th>
<th>Litter Wt. Gain (g) 0-21 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>Controls</td>
<td>74.0±7.7</td>
<td>566.2±41.6</td>
<td>492.2±35.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mating</td>
<td>83.3±3.3*</td>
<td>574.1±53.4</td>
<td>490.8±52.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td></td>
</tr>
<tr>
<td>During</td>
<td>Controls</td>
<td>71.5±4.2</td>
<td>499.5±11.4</td>
<td>428.1±12.7</td>
<td></td>
</tr>
<tr>
<td>gestation</td>
<td>AROCLOR</td>
<td>77.6±11.0</td>
<td>563.1±80.7</td>
<td>485.5±69.9</td>
<td></td>
</tr>
<tr>
<td>During</td>
<td>Controls</td>
<td>70.4±12.5</td>
<td>447.8±67.2</td>
<td>377.4±55.5</td>
<td></td>
</tr>
<tr>
<td>lactation</td>
<td>AROCLOR</td>
<td>67.2±5.7</td>
<td>416.5±94.6</td>
<td>349.3±95.3</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean of 4 litters+sd, except where shown otherwise in brackets.

* Values significantly different from controls (p<0.05)
4.3 Pup Weight

PCB treatment had no effect on the mean weight of male or female pups at birth (table 4.3) or at weaning (table 4.4).

Table 4.3. The effect of AROCLOR 1254 on mean pup weight per litter at birth.

<table>
<thead>
<tr>
<th>Dosing Regimen</th>
<th>Treatment</th>
<th>Mean Weight of Neonates (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Males</td>
</tr>
<tr>
<td>Before Controls</td>
<td></td>
<td>6.34±0.17</td>
</tr>
<tr>
<td>Mating AROCLOR</td>
<td></td>
<td>6.43±0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5)</td>
</tr>
<tr>
<td>During Controls</td>
<td></td>
<td>5.85±0.33</td>
</tr>
<tr>
<td>Gestation AROCLOR</td>
<td></td>
<td>6.07±0.13</td>
</tr>
</tbody>
</table>

Values represent mean of 4 litters±sd except where shown otherwise in brackets.
Table 4.4. The effect of AROCLOR 1254 on mean pup weight per litter at age 21 days.

<table>
<thead>
<tr>
<th>Dosing Regimen</th>
<th>Treatment</th>
<th>Mean Weight of Pups (g)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
<td></td>
</tr>
<tr>
<td>Before Controls</td>
<td>49.1±1.7</td>
<td>47.5±1.0</td>
<td></td>
</tr>
<tr>
<td>mating AROCLOR</td>
<td>43.9±5.1</td>
<td>43.4±4.0</td>
<td>(5) (5)</td>
</tr>
<tr>
<td>During Controls</td>
<td>41.7±3.4</td>
<td>38.6±1.5</td>
<td></td>
</tr>
<tr>
<td>gestation AROCLOR</td>
<td>43.9±1.7</td>
<td>41.1±3.5</td>
<td></td>
</tr>
<tr>
<td>During Controls</td>
<td>38.4±3.2</td>
<td>37.1±1.6</td>
<td></td>
</tr>
<tr>
<td>lactation AROCLOR</td>
<td>42.2±5.5</td>
<td>40.7±6.5</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean of 4 litters±sd except where shown otherwise in brackets

4.4 Pup Survival

The survival rate of pups between birth and weaning (95-98% in control groups) was unchanged by PCB treatment in all three dosing regimens.

4.5 Organ Weights

Tables 4.5 and 4.6 show the effects of AROCLOR 1254 on the
liver:body weight ratio and the absolute liver weight, respectively, in 21-day old pups. The ratio was significantly increased (p<0.05) by AROCLOR 1254 treatment in all groups. Absolute liver weight was significantly increased (p<0.001) when dams were dosed during gestation or lactation, but the increase seen with dosing before mating was not significant in either sex.

Table 4.5. The effect of AROCLOR 1254 on liver:body weight ratio in 21-day old rat pups.

<table>
<thead>
<tr>
<th>Dosing Regimen</th>
<th>Treatment</th>
<th>Liver : Body Weight Ratio</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before mating</td>
<td>Controls</td>
<td>0.037±0.002</td>
<td>0.036±0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AROCLOR</td>
<td>0.042±0.006*</td>
<td>0.043±0.007*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10)</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td>During gestation</td>
<td>Controls</td>
<td>0.035±0.002</td>
<td>0.033±0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AROCLOR</td>
<td>0.045±0.004**</td>
<td>0.045±0.004**</td>
<td></td>
</tr>
<tr>
<td>During lactation</td>
<td>Controls</td>
<td>0.033±0.003</td>
<td>0.034±0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AROCLOR</td>
<td>0.049±0.008**</td>
<td>0.047±0.007**</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean of 8 rats+sd, except where shown otherwise in brackets

Values significantly different from controls:

* p<0.05;  ** p<0.001.
Table 4.6. The effect of AROCLOR 1254 on absolute liver weight in 21-day old rat pups.

<table>
<thead>
<tr>
<th>Dosing Regimen</th>
<th>Treatment</th>
<th>Absolute Liver Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Before Control</td>
<td>1.89±0.15</td>
<td>1.72±0.11</td>
</tr>
<tr>
<td>Mating AROCLOR</td>
<td>1.94±0.20</td>
<td>1.82±0.22</td>
</tr>
<tr>
<td>(10)</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>1.52±0.27</td>
<td>1.30±0.18</td>
</tr>
<tr>
<td>During (9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestation AROCLOR</td>
<td>2.08±0.15*</td>
<td>1.99±0.18*</td>
</tr>
<tr>
<td>Lactation AROCLOR</td>
<td>2.14±0.28*</td>
<td>1.95±0.29*</td>
</tr>
</tbody>
</table>

Values represent mean of 8 rats±sd, except where shown otherwise in brackets

* Values significantly different from controls (p<0.001)

In 21-day old rat pups the weights of the thymus gland, spleen and both kidneys (expressed as the organ weight:body weight ratio) were found to be unaffected by PCB treatment given before mating or during lactation. Heart weight in male offspring was decreased (p<0.05) by PCBs given before mating but not in females, and no difference was found when dosing was carried out during lactation. No organ weights were recorded for rats dosed during gestation.
Table 4.7 shows the mean liver microsome protein levels in 21-day old rats. These were significantly increased \((p<0.02)\) in all groups, indicating that protein synthesis had occurred.

Table 4.7. The effect of AROCLOR 1254 on liver microsome protein concentration in 21-day old rat pups.

<table>
<thead>
<tr>
<th>Dosing Regimen</th>
<th>Treatment</th>
<th>mg protein/g liver wet weight</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>17.0±3.0</td>
<td>19.1±4.5</td>
</tr>
<tr>
<td>Before mating</td>
<td>AROCLOR</td>
<td>25.2±2.4**</td>
<td>25.0±4.0*</td>
<td></td>
</tr>
<tr>
<td>During gestation</td>
<td>AROCLOR</td>
<td>27.8±2.5**</td>
<td>28.1±3.0**</td>
<td></td>
</tr>
<tr>
<td>During lactation</td>
<td>AROCLOR</td>
<td>25.3±3.4**</td>
<td>29.2±4.3**</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean of 8 rats+sd, except where shown otherwise in brackets.

Values significantly different from controls:

* \(p<0.02\); ** \(p<0.001\).
4.7 Aniline Hydroxylase Activity

Table 4.8 shows the activity of aniline hydroxylase in 21-day old pups. This was significantly increased (p<0.01) in all groups, suggesting that induction of both cytochrome P-450- and P-448-dependent metabolic pathways had occurred.

Table 4.8. The effect of AROCLOR 1254 on microsome aniline hydroxylase activity in 21-day old rat pups.

<table>
<thead>
<tr>
<th>Dosing Regimen</th>
<th>Treatment</th>
<th>Males (nmol PAP/mg protein/minute)</th>
<th>Females (nmol PAP/mg protein/minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Controls</td>
<td>0.46±0.09</td>
<td>0.48±0.03</td>
<td></td>
</tr>
<tr>
<td>mating AROCLOR</td>
<td>0.72±0.10**</td>
<td>0.67±0.09**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td>During Controls</td>
<td>0.51±0.09</td>
<td>0.50±0.14</td>
<td></td>
</tr>
<tr>
<td>gestation AROCLOR</td>
<td>0.71±0.09**</td>
<td>0.77±0.12**</td>
<td></td>
</tr>
<tr>
<td>During Controls</td>
<td>0.59±0.10</td>
<td>0.56±0.15</td>
<td></td>
</tr>
<tr>
<td>lactation AROCLOR</td>
<td>0.85±0.11**</td>
<td>0.78±0.13*</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean of 8 rats±sd, except where shown otherwise in brackets.

Values significantly different from controls:

* p<0.01;  ** p<0.001.
4.8 Aminopyrine-N-Demethylase Activity

Table 4.9 shows the activity of aminopyrine-N-demethylase in 21-day old pups. This was significantly increased (p<0.001) in both sexes in all three dosing regimens, suggesting that induction of cytochrome P-450-dependent metabolic pathways had occurred.

**Table 4.9. Effect of AROCLOR 1254 on microsome aminopyrine-N-demethylase activity in 21-day old rat pups.**

<table>
<thead>
<tr>
<th>Dosing Regimen</th>
<th>Treatment</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>Controls</td>
<td>0.67±0.31</td>
<td>0.61±0.09</td>
</tr>
<tr>
<td>mating</td>
<td>AROCLOR</td>
<td>2.07±0.93*</td>
<td>2.19±0.89*</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>During</td>
<td>Controls</td>
<td>0.60±0.25</td>
<td>0.82±0.30</td>
</tr>
<tr>
<td>gestation</td>
<td>AROCLOR</td>
<td>2.72±0.44*</td>
<td>3.07±0.42*</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>During</td>
<td>Controls</td>
<td>1.08±0.28</td>
<td>0.82±0.50</td>
</tr>
<tr>
<td>lactation</td>
<td>AROCLOR</td>
<td>2.80±0.68*</td>
<td>2.63±0.64*</td>
</tr>
</tbody>
</table>

Values represent mean of 8 rats±sd, except where shown otherwise in brackets.

* Values significantly different from controls (p<0.001)
4.9 Discussion

AROCLOR 1254 caused no significant changes (p<0.05) in litter size, litter weight at birth and 21 days, mean pup weight at 21 days, pup survival rate or organ:body weight ratios (other than for liver). In contrast, the parameters of liver microsomal protein, aniline hydroxylase activity and aminopyrine-N-demethylase activity were all increased by AROCLOR 1254 (p<0.001).

Villeneuve et al. (69) also observed no changes in litter size or weight at birth when oral doses of AROCLOR 1254 of up to 100 mg/Kg/day were given to pregnant Wistar rats from day 6 to 15 of gestation. This provided a much higher total PCB dose (1000 mgKg⁻¹) than was used in this study (180 mgKg⁻¹) although this higher dose was given over a shorter period of time. However in the same study mean neonatal weight at birth was found to be significantly decreased (p=0.05) by the 100 mg/Kg/day dose. Spencer (66) fed AROCLOR 1254 in the diet to pregnant Holtzman rats also from day 6 to 15 of gestation. He found that a dietary concentration of 300 ppm was needed to cause a significant reduction in foetal survival rate and a consequent reduction in litter size at birth, and that 100 ppm or more in the diet caused a reduction in the mean neonatal weight at birth. Linder et al. (48) reported that 100 ppm of AROCLOR 1254 in the diet (equivalent to 7.6 mg/Kg/day) of Sherman rats over a period of 62 days prior to mating caused a reduction in litter size at birth, whereas 20 ppm did not. They also gave pregnant rats 100 mg/Kg/day AROCLOR 1254 orally from days 7 to 15 of gestation and found no effect on litter size at birth.
The lack of any effect due to AROCLOR 1254 on the mean pup weight at weaning observed in this study agrees with the studies reported by Linder et al. (48). A similar observation was reported by Curley et al. (84) who gave pregnant Sherman rats 50 mg/Kg/day AROCLOR 1254 orally from day 7 to 15 of gestation. McCormack et al. (147) reported that 50 ppm AROCLOR 1254 in the diet of Sprague Dawley rats from day 8 of gestation until 14 days after delivery caused no significant change in the mean weight of their 14-day old pups. However, AROCLOR 1254 has been reported to decrease (p=0.0001) the mean weight of 4-week old mouse pups (149). The pups were suckled by dams fed 10 ppm of the PCBs in their diet from the start of gestation onwards.

In addition, Overmann et al. (150) exposed female rats to AROCLOR 1254 in their diet continuously from mating until weaning of their pups. Pup birth weight and pregnancy success (the number of impregnated dams that delivered a litter) were decreased by a dietary PCB concentration of 269 ppm but not by 26 ppm, although the lower concentration did reduce the rate of pup growth before weaning. Litter size and sex ratio at birth were unaffected by either of these dietary concentrations.

AROCLOR 1254 had no effect on pup survival rate between birth and weaning in this study. This agrees with the studies by Curley et al. and Linder et al. described above although the latter workers also found that doubling the dose to 100 mg/Kg/day during gestation caused a marked decrease in pup survival. However, the body weights of those that did survive were not significantly different from
controls. In a brief communication Keplinger et al. (148) reported a decrease in the survival rate of pups raised by dams fed AROCLOR 1254, but no experimental details were included.

Generally, the literature indicates that AROCLOR 1254 can reduce the parameters of litter size and mean pup weight at birth, and pup survival rate between birth and weaning. The body weight of pups surviving to weaning appears to be unaffected. However, the dosing regimens that were used in the various published studies vary greatly, making comparisons of the results difficult. Factors such as the duration and rate of PCB administration to dams appear to influence the magnitude of the effects of a given dose in their suckled offspring. It is therefore not possible to define a minimum dose of AROCLOR 1254 necessary to produce each of these effects. In this study no effects on these parameters were seen, except for an increase in total litter weight at birth when dams were dosed before mating. It can therefore be concluded that these parameters are not likely to be useful in the detection of PCB effects in this type of study, unless the total dose is increased or a more toxic PCB is used. However, as the data are easily obtained it would be wise to monitor these parameters, and this was done in the subsequent work using single PCB congeners which are reported in chapter five.

Organ weights other than liver were mainly unaffected by PCB treatment in this study. A loss in the weight of the thymus gland and kidneys and an increase in heart weight are considered to be typical effects of PCBs and related compounds (McConnell, 151). Spleen weight is generally increased by low doses and decreased by
higher doses. Parkinson et al. (152) reported a significant decrease in thymus:body weight ratio in three-week old male Long Evans rats killed four days after a single i.p. dose of AROCLOR 1254 1.5 mmol Kg\(^{-1}\) (assuming an average molecular weight of 326).

A significant increase in the liver:body weight ratio \(p<0.05\) was observed in the present study (table 4.5) in all treatment groups. Liver:body weight ratio was a more sensitive indicator of PCB effects in pups than the other organ:body weight ratios measured. It is interesting that while no change in absolute liver weight was seen in pups from dams dosed before mating, a highly significant increase \(p<0.001\) was seen in the other two dosing regimens in both sexes. The liver:body weight ratio was also increased in the study reported by Parkinson et al. (152) already described. This effect has been observed by numerous other workers using AROCLOR 1254 in rats dosed directly \(153, 154, 155\).

Increases in microsome protein concentration and aniline hydroxylase activity were seen in this study \(p<0.001\) and have also been reported in rats dosed directly with AROCLOR 1254 25 mgKg\(^{-1}\) day\(^{-1}\) orally for six days \(154\) and in rats fed AROCLOR 1254 10 ppm in their diet for 12 weeks \(156\). Increases in aminopyrine demethylase were seen in this study \(p<0.001\) and have also been reported in rats dosed directly with AROCLOR 1254 50 mgKg\(^{-1}\) day\(^{-1}\) i.p. for 3 days \(122\) and in rats fed AROCLOR 1254 5 ppm in their diet for 35 days \(157\). As discussed in chapter one, the use of these two enzymes should allow distinction between pure MC-type enzyme inducers on the one hand and mixed- or PB-type inducers on the other. In this study,
the induction of both of these enzymes agrees with published reports that have confirmed AROCLOR 1254 as a mixed-type enzyme inducer (122), increasing the activities of enzymes dependent on both cytochromes P-450 and P-448.

The parameters of liver:body weight ratio, microsome protein concentration and the activities of aniline hydroxylase and aminopyrine-N-demethylase were the most sensitive indicators of PCB effects used in this study. The effects of AROCLOR 1254 on these parameters are summarised in table 4.10. A noticeable feature is the similarity between the results for male and female rats. There is no indication here of any difference in sensitivity between the sexes to the effects of AROCLOR 1254 although sex differences in the effect of inducers on hepatic microsomal enzymes have been reported (160,161).

Table 4.10 shows that the parameters of liver:body weight ratio, microsome protein concentration and aniline hydroxylase activity were all increased to approximately equal extents. This differs from the conclusions of Baker et al. (158) who fed Wistar dams AROCLOR 1254 70 ppm in their drinking water continuously for several weeks starting before mating. They reported an increase in the activity of liver aniline hydroxylase in the 20 day old pups of these dams, but no increases were found in liver weight or microsome protein, suggesting that aniline hydroxylase activity is a more sensitive indicator of PCB effects. However, the exact experimental protocol is not clearly described and raises doubts over the duration of dosing and the total dose received.
Table 4.10. Summary of changes in liver parameters in 21-day old pups due to administration of AROCLOR 1254 to dams using three different dosing regimens

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before</th>
<th>During</th>
<th>During</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mating</td>
<td>Gestation</td>
<td>Lactation</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver:body weight ratio</td>
<td>114 *</td>
<td>129</td>
<td>148</td>
</tr>
<tr>
<td>Absolute liver weight</td>
<td>102 NS</td>
<td>137</td>
<td>165</td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>148</td>
<td>173</td>
<td>161</td>
</tr>
<tr>
<td>Aniline hydroxylase</td>
<td>157</td>
<td>139</td>
<td>144</td>
</tr>
<tr>
<td>Aminopyrine-N-demethylase</td>
<td>309</td>
<td>453</td>
<td>259</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver:body weight ratio</td>
<td>119 *</td>
<td>136</td>
<td>138</td>
</tr>
<tr>
<td>Absolute liver weight</td>
<td>106 NS</td>
<td>153</td>
<td>151</td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>131 **</td>
<td>180</td>
<td>176</td>
</tr>
<tr>
<td>Aniline hydroxylase</td>
<td>140</td>
<td>154</td>
<td>139 ***</td>
</tr>
<tr>
<td>Aminopyrine-N-demethylase</td>
<td>359</td>
<td>323</td>
<td>321</td>
</tr>
</tbody>
</table>

Values are percentages of the control values measured in the same dosing regimen. All values are significantly greater than controls (p<0.001) except for: * p<0.05; ** p<0.02; *** p<0.01; NS not significant.

None of the three dosing regimens gave consistently greater differences than either of the others although there was a tendency
for dosing before mating to cause lower increases. The size of the
dose given may however be important in the interpretation of these
results. If the tendency for PCB effects to appear differs only
slightly depending on the timing of dosing, then perhaps only with
small doses (just above the no-effect level) will these differences
be detected. If so, a smaller dose of AROCLOR 1254 would have been
appropriate in this study.

A comparison of all the parameters measured in this study shows
that by far the greatest percentage increase occurred in the case of
aminopyrine-N-demethylase activity. Narbonne (159) concluded that
the activity of this enzyme was a more sensitive index of PCB
effects than aniline hydroxylase activity, microsome protein
concentration, cytochrome P-450 concentration and cytochrome b₅
concentration.

In order to meaningfully compare the effects of different maternal
treatments on the suckled pups, the pups should be tested at the
time when the PCB effects are maximal. The time-course of induction
is therefore important. The changes in enzyme activity during
continuous exposure to PCBs in the diet have been measured in adult
(162) and weanling (159) rats. Other workers have compared young
with old adults (161,163). However none of these studies takes
account of the changes in the maturing enzyme systems that occur
between birth and weaning. Macleod et al. (164) observed changes in
the activities of various liver microsomal enzymes in rats which had
not received any drug treatment. The activity of aminopyrine
demethylase increased steadily from birth until adult values were
attained at age ten weeks. Aniline hydroxylase activity increased from birth until adult values were attained at age five weeks, and cytochrome P-450 levels increased from birth to age three weeks by which time adult values had been attained. This pattern was seen in both sexes.

The effect of inducers on liver enzymes was examined by Cresteil et al. (160). They observed that both PB and MC had no effect on aniline hydroxylase activity in five-day old rats and caused inhibition in 15-day old rats and marked induction in adults. PB induced benzphetamine demethylase activity in five and 15-day old rats and in adults, so that activity in the induced animals increased steadily between birth and adulthood. MC inhibited benzphetamine demethylase in 15-day old rats but had no effect at other ages. However the dosing regimens used in this study are not clearly indicated. Nunnink et al. (165) reported that AHH activity in liver nuclei (rather than microsomes), following a single dose of MC given 24 hours before sample preparation, increased with age until age 3-4 weeks and then declined. Carlstedt-Duke et al. (166) measured Ah receptor levels in rat liver cytosol in the absence of any drug treatment, from animals aged 0, 7, 21, 42 and 56 days. The levels showed a peak at 21 days and a decline thereafter.

Masuda et al. (82) fed seven different PCB congeners to mice at low concentrations in their diet (about 0.4 ppm) from days 1 to 18 of pregnancy and measured whole body PCB levels during the lactation period. The percentage of the total dose which was transferred to the suckling pups varied greatly between different congeners.
However of those congeners which were transferred to any great extent (e.g. 53% of a hexachlorobiphenyl transferred by two weeks postpartum) the amount transferred reached a peak between 14 and 21 days postpartum and then declined gradually over a period of weeks.

The preceding discussion only allows an informed guess as to the time-course of induction that occurred in the present study with AROCLOR 1254. However, Vodicnik et al. (94) administered a single i.p. dose of 2,2',4,4',5,5'-hexachlorobiphenyl 100 mgKg\(^{-1}\) to mice two weeks before mating and measured enzyme activities in the suckled pups at 5, 10, 15 and 20 days of age. At all time points ethoxycoumarin-O-deethylase (which responds to induction by MC-type inducers) and benzphetamine-N-demethylase activities and cytochrome P-450 levels were raised, most of the increases being statistically significant (p<0.05). The maximum values for all three parameters occurred at age 20 days, and all parameters increased between 10, 15 and 20 days. It was therefore concluded that in the present study the maximal inductive effects occurred in the pups at around 21 days of age, which was the time of microsome preparation.

In this study, PCB effects were readily detected by measurements of absolute liver weight, liver:body weight ratio, liver microsome protein concentration and the activities of aniline hydroxylase and aminopyrine-N-demethylase in liver microsomes. These parameters were therefore used in the congener-specific studies reported in chapter five. Since dosing before mating appeared slightly less suitable for detecting PCB effects in pups, and dosing during lactation provides the shortest experimental protocol, the latter regimen was adopted
for subsequent studies. This choice also means that any problems that arise during gestation or parturition can be avoided which is an important consideration when administering scarce PCB congeners.
CHAPTER FIVE

PCB EFFECTS IN PUPS AFTER MATERNAL DOSING WITH
DIFFERENT CONGENERS: RESULTS AND DISCUSSION

This experiment compared the effects of the different PCB congeners in the offspring of rats dosed twice a week during lactation, and also in a second litter born subsequently to the same dams without further dosing. Measurements were made of litter size and weight, pup weight and pup survival. In 21-day old pups, measurements were made of liver weight, liver microsomal protein and cytochrome P-450 concentrations, and the activities of aniline hydroxylase and aminopyrine-N-demethylase in liver microsomes. For each parameter measured, the unpaired student's t-test was used at the 5% confidence level to detect any differences due to PCB treatment compared to controls. The total dose given to each dam was 0.5 mmolKg\(^{-1}\) except in the case of PCB 169 for which a dose of 0.05 mmolKg\(^{-1}\) was used. The results are presented and discussed below.

5.1 Litter Size

Table 5.1 shows the total size of second litters at birth. First litter sizes at birth could not be affected by the PCB treatment given as dosing occurred after parturition, and in fact no significant differences from controls were seen. In second litters at birth the numbers of male pups in the groups treated with PCBs 157 and 169 were noticeably lower than controls, although the decrease was not statistically significant. No significant
differences were seen in the numbers of females or in total litter size.

Table 5.1. Effects of PCB congeners on size of second litters at birth.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Numbers of Pups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls PCB 157 PCB 169 PCB 189 PCB 118 PCB 97</td>
</tr>
<tr>
<td>Males</td>
<td>5.0 2.0 2.7 6.8 5.8 6.8</td>
</tr>
<tr>
<td></td>
<td>+2.8 +1.0 +1.5 +2.1 +1.7 +1.6</td>
</tr>
<tr>
<td>Females</td>
<td>4.8 3.7 4.7 6.0 6.3 7.0</td>
</tr>
<tr>
<td></td>
<td>+2.3 +2.1 +4.7 +2.8 +1.3 +2.1</td>
</tr>
<tr>
<td>Whole</td>
<td>9.8 5.7 7.3 12.8 12.0 13.8</td>
</tr>
<tr>
<td>Litter</td>
<td>+4.8 +2.5 +6.1 +2.9 +1.8 +1.5</td>
</tr>
<tr>
<td>n=5</td>
<td>n=3 n=3 n=4 n=4 n=5</td>
</tr>
</tbody>
</table>

Values represent mean of n litters+sd

5.2 Litter Weight

Tables 5.2 and 5.3 show the total weights of first litters 21 days postpartum and the weight gain of first litters between birth and age 21 days. In first litters, no difference in mean litter weight at birth was found between controls and any of the PCB treatment groups. Administration of PCBs during lactation led to significantly
lighter first litter weights 21 days postpartum only in the case of PCBs 157 and 169. The weight gain over the same 21-day period was significantly less than controls only in the case of PCBs 157 (in both sexes) and 169 (in males only).

Table 5.2. Effects of PCB congeners on weights of first litters 21 days postpartum.

<table>
<thead>
<tr>
<th></th>
<th>Total Weight (g)</th>
<th>Litter Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>484.8</td>
<td>313.2</td>
</tr>
<tr>
<td>PCB 157</td>
<td>314.5</td>
<td>405.2</td>
</tr>
<tr>
<td>PCB 169</td>
<td>414.0**</td>
<td>496.0</td>
</tr>
<tr>
<td>PCB 189</td>
<td>451.4</td>
<td>536.4</td>
</tr>
<tr>
<td>PCB 118</td>
<td>489.2</td>
<td>569.2</td>
</tr>
<tr>
<td>PCB 97</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean of 5 litters ± sd except where shown otherwise in brackets.

Values significantly different from controls:

* p<0.05; ** p<0.02.
Table 5.3. Effects of PCB congeners on 21-day weight gain of first litters

<table>
<thead>
<tr>
<th>Sex</th>
<th>Litter weight gain (g)</th>
<th>Controls PCB 157</th>
<th>PCB 169</th>
<th>PCB 189</th>
<th>PCB 118</th>
<th>PCB 97</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>198.2 113.6 94.9 175.5 193.6 210.5</td>
<td>+61.0 +47.0* +48.7** +57.0 +55.0 +24.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>219.7 134.7 146.1 168.3 184.9 206.0</td>
<td>+66.6 +25.8* +64.0 +31.6 +85.0 +57.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td>417.9 248.2 241.0 343.8 378.5 416.5</td>
<td>+86.2 +68.7*** +109.3* +83.7 +33.2 +68.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litter</td>
<td>(4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean of 5 litters±sd except where shown otherwise in brackets.

Values significantly different from controls:

* p<0.05; ** p<0.02; *** p<0.01.

Tables 5.4 and 5.5 show the total weights of second litters 21 days postpartum and the weight gain of second litters between birth and age 21 days. In second litters there was again no difference between controls and any of the PCB treatment groups in mean litter weight at birth. No differences were seen in second litter weights at age 21 days or in litter weight gains over the 21-day period following parturition.
Table 5.4. Effects of PCB congeners on weights of second litters 21 days postpartum.

<table>
<thead>
<tr>
<th>Controls</th>
<th>PCB 157</th>
<th>PB 169</th>
<th>PCB 189</th>
<th>PCB 118</th>
<th>PCB 97</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Litter Weight (g) *</td>
<td>418.7</td>
<td>320.0</td>
<td>361.3</td>
<td>493.7</td>
<td>470.2</td>
</tr>
<tr>
<td>+167.6</td>
<td>+129.0</td>
<td>+17.2</td>
<td>+57.8</td>
<td>+56.5</td>
<td>+95.0</td>
</tr>
<tr>
<td>n=5</td>
<td>n=3</td>
<td>n=2</td>
<td>n=4</td>
<td>n=4</td>
<td>n=5</td>
</tr>
</tbody>
</table>

Table 5.5. Effects of PCB congeners on 21-day weight gain of second litters

<table>
<thead>
<tr>
<th>Sex</th>
<th>Litter weight gain (g) *</th>
<th>Controls</th>
<th>PCB 157</th>
<th>PCB 169</th>
<th>PCB 189</th>
<th>PCB 118</th>
<th>PCB 97</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>187.0</td>
<td>102.5</td>
<td>139.2</td>
<td>235.4</td>
<td>197.2</td>
<td>224.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+93.2</td>
<td>+60.7</td>
<td>+22.9</td>
<td>+71.0</td>
<td>+37.5</td>
<td>+73.9</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>170.6</td>
<td>178.4</td>
<td>162.2</td>
<td>183.1</td>
<td>203.3</td>
<td>218.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+72.7</td>
<td>+81.2</td>
<td>+10.0</td>
<td>+62.3</td>
<td>+51.9</td>
<td>+47.6</td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>357.6</td>
<td>280.9</td>
<td>301.4</td>
<td>418.5</td>
<td>400.5</td>
<td>443.5</td>
<td></td>
</tr>
<tr>
<td>Litter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+141.8</td>
<td>+111.8</td>
<td>+12.9</td>
<td>+42.7</td>
<td>+44.4</td>
<td>+83.3</td>
<td></td>
</tr>
<tr>
<td>n=5</td>
<td>n=3</td>
<td>n=2</td>
<td>n=4</td>
<td>n=4</td>
<td>n=5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values represent mean of n litters + sd
Table 5.6 shows the mean weights of 21-day old pups in first litters. As expected first litters showed no differences from controls in either sex in the mean birthweight of pups as dosing occurred after parturition. At age 21 days the mean weight of both male and female pups was significantly lower in groups given PCBs 157 and 169.

Table 5.6. Effects of PCB congeners on mean weight of 21-day old pups from first litters.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Mean Pup Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls PCB 157 PCB 169 PCB 189 PCB 118 PCB 97</td>
</tr>
<tr>
<td>Males</td>
<td>46.1 30.4 25.2 44.7 40.5 43.1</td>
</tr>
<tr>
<td></td>
<td>+4.2 +4.5** +7.3** +4.9 +6.9 +5.6 (4)</td>
</tr>
<tr>
<td></td>
<td>Females 45.3 31.9 24.4 43.7 38.6 38.7</td>
</tr>
<tr>
<td></td>
<td>+5.8 +6.4* +7.8* +5.7 +6.4 +6.4 (4)</td>
</tr>
</tbody>
</table>

Values represent mean of 5 litters±sd except where shown otherwise in brackets.

Values significantly different from controls:

* p<0.01; ** p<0.001.
Tables 5.7 and 5.8 show the mean weights of newborn and 21-day old pups respectively, in second litters. Neither of these parameters was affected by PCB treatment in either sex.

**Table 5.7. Effects of PCB congeners on mean weight of newborn pups from second litters.**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Mean</th>
<th>Pup Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>PCB 157</td>
<td>PCB 169</td>
</tr>
<tr>
<td>Males</td>
<td>6.72</td>
<td>7.24</td>
</tr>
<tr>
<td></td>
<td>+0.85</td>
<td>+0.27</td>
</tr>
<tr>
<td>Females</td>
<td>6.31</td>
<td>6.68</td>
</tr>
<tr>
<td></td>
<td>+0.94</td>
<td>+0.52</td>
</tr>
<tr>
<td>n=5</td>
<td>n=3</td>
<td>n=3</td>
</tr>
</tbody>
</table>

Values represent mean of n litters ± sd
### Table 5.8. Effects of PCB congeners on mean weight of 21-day old pups from second litters.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Mean Weight (g)</th>
<th>Controls PCB 157</th>
<th>PCB 169</th>
<th>PCB 189</th>
<th>PCB 118</th>
<th>PCB 97</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>46.4</td>
<td>57.2</td>
<td>47.5</td>
<td>41.3</td>
<td>41.2</td>
</tr>
<tr>
<td></td>
<td>Pup Weight (g)</td>
<td>+6.2</td>
<td>+5.7</td>
<td>+15.3</td>
<td>+5.6</td>
<td>+4.9</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>44.4</td>
<td>57.2</td>
<td>40.4</td>
<td>38.0</td>
<td>38.1</td>
</tr>
<tr>
<td></td>
<td>Pup Weight (g)</td>
<td>+9.4</td>
<td>+6.0</td>
<td>+25.0</td>
<td>+8.0</td>
<td>+5.7</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Values represent mean of n litters ± sd

### 5.4 Pup Survival

None of the PCB treatments had any significant effect on pup survival from birth to weaning in first or second litters. The mean percentage of pups surviving in each litter treated with PCB 169 was 87.6±17.0 (against 100% in controls) for first litters, and 59.7±52.7 (against 95.8±9.4 in controls) for second litters, but these differences were not significant. There were however some signs of a fetotoxic effect during the second pregnancies. In the PCB 169 group, two out of the five dams (all of which had mated successfully as indicated by the presence of vaginal plugs) aborted their litters during pregnancy, while a third dam produced only two pups. In the PCB 157 group one out of the four dams aborted, the
fifth dam having died suddenly during mating.

5.5 Liver Weight

Tables 5.9 and 5.10 show the liver weight:body weight ratio in 21-day old pups in first and second litters respectively. In first litters this ratio was significantly increased (p<0.05) over controls in all groups except PCB 97. The increase was greatest for PCB 157. In second litters, a significant increase over controls was seen only for PCBs 157 and 169, in both sexes.

Table 5.9. Effects of PCB congeners on liver:body weight ratio in 21-day old pups from first litters.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Liver weight : Body weight Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls  PCB 157  PCB 169  PCB 189  PCB 118  PCB 97</td>
</tr>
<tr>
<td></td>
<td>Males 0.038  0.064  0.052  0.041  0.048  0.040</td>
</tr>
<tr>
<td></td>
<td>females 0.037  0.059  0.054  0.043  0.049  0.039</td>
</tr>
</tbody>
</table>

Values represent mean of 10 rats±sd except where shown otherwise in brackets

Values significantly different from controls:

* p<0.05;  ** p<0.001.
Table 5.10. Effects of PCB congeners on liver:body weight ratio in 21-day old pups from second litters.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Liver weight : Body weight Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls  PCB 157  PCB 169  PCB 189  PCB 118  PCB 97</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.038</td>
<td>0.037</td>
</tr>
<tr>
<td></td>
<td>0.041</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td>0.045</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>0.036</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>0.036</td>
<td>0.037</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>+0.003</th>
<th>+0.002**</th>
<th>+0.002</th>
<th>+0.004</th>
<th>+0.004</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=8</td>
<td>n=6</td>
<td>n=8</td>
<td>n=8</td>
<td>n=10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+0.002</td>
<td>+0.006*</td>
<td>+0.002</td>
<td>+0.003</td>
<td>+0.003</td>
</tr>
<tr>
<td></td>
<td>n=7</td>
<td>n=6</td>
<td>n=8</td>
<td>n=8</td>
<td>n=10</td>
</tr>
</tbody>
</table>

Values represent mean of n rats+sd

Values significantly different from controls:

* p<0.05; ** p<0.01.

5.6 Liver Microsome Protein Concentration

Tables 5.11 and 5.12 show microsomal protein concentrations in 21-day old pups in first and second litters respectively. A significant increase (p<0.05) in microsome protein was seen in first litters in all PCB treatment groups with the exception of female pups in the PCB 97 group. In second litters this parameter was significantly increased (p<0.05) over controls in all groups except males in the PCB 118 group. However values were lower in second litter controls than in first litter controls, the difference being
significant \((P<0.001)\) in females but not in males. The reason for this difference is not clear.

**Table 5.11. Effects of PCB congeners on microsome protein levels in 21-day old pups from first litters.**

<table>
<thead>
<tr>
<th>Sex</th>
<th>mg protein / g liver wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls PCB 157 PCB 169 PCB 189 PCB 118 PCB 97</td>
</tr>
<tr>
<td>Males</td>
<td>19.58 30.92 27.68 24.86 34.12 26.52</td>
</tr>
<tr>
<td></td>
<td>+2.35 +4.75*** +7.37** +3.03*** +3.29*** +3.24***</td>
</tr>
<tr>
<td></td>
<td>n=10 n=4 n=10 n=10 n=8 n=10</td>
</tr>
<tr>
<td>Females</td>
<td>22.68 32.48 28.25 26.23 31.09 24.86</td>
</tr>
<tr>
<td></td>
<td>+3.02 +1.32*** +4.49** +3.94* +2.33*** +2.89</td>
</tr>
<tr>
<td></td>
<td>n=10 n=4 n=8 n=10 n=8 n=10</td>
</tr>
</tbody>
</table>

Values represent mean of \(n\) rats + sd

Values significantly different from controls:

\[* p<0.05; \quad ** p<0.01; \quad *** p<0.001.\]
**Table 5.12. Effects of PCB congeners on microsome protein levels in 21-day old pups from second litters.**

<table>
<thead>
<tr>
<th>Sex</th>
<th>mg protein / g liver wet weight</th>
<th>Controls</th>
<th>PCB 157</th>
<th>PCB 169</th>
<th>PCB 189</th>
<th>PCB 118</th>
<th>PCB 97</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.83</td>
<td>26.81</td>
<td>27.29</td>
<td>21.20</td>
<td>17.60</td>
<td>19.63</td>
</tr>
<tr>
<td></td>
<td>+1.66</td>
<td>+2.37***</td>
<td>+4.29***</td>
<td>+0.99***</td>
<td>+2.47</td>
<td>+1.38*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=8</td>
<td>n=5</td>
<td>n=6</td>
<td>n=8</td>
<td>n=8</td>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td>15.87</td>
<td>24.84</td>
<td>25.22</td>
<td>20.55</td>
<td>18.08</td>
<td>18.42</td>
</tr>
<tr>
<td></td>
<td>+1.95</td>
<td>+3.98***</td>
<td>+3.74***</td>
<td>+1.38***</td>
<td>+1.71*</td>
<td>+1.53**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=7</td>
<td>n=6</td>
<td>n=6</td>
<td>n=8</td>
<td>n=8</td>
<td>n=10</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean of n rats + sd

Values significantly different from controls:

* p<0.05;  ** p<0.01;  *** p<0.001.
5.7 Liver Cytochrome P-450 Concentration

The cytochrome P-450 assay was carried out by recording the absorption spectrum of the reduced cytochrome P-450:CO complex. A sample spectrum is shown in appendix 2.

Tables 5.13 and 5.14 show the effect of PCB congeners on cytochrome P-450 concentration in first and second litters. Both sexes in first litters showed marked induction of cytochrome P-450 levels in all PCB groups except PCB 97. Again the effect was greatest in the PCB 157 group.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Controls</th>
<th>PCB 157</th>
<th>PCB 169</th>
<th>PCB 189</th>
<th>PCB 118</th>
<th>PCB 97</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>0.37</td>
<td>2.06</td>
<td>1.70</td>
<td>1.04</td>
<td>1.61</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>$\pm 0.07$</td>
<td>$\pm 0.51^*$</td>
<td>$\pm 0.19^*$</td>
<td>$\pm 0.20^*$</td>
<td>$\pm 0.27^*$</td>
<td>$\pm 0.07$</td>
</tr>
<tr>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
<td>n=8</td>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>0.32</td>
<td>2.08</td>
<td>1.72</td>
<td>1.03</td>
<td>1.49</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>$\pm 0.10$</td>
<td>$\pm 0.44^*$</td>
<td>$\pm 0.29^*$</td>
<td>$\pm 0.27^*$</td>
<td>$\pm 0.51^*$</td>
<td>$\pm 0.06$</td>
</tr>
<tr>
<td>n=9</td>
<td>n=8</td>
<td>n=8</td>
<td>n=10</td>
<td>n=8</td>
<td>n=10</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean of n rats $\pm$ sd

* Values significantly different from controls (p<0.001)
In second litters, only PCBs 157 and 169 caused significant increases in P-450 levels over controls, an effect which was seen in both sexes. PCB 97 caused a significant decrease (p<0.05) in P-450 levels in females, in contrast to its effect in males and to its effect in first litters. However, the control values in second litters were significantly higher than in first litters in females (p<0.02).

Table 5.14. Effects of PCB congeners on cytochrome P-450 levels in 21-day old pups from second litters.

<table>
<thead>
<tr>
<th>Sex</th>
<th>mmoles cytochrome P-450 / mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls PCB 157 PCB 169 PCB 189 PCB 118 PCB 97</td>
</tr>
<tr>
<td>Males</td>
<td>0.47 0.75 0.92 0.48 0.46 0.43 +0.05 +0.11**** +0.47** +0.06 +0.04 +0.08</td>
</tr>
<tr>
<td></td>
<td>n=8 n=5 n=6 n=8 n=8 n=10</td>
</tr>
<tr>
<td>Females</td>
<td>0.50 0.90 0.97 0.43 0.44 0.38 +0.15 +0.19*** +0.53* +0.06 +0.06 +0.05*</td>
</tr>
<tr>
<td></td>
<td>n=7 n=6 n=6 n=8 n=8 n=10</td>
</tr>
</tbody>
</table>

Values represent mean of n rats+sd

Values significantly different from controls:

* p<0.05; ** p<0.02; *** p<0.01; **** p<0.001.
5.8 Liver Cytochrome P-450 Absorption Maximum

Tables 5.15 and 5.16 show the absorption maxima of the reduced cytochrome P-450:CO complex in first and second litters respectively. These were measured in the liver microsome suspensions prepared from 21-day old pups. In first litters, the wavelength of the absorption maximum was shifted to the greatest extent, in both sexes, by PCBs 157 and 169. PCB 97 had no significant effect on this parameter. In second litters, only PCBs 157 and 169 caused a significant shift in the absorption maximum, and this occurred in both sexes.

Table 5.15. Effects of PCB congeners on cytochrome P-450 absorption maximum in first litters

<table>
<thead>
<tr>
<th>Sex</th>
<th>Absorption Maximum (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>Males</td>
<td>450.8</td>
</tr>
<tr>
<td></td>
<td>+0.4</td>
</tr>
<tr>
<td></td>
<td>n=10</td>
</tr>
<tr>
<td>Females</td>
<td>451.2</td>
</tr>
<tr>
<td></td>
<td>+0.8</td>
</tr>
<tr>
<td></td>
<td>n=9</td>
</tr>
</tbody>
</table>

Values represent mean of n rats + sd

* Values significantly different from controls (p<0.001)
Table 5.16. Effects of PCB congeners on cytochrome P-450 absorption maximum in second litters.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Absorption</th>
<th>Maximum (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls PCB 157</td>
<td>PCB 169</td>
<td>PCB 189</td>
</tr>
<tr>
<td>Males</td>
<td>450.4</td>
<td>448.6</td>
</tr>
<tr>
<td></td>
<td>+0.50</td>
<td>+0.38*</td>
</tr>
<tr>
<td>n=8</td>
<td>n=5</td>
<td>n=6</td>
</tr>
<tr>
<td>Females</td>
<td>450.6</td>
<td>448.3</td>
</tr>
<tr>
<td></td>
<td>+0.53</td>
<td>+0.26*</td>
</tr>
<tr>
<td>n=7</td>
<td>n=6</td>
<td>n=6</td>
</tr>
</tbody>
</table>

Values represent mean of n rats±sd

* Values significantly different from controls (p<0.001)

5.9 Liver Aniline Hydroxylase Activity

Tables 5.17 and 5.18 show the liver aniline hydroxylase activities in 21-day old pups from first and second litters respectively. In first litters PCBs 169 and 97 caused a significant inhibition of aniline hydroxylase activity in male pups, but in females only a slight inhibition was seen which was not significant. The other three PCBs caused significant induction of this enzyme in first litters in both sexes.

In second litters no significant changes in aniline hydroxylase
activity were seen for any of the PCBs used. However, the values for PCB 97 were noticeably depressed below those for controls in contrast to values for the other PCB groups.

Table 5.17. Effects of PCB congeners on aniline hydroxylase activity in 21-day old pups from first litters.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Sex</th>
<th>mmoles PAP / mg protein / minute</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>PCB 157</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=10</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=10</td>
</tr>
</tbody>
</table>

Values represent mean of n rats + sd

Values significantly different from controls:

* p<0.05; ** p<0.01; *** p<0.001.
Table 5.18. Effects of PCB congeners on aniline hydroxylase activity in 21-day old pups from second litters.

<table>
<thead>
<tr>
<th>Sex</th>
<th>nmoles PAP / mg protein / minute</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls PCB 157 PCB 169 PCB 189 PCB 118 PCB 97</td>
</tr>
<tr>
<td>Males</td>
<td>0.64 0.71 0.67 0.75 0.67 0.56</td>
</tr>
<tr>
<td></td>
<td>+0.14 +0.12 +0.07 +0.09 +0.13 +0.16</td>
</tr>
<tr>
<td></td>
<td>n=8 n=5 n=6 n=8 n=8 n*10</td>
</tr>
<tr>
<td>Females</td>
<td>0.67 0.70 0.68 0.66 0.66 0.55</td>
</tr>
<tr>
<td></td>
<td>+0.19 +0.11 +0.03 +0.11 +0.09 +0.11</td>
</tr>
<tr>
<td></td>
<td>n=7 n=6 n=6 n=8 n=8 n=10</td>
</tr>
</tbody>
</table>

Values represent mean of n rats + sd

5.10 Liver Aminopyrine-N-Demethylase Activity

Tables 5.19 and 5.20 show the activities of aminopyrine demethylase in 21-day old pups from first and second litters respectively. Statistically significant induction of aminopyrine-N-demethylase activity over controls was evident in first litters in all PCB treatment groups of both sexes. The greatest activity was seen in the PCB 157 group and the least in the PCB 97 group.

In second litters, induction was evident to a lesser extent than in first litters, being significantly greater than controls in both sexes only in the PCB 157 group which again showed the highest
activity. Again the PCB 97 group showed the least activity, the enzyme being inhibited compared to controls. Significant inhibition was also seen in the PCB 118 group in males. However, this apparent inhibition must be interpreted with caution as the control values were higher than those in first litter controls, and the difference was significant in both sexes.

Table 5.19. Effects of PCB congeners on aminopyrine demethylase activity in 21-day old pups from first litters.

<table>
<thead>
<tr>
<th>Sex</th>
<th>nmoles HCHO / mg protein / minute</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls PCB 157 PCB 169 PCB 189 PCB 118 PCB 97</td>
</tr>
<tr>
<td>Males</td>
<td>0.66  2.23  1.19  1.91  1.94  0.95</td>
</tr>
<tr>
<td></td>
<td>+0.20  +0.49**  +0.21**  +0.37**  +0.31**  +0.19*</td>
</tr>
<tr>
<td></td>
<td>n=10  n=10  n=10  n=10  n=8  n=10</td>
</tr>
<tr>
<td>Females</td>
<td>0.58  2.61  1.02  2.16  1.92  0.90</td>
</tr>
<tr>
<td></td>
<td>+0.14  +0.39**  +0.34*  +0.27**  +0.40**  +0.14**</td>
</tr>
<tr>
<td></td>
<td>n=10  n=9  n=9  n=10  n=8  n=10</td>
</tr>
</tbody>
</table>

Values represent mean of n rats±sd

Values significantly different from controls:

* p<0.01;  ** p<0.001.
Table 5.20. Effects of PCB congeners on aminopyrine demethylase activity in 21-day old pups from second litters.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Controls</th>
<th>PCB 157</th>
<th>PCB 169</th>
<th>PCB 189</th>
<th>PCB 118</th>
<th>PCB 97</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>0.87</td>
<td>1.34</td>
<td>0.92</td>
<td>0.79</td>
<td>0.73</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>+0.11</td>
<td>+0.17***</td>
<td>+0.21</td>
<td>+0.06</td>
<td>+0.13*</td>
<td>+0.11***</td>
</tr>
<tr>
<td></td>
<td>n=8</td>
<td>n=5</td>
<td>n=6</td>
<td>n=8</td>
<td>n=8</td>
<td>n=10</td>
</tr>
<tr>
<td>Females</td>
<td>0.81</td>
<td>1.40</td>
<td>1.09</td>
<td>0.74</td>
<td>0.67</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>+0.23</td>
<td>+0.31**</td>
<td>+0.15*</td>
<td>+0.10</td>
<td>+0.16</td>
<td>+0.03*</td>
</tr>
<tr>
<td></td>
<td>n=7</td>
<td>n=6</td>
<td>n=6</td>
<td>n=8</td>
<td>n=8</td>
<td>n=10</td>
</tr>
</tbody>
</table>

Values represent mean of n rats+sd

Values significantly different from controls:

* p<0.05; ** p<0.01; *** p<0.001.
5.11 Discussion

5.11.1 Litter Size and Pup Weight

Measurements of litter size and weight following administration of single PCB congeners to the dams do not appear to be well reported in the literature. In this study, PCB congener numbers 97, 118, 157 and 189 were administered to rat dams at doses of 0.5 mmol Kg\(^{-1}\) during lactation. PCB 169 was administered at a dose of 0.05 mmol Kg\(^{-1}\). Following the results obtained with AROCLOR 1254 (chapter four) it was not expected that such measurements would provide a very sensitive indicator of PCB toxicity. However a trend can be discerned from the data. Generally PCBs 157 and 169 (the dose of 169 having been one tenth that of the other congeners) tended to decrease litter size and decrease litter and pup weight gains. PCB 169 is reported to be one of the most potent of all the PCB congeners (70) and so would be expected to show toxic effects most readily. There was no sign of toxicity due to PCBs 97, 118 or 189 in these parameters.

In addition, the possible foetotoxic effects observed with PCB 169 (and to a lesser extent with PCB 157) reinforce the apparent trend of these results. As PCBs 169 and 157 decreased the number of male rather than female pups born in second litters, this effect may be sex-specific. These results recall those of Spencer (66) who reported a decreased foetal survival rate at birth after rat dams had been fed AROCLOR 1254 300 ppm in their diet for ten days during gestation. As the number of conceptuses at day 12 of pregnancy was
not affected by PCB treatment, foetal resorption rather than inhibition of implantation may have occurred. Perhaps the alleged effects of PCBs on sex hormone metabolism (68) caused an increase in resorption of male foetuses in this study. No reports of these particular PCB congeners causing such an effect have been found in the literature.

5.11.2 Liver Parameters

The effects of the different PCB congeners on the liver parameters measured in first and second litters are illustrated in figures 5.1-5.6. Generally, PCBs 157, 189 and 118 increased all parameters measured, i.e. liver:body weight ratio, microsome protein concentration, cytochrome P-450 concentration and the activities of aniline hydroxylase and aminopyrine demethylase. Parkinson et al. (152) also observed increases in cytochrome P-450 and liver:body weight ratio in three-week old male Long Evans rats dosed directly with a single i.p. dose of 0.5 mmol Kg\(^{-1}\) of PCBs 157, 189 or 118. Parkinson et al. (123) dosed one-month old male Wistar rats directly with PCBs 157 or 118 in two separate doses, each of 0.15 mmol Kg\(^{-1}\), two days apart. They found PCB 118 to be more potent than PCB 157 on the basis of induction of microsomal protein, cytochrome P-450 and aminopyrine demethylase but less potent as an inducer of AH. They did not measure aniline hydroxylase activity. They concluded that both these PCBs are mixed-type enzyme inducers. On this basis they would be expected to increase the activities of both aniline hydroxylase and aminopyrine demethylase.
Figure 5.1. Effects of PCB congeners on liver:body weight ratio and microsome protein levels in 21-day old male rat pups.
Figure 5.2. Effects of PCB congeners on cytochrome P-450 levels and aniline hydroxylase activity in 21-day old male rat pups
Figure 5.3. Effects of PCB congeners on aminopyrine-N-demethylase activity in 21-day old male rat pups.
Figure 5.4. Effects of PCB congeners on liver:body weight ratio and microsome protein levels in 21-day old female rat pups.
Figure 5.5. Effects of PCB congeners on cytochrome P-450 levels and aniline hydroxylase activity in 21-day old female pups.
Figure 5.6. Effects of PCB congeners on aminopyrine-N-demethylase activity in 21-day old female rat pups.
In this study the wavelength of the reduced P-450:CO absorption maximum was shifted down to 448.2 nm by PCB 157 and 448.3 nm by PCB 118 (against 450.8 in controls), which agrees quite well with the results of Parkinson et al. of 448.0 nm for both of these PCBs.

The increase in the liver:body weight ratio and cytochrome P-450 levels by PCB 189 seen in this study was also observed by Parkinson et al. (152). An increase in the activity of aminopyrine demethylase and P-450 levels, but without any shift in the wavelength of the P-450 absorption maximum, was reported by Parkinson et al. (124). In this study the wavelength was moved slightly down to 449.4 nm. PCB 189 is considered to be a mixed-type enzyme inducer (124,167) and so again an increase in the activity of both the enzymes measured would be expected. Interestingly, Goldstein et al. (122) observed an inhibition of aminopyrine-N-demethylase activity (without any effect on P-450 concentration) by PCB 189 at a lower dose than that used by Parkinson et al. (124).

In contrast to PCBs 157, 189 and 118, PCB 169 inhibited the activity of aniline hydroxylase but increased all the other liver parameters measured in first litters. Yoshimura et al. (168) observed an increase in liver weight in four-week old male Wistar rats, but no increase in P-450 levels although AHH activity was increased. Goldstein et al. (122) found that the liver effects of PCB 169 varied with the magnitude of the dose in one-month old Sprague Dawley rats dosed directly. With a higher dose (0.14 mmolKg⁻¹ i.p. daily for 3 days) microsome protein and P-450 concentrations and aminopyrine demethylase activity were all decreased, although AHH activity and
liver:body weight ratio were both increased. With a lower dose (0.03 mmol Kg\(^{-1}\) i.p. daily for 3 days) microsome protein was unchanged and liver:body weight ratio, P-450 concentration and AHH activity were all increased, while aminopyrine demethylase activity was still decreased. Parkinson et al. (152) gave three-week old male Long Evans rats PCB 169 0.125 mmol Kg\(^{-1}\) as a single i.p. dose and observed an increase in P-450 levels but no change in the liver:body weight ratio.

The inhibition of aniline hydroxylase by PCB 169 seen in this study is surprising, since this enzyme is reported to be induced by MC (120) and PCB 169 has been shown to be a MC-type enzyme inducer (121,122). Yoshimura et al.(120) observed an increase in the activity of aniline hydroxylase but not aminopyrine demethylase in young adult rats given a single i.p. dose of 1 mg Kg\(^{-1}\) PCB 169. Fujita et al.(119) reported an increase in all the liver parameters measured in this study, including aniline hydroxylase and aminopyrine demethylase activities, after administration of PCB 169 to rats. In this study, the wavelength of the P-450 absorption maximum was shifted from 450 to 448.1 nm by PCB 169, a result similar to that of Kohli et al.(121) who reported a value of 448.4 nm for both PCB 169 and MC.

All of the studies reported in the literature and referred to above have used immature (weaned) rats dosed directly with a single PCB congener, rather than pups that were suckled by dams exposed to PCB. There still seems to be a shortage of literature on the latter type of congener-specific study, with most reports being concerned with
commercial PCB mixtures.

PCB 97 caused the least changes in liver parameters in first litters than any of the other congeners used. Liver:body weight ratio and P-450 concentration were unchanged, and microsome protein was increased only in males. The wavelength of the P-450 absorption maximum was virtually unchanged. Aniline hydroxylase activity was decreased in males only while aminopyrine demethylase activity was increased in both sexes. No reports of the effects of PCB 97 on these parameters have been found in the published literature.

The histograms presented in figures 5.1-5.6 allow some attempt at establishing a rank order of potency for the different PCB congeners used in this study. In both male and female rat pups, PCB 97 seemed on the whole fairly inactive. PCB 169, having been administered at only one tenth of the dose used for the other congeners, stands out as the most potent. This conclusion would be expected from the numerous reports that have highlighted PCB 169 as one of the most toxic of all the 209 possible PCB congeners. There is not a great difference between the remaining three congeners in the magnitude of the changes produced. There is a tendency for slightly greater increases with PCB 157 and slightly smaller increases with PCB 189 when compared to PCB 118. Therefore a tentative rank order of potency is PCB 169 > PCB 157 > PCB 118 > PCB 189 > PCB 97. However, attempts to compare the different congeners are complicated by any differences in their characteristic induction patterns. An example of this is the tendency of PCBs 169 and 97 to inhibit aniline hydroxylase while the other congeners induce this
enzyme, at least in the type of protocol used here. Thus any comparisons between congeners must be based on as many parameters as possible.

The conclusions based on litter size and body weights (section 5.11.1) support the impression that PCBs 169 and 157 are the most potent of the congeners used.

Consideration of the wavelength changes observed in the P-450 absorption maxima in this study suggests the same rank order of potency as do the other results. As discussed in chapter one, MC-type induction and a shift of the P-450 absorption maximum from 450 nm towards 447 nm are associated with greater toxicity than mixed- or PB-type induction with little or no change in the wavelength. Although no firm correlation has been established between the wavelength of the P-450 absorption maximum and toxicity, it is noticeable that in this study the greatest shifts in this parameter were caused by PCBs 169 and 157 and the least shift by PCB 97, showing (for first litter males) a sequence of:

PCB 169 > PCB 157 > PCB 118 > PCB 189 > PCB 97 (controls 450.8)

448.1 448.2 448.3 449.4 450.9
5.11.3 **PCB Effects in Second Litters**

The effects of the PCB congeners on liver parameters in second litters are illustrated in figures 5.1-5.6. In both sexes, it was generally only in the case of PCBs 157 and 169 that any marked or statistically significant changes appeared in second litters. Such persistence must be a function of both the potency of the congener and its tendency to be retained in the body, i.e., its pharmacodynamic and its pharmacokinetic properties. The elimination of the PCBs from animal tissues and the possible implications for this study are discussed in section 8.3.2.

5.11.4 **Sex Differences in Response to PCBs**

Apart from the tendency of PCBs 157 and 169 to reduce the numbers of male pups born to dosed dams, there was no indication of sex differences in response to any of the PCB treatments. This is despite reports of sex differences in the response of hepatic microsomal enzymes to inducers (160,161).

5.11.5 **Effect of Contaminants in the PCB Congeners**

As reported in chapter three, PCB 157 was contaminated with approximately 0.6% PCB 169. Judging from the effects seen in this study, this may have resulted in an enhancement of the apparent effect of PCB 157 on all parameters measured except for aniline hydroxylase activity which would be decreased. The effect of such bias would however be small. The effects of PCB 157 were in most
cases sufficiently greater than those of the next most potent congeners (PCB 118) for such bias to have no effect on the rank order of potency indicated in section 5.11.2.

PCB 118 was contaminated with a small percentage of (possibly) a hexa- or a heptachlorodibiphenyl which was not identified. This may have affected its potency ranking with respect to PCB 189 as these two congeners appeared very similar in potency. However this is conjecture.
CO-ADMINISTRATION OF PCBs 97 AND 169

RESULTS AND DISCUSSION

This experiment was designed to assess the effects of administering PCB 97 several days prior to the administration of PCB 169, in order to detect any synergism that may occur between these two congeners. Female weanling rats were dosed directly, by the oral (study 1) and the intraperitoneal (study 2) routes. For each parameter measured, the unpaired student’s t-test at the 5% level of significance was used to detect any differences due to PCB treatment compared to controls.

6.1 Study 1: Oral Dosing

The effects of the oral administration of 0.3 mmolKg⁻¹ PCB 97 seven days prior to 0.025 mmolKg⁻¹ PCB 169 are shown in table 6.1. Most of the parameters measured do not show any significant increase over controls, indicating that minimal induction had occurred. Liver:body weight ratio was significantly increased by either PCB given alone, but in combination their effects were not additive. Cytochrome P-450 concentration was increased by PCB 169 given alone and to a greater extent with both PCBs combined.
Table 6.1. Effects of an oral dose of PCB 97 given seven
days prior to an oral dose of PCB 169. Rats
were killed five days after PCB 169 dosing.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured Controls</td>
</tr>
<tr>
<td>Liver:body weight ratio</td>
<td>0.038</td>
</tr>
<tr>
<td>Microsome protein concentration mg protein/g liver</td>
<td>29.28</td>
</tr>
<tr>
<td>Aniline hydroxylase activity: nmol PAP/mg protein/min</td>
<td>0.32</td>
</tr>
<tr>
<td>Aminopyrine demethylase activity: nmol HCHO/mg protein/min</td>
<td>1.16</td>
</tr>
<tr>
<td>Cytochrome P-450 concentration nmol P-450/mg protein</td>
<td>0.30</td>
</tr>
<tr>
<td>Cytochrome P-450 absorption max, nm</td>
<td>451.4</td>
</tr>
</tbody>
</table>

Values represent mean of 4 rats±sd
Values significantly different from controls:
* p<0.01; ** P<0.001.

6.2 Study 2: Intraperitoneal Dosing

The effects of 0.5 mmol Kg⁻¹ PCB 97 administered six days prior to
0.05 mmol Kg⁻¹ PCB 169 are shown in table 6.2.
Table 6.2. Effects of an i.p. dose of PCB 97 given six days prior to an i.p. dose of PCB 169. Rats were killed three days after PCB 169 dosing.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Treatment Control</th>
<th>PCB 97</th>
<th>PCB 169</th>
<th>97+169</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver:body weight ratio</td>
<td>0.040</td>
<td>0.045</td>
<td>0.051</td>
<td>0.047</td>
</tr>
<tr>
<td>Microsome protein concentration</td>
<td>33.15</td>
<td>36.47</td>
<td>39.59</td>
<td>39.37</td>
</tr>
<tr>
<td>Aniline hydroxylase activity: nmol PAP/mg protein/min</td>
<td>0.37</td>
<td>0.36</td>
<td>0.37</td>
<td>0.47</td>
</tr>
<tr>
<td>Aminopyrine demethylase activity: nmol HCHO/mg protein/min</td>
<td>1.41</td>
<td>1.88</td>
<td>1.59</td>
<td>1.81</td>
</tr>
<tr>
<td>Cytochrome P-450 concentration: nmol P-450/mg protein</td>
<td>0.34</td>
<td>0.35</td>
<td>1.07</td>
<td>1.12</td>
</tr>
<tr>
<td>Cytochrome P-450 absorption max, nm</td>
<td>451.0</td>
<td>451.7</td>
<td>449.3</td>
<td>449.0</td>
</tr>
</tbody>
</table>

Values represent mean of 4 rats±sd
Values significantly different from controls:
* p<0.05; ** p<0.02; *** P<0.01; **** P<0.001.

Both PCBs were administered by the intraperitoneal route. Again little evidence of enzyme induction was seen with PCB 97, and PCB 169 caused significant changes in only three parameters. Most parameters were significantly increased by the PCB combination. Liver:body weight ratio and cytochrome P-450 concentration were both
increased by PCB 169 alone and to an almost identical extent by combination treatment. Microsomal protein showed a slight but non-significant increase due to PCB 169 alone, and an almost identical increase due to both congeners together.

6.3 Discussion

The liver effects of the combination of PCBs 97 and 169, given either by the oral or the i.p. route, do not suggest that synergism was occurring. In other words, the effects of the combination were no greater, and in many cases less than, those that would be expected from a consideration of the effects of either PCB given alone. However, the degree of induction caused by these doses was generally very low. Therefore a further study with higher doses of both PCBs is desirable, in order to achieve a small but significant degree of induction of most parameters with either PCB alone.

The possibility of synergism occurring between PCB congeners was discussed by Leece et al. (97) who pretreated one month old male Wistar rats with a single dose of 2,4,5,2',4',5'-hexachlorobiphenyl (HCB) 0.3 mmolKg\(^{-1}\) and then administered a single sub-maximal inducing dose of PCB 169 seven days later. They also administered either PCB alone. The combination induced AHH and ethoxyresorufin-O-deethylase activities to a greater extent than would be expected if the combined effects were additive. They suggest this effect may be due to increases in the hepatic cytosolic Ah receptor levels, an effect which they demonstrated in the same study using HCB alone, and which has been shown to be caused by
di-ortho substituted PCBs (118). However, Okey and Vella (169) reported that although PB treatment can double Ah receptor levels, the degree of AHH induction caused by the subsequent administration of MC-type inducers is not doubled. The situation is therefore far from clear at present.

PCB 97 was used in this study because it was the only di-ortho substituted congener available. Although the dose of PCB 97 used was inadequate to cause induction when given alone, it was large enough to suggest that there is unlikely to be a significant increase in PCB toxicity due to any interaction between PCBs 97 and 169. Further work is required on this subject, as the detection of synergism may depend on selection of the right enzymes, doses and congeners.

The toxicological implications of synergy between PCBs are enormous. As discussed in chapter one, a large number of different congeners occur in human milk. The relative concentrations and identities of each will vary between different mothers depending on factors such as their history of exposure to PCBs. The PCB composition in the milk from the same mother can also vary with time and is influenced by lactation history (170). If a significant degree of synergism was occurring amongst some of the congeners present, not only would the total toxicity of a given milk sample be very difficult to interpret but slight changes in the congener composition of the milk could cause disproportionately large changes in toxicity. These considerations call for carefully designed studies.
These studies were carried out in order to compare the patterns of enzyme induction results from chapters four and five with those obtained from studies involving a variety of other toxic effects. This was done to allow comparison of the rank order of toxic potency for the different congeners that had emerged from the enzyme induction work with the rank order indicated by non-enzyme based measurements in immature animals dosed directly. The effects chosen were effects on organ weights, dermal toxicity and neurotoxicity.

7.1 Organ Weight Study

This study was carried out to compare the effects of different PCB congeners on organ weights and body weight gains 14 days after a single dose of 0.5 mmolKg$^{-1}$ PCB. Sixteen-day old female rats were used. The organ weights recorded are shown in table 7.1. The greatest changes in organ weights were caused by PCBs 157 and 156. Rats in these groups gained very little in body weight over the 14 days of the study and were the only groups to experience a significant decrease (p<0.05) in liver weight. These animals also showed marked atrophy of the thymus gland and spleen, as well as substantial decreases in heart and kidney weights.
Table 7.1. Organ weights and body weight gains in female weanling rats 14 days after a single i.p. dose of 0.5 mmol Kg$^{-1}$ PCB

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control</th>
<th>PCB 157</th>
<th>PCB 156</th>
<th>PCB 189</th>
<th>PCB 118</th>
<th>PCB 97</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt.</td>
<td>68.5</td>
<td>10.4****</td>
<td>11.0****</td>
<td>67.2</td>
<td>50.9***</td>
<td>64.1</td>
</tr>
<tr>
<td>gain, g</td>
<td>+8.4</td>
<td>+12.7</td>
<td>+11.6</td>
<td>+1.9</td>
<td>+3.4</td>
<td>+1.9</td>
</tr>
<tr>
<td>Liver, g</td>
<td>6.66</td>
<td>4.69*</td>
<td>5.20*</td>
<td>6.40</td>
<td>7.41</td>
<td>6.41</td>
</tr>
<tr>
<td>Thymus, mg</td>
<td>425</td>
<td>58****</td>
<td>59****</td>
<td>389</td>
<td>335</td>
<td>395</td>
</tr>
<tr>
<td>Heart, mg</td>
<td>515</td>
<td>258****</td>
<td>299****</td>
<td>501</td>
<td>432**</td>
<td>505</td>
</tr>
<tr>
<td>Spleen, mg</td>
<td>486</td>
<td>164****</td>
<td>189****</td>
<td>678***</td>
<td>506</td>
<td>489</td>
</tr>
<tr>
<td>Kidneys, mg</td>
<td>1065</td>
<td>655****</td>
<td>683****</td>
<td>1092</td>
<td>942</td>
<td>1070</td>
</tr>
</tbody>
</table>

Values represent mean of 4 rats+sd (7 rats in controls)

Values significantly different from controls:

* p<0.05; ** p<0.02; *** p<0.01; **** p<0.001.
PCB 189 produced a significant change (p<0.01) only in spleen weight which was increased over controls. Rats in the PCB 97 group showed no significant changes, with mean values very close to those of the control group. The responses to PCB 118 were on the whole somewhere between those of PCBs 157 and 189, producing a significant (but not a major) decline in body weight gain and in heart weight. Thymus weight was noticeably depressed, but not to a statistically significant extent.

The results of this study are expressed in terms of the organ weight:body weight ratio in table 7.2. The overall pattern of response shown by the organ:body weight ratios is similar to that shown by the organ weight results already discussed. The ratio results however suggest a greater similarity between PCBs 189 and 118.
Table 7.2. Organ:body weight ratios in female weanling rats 14 days after a single i.p. dose of 0.5 mmol/Kg⁻¹ PCB

<table>
<thead>
<tr>
<th>Organ</th>
<th>Organ Weight : Body Weight Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>Liver</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>+0.004</td>
</tr>
<tr>
<td>Thymus</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>+0.55</td>
</tr>
<tr>
<td>Heart</td>
<td>4.85</td>
</tr>
<tr>
<td></td>
<td>+0.17</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.63</td>
</tr>
<tr>
<td></td>
<td>+0.81</td>
</tr>
<tr>
<td>Kidneys</td>
<td>10.03</td>
</tr>
<tr>
<td></td>
<td>+0.58</td>
</tr>
</tbody>
</table>

Values represent mean of 4 rats+sd (7 rats in controls)

Values significantly different from controls:

* p<0.02; ** p<0.01; *** p<0.001.
7.2 Dermal Toxicity Study

The effects of the application of PCBs 157, 189 and 97 to the skin of hairless mice were observed in this study. The PCBs were applied, dissolved in acetone, at a rate of 1 mg daily on five days each week for six weeks after which skin histology was compared to controls which had received acetone only.

The appearance of the live animals' skin showed the effect of PCB 157 application after three weeks of treatment in the case of three mice, the skin becoming dry and scaly in appearance. A fourth mouse showed no such effects even after six weeks. Mice treated with PCBs 189 and 97 and control mice all appeared normal throughout the experiment.

The skin histology of the four treatments is shown in the photographs in figures 7.1-7.4. These show marked differences between PCB 157 treated mice and controls. Control mice showed an epidermis of approximately two cell layers thick covered by a thin stratum corneum. Sebaceous glands and sebaceous follicles were abundant, the latter being associated with minimal amounts of keratin. In the dermis, numerous pilar cysts were seen which appeared empty.

The PCB 157 treated samples in contrast showed marked epidermal hyperplasia producing an epidermis of 8-9 cell layers in places, and occluding some of the sebaceous follicles which were much reduced in number. Follicular hyperkeratosis was observed in the sebaceous
Figure 7.1. Skin section from hairless mouse (control) x250. Note thin stratum corneum and epidermis, and the presence of sebaceous glands.

Figure 7.2. Skin section from hairless mouse treated with PCB 157, x250. Note hyperkeratinisation of the stratum corneum, epidermal hyperplasia, absence of sebaceous glands, and keratin rings in intradermal cysts.
Figure 7.3. Skin section from hairless mouse treated with PCB 189, x250. Note mild epidermal hyperplasia and the presence of sebaceous glands.

Figure 7.4. Skin section from hairless mouse treated with PCB 97, x250. Note mild epidermal hyperplasia and the presence of sebaceous glands.
follicles not occluded by epidermis. Hyperkeratinisation of the stratum corneum was noticeable. Very few sebaceous glands were seen in any of the PCB 157 samples. The pilar cysts in the dermis appeared reduced in number and contained numerous concentric keratin rings. These observations were seen in three out of the four mice treated with PCB 157. The fourth mouse, whose skin had appeared normal in the live animal, showed these features but to a slightly lesser extent.

The mice treated with PCBs 97 and 118 generally showed the same responses as the PCB 157 treated mice but to a noticeably lesser extent. Hyperplasia of the epidermis was seen resulting in an epidermis of about 4 cell layers in places, with some tendency to occlude the sebaceous follicles. The stratum corneum showed mild hyperkeratinisation. Follicular hyperkeratosis was not evident. Sebaceous glands were abundant. Pilar cysts appeared as for controls, being abundant and empty. It was not possible to distinguish between the responses to PCBs 189 and 97. There was close similarity between different mice in their responses to the same PCB.

7.3 Neurotoxicity Study

This study was designed to detect neurotoxicity in the form of movement disorders ("spinning syndrome") in the offspring of mice dosed with PCB 169 30 mgKg⁻¹ day⁻¹ on days 10-16 of gestation.

The five control dams used in this study each successfully gave
birth to and suckled their litters. No abnormalities or mortalities were noted throughout the experiment which was terminated for each litter at the age of six weeks.

The offspring of the five dams which each received PCB 169 showed a high mortality rate. One of the dams died on the day before delivery was due. Two dams produced litters but killed the pups at birth. One dam killed her pups on the third day after birth. The fifth dam produced a litter of seven pups of which she killed two a few days after birth and suckled five successfully. These five pups were the only offspring from PCB-dosed dams to survive until the end of the experiment, during which time no abnormalities of movement were detected. The tendency of the PCB-dosed dams to kill their offspring was ascribed to a general feeling of "illness" caused by the PCB. This conclusion is supported by the condition of one of the dams which killed its litter at birth, after which it was examined and found to have accumulated a large quantity of ascitic fluid.

7.4 Discussion

7.4.1 Organ Weight Study

In this study, PCBs 157 and 156 showed the greatest effects, depressing body weight gain and reducing all the organ weights measured. They caused marked thymic atrophy. PCB 118 depressed only body weight gain and heart weight, and increased liver:body weight ratio. PCB 189 only increased spleen weight. PCB 97 had no significant effects (p<0.05).
Although the effects of the chlorinated hydrocarbons on organ weights have been fairly well studied, of the reports that concern PCBs, relatively few are of congener-specific studies. Parkinson et al.(152) gave four-week old Long Evans rats a single i.p. dose of a single PCB congener and killed the animals four days later. They found that PCBs 157, 156, 189 and 118 caused no significant loss in body weight but all of them caused an approximately equal increase in the liver:body weight ratio, that due to PCB 189 being slightly less than the others. A significant decrease in the thymus:body weight ratio was caused only by PCBs 156 and 157. They also tested PCB 169 and observed a marked decline in both body and thymus weight, as expected from this relatively toxic congener. It is interesting that no significant change in the liver:body weight ratio was caused by PCB 169. Their data suggests a rank order of congener toxicity of PCB 169 >PCB 156 >PCB 157 >PCB 118=PCB 189.

Leece et al.(171) determined the doses required to cause reductions of 25% and 50% in body and thymus weights of 30-day old female Wistar rats 14 days after a single i.p. dose. Their results agree with those of Parkinson et.al. with respect to the order of potency of PCBs 169, 156, 157 and 118; they did not test PCB 189.

PCB 169 was not used in this study, as its effects on organ weights in rats have already been well established. PCB 156 was included to provide another mono-ortho substituted hexachlorobiphenyl for comparison with PCB 157, and to facilitate comparison of this study with similar published work.
As Parkinson and Safe (74) have pointed out, the time courses of these different effects are not all the same, body weight loss taking longer to develop than thymic atrophy. In fact the studies of Parkinson et al. and Leece et. al. referred to above indicate that it is preferable to allow at least two weeks for the effects of the less toxic congeners on both body and thymus weights to show. This is in contrast to the increase in liver weight which occurs within a few days. It does not appear that thymic atrophy is complete within five days, as suggested by Parkinson and Safe (74).

In this study, it was felt necessary to allow about two weeks for the effects on organ weights to show. The results allow some distinction to be made between the less toxic congeners, suggesting an order of potency of PCB 157 = PCB 156 > PCB 118 > PCB 189 > PCB 97. However, the distinction between PCBs 118 and 189 is tenuous.

The effects of the chlorinated hydrocarbons, as a broad chemical class, on organ weights have been reviewed by McConnell (151) and the changes found in this study were mainly those that would be expected with this class of chemicals. One anomaly was the decrease in absolute heart weight seen with PCBs 157, 156 and 118. The expected increase in heart:body ratio was only seen with PCB 156. Absolute liver weight also declined with PCBs 157 and 156. Although the decline in absolute kidney weight seen with these two PCBs was expected, the increase which they caused in the kidney:body weight ratio was not. It must be borne in mind however that these expected effects are based mainly on studies with compounds such as TCDD, rather than specifically on studies with PCBs.
A single PCB congener was however used by Yamamoto et al. (71) who administered a single oral dose of 150 mgKg⁻¹ of 2,3,3',4,4'-pentachlorobiphenyl to rats. After eight days, liver and spleen weights had declined both as absolute weight and as organ:body weight ratio, while kidneys and heart weights had declined as the absolute weight and increased as the ratio. The results for this pentachlorobiphenyl generally reflect those for PCBs 156 and 157 recorded in this study, except for their effect on the liver:body weight ratio.

The spleen results were of particular interest. As discussed in chapter four, the weight of this organ (and the organ:body weight ratio) seem generally to be decreased by higher doses and increased by lower doses of a given chlorinated hydrocarbon. Both these parameters were decreased by PCB 157. PCB 156 decreased absolute spleen weight but the ratio was unchanged. PCB 189 increased both measurements. PCBs 118 and 97 had no effect on either of them. However as the effect of high toxicity seems to be the reverse to that of low toxicity, there must be a cross-over point (of intermediate toxicity) somewhere in between at which no effect on spleen weight is seen. The effect of PCBs 118 or 97 (or both) could therefore lie on this point. Although this reasoning is conjecture, it does support the tentative rank order of potency proposed above, i.e. PCB 157 > PCB 156 > PCB 118 > PCB 189 > PCB 97.
7.4.2 Dermal Toxicity Study

Substantial differences in the histology of hairless mouse skin were observed after topical application of PCB 157 for six weeks, in comparison with controls. Epidermal hyperplasia, hyperkeratoses of the stratum corneum, disappearance of sebaceous glands, follicular hyperkeratosis, reduction in the number of sebaceous follicles and the appearance of concentric keratin rings within the pilar cysts of the dermis were all noticeable. PCBs 189 and 97 produced the same effects but to a much lesser degree than PCB 157. PCBs 189 and 97 showed equal potency in eliciting these effects.

Dermal toxicity due to PCBs has been observed in cows, monkeys and man as well as in rabbit ears and hairless mice (10,172,173,174). Vos and Beems (175) applied commercial PCB mixtures to the backs of adult New Zealand rabbits and observed skin lesions consisting of hyperplasia and hyperkeratoses of the epidermal and follicular epithelium, with the keratinisation of the hair follicles.

Puhvel et al. (176) applied AROCLOR 1254 dissolved in acetone to the backs of hairless mice. They observed no gross or histological changes after applying 3mg four times a week for six weeks, in either Skh:HR-1 or HRS/J strains. Phenoclor 54 (0.2 mg five times a week for ten weeks) produced no gross changes but histological examination showed hyperkeratosis of the stratum corneum, epidermal hyperplasia, disappearance of sebaceous glands and the presence of keratinised cysts in the dermis. Both mouse strains showed these effects.
Puhvel and his coworkers also applied 3,3',4,4'-tetrachlorobiphenyl (TCB) 0.2 mg five times a week for 10 weeks to his hairless mice. In the Skh:HR-1 strain they observed marked follicular hyperkeratosis, epidermal hyperplasia, hyperkeratinisation of the stratum corneum and the disappearance of sebaceous glands. Intradermal pilar cysts had filled with keratin, appearing as concentric rings within the cysts. These cysts had ruptured in places, giving rise to a local inflammatory reaction which was visible on gross inspection as white pinhead-sized spots on the skin. However the HRS/J strain showed no such white spots on the skin which was shiny and scaly. A similar pattern of histological changes was noted, but with no rupturing of dermal cysts.

The study of Puhvel et al. described above was notable in that it produced some differences in response between the two strains of mouse used and which the authors were unable to explain. However, their descriptions suggest that the differences may only have been of the degree of response, with the Skh:HR-1 mice showing greater sensitivity to TCB. In the present study, the changes produced in MFl-hr mice were more consistent with those seen by Puhvel et al. in the HRS/J strain, in that no rupturing of the keratinised intradermal cysts occurred and the skin became scaly without any spots. The other histological changes were of the type observed by Puhvel et al.

The results from this study indicate that PCB 157 is markedly more toxic than either PCBs 189 or 97, and that the latter two show broadly similar potencies under the conditions used in this study.
7.4.3 Neurotoxicity Study

It has been established that PCBs can cause neurotoxicity in several species of animals and birds (10). Aroclor 1254, for example, has been reported to suppress the avoidance response of quail chicks (177), and to depress spontaneous motor activity in mice while inhibiting uptake and release of neurotransmitters (178). Neurobehavioral effects were detected in rat pups suckled by dams exposed to AROCLOR 1254 in their diet throughout gestation and lactation. The effects included delayed auditory startle and air righting reflex caused by 26 ppm PCBs in the diet (150). Mele et al. (179) concluded that the perinatal exposure of rhesus monkeys to AROCLOR 1248 had caused functional changes in response rate and locomotor activity when the animals were tested at age five years. Chen et al. (56) observed symptoms of peripheral neuropathy, including paresthesia of the limbs and pain in the limbs, amongst victims of the PCB poisoning incident which occurred in Taiwan in 1979. Tests revealed abnormally low conduction velocities in motor and sensory nerves.

Chou et al. (180) observed a permanent motor disturbance ("spinning syndrome") in weanling CD-1 mice suckled by dams dosed with 3,4,3',4'-tetrachlorobiphenyl (TCB) daily from day 10 to day 16 of gestation, and found that this syndrome was associated with histological abnormalities in certain CNS nerve roots. The syndrome was characterised by bouts of rapid circling movements of up to 150 turns per minute with general restlessness and hyperactivity. Based on this report it was hoped to use the spinning syndrome as a model
for PCB induced neurotoxicity to compare different PCB congeners administered to dams during gestation.

The study demonstrated the general toxicity of PCB 169 in the dams, but failed to demonstrate any neurotoxicity in the offspring which survived. It was concluded that since PCB 169 had failed to cause the "spinning syndrome" at this relatively high dose, it was very unlikely that any of the other PCB congeners would do so, at least in CILP mice, and so no other congeners were tested in this study. As different mouse strains may vary in their susceptibility to this syndrome, further work could usefully be carried out with PCB 169 (or better still with TCB) to establish the most suitable animal model for such studies before going on to look at the effects of different congeners.
CHAPTER EIGHT

GENERAL DISCUSSION

8.1 Summary of Main Results

In this study, a series of PCB congeners were synthesized, purified and administered to lactating rats. Their effects were measured in the suckled pups at weaning, in order to compare their toxic potencies after transfer in milk. On the basis of the liver parameters measured (cytochrome P-450 concentration, aniline hydroxylase and aminopyrine demethylase activities, microsomal protein concentration and liver:body weight ratios) and the litter size and body weight changes, the order of potency was found to be PCB 169 > PCB 157 > PCB 118 > PCB 189 > PCB 97. PCB 169 produced marked changes in the pups while PCB 97 had very mild effects. This rank order was in agreement with that indicated by the effect of these congeners on organ weights in sixteen-day old rat pups dosed directly and in agreement with published reports on the effects of some of these congeners on organ weights in weanling rats. The greater potency of PCB 157 compared to PCBs 189 and 97 was confirmed by their effects on skin histology in hairless mice after direct topical application.

Such a result indicates that the concentrations of PCBs 169, 157, 118 and 189 will have a greater effect on the total toxicity of the PCB burden in breast milk than will the levels of PCB 97.

171
No sex differences in the inductive effects of the congeners used were observed.

In contrast to the other congeners used, PCBs 169 and 157 caused changes in second litters, although no further exposure of the dam to PCBs had occurred.

8.2 PCB Mode of Action: Receptor Binding

As mentioned in section 1.8.4, three main structural groups of PCBs (coplanar, mono-ortho and di-ortho congeners) have been identified with respect to their biological effects, which have been explained in terms of the steric interaction of the PCB molecule with the Ah receptor (70). PCBs show effects in common with those of the polychlorinated dibenzo-p-dioxins, the most potent of which is the coplanar 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The polychlorinated dibenzofurans, of which the most potent is the coplanar 2,3,7,8-tetrachlorodibenzo-furan (TCDF) also show similar toxic effects to the PCBs. TCDD and TCDF are however much more potent than any of the PCBs (181,182), the former being regarded as one of the most toxic substances known (183,184). Both TCDD and TCDF are thought to exert their effects by binding to the Ah receptor, their high binding affinities being due in part to the planarity of the molecule. Their structural similarities with the coplanar PCBs have been noted (185). The molecular structures of TCDD and the coplanar PCB 169 are shown in figure 8.1.
Similarly, the lack of ortho substituents in the molecule of PCB 169 results in planarity, so that the affinity for the receptor is relatively high. Conversely, the presence of ortho substituents disrupts molecular planarity and decreases binding. This theory was put forward by Poland and Glover (186) who determined the affinities of a series of halogenated compounds by measuring the concentration of the compound required to cause a 50% reduction in the binding of $[^3H] \text{TCDD}$ to the Ah receptor in the chicken embryo, i.e. to competitively displace the $[^3H] \text{TCDD}$. They found that 3,3',4,4'-tetrachlorobiphenyl displayed a high affinity. They were unable to measure the exact affinity of PCB 169 by this method because of its poor aqueous solubility but they did find that it displaced TCDD to a marked extent. The same workers also found that only 3,3',4,4'-tetrachlorobiphenyl and PCB 169 induced AHH in the chicken embryo, while the di-ortho substituted congeners they tested did not. The same coplanar PCBs induced AHH in the rat to a marked extent while the di-ortho congeners did so only to a very mild extent. The congeners which were active inducers of AHH did not induce aminopyrine demethylase and vice-versa. In other words they were either MC- or FB-type inducers.
8.3 Structure-Activity Relationships

8.3.1 Correlations between Ah Receptor Binding, Toxicity and Enzyme Induction

The choice of congeners used in this study provided a range of the higher (penta-, hexa- and hepta-) chlorinated biphenyls and a variety of substitution patterns. Higher chlorinated congeners were chosen because they are in general more abundant in human milk than the lower chlorinated PCBs (34,36). Also, all the congeners used have been identified in human milk with the exception of PCB 169 (34). As table 2.1 in chapter two shows, the congeners consisted of one di-ortho substituted pentachlorobiphenyl (PCB 97), one mono-ortho substituted pentachlorobiphenyl (PCB 118), two mono-ortho substituted hexachlorobiphenyls (PCBs 156 and 157), one coplanar hexachlorobiphenyl (PCB 169) and one mono-ortho substituted heptachlorobiphenyl. PCB 156 was not used in the milk transfer studies described in chapter five due to a shortage of material.

PCB 169, the only coplanar congener used, was clearly the most toxic considering that it was used at a dose level one tenth that of the other congeners. This was expected on the basis of current quantitative structure-activity relationship (QSAR) theory, which has been described by Safe et al.(70). Those coplanar PCBs which are substituted in both para and in two or more meta positions, such as 3,4,4',5-tetrachlorobiphenyl, 3,3',4,4'-tetrachlorobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169) display the greatest toxicities in a variety of tests. They are also MC-type enzyme
inducers (see section 1.8.2). These congeners show the highest affinities for the Ah receptor in hepatic cytosol (145).

Yoshimura et al. (168) found that PCBs 169 and 3,3',4,4',5-pentachlorobiphenyl (both coplanar congeners) at doses of 10 mgKg\(^{-1}\) caused significant changes (p<0.05) in the weights of liver, spleen and thymus gland in adult rats whereas similar or greater doses of mono- and di-ortho congeners did not. McKinney et al.(181) fed various hexachlorobiphenyls to day-old chicks at a dose of 400 ppm in their diet, and found that PCB 169 caused 100% mortality, marked thymic involution, depletion of lymphocytes in the spleen, mild hepatotoxicity and a generalised oedema. By comparison, four congeners each possessing two or more ortho substituents caused mortality which did not exceed 10% for any congener, only slight thymic involution, no toxicity in the spleen and no oedema except for oedema of the endocardium caused by one congener. However, hepatotoxicity was greater than with PCB 169.

Biocca et al.(187) fed mice PCB 169 or some of the ortho substituted congeners used by McKinney et al. at a dose of 300 ppm in their diet for 28 days (30 ppm for PCB 169). They found a similar difference in toxicity to that observed by McKinney et al., with PCB 169 being far more toxic to thymus and spleen but also to the liver. In addition, PCB 169 was the only congener used that caused porphyria.

PCBs 157, 156, 189 and 118 are mono-ortho substituted congeners. Although this structural group shows a range of toxic effects similar to the coplanar PCBs (71,188) they appear to be generally
less toxic, for example in their effects on the thymus (152, 171). In the study by Yoshimura et al. (168) already described it was found that 2,3,4,4'-tetrachloro- and 2,3,3',4,4'-pentachlorobiphenyl failed to significantly change (p<0.05) the weights of the liver, spleen and thymus in adult rats whereas coplanar congeners did. They show a lower affinity for the Ah receptor (145) and are mixed-type enzyme inducers (123, 124).

PCB 97 is a di-ortho substituted congener. This group shows the lowest Ah receptor affinities of all the PCBs (145) and they are mixed-type enzyme inducers (146). They are generally considered to be of lower potency than the mono-ortho congeners but there is little information available to support this. They are however known to be markedly less toxic than some of the coplanar congeners as already indicated. Stonard and Greig (189) reported that di-ortho substituted congeners caused porphyria in rats.

The QSAR theory suggests there is a correlation between the enzyme induction pattern (particularly AhH induction), receptor binding and general toxic potency of the PCBs. This represents a major part of the current thinking about the toxicology of the halogenated hydrocarbons in general and the PCBs in particular and has been enthusiastically supported by Safe and co-workers (10, 190, 70). However, there is still a paucity of toxicity data to support this theory for the PCBs except in broad terms. Neal (191) has pointed out that the wide variation in toxicity to TCDD shown by different species (single oral LD_{50} values being 2 mcgKg^{-1} in the guinea pig compared to 5051 mcgKg^{-1} in the hamster) is not associated with a
similar variation in the concentration of the hepatic Ah receptor, which is of the same order of magnitude in each of these different species. The differences between species in their sensitivity to TCDD cannot be explained by different receptor affinities or by different rates of elimination of TCDD. A similar wide variation can be seen in the sensitivity of different species to thymic atrophy. However, the validity of comparing different species in this way seems questionable.

8.3.2 QSAR: Metabolism

Early work by Grant et al. (98) indicated that the rate of elimination of PCBs from various organs in rats was faster for the lower than for the higher congeners. The situation was clarified by the study of Gage and Holm (102) who administered single oral doses of a series of 14 PCB congeners (ranging from dichloro- to hexachlorobiphenyls) to mice and measured the concentrations in fat, 7 and 21 days after dosing. They concluded that the retention of a congener in fat is dependent on its resistance to metabolic degradation and that such resistance depends on the position of the chlorine atoms in the molecule (i.e. the substitution pattern) rather than on the total number of chlorine atoms present. They found that the most rapid rate of elimination occurred with congeners in which the adjacent positions 2, 3 and 4 (on at least one ring) were unsubstituted (i.e. free of chlorine atoms) and that the presence of chlorine atoms in ortho positions elsewhere in the molecule did not noticeably retard elimination. Moderately rapid loss from fat occurred if only an adjacent 2,3 pair was
unsubstituted but the presence of chlorines in the remaining ortho positions slowed this down. In contrast, fairly rapid elimination occurred if only a 3,4 pair was free but the presence of ortho chlorines had no effect. The same workers also compared the retention of radioactivity from two $^{14}$C-labelled hexachlorobiphenyls with the retention of each congener as indicated by GC analysis, and their results suggested that PCBs are stored unchanged in fat.

The results of other studies tend to support the conclusions of Gage and Holm. Matthews and Anderson (192) also concluded that very little of the PCBs stored in animal tissues was present as metabolites, storage of the unchanged parent PCB being the rule. They found that following oral or intravenous administration to rats, PCBs are stored in the liver and muscle because these tissues are well perfused, but over the succeeding days and weeks a gradual redistribution occurs into adipose tissue and skin, which are sites of higher affinity for PCBs but are less well perfused. Their results show that after a redistribution period of 42 days, 85% of the radioactivity derived from a single tracer dose of $^{14}$C-labelled 2,2',4,4',5,5'-hexachlorobiphenyl was found in fat and 15% in skin. They estimated that less than 20% of the dose ingested would ever be excreted. In contrast, minimal amounts of 4-, 4,4'- or 2,2',4,5,5'-substituted congeners were retained in these tissues, most having been eliminated. The same workers judged that oral absorption of PCBs is good, since tissue levels in different organs were very similar after oral and intravenous administration.
Good oral absorption of PCBs in the rat was also reported by Albro and Fishbein (193) who measured the appearance of a range of congeners in faeces and urine. In most cases 95%+ of the dose was absorbed orally.

Hutzinger et al. (99) observed an apparent inability of rats and pigeons to metabolise 2,2',4,4',5,5'-hexachlorobiphenyl, whereas mono-hydroxy metabolites of 4-, 4,4'- and 2,2',5,5'- substituted congeners were detected in faeces and urine. Kato et al. (194) gave tracer doses of [14C]-labelled hexachlorobiphenyls to rats and measured the appearance of radioactivity in faeces and urine. Rapid metabolism and elimination of 2,2',3,3',6,6'-hexachlorobiphenyl was observed. Using mass spectrometry and NMR they showed that negligible excretion of the unchanged parent compound occurred. In other words, the molecule was eliminated almost entirely as metabolites.

In contrast, in the same study congeners substituted in the 2,2',3,3',5,5'-, 2,2',4,4',5,5'- and 2,2',4,4',6,6'- positions were excreted only slowly. The metabolites which they identified consisted of hydroxylated PCBs. Dechlorination or chlorine shifts had occurred in places. They concluded from the nature of the metabolites that hydroxylation had occurred via an arene oxide intermediate in the manner suggested by Daly et al. (195). Metabolism by the direct insertion of a hydroxyl group was also considered possible, according to the mechanism proposed by Jensen and Sundström (196). The latter workers suggested that hydroxylation of isolated unsubstituted carbon atoms in PCBs might proceed by a
different pathway which is slower than the arene oxide route.

Studies of PCB metabolism in man have of necessity been limited, but the Yusho and Taiwan poisonings have provided some information. Kuroki and Masuda (197) measured some PCB congeners in adipose tissue and blood of Yusho patients nine years after they had been poisoned, and compared these levels to those in the normal (non-poisoned) population as controls. The level of 2,3',4,4',5-pentachlorobiphenyl (PCB 118) had declined in these patients to that of the normal population. The levels of 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156) and 2,2',3,3',4,4',5-heptachlorobiphenyl were rather elevated (the latter being 0.28 ppb compared to 0.11 ppb in controls). However the levels of 2,2',4,4',5,5'- and 2,2',3,4,4',5-hexachlorobiphenyls and 2,2',3,4,4',5,5'-heptachlorobiphenyl were more elevated still. Thus the congeners with adjacent 2,3 positions free were present at the lowest levels. Those without 2,3 or 3,4 or 2,3,4 positions free, or those in which the 2,3 positions were free but another ortho chlorine was present, appeared to be more persistent. However, the levels of these congeners in the toxic rice oil, which was the vehicle of the poisoning, were not measured in this study and so the results could be misleading.

Chen et al. (198) did however compare PCB congener levels in the blood of the Taiwan poisoning victims with those in the rice oil implicated in that incident. They concluded that tetra- and pentachlorobiphenyls with 3,4 positions free are eliminated quickly, but elimination is slower if only the 2,3 positions are free. The elimination rate of hexa- and hepta- congeners with only 2,3
positions free was very slow.

Wolff et al. (199) reported that of 37 congeners measured in blood and adipose tissue of workers occupationally exposed to various PCB mixtures, those with unsubstituted 3,4 positions were present in much lower concentrations than congeners in which the 2,3 rather than the 3,4 positions were free.

The metabolism of the PCBs has been reviewed by Safe et al. (200).

8.3.3 QSAR: Enzyme Induction

The studies discussed in section 8.3.2 indicate that the rate of elimination of PCB congeners depends on their rate of metabolism to more polar compounds prior to excretion in urine and faeces. It follows that the elimination rate will influence the extent to which PCBs exert their effects, and there is evidence that this is the case. Hansell et al. (201) administered PCB congeners in single i.p. doses of $0.2 \text{ mmolKg}^{-1}$ to young adult male Wistar rats and reported that $4,4'$- and $2,2',5,5'$- substituted congeners were rapidly eliminated from liver. These congeners also caused very little induction of $p$-nitroanisole-O-demethylase and aniline hydroxylase, and very little change in liver morphology. However the $2,2',4,4'$- and $2,2',4,4',5,5'$- substituted congeners were eliminated far more slowly and also caused marked changes in these parameters within three days. The changes due to the hexachlorobiphenyl lasted for over 35 days.
Ecobichon and Comeau (202) studied the effects of a series of PCB congeners ranging from mono- to octachlorobiphenyls administered to weanling male Wistar rats at a dose of 50 mgKg\(^{-1}\)day\(^{-1}\) for three consecutive days. They observed that induction of p-nitroanisole-O-demethylase and aniline hydroxylase was greatly enhanced by the presence of chlorine substituents at both of the para (4,4') positions. They postulated that this was due to a decreased rate of metabolism caused by chlorines in these positions. The exception to this was 2,2',4,4',6,6'-hexachlorobiphenyl which did not induce the demethylase enzyme. Presumably the presence of four ortho substituents in this molecule decreases its receptor binding to such an extent that it is a weak inducer regardless of its rate of elimination. The same workers noticed an interesting difference between the mixed function oxidases (demethylase and aniline hydroxylase) which are located bound to the endoplasmic reticulum in hepatocytes and some enzymes less localized within the cell (hepatic carboxylesterases and BSP-GSH conjugating enzyme). Induction of the former group was influenced by a 4,4'-substitution pattern in PCBs as described, but in the latter group the position of the chlorine atoms in the molecule appeared to be less important for induction.
Based on the discussion of toxicity in section 8.3.1, the order of toxic potency of the congeners used in this study would be expected to show the coplanar PCB 169 as the most toxic and the di-ortho substituted PCB 97 as the least toxic in studies involving direct PCB administration to rats. The mono-ortho substituted PCBs 157, 189 and 118 would be expected to lie somewhere in between these two. There is some evidence in the literature to indicate that this is the case, although no reports concerning PCB 97 have been seen. Yoshihara (188) administered a single i.p. dose of PCBs 156 or 118 50 mgKg\(^{-1}\) (equivalent to 0.14 mmolKg\(^{-1}\) of PCB 156 and 0.15 mmolKg\(^{-1}\) of PCB 118) to four-week old male Wistar rats. They found that PCB 156 was clearly more potent than PCB 118 as an inducer of AHH, but equally potent as an inducer of benzphetamine demethylase and cytochrome P-450. Measurements of liver, spleen and thymus weights showed no differences between them, but PCB 156 caused a greater increase in liver lipids.

As mentioned in section 5.11.2, Parkinson et al. (123) using two doses of 0.15 mmolKg\(^{-1}\) in one-month old male Wistar rats found that PCB 118 was more potent than PCB 157 based on their effects on cytochrome P-450, aminopyrine demethylase activity and microsomal protein. However this order was reversed if comparison was based on their effects on AHH activity and liver:body ratio, although the differences between the two congeners were then small. In the same study, PCB 156 showed equal potency to PCB 118 in all measurements except microsomal protein which was increased slightly more by PCB
118. There was very little difference between the three congeners in the wavelength of the P-450 absorption maximum.

Goldstein et al. (122) compared the effects of PCBs 169 and 189 in one-month old female rats given $0.014 \text{ mmol Kg}^{-1} \text{ day}^{-1}$ for three days and found that PCB 169 was considerably more potent as an inducer of cytochrome P-450 and AHH. PCB 189 inhibited aminopyrine demethylase, whereas PCB 169 had no effect.

It can be seen from the preceding discussion that comparisons of the toxic potencies of different PCB congeners depend on the criteria used to measure toxicity. AHH induction is the most widely used yardstick because it is believed to be implicated in the formation of toxic reactive metabolites (arene oxides) from various polycyclic aromatic hydrocarbons (PAHs), such as benzpyrene, and as such to be fundamental in expressing the toxicity of the PAHs (200). As already discussed, AHH induction is also a feature of those (MC-type) chlorinated hydrocarbons which show the greatest toxicity as judged by other (non-enzyme) tests.

However, in spite of the undoubted usefulness of AHH as a toxicity indicator, there is no firm indication in the literature that the induction of other enzymes is not equally harmful in the long term. Therefore the assessment of toxicity based on enzyme induction should use a variety of different classes of enzymes to detect a broader range of effects than is afforded by AHH induction alone. If AHH induction alone is used to compare the congeners used in this study, the rank order of potency indicated by the published
literature is PCB 169 >PCB 157 >PCB 118 >PCB 189, which agrees with the conclusion reached in chapter five based on all the results obtained in this study. If however demethylase induction is used then the order is PCB 118 >PCB 157 >PCB 169.

This rank order of potency based on published reports of demethylase induction is quite different from that indicated by the aminopyrine demethylase results in this study, which show an order of PCB 169 >PCB 157 >PCB 118 >PCB 189 >PCB 97. This discrepancy may reflect pharmacokinetic differences between the congeners (see section 1.9). The results quoted above from the published literature refer to studies in which rats were dosed directly with the PCBs, instead of ingesting them in their mother's milk as occurred in this study. Five of the congeners used in this study were identified in human milk by Safe et al. (34) who did not detect PCB 169. Although these congeners can be transferred in milk there is no indication in the literature of the extent to which they are excreted in milk relative to the amounts ingested.

However Masuda et al. (82) fed seven different PCB congeners to mice at low concentrations in their diet (about 0.4 ppm) from day one to day 18 of pregnancy and measured whole body PCB levels during the lactation period. They found that very little (<0.3%) of the total dose of any of the congeners ingested by dams was transferred to the foetus by day 18 of pregnancy. The amounts appearing in the suckled pups varied greatly. Two hexachlorobiphenyls and one octachlorobiphenyl were readily transferred in milk, so that by the time the pups were two weeks old they had accumulated 44-53% of the
dose ingested by the dams. In contrast a trichlorobiphenyl, a tetrachlorobiphenyl, a pentachlorobiphenyl and a heptachloro-
biphenyl were transferred to a minor extent only, less than 3% of the dose of each accumulating by two weeks of age. In all cases the dams contained only trace amounts of PCB after the lactation period.

Masuda et al. concluded that the lower chlorinated and the heptachlorinated congeners had been eliminated from the dams' bodies before transfer in milk could occur, while the hexachloro- and octachlorobiphenyls were retained in the dams' bodies and so were available for milk transfer. As the heptachlorobiphenyl had been eliminated before milk transfer as well as the lower chlorinated congeners, the position as well as the total number of chlorine atoms in the PCB molecule was thought to be important in determining elimination rates.

In this study, dosing with single congeners was carried out during lactation instead of during gestation as in the study of Masuda et al. However, similar kinetic considerations would be expected to apply. The very weak inductive effects of PCB 97 compared to the other congeners used may have been due partly to faster elimination from the lactating dams by metabolic routes rather than by transfer of the unchanged congeners in milk. PCB 97 is 2,2',3',4,5-pentachlorobiphenyl and so possesses unsubstituted carbon atoms in positions 4',5' and 6' (which is equivalent to positions 2, 3 and 4 being free). As described in section 8.3.2, the presence of this substitution pattern is considered to favour hydroxylation of the ring, and therefore to promote elimination of
the PCB from the body in the faeces and urine.

PCB 118 is 2,3',4,4',5-pentachlorobiphenyl and so is unsubstituted in a 2,3 position on one ring. This substitution pattern favours moderately fast elimination but not as fast as in the case of PCB 97. However the presence of the ortho substituent would slow down its metabolism. PCB 189 is 2,3,3',4,4',5,5'-heptachlorobiphenyl and so all of the unsubstituted positions are isolated from each other. This structure should not favour rapid metabolism, which would be expected to proceed via the slower process of direct insertion of a hydroxyl group (see section 8.3.2). This in turn should favour expression of the enzyme inducing effects of the molecule.

PCB 157 is 2,3,3',4,4',5'-hexachlorobiphenyl which has an unsubstituted 2,3 position and an ortho chlorine atom. The same considerations apply as in the case of PCB 118. PCB 169 is 3,3',4,4',5,5'-hexachlorobiphenyl which like PCB 189 contains only isolated unsubstituted positions and so metabolism is expected to be slow.

It appears that structure-activity theory can be used to explain the comparative toxicity of different congeners in terms of their receptor binding and metabolism. Biocca (187) found that the toxicity of four symmetrical hexachlorobiphenyls fed to five-week old mice for 28 days at dietary concentrations of up to 300 ppm decreased in the order of 3,4,5 substitution >2,4,6 >2,4,5 >2,3,6. The metabolism of the 3,4,5 substituted congener (PCB 169) would be expected to be the slowest and that of the 2,3,6 congener the
fastest, of all the congeners used. The binding of PCB 169 to the Ah receptor would be greatest since it is a coplanar congener. The binding of the other three molecules would be relatively weak as they each contain at least two ortho groups. QSAR does not yet allow prediction of the relative toxicity of the 2,4,6 and the 2,4,5 substituted congeners.

From this discussion, the structures of the PCB congeners used in this study would be expected to show elimination rates increasing in the following order: PCB 169 > PCB 189 > PCB 118 > PCB 157 > PCB 97. This rank order partially explains the relative potencies observed in this study of PCB 169 > PCB 157 > PCB 118 > PCB 189 > PCB 97. PCB 169 with its coplanar structure is also slowly metabolised and is therefore the most toxic congener. PCB 97 with its di-ortho substituents would be expected to show weak receptor binding and to be metabolised the fastest, and therefore would be the least toxic congener. PCB 189 however would be expected to be more toxic than PCBs 157 and 118 on the basis of its expected slower elimination rate.

PCBs 169 and 157 were the only two congeners to cause noticeable changes in pups of second litters. The expected slow elimination rate of PCB 169 would favour its retention in the dams' tissues more than in the case of the other congeners. This must have resulted in enough residual PCB remaining to be transferred in milk during the second lactation. The lack of direct measurements of PCB tissue levels in this study makes it impossible to distinguish between pharmacokinetic and pharmacodynamic effects. Some of the other
congeners apart from PCBs 169 and 157 may have been transferred to second litter pups, but if they were it was generally in amounts that were too small to have an effect.

When comparing the relative potencies of PCB congeners using direct single dosing studies with those using milk transfer studies, two further aspects need consideration. The first is the kinetic fate of each congener after transfer to pups in milk. The published reports concerning PCB metabolism and inductive effects refer mainly to studies in the rat or mouse with weanling or adult animals rather than suckling pups. There may be differences in the handling of PCBs in animals of this age which can enhance or diminish the effects of certain PCB congeners depending on their structures.

The other factor which may be important is the effect of continuous PCB exposure throughout the first 21 days postpartum. This is a period of liver enzyme development (see section 4.9). The PCB-enzyme interaction during this development period has not been closely studied. The extent to which the effect of a given dose may be magnified or diminished as a result of this interaction is unknown. Consequently it would seem unreasonable to assume that a single dose of a PCB congener administered directly to a 21-day old rat would have the same effect, relative to another congener, as administration of the same dose slowly over the first 21 days postpartum. Both pharmacokinetic and pharmacodynamic influences may be involved here, and this effect is distinct from possible pharmacokinetic differences between congeners in the extent to which they appear in milk.
8.5 *Induction Patterns seen in this Study*

As discussed in section 5.11.2, some unexpected results were seen in this study in the pups suckled by dams that had received PCB congeners. PCB 169 induced aminopyrine demethylase contrary to reports already mentioned that it does not induce this enzyme and in fact can inhibit it in one-month old rats dosed directly (122,171). PCB 169 also inhibited aniline hydroxylase, although an increase had been expected. These results indicate that PCB 169 behaved more as a mixed-type enzyme inducer rather than a MC-type as has been reported (120). In addition the wavelength of the absorption maximum was very similar to that of PCBs 157 and 118, which have been shown to be mixed-type inducers as discussed in section 5.11.2. Perhaps an explanation of these observations lies again in the pattern of exposure of the pups to the PCB, as discussed in section 8.4.

The other congeners used in this study behaved on the whole as mixed-type enzyme inducers, as they are reported to be, since they induced both aniline hydroxylase and aminopyrine demethylase. (This induction pattern would also be produced by pure FB-type inducers).

8.6 *Is Breast-Feeding Hazardous?*

The decision of whether or not to breast-feed, based on an assessment of the risk of transferring a toxic burden of chemicals to the infant, is difficult to make. The advantages of breast-feeding are now well recognized and have been discussed by Garfield (203). They include benefits to the mother such as weight
loss, the speedy return of the uterus to its pre-pregnancy size (due to stimulation of oxytocin secretion) and the suppression of reproductive function. Benefits to the infant include a more favourable protein composition in human milk (compared to cow's milk or infant formulae), increased resistance to infections due to the presence of immunoglobulins in breast milk, and a possible decrease in hereditary allergies. There is some evidence that breast-fed babies have a lower incidence of learning disabilities, and psychological benefits to both mother and child have been noted. In the last few decades the advantages of breast-feeding have become more widely appreciated and the earlier post-war trend towards bottle-feeding has been gradually reversed, at least in the industrialised countries.

On the other hand, it is now known that unwanted compounds such as PCBs can be transferred to the breast-fed infant in milk (see section 1.6). Certain drugs can also be transferred (204,205). Garfield (206) indicated that the transfer of xenobiotics was the main disadvantage of breast-feeding. Toxic effects in human infants have been attributed to PCBs transferred in human milk (see section 1.6), although some surveys of human infants have failed to detect any clinically significant consequences of such exposure (207). Because of the resurgence in popularity of breast-feeding the disadvantages need to be considerable before mothers are discouraged. For trace levels of xenobiotics in milk to constitute a contra-indication, convincing toxicological evidence is required.

The situation at present is that although contaminants such as PCBs
are recognised as a potential hazard, the hazard needs to be quantified in some way before recommendations to restrict breast-feeding can be justified. The transfer of PCBs in milk varies according to lactation history and duration of lactation (38,170). Generally, PCB levels tend to be higher earlier in lactation and during a mother's first lactation, because at such times the total body burden of PCBs is greatest. If those PCB congeners most likely to cause toxicity are identified, congener-specific milk analyses can be used to screen human milk for the presence of these "high risk" congeners. If such congeners were present above a certain pre-determined concentration, this would form a reasonable basis for recommending that bottle feeding be used with manual expression of milk to reduce the PCB load, for a limited period until milk PCB levels had declined.

8.7 Criticisms

As mentioned in section 8.4 any assessment of toxicity must be based on as many parameters as possible. The scope of this study is limited in this respect, as only two enzymes were studied. Particularly, a purely cytochrome P-448 dependent enzyme such as ethoxyresorufin-O-deethylase should be included.

In order to complete any comparisons between different PCB congeners in milk transfer studies, pharmacokinetic as well as pharmacodynamic measurements are desirable. This study did not include any assays of PCBs in the tissues of the dams or suckled pups, and so the fate of each congener can only be inferred.
The attempts to detect synergism between the congeners used were too limited, and would have been more useful if a range of doses and congener combinations had been used. Again, other enzymes may be more suitable for detecting synergism.

8.8 Conclusions

The objectives of the study adopted in chapter one (section 1.9) were broadly met, i.e.:

- the timing of maternal PCB exposure appeared unimportant at the dose level used, to detect effects in suckled pups;

- the general method used seems suitable for the comparison of different PCB congeners with respect to their effects in rat pups suckled by PCB-exposed mothers;

- when single PCB congeners were administered to lactating rats, their order of toxic potency observed in suckled pups was PCB 169 >PCB 157 >PCB 118 >PCB 189 >PCB 97;

- no synergism was detected between PCBs 169 and 97 at the doses used, but synergism may still be important in PCB toxicity;

- the order of potency indicated by milk transfer studies was broadly confirmed by limited toxicity tests involving skin histology and organ weights;
- no differences between the sexes were seen in the effects of any of the PCB congeners used;

- PCBs 169 and 157 persist in dams beyond the first lactation following exposure and cause effects in second litters.

8.9 Recommendations for Future Work

As indicated in the criticism in section 8.7, the method for comparing the effects of different PCB congeners transferred to rat pups in milk should be carried out with other enzymes in addition to those used in this study. The kinetics of the congeners used should be examined using appropriate gas chromatographic assays to measure PCB levels in tissues. Investigations into the time-course of induction would be useful, especially if linked to PCB assays.

All studies should be carried out in more than one species. As the ultimate objective is extrapolation to man, inter-species variation needs to be assessed. More congeners need to be used, concentrating on those that appear in milk. The objective would be to accumulate data using this method for as many congeners as possible, to assist in toxicological interpretation of congener-specific milk analyses.

Lastly, the relationship between toxic potencies indicated by milk transfer studies and other, non-enzyme based toxicity measurements needs to be examined. As indicated in section 8.3.1, such toxicity data is still scanty and that which concerns developing neonates is particularly needed.
REFERENCES


195


34. Safe, S., Safe, L., Mullin, M. (1985) 

   Ambio 3, 70-76.


   Arch. Environ. Contam. Toxicol. 17, 55-63.

38. Yakushiji, T., Watanabe, I., Kuwabara, K., et al. (1979) 

   Environ. Health Perspect. 60, 121-126.

   Human Toxicol 1, 425-431.

41. Price, R., Welch, R. (1972) 
   Environ. Health Perspect. 1, 73-78.

42. World Health Organisation (1976) 
   Environmental Health Criteria 2. Polychlorinated Biphenyls 


   J. Dermatol. (Tokyo) 7, 435-441.

45. Kuratsune, M., Yoshimura, T., Matsuzaka, J., et al. (1972) 
   Environ. Health Perspect. 1, 119-128.


47. Gillette, D., Corey, R., Lowenstein, L., Shull, L. (1987) 

   Food Cosmet. Toxicol. 12, 63-77.

49. Fishbein, L. (1972) 
   J. Chromatogr. 68, 345-426.

   Environ. Res. 5, 249-362.


197
    Bull. Environ. Contam. Toxicol. 12, 145-152.


    J. Formosan Med. Assoc. 80, 47-54.


58. Rogan, W., Gladen, B., Wilcox, A. (1985)
    Environ. Health Perspect. 60, 233-239.


60. Young, S. (1985)

    Food Cosmet. Toxicol. 12, 323-330.


    Arch. Environ. Contam. Toxicol. 17, 47-53.

64. Collins, W., Kasza, L., Capen, C. (1979)
    In Animals as Monitors of Environmental Pollutants. pp.327-338
    National Academy of Sciences, Washington DC.


    Environ. Res. 31, 76-94.

68. Nowicki, H., Norman, A. (1972)
    Steroids 19, 85-99.

69. Villeneuve, D., Grant, D., Khera, K. et al. (1971)
Xenobiotica 17, 299-310.

89. Takagi, Y., Otake, T., Kataoka, M., et al. (1976)  


Environ. Health Perspect. 59, 41-45.

92. Rogan, W., Gladen, B., McKinney, J., et al. (1986)  
J. Pediatr. 109, 335-341.

93. Kuwabara, K., Yakuishi, T., Watanabe, I., et al. (1978)  


Arch. Environ. Contam. Toxicol. 16, 375-381.


Bull. Environ. Contam. Toxicol. 6, 102-112.

99. Hutzinger, O., Nash, D., Safe, S. et al. (1972)  
Science 178, 312-314.


Toxicol. Appl. Pharmacol. 54, 293-300.


108. Omura, T., Sato, R. (1964) 
J. Biol. Chem. 239, 2379-2385.

Xenobiotica 12, 701-716.

Xenobiotica 12, 727-744.

111. Johnson, E. (1979) 
In Reviews in Biochemical Toxicology. pp.1-26. Hodgson, 
Bend, Philpot. (Eds.) 
Elsevier/ North Holland, Inc.

J. Biochem. 100, 1359-1371.

113. Conney, A. (1967) 

J. Biol. Chem. 250, 2157-2163.

J. Biol. Chem. 254, 1365-1374.


Fukuoka Acta Med. 62, 30-34.


Life Sciences 26, 945-952.


<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
</tr>
</thead>
</table>


Environ. Pollut. 6, 21-29.

Neurotoxicol. 2, 749-764.


180. Chou, S., Mike, T., Payne, W., Davis, G. (1979)  


183. McConnell, E., Moore, J. (1979)  


188. Yoshihara, S., Kawano, K., Yoshimura, H. (1979)  
Chemosphere, 8, 531-538.

189. Stonard, M., Greig, J. (1976)  


Environ. Health Perspect. 60, 41-46.

Drug Metab. Dispos. 3, 371-380.

Bull. Environ. Contam. Toxicol. 8, 26-31.

195. Daly, J., Jerina, D., Witkop, B. (1972) 
    Experientia 28, 1129-1149.

    Nature 251, 219-220.

    Chemosphere 8, 469-474.


    In Hydrocarbons in the Aquatic Environment. pp. 537-544. 

201. Hansell, M., Ecobichon, D., Comeau, A., Cameron, P. (1977) 
    Exp. Mol. Pathol. 26, 75-84.


    Current Contents 17(20), 3-12.

204. Rasmussen, F. (1971) 
    In Handbook of Experimental Pharmacology. Volume XXVIII, 
    part 1, chapter 20. Brodie, B., Gillette, J. (Eds.) 
    Springer-Verlag, New York.

205. Knowles, J. (1965) 
    J. Pediatr. 66, 1068-1082.

    Current Contents 17(21), 3-13.

    Am. J. Public Health 77, 1294-1297.

### Appendix One

**Composition of Buffers**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Buffer No.1</th>
<th>Buffer No.2</th>
<th>Buffer No.3</th>
<th>Buffer No.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$.2H$_2$O</td>
<td>8.90g</td>
<td>8.90g</td>
<td>8.90g</td>
<td>8.90g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>2.50g</td>
<td>2.50g</td>
<td>2.50g</td>
<td>2.50g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>200ml</td>
<td>200ml</td>
<td>_</td>
<td>200ml</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.37g</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>CHAPS *</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>6.15g</td>
</tr>
<tr>
<td>Water to</td>
<td>1000ml</td>
<td>1000ml</td>
<td>1000ml</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

**Final pH** ** 7.35  7.35  7.40  7.40**

*Full name: 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate*

**Final pH adjusted by addition of further quantities of either phosphate salt as necessary."
APPENDIX TWO

Spectra Recorded in Determination of Cytochrome P-450 Concentration

The spectra shown above were recorded using a liver microsome suspension prepared from a 21-day old rat pup suckled by a dam which had been exposed to PCB 169 during lactation. The protein concentration in the cuvettes was 1.0 mg/ml. For method see section 2.6.3.

Spectrum No.1 was recorded after gassing the sample cuvette with carbon monoxide. It shows a maximum at 420 nm due to the haemoglobin:CO complex.

Spectrum No.2 was recorded after addition of sodium dithionite to both cuvettes followed by a second gassing of the sample cuvette. It shows a minor maximum at 420 nm due to both the reduced cytochrome P-420:CO and the haemoglobin:CO complexes. The spectrum also shows a major maximum at about 450 nm due to the reduced cytochrome P-450:CO complex.
The absorbance of the reduced cytochrome P-450:CO complex was recorded using dilutions of the same microsome suspension, as described in section 2.6.3.
**APPENDIX FOUR**

Aniline Hydroxylase Activity Determination: Solutions added to incubation tubes (in order of addition) before incubating at 37°C for 25 minutes. For full procedure see section 2.6.4.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume added, ml</th>
<th>Test incubations</th>
<th>Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer*</td>
<td>to give final</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>volume of 1.0ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCA 25%w/v aqueous solution</td>
<td>-</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>PAP in buffer:* 0, 0.5, 1.0, 2.0, 4.0, 6.0 mcg in 0.2ml</td>
<td>-</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Aniline 46.6 mg/100ml buffer*</td>
<td>0.2</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Microsome suspension</td>
<td>equiv. to 1.0mg protein</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>NADPH generating system**</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Buffer No. 3, Appendix 1.

**Composed of: NADPH

- Glucose-6-phosphate 113.5 mg
- MgCl₂6H₂O 63.5 mg
- Glucose-6-phosphate dehydrogenase 50.0 units
- Buffer* to 10.0 ml

Notes.

1. All solutions were freshly prepared before use except for TCA.

2. The NADPH generating system was pre-incubated for 15 minutes at 37°C immediately before adding to incubation tubes.

3. The incubation tubes were pre-incubated for five minutes at 37°C immediately after addition of microsomal suspension i.e. just before addition of NADPH generating system.
Aniline Hydroxylase Activity Determination: Change in absorbance with incubation time at 37°C for incubations containing 1.0 mg protein. For method see section 2.6.4.
Aniline Hydroxylase Activity Determination: Change of absorbance with volume of microsome suspension incubated (37°C for 25 minutes). For method see section 2.6.4.
**APPENDIX SEVEN**

**Aminopyrine-N-Demethylase Activity Determination:** Solutions added to incubation tubes (in order of addition) before incubating at 37°C for 25 minutes. For full procedure see section 2.6.5.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume added, ml</th>
<th>Test incubations</th>
<th>Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer*</td>
<td>to give final</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>volume of 1.0ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc sulphate 15%w/v aqueous solution</td>
<td>-</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>HCHO in buffer:* 0,0.5,1.0, 2.0,4.0 or 6.0 mcg in 0.2ml</td>
<td>-</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Aminopyrine 28.9mg</td>
<td></td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Semicarbazide HCl 156.3mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in buffer* to 25.0ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsome suspension</td>
<td>equiv. to 1.0mg</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH generating system**</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Buffer No. 3, Appendix 1.

**Composed of:**
- NADP 14.9 mg
- Glucose-6-phosphate 113.5 mg
- MgCl₂·6H₂O 63.5 mg
- Glucose-6-phosphate dehydrogenase 50.0 units
- Buffer* to 10.0 ml

**Notes.**

1. All solutions were freshly prepared before use except for zinc sulphate.

2. The NADPH generating system was pre-incubated for 15 minutes at 37°C immediately before adding to incubation tubes.

3. The incubation tubes were pre-incubated for five minutes at 37°C immediately after addition of microsomal suspension i.e. just before addition of NADPH generating system.
Aminopyrine-N-Demethylase Activity Determination: Change in final absorbance with incubation time at 37°C for incubations using 1.0 mg protein. For method see section 2.6.5.
Aminopyrine-N-Demethylase Activity Determination: Change in final absorbance with volume of microsome suspension incubated (37°C for 25 minutes). For method see section 2.6.5.