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AN EVALUATION OF THE CENTRAL NEUROTOXIC EFFECTS OF AROCLOR 1254, A COMMERCIAL MIXTURE OF POLYCHLORINATED BIPHENYLS, IN MICE

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AN EVALUATION OF THE CENTRAL NEUROTOXIC EFFECTS OF AROCLOR

1254, A COMMERCIAL MIXTURE OF POLYCHLORINATED BIPHENYLS, IN MICE

By

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Thesis

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CURRICULUM VITAE

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DEDICATION

To my family,

for their encouragement and confidence in

me through all my endeavors

and

To Mark,

for his love and friendship

and for giving me a new perspective on my life

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LIST OF ABBREVIATIONS

Ach	acetylcholine
ACS	Aqueous Counting Scintillant. Registered trademark of Amersham Searle Corp.
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
ATPase	adenosine-5'-triphosphatase
BSA	bovine serum albumin
BSS	bufferred salt solution
Ca^{++}	calcium
$[Ca^{++}]_i$	intracellular calcium concentration
$[Ca^{++}]_o$	extracellular calcium concentration
Ch	choline
Ci	Curie, 3.7×10^{10} disintegrations per second
cm	centimeter (10^{-2} meter)
CNS	central nervous system
CO_2	carbon dioxide
cpm	counts per minute
DA	dopamine; 3,4-dihydroxyphenyl-ethylamine
DOPA	dihydroxyphenylalanine
dpm	disintegrations per min
EDTA	(ethylenedinitrilo)-tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N, N'-tetraacetic acid
EtOH	ethanol
fmol	fentomole (10^{-15} moles)

$\underline{g}$	units of relative centrifugal force
gm	gram
HCl	hydrochloric acid
HEPES	N-2-hydroxyethyl-N-2-ethane sulfonic acid
hr	hour(s)
5-HT	serotonin; 5-hydroxytryptamine
IC_{50}	median inhibitory concentration; concentration at which a given response is inhibited by 50%
i.p.	intraperitoneal route of administration
K^+	potassium
$[K^+]_i$	intracellular potassium concentration
$[K^+]_o$	extracellular potassium concentration
kg	killogram (10^3 gm)
K-H	Krebs-Henseleit (buffer)
Km	Michaelis-Menten constant
LD_{50}	median lethal dose; dose at which one-half of the animals die
LDH	lactate dehydrogenase
LSC	liquid scintillation counting
M	molar
MAO	monoamine oxidase
Mg^{++}	magnesium
mg	milligram (10^{-3} gm)
min	minute(s)
ml	milliliter (10^{-3} liter)
mM	millimolar (10^{-3} M)
μCi	microcurie (10^{-6} Ci)

μg	microgram (10^{-6} gm)
μl	microliter (10^{-6} liter)
Na^+	sodium
$[Na^+]_i$	intracellular sodium concentration
$[Na^+]_o$	extracellular sodium concentration
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
nCi	nanocurie (10^{-9} Ci)
NE	norepinephrine; 3,4-dihydroxyphenylethanolamine
nmol	nanomole (10^{-9} mole)
PCBs	polychlorinated biphenyls
PCDFs	polychlorinated dibenzofurans
PCNs	polychlorinated naphthalenes
PCQs	polychlorinated quarterphenyls
PEP	phospho(enol)pyruvate
PK	pyruvate kinase
pmol	picomole (10^{-12} mole)
PNS	peripheral nervous system
p.o.	per os; oral route of administration
ppb	parts per billion
ppm	parts per million
S.A.	specific activity
S.E.	standard error of the mean
sec	second(s)
SER	smooth endoplasmic reticulum

TH	tyrosine hydroxylase
TLC	thin layer chromatography
TP	tryptophan
TPP	scintillation cocktail: toluene containing 0.4% diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene
Tris	2-amino-2-hydroxymethyl propane-1:3-diol
U	uniformly labeled
% w/v	percent weight per unit volume (grams per 100 ml)
wk	week(s)

ABSTRACT

AN EVALUATION OF THE CENTRAL NEUROTOXIC EFFECTS OF AROCLOR 1254, A
COMMERCIAL MIXTURE OF POLYCHLORINATED BIPHENYLS, IN MICE

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Polychlorinated biphenyls (PCBs) are industrial compounds whose
ubiquitous environmental contamination has been known since the late
1960's. Incidents of human intoxication have been reported, most nota-
bly, the "Yusho" incident that affected over 1600 people in Japan in
1968. A broad spectrum of adverse effects are produced by PCBs, inclu-
ding some neurological symptoms. While examination of the toxic effects
of PCBs has received much attention, there has been little work on the
neurotoxicity of PCBs. The intent of the experiments presented in this
thesis is to provide further information on the central neurotoxicity
of PCBs in mice.

A commercial mixture of PCBs (Aroclor 1254) was used in these ex-
periments. The first set of experiments was initiated to evaluate the
behavioral effects of orally administered Aroclor 1254 in mice. A dose
of 500 mg/kg depressed spontaneous motor activity at times of 15 min to
3 hr after gavage, with the peak effect observed at 45 min. A dose-re-
sponse curve generated at 45 min revealed statistically significant
effects only at the highest dose, 500 mg/kg. Aroclor 1254 had no
effect on the other behavioral measures evaluated - performance on the
rotor rod and inverted screen and effect on pentylenetetrazol-induced

convulsions. These results suggest that Aroclor 1254 may be exerting a CNS depressant effect as evidenced by a suppression of spontaneous activity in the absence of impaired motor coordination.

In order to examine further the suggested CNS depressant effect, the interaction of Aroclor 1254 with a known CNS depressant, pentobarbital, was investigated. Pretreatment with a single dose of Aroclor 1254 (500 mg/kg) significantly enhanced pentobarbital-induced sleep time at pretreatment times of 0, 0.75, 2, 4 and 8 hr. A dose-response relationship was demonstrated with doses of 5 to 500 mg/kg of Aroclor 1254 given at the pretreatment time of peak effect, 2 hr. By contrast, subchronic (14 day) administration of Aroclor 1254 (30 or 100 mg/kg) reduced pentobarbital-induced sleep time in a dose-dependent fashion when pentobarbital was given 45 min after the last dose of Aroclor 1254 with a further reduction when given 24 hr after Aroclor 1254. Results of studies of the disposition of ^{14}C-pentobarbital following Aroclor 1254-pretreatment suggested that acute Aroclor 1254-pretreatment inhibits pentobarbital metabolism while subchronic PCB-pretreatment enhances pentobarbital metabolism as evidenced by tissue levels of pentobarbital and metabolites. Therefore, it appears that the observed increase in pentobarbital-induced sleep time is not a result of altered CNS sensitivity or combined depressant effects of the two compounds, but rather is due to Aroclor 1254-induced alterations in the pharmacokinetics of pentobarbital.

The next series of experiments was aimed at evaluating the effects of Aroclor 1254 on neurochemistry. Aroclor 1254 produced concentration-dependent alterations in the following parameters measured in vitro: inhibition of neurotransmitter and precursor uptake, enhancement

of neurotransmitter release, enhancement of synaptosomal $^{45}Ca^{++}$ uptake, stimulation of ATP-supported mitochondrial $^{45}Ca^{++}$ uptake, inhibition of synaptosomal Na^+/K^+- and Mg^{++}-ATPases, and inhibition of mitochondrial Mg^{++}-ATPases. Effective concentrations of Aroclor 1254 in these <u>in vitro</u> systems ranged from 10^{-6} to 10^{-4} M. Acute (up to 500 mg/kg) and subchronic (90 day) administration of Aroclor 1254 had no effect on uptake of neurotransmitters or precursors by synaptosomes isolated from Aroclor 1254-treated mice. Nor was there any effect on ATPase activities in synaptosomes or mitochondria isolated from mice exposed to acute or subchronic (14 day) Aroclor 1254 regimens.

This disparity between the results of <u>in vitro</u> and <u>in vivo</u> exposure to Aroclor 1254 prompted studies of the disposition of ^{14}C-PCBs. A time course of radioactivity in plasma and brain following a 500 mg/kg dose of ^{14}C-PCBs (a mixture of PCBs with 50% Cl by weight and uniformly labeled with ^{14}C) compared favorably with that of the behavioral activity of Aroclor 1254. No differences in brain regional distribution of ^{14}C-PCBs was observed. Most importantly, levels of radioactivity in synaptosomes and mitochondria of subfractionated brains of mice that had been exposed to a behaviorally active dose of ^{14}C-PCBs (500 mg/kg) were comparable to tissue levels in isolated synaptosomes and mitochondria that had been incubated with concentrations of ^{14}C-PCBs (10^{-5} to 10^{-4} M) that altered neurochemistry <u>in vitro</u>. Thus, effective concentrations of ^{14}C-PCBs are apparently achieved in subcellular organelles of brain following oral administration of a behaviorally active dose. The lack of observable neurochemical effects in Aroclor 1254-treated mice may be due to compensatory mechanisms that maintain homeostasis in the intact brain.

In summary, Aroclor 1254 has been shown to produce a depression of spontaneous motor activity in mice, changes in pentobarbital-induced sleep time (an effect that is probably a manifestation of changes in the pharmacokinetic behavior of pentobarbital rather than a direct effect of Aroclor 1254 on brain), and alterations in a number of neuro-chemical events in vitro. These results may help to expand the limited data base on the neurotoxicity of PCBs.

I. INTRODUCTION

A. Polychlorinated Biphenyls

1. Production, properties, and use

Polychlorinated biphenyls (PCBs) are a group of synthetic chlori-
nated hydrocarbons which have been used commercially for over 40 years
and recently have received much attention from both the lay and scien-
tific communities. They were first synthesized in the late 19th cen-
tury (Griefs, 1867; Schmidt and Schultz, 1881) but did not become
available for commercial use until the early 1930's (Hubbard, 1964).
The structure of PCBs is shown in Figure 1. The synthetic process
involves the progressive chlorination of this biphenyl ring system.
Theoretically, 210 possible chlorinated biphenyl molecules could be
formed with from 1 to 10 chlorines at positions indicated in the figure.
The individual biphenyls are often incorrectly referred to in the
literature as "isomers." This term is inappropriate, as it is used to
describe compounds of identical molecular composition. Perhaps a more
appropriate term would be "congeners, although when possible, they may
simply be called individual "chlorinated biphenyls."

Commercial use of PCBs dose not attend to these individual con-
geners, but rather involves a product of manufacturing which is a
mixture of chlorinated biphenyls. These commercial mixtures were
manufactured in several different countries, and each has its own trade
name (by which it is referred to in the literature): Aroclor (U.S.A.),
Phenoclor (France), Kanechlor (Japan), Clophen (West Germany), Fenchlor
(Italy), and Sovol (U.S.S.R.). The composition of the mixture and its
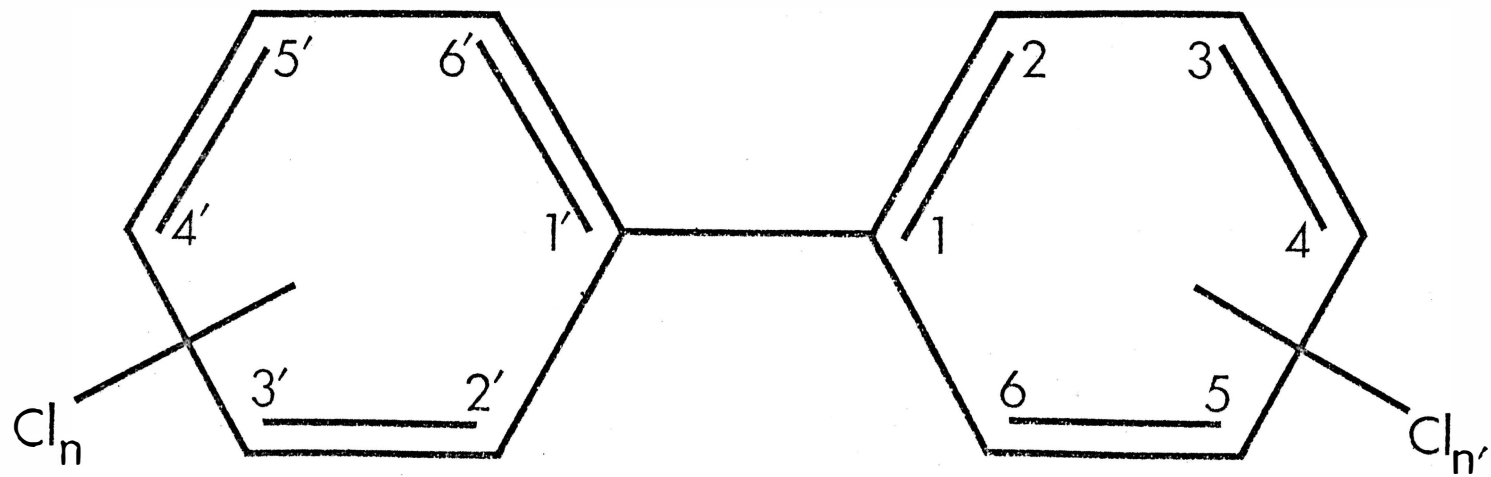
Figure 1 Structure of PCBs

associated identification number are generally defined on the basis of the chlorine content. For example, the Aroclors (produced by the Monsanto Chemical Co., St. Louis, MO) are identified by a four digit code. The first two digits define the ring system, e.g. 12__ for biphenyls, 54__ for terphenyls. The chlorine content of the mixture, in % chlorine by weight, is given by the second two digits. Therefore, Aroclor 1254, which is the commercial mixture used in the experiments to be described in this thesis, is a mixture of chlorinated biphenyls with 54% chlorine content (w/w). Analysis of some batches of several different commercial mixtures of PCBs has revealed the presence of impurities, such as the polychlorinated dibenzofurans (PCDFs) and polychlorinated naphthalenes (PCNs), the structures of which are shown in Figure 2. This finding becomes particularly important in interpreting evaluations of the toxicity of these mixtures and will be discussed in more detail in a subsequent section (Toxicology, Human Studies).

The physical and chemical properties of PCBs contribute both to their widespread industrial applications and to their ubiquitous environmental contamination and subsequent biological effects. PCBs see their primary application in the electrical industry owing to their excellent dielectric properties (i.e., they do not conduct electricity and therefore, are good insulators) and resistance to heat. The majority of the PCBs produced worldwide have been used as dielectric fluids in transformers and large capacitors. PCBs are also very stable chemically and are non-flammable. Water solubility of PCBs is quite low and decreases with increasing chlorine content. Monsanto gives the following water solubility figures for some of its preparations:
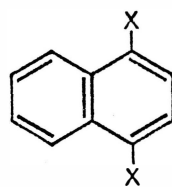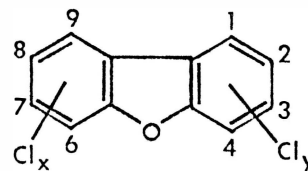
PCNs   PCDFs

Figure 2

Structures of polychlorinated naphthalenes (PCNs)

and polychlorinated dibenzofurans (PCDFs)

Aroclor 1242, 200 ppb; Aroclor 1254, 40 ppb; Aroclor 1260, 25 ppb
(Panel on Hazardous Trace Substances, 1972). Non-ionic surfactants,
such as Tween® 80, will increase the solubility of PCBs in water, and
if an aqueous medium is not required, PCBs are soluble in many organic
solvents and oils.

With these chemical and physical porperties in mind, it is under-
standable that PCBs met with such a receptive market and saw their way
into so many uses. Their uses can be grouped into the following three
categories: 1) large closed systems - PCBs used as dielectric fluids in
transformers and large capacitors. When this equipment is replaced,
the PCBs can be reclaimed; 2) small closed systems - PCBs used in
smaller quantities in hydraulic and heat transfer systesm, gas tur-
bines, and vacuum pumps. Because small quantities of fluid are used,
PCBs are rarely recovered. These types of equipment are very widely
used and are serviced frequently, therefore providing increased oppor-
tunity for accidental contamination. Proper disposal of spent equip-
ment has been difficult to enforce; and 3) dissipative uses - applica-
tions in which PCBs can easily gain access to the environment, such as
their use as fire retardants, lubricating and cutting oils, plastici-
zers (in adhesives, textiles, surface coatings, sealants, carbonless
copy paper, and paint), and as an additive in pesticides to reduce
volatilization. Leakage and improper disposal of PCBs or PCB-contain-
ing equipment and direct contact between open-ended uses of PCBs and
the environment may all contribute to contamination of the environment
by PCBs.

With all of these attractive applications, it is no wonder that
PCBs production and use escalated in the 40 years from their introduc-

tion on the market. It was not until the late 1960's that attention was called to the matter, when Jensen (1966) reported that PCBs could be detected in birds and mammals worldwide. Public concern began to develop regarding the health effects of PCBs, and by the early 1970's, both voluntary and goverment control of PCB usage had been instituted in several countries. By 1971, U.S. production was voluntarily limited to closed systems. Japan banned all production of PCBs in 1972. Throughout the early to mid-1970's, various U.S. governmental agencies became involved in the investigation of the PCB problem, and numerous restrictions were placed on PCB use and disposal. Finally, on November 1, 1979, the Environmental Protection Agency declared that PCBs would no longer be approved for new systems, and that existing equipment was permitted to remain in use only until July 1, 1984 (Fed. Regis., 1980).

Despite these controls, there is still the potential for continued PCB exposure. Workers who maintain capacitors and transformers may become exposed, as well as those who dispose of old equipment. In addition, the general population comes in contact with PCBs through environmental contamination. Therefore, because human exposure to PCBs is still a potential problem, evaluation of the toxicity of PCBs continues to provide useful information.

Additional information on the properties and uses of PCBs can be found in several reviews (Higuchi, 1976; Hutzinger, 1976; IARC, 1978; Panel on Hazardous Trace Substances, 1972; Rappé and Buser, 1980; WHO, 1976).

2. Environmental levels of PCBs in air, water, soil, and living organisms

A discussion of environmental contamination highlights the nature of the PCB problem, i.e. the source and extent of contamination and the impact of these factors on the toxicity of PCBs. As mentioned previously, the properties of PCBs, particularly chemical stability, which led to their widespread industrial application have also contributed to the gravity of environmental contamination and toxicity.

The fate of PCBs in the environment is determined by a number of factors. Physical and chemical properties play a role in transport and transformation of PCBs in the environment. While their water solubility is relatively low, on the order of 25-200 ppb (Panel on Hazardous Trace Substances, 1972), PCBs are readily absorbed onto water sediments. Likewise, despite low vapor pressures, levels of PCBs in air have been estimated to range form 1 to 50 ng/m^3 in the United States (Panel on Hazardous Trace Substances, 1972) and may even approach the $\mu g/m^3$ level in urban areas, as has been found in some Japanese cities (Taksukawa, 1976). Whatever the medium with which they ar associated, the composition of PCBs in the environment can be altered by one or more processes. As would be expected, uptake of PCBS by living organisms and metabolic transformation will alter their chemical composition. If not taken up by an organism, PCBs in the environment are generally chemically stable, however they may be light sensitive. Hutzinger and Safe (1972) have demonstrated that PCBs can undergo a photolytic process in which dechlorination results in the formation of different congeners of unidentified chemical, physical, or toxicological properties.

One of the problems associated with PCBs pollution is their accumulation in tissue and magnification in food chains. PCBs are highly

lipid soluble and chemically stable thus setting the stage for uptake by and persistant storage in fatty tissue (see the next section, Pharmacokinetics, for details). Looking at total body levels of PCBs in organisms at various trophic stages of a food chain, one finds increasing levels at higher stages in the food chain. Biomagnification of PCB levels of 10- to 100-fold at each step can be found in both marine and terrestrial ecosystems (Risebrough et al., 1968). This becomes particularly important when considering the contribution of diet to the total body burden of PCBs in humans.

Contaminated foodstuffs can be divided into two groups, unprocessed and processed foods. The former category includes plants or animals that have come directly in contact with environmental contamination, e.g., fish in contaminated bodies of water, or foodstuffs that have been contaminated as a consequence of industrial accidents, such as a spill into animal feed. Packaging materials are the source of contamination for processed foods. PCBs have been used in pressure-sensitive duplicating paper, and when this paper is recycled, PCBs are transferred to food packaging materials (Masuda et al., 1972).

Since the early 1970's, the use of PCBs has been confined to closed systems, tighter controls on disposal have been instituted and enforced, and thus, levels of PCBs in most foods have declined to below detectable levels. The exceptions are meat, poultry, and fish in which trace levels can still be detected (Cordle et al., 1978).

3. Pharmacokinetics

Experimental evaluation of the pharmacokinetics of PCBs has been a difficult problem because of the complexity of the commercial PCB mix-

tures. Because PCBs are used industrially as mixtures and gain access to the environment in that form, it would be useful to have data on the pharmacokinetics of the commmercial PCB products. While this approach may seem valid, there are also several drawbacks. With so many mixtures available on the market, it is difficult to make a statement concerning PCBs as a whole. For one thing, each mixture contains a large number of different constituents, e.g. over 50 components of Aroclor 1254 have been identified (Sissons and Welti, 1971), and another mixture may contain a completely different array of congeners. Theoretically, each congener could behave differently in the body. Ideally, one would like to be able to identify and quantitate each separate component and its metabolites in biological samples, however the structures of many of these compounds are unknown. For these reasons, many investigators have taken the course of evaluating individual chlorinated biphenyls. One advantage of this approach is that it is much easier to evaluate one single compound, however this does not answer the question of what happens when a person is exposed to a PCB mixture. Furthermore, the task of evaluating all the possible PCB congeners is formidable, if not impossible. Nevertherless, both appraoches have been taken, and examples will be given of each.

A number of recent reviews provide further details on the pharma-cokinetics of PCBs (Safe, 1980; WHO, 1976; Yoshimura and Yoshihara, 1976).

a. Absorption

There are three possible routes by which humans may be exposed to PCBs: dermal, oral, and inhalation. The first indication that PCBs

could be absorbed through the skin appeared in the literature in the early 1930's. A number of cases of human toxicity from occupational exposure were reported (Drinker et al., 1937; Greenburg et al., 1939; Schwartz, 1936). Later, Vos and Beems (1971) concluded that systemic effects observed in rabbits following dermal exposure were due to absorption of PCBs through the skin. Inhalation is the second major route of exposure in the industrial setting, and Benthe et al. (1972) have demonstrated absorption of a PCB-containing aerosol through the lungs. Finally, the general population is exposed to PCBs through contaminated food and water sources. Both Albro and Fishbein (1972) and Allen et al. (1974) have shown that PCBs are readily absorbed from the gastrointestinal (GI) tract.

b. Distribution

PCBs are highly lipophilic compounds, therefore one would expect to find highest concentrations in fatty tissues. Following oral or intravenous administration, PCBs are found distributed throughout the body with highest concentrations in adipose tissue. For example, Grant et al. (1974) administered Aroclor 1254 orally to rats at a dose of 500 mg/kg for 4 days and then looked at tissue distributions. Highest levels were found in fat, followed by liver, then lower levels in heart, testes, spleen, kidney, and brain, and lowest concentrations in blood. Other investigators have detected high concentrations of PCBs in lung, adrenals, skin, glandular secreting tissue, and muscle (Yoshimura and Yoshihara, 1976).

Placental transport of PCBs has been demonstrated in a variety of animal species and humans. PCBs administered to pregnant animals were

detected in the fetuses of rabbits (Grant et al., 1971), rats (Curley et al., 1973), and mice (Berlin et al., 1975; Masuda et al., 1978a, b; 1979). Babies born to mothers who were exposed to PCBs in the Yusho incident also showed signs of toxicity; a brief description of these cases is given by Kuratsune (1980).

c. Metabolism

A huge body of literature exists on the metabolism of PCBs, and several reviews have appeared on the subject (Hutzinger et al., 1974; Matthews et al, 1978; Safe, 1980; WHO, 1976). Particularly noteworthy is the review by Yoshimura and Yoshihara (1976) in which they give a thorough discussion of the metabolism of individual chlorobiphenyls.

There are several issues to be addressed concerning problems and goals associated with the study of PCBs metabolism. As mentioned previously, studies involving the commercial PCB mixtures, particularly metabolic studies, are extremely difficult due to the complexity of the mixture. Analysis of the structure of metabolites has been the focus of many of these studies. Because there exists such a diversity of PCB congeners, many investigators have examined the effect of position and number of chlorines on metabolism. Taken altogether, determination of metabolic pathways for PCBs has been an arduous task.

A few general points can be made about PCB metabolism. PCBs, like many other endogenous compounds and xenobiotics, are metabolized in the liver to more polar derivatives (Goldstein et al., 1974). The rate of metaoblism is inversely related to the number of chlorines per molecule (Matthews and Anderson, 1975). The position of the chlorines also becomes important, particularly when there are four or more halogens

per molecule, because oxidative metabolism of PCBs is more likely when there are two adjacent, unsubstituted carbons.

Although ring hydroxylation is the major route of metabolism, a number of other products have also been identified. The following PCB metabolites have been identified in vivo: dihydrodiols, phenols, phenol acetates and methyl ethers, dechlorinated derivatives, and sulfur-containing metabolites (see Safe, 1980).

The impact of these metabolic properties on PCB toxicity is a complex problem. In rats, the lower chlorinated congeners, which are metabolized and excreted more rapidly, are also less toxic than the more highly chlorinated congeners, however there is no association between the degree of chlorination and the acute toxicity in rabbits (Panel on Hazardous Trace Substances, 1972). Investigation of the toxic effects of the PCB metabolites has received little attention. While it is often the case that metabolism to more polar derivatives is a detoxifying mechanism, it may not be applicable to PCB matabolites. Yamamoto and Yoshimura (1973) evaluated the toxicity of the 5-hydroxy-derivative of 2,4,3'4'-tetrachlorobiphenyl in mice. The LD_{50}'s for the parent and the metabolite (given i.p.) were 2.15 and 0.43 g/kg, respectively. Therefore, the acute toxicity of PCBs may be due, in part, to the production of phenolic metabolites.

In line with this thinking is the possility that arene oxide intermediates may be formed when PCBs are metabolized by the hepatic mixed-function oxidases. Gardner et al. (1973) were the first to report evidence for the existence of an arene oxide. The implication of the formation of such as intermediate lies in the electrophilic nature and highly reactive properties of the arene oxides. Thus, they

can potentially bind covalently to critical cell molecules - protein, RNA, and DNA - and produce a cytotoxic, mutagenic, or carcinogenic response. Binding of two hexachlorobiphenyls to liver macromolecules has been demonstrated by Morales and Matthews (1978). The carcinogenic potential of PCBs will be discussed in a subsequent section.

d. Elimination

Fecal excretion is the major route of elimination for PCBs, and since greater than 90% of the excreted PCBs is in the form of metabolites, excretion is dependent upon the rate of metabolism. As in the metabolism of PCBs, the degree of chlorination has an effect on elimination, i.e. the rate of elimination decreases with increasing chlorination. Furthermore, the route of excretion is also determined by this parameter; Matthews and Anderson (1975) showed that monochlorinated biphenyls were excreted primarily in the urine, whereas with increasing chlorination the majority of the compound was excreted in the feces.

Because the majority of the excreted PCBs is in the form of metabolites, the source of fecal excretion products can not solely be unabsorbed PCBs from the GI tract, expecially if the dose has been given by a parenteral route. Yoshimura and Yamamoto (1975) demonstrated the reabsorption of unchanged compound through the intestinal wall and excretion into the small intestine of rats. Tuey and Matthews (1980) have also provided evidence that PCB metabolites undergo biliary excretion in mice.

While alternative modes of excretion may not contribute significantly to the bulk of elimination, there are important consequences. Of particular concern in terms of human health effects has been the detection of PCBs in human milk. PCBs have been detected in Japanaese

women who were exposed to PCBs occupationally (Yakushiji et al., 1978) and non-occupationally (Yakushiji, 1979a, b) and in populations throughout the world in which no known PCB exposure has occurred. A summary of this literature is given by Landrigan (1980).

4. Toxicology

a. Animal studies

The acute toxicity of orally administered PCBs (generally the commercial mixtures) has typically been evaluated by using lethality as the endpoint. Acute oral LD_{50}'s in rats for a series of Aroclors were determined by Monsanto Corp. (Panel on Hazardous Trace Substances, 1972) and values ranged from 4 to 11 g/kg. Similar findings in rats were reported in an FDA study (see Fishbein, 1974) with Aroclor LD_{50}'s between 3 and 19 g/kg. A survey of other studies in rats, rabbits, and mice shows the same range of values, from 1 to 10 g/kg (Kimbrough et al., 1978). These values indicate that PCBs have a relatively low order of acute toxicity. Yamamoto and Yoshimura (1973) determined the acute toxicity of a single dose (i.p.) of a pure tetrachlorobiphenyl and its major metabolite, the 5-hydroxy derivative. The LD_{50}'s were 2.15 and 0.43 g/kg for parent compound and metabolite, respectively. This study would suggest that further evaluation of the toxicity of PCB metabolites is warranted.

As for specific effects of PCBs, Bruckner et al. (1973) examined the response of rats to a single oral dose of Aroclor 1254. They reported that a dose of 4 g/kg (the LD_{50} was ~ 4.25 g/kg) resulted in loss of body weight, diarrhea, ataxia, lack of response to painful stimuli, anorexia, weakness, and decreased exploratory behavior. Coma and death

ensued. Pathological alterations were observed only in the liver and kidney.

Because dermal exposure to PCBs is a problem, particularly in the industrial setting, Vos and Beems (1971) applied a mixture of 3 commercial PCBs (all with 60% Cl by weight) to the skin of rabbits, 5 times each day for 38 days. Skin lesions were observed as well as liver and kidney damage, thus indicating that PCBs were readily absorbed through the skin.

Subchronic exposure to PCBs yields a much greater degree of toxicity than acute doses and implies that repeated dosing produces cumulative toxic effects. For example, Bruckner et al. (1973) extended dosing for a period of 3 wk on a regimen of 100 mg/kg dose (p.o.) every other day. Histopathological changes were more pronounced than those described above for acute exposure and included liver enlargement, fatty infiltration, and hepatic necrosis. Hepatic microsomal enzyme activity was also elevated. Rhesus monkeys exhibited facial edema, alopecia, and acne following a 2 month exposure to 25 ppm of PCBs in the diet (Allen et al., 1974). Mink is the most sensitive species to the effects of PCBs. Exposure to 30 ppm of PCBs in the diet resulted in 100% mortality within 6 months (Aulerich et al., 1973).

Toxicity has also been evaluated following longer periods of exposure, i.e., chronic administration of PCBs. Rats were exposed to Aroclor 1254 or 1260 for 8 months at concentrations of 20, 100, 500, or 1000 g of PCBs/kg of diet. At the highest dose, separation of effects by sex was observed. Whereas all the male rats survived, 80% mortality in the females was observed. In male rats, a dose-dependent increase in liver weight occurred (significant even at the lowest dose of PCBs),

and associated liver morphological changes were found (Kimbrough et al., 1972).

Rhesus monkeys are the only species that exhibit a symptomological picture that is similar to Yusho patients (Allen, 1975; Allen and Norback, 1973; Allen et al., 1974). Administration of Aroclor 1248 at levels of 2.5 and 5 mg/kg of diet for 1 year produced facial edema, alopecia, and acne within 1 to 2 months. Higher doses (100 to 300 mg/kg of diet) resulted in 100% mortality within 2 to 3 months.

The reproductive effects of several Aroclors were evaluated in rats (Keplinger et al., 1971). Decreased pup survival was observed following exposure of mothers to dietary levels of 100 ppm of Aroclor 1254 for 18 months. Comparison of the PCB mixtures indicated that decreased reproductive effects were produced with increasing chlorination. Lower doses of Aroclor 1254 (20 ppm) produced decreased litter size in rats in another study (Linder et al., 1974), but offspring mortality was again observed only with 100 ppm in the diet of mothers. Rabbits appear to be more sensitive to these effects than rats. Doses of 12.5 to 50 mg/kg/day produced abortions and stillbirths, but no fetal abnormalities were observed (Villeneuve et al., 1971). Neither has there been any evidence of teratogenicity in rats (Kiplinger et al., 1971).

The carcinogenic potential of PCBs has been the subject of some controversy, but the consensus is that the liver is apparently the only site of neoplastic lesions, as indicated by the following examples. Liver nodules and hepatocellular carcinomas were detected in mice exposed to 500 ppm of Kanechlor 500 in the diet for 32 wk; no tumors were found in other organs (Ito et al., 1973). Dietary exposure to 300

ppm of Aroclor 1254 for 6 or 11 months resulted in adenofibrosis in livers of mice in the 11 month exposure group and hepatomas in both groups (Kimbrough and Linder, 1974). Similar results have been observed in rats (Kimbrough et al., 1972, 1975).

Mutagenicity has been evaluated in a number of different systems. Green et al. (1975a) were unable to demonstrate any mutagenic effects as measured by the dominant lethal test in rats following a variety of dosing regimens of Aroclors 1242 and 1254. Nor was there any evidence of an increase in chromosomal aberrations in bone marrow and spermatogonial cells from rats treated with either Aroclor (Green et al., 1975b).

PCBs produce a number of biochemical effects in animals. One of the best characterized effects of PCBs is the inductive effect on the hepatic microsomal enzyme system (Bruckner et al., 1974, 1977; Goldstein, 1979, 1980; Grant et al., 1974; Litterst et al., 1972; Litterst and van Loon, 1974). Low doses of PCBs will produce this inductive response, for example, as little as 1 mg/kg (p.o.) of Aroclor 1242 given daily for 21 days produced an increase in liver enzyme activity in the rat (Iverson et al., 1975). The implication of this effect is that PCB exposure can produce changes in the response to other drugs or toxic compounds that are metabolized in the liver. For example, PCB-pretreatment enhances pentobarbital elimination (Chu et al., 1977; Stella and Chu, 1980) and reduces pentobarbital-induced sleep time (Sanders and Kirkpatrick, 1975; Sanders et al., 1974; Villeneuve et al., 1972; Zepp et al., 1974).

Another biochemical effect of PCBs is the production of porphyria, a disorder in porphyrin metabolism manifested by excess porphyrin

production and excretion in the urine. Porphyria has been observed in PCB-treated rabbits (Vos and Beems, 1971), quail (Vos et al., 1971), chickens (Vos and Koeman, 1970), and rats (Goldstein et al., 1974, 1975b) and is probably associated with the biochemical effects of PCBs on the liver. This subject has recently been reviewed by Strik et al. (1980).

Lastly, PCBs have been shown to have immunosuppressive properties. Atrophy of lymphoid tissue has been demonstrated in chickens (Vos and Koeman, 1970), rabbits (Vos and Beems, 1971) and guinea pigs (Vos and van Driel-Grootenhuis, 1972). Suppression of humoral immunity, as evidenced by decreased antibody production in response to antigenic challenge, was shown in guinea pigs (Vos and de Roij, 1972; Vos and van Driel-Grootenhuis, 1972) and mice (Loose et al., 1977). This latter study also reported a decrease in circulating immunoglobulins. PCB-pretreated mice also showed an increased susceptibility to infection when presented with a bacterial challenge (Loose et al., 1978). Further details of immune alterations by PCBs can be found in a recent review (Vos et al., 1980).

As can be seen by this brief review, PCBs produce a variety of toxic effects in animals exposed to either acute or chronic dosing regimens. Studies of the neurotoxic effects will be handled separately in a subsequent section of this chapter. Additional information on the toxicity of PCBs can be found in a number of reviews (Fishbein, 1974; Kimbrough, 1974; Kimbrough et al., 1978; McConnell, 1980; Ueda, 1976; WHO, 1976).

b. <u>Human studies</u>

Although tragic to those individuals involved, there have been several incidents of PCB poisoning which have provided a plethora of valuable information on the human toxicity of PCBs. Probably the most widely investigated incident was one that occurred in Japan in 1968. A second population from whom toxicological data has been drawn includes workers who have been occupationally exposed to PCBs. Finally, general population exposure through environmental contamination is a problem worldwide.

1). Yusho

In early 1968 in Kyusho, Japan, physicians began to see an ever-increasing number of cases of an unidentified disease that was likened to chloracne. Actually, the first symptoms to appear were discharges from the eye, swelling of the upper lid, and weakened eyesight. About 2 to 3 months later, dermal signs began to appear and included comedo formation, acneform eruptions, enlarged follicular openings, and a blackish-brown pigmentation of the nails, skin, lips, and gingivae. Other early symptoms included swelling and numbness of the limbs, nausea, vomiting, abnormal menstruation, and impotence. Laboratory tests detected increased serum alkaline phosphatase and triglycerides and elevated SGOT and SGPT in severe cases. Earliest reports of this curious disease appeared in the Japanese literature (Kuratsune <u>et al</u>., 1969; Goto and Higuchi, 1969; Yoshimura, 1971) but have been summarized in English by Kuratsune <u>et al</u>. (1972). Reviews of the Yusho incident and subsequent follow-up reports have appeared more recently (Cordle <u>et al</u>., 1978; Hirayama, 1976; Kuratsune, 1976, 1980; Urabe, 1979; WHO, 1976).

With hundreds of cases appearing throughout 1968, a Study Group was formed at Kyusho University to investigate the etiology of Yusho. The results of their epidemiological study revealed that all affected persons had used a specific lot of rice oil produced or shipped by the Kanemi Soko Co. on February 5 or 6, 1968. This suggested causal relationship was corroborated by a careful analytical study. Analysis of the identified batches of rice oil showed that they were contaminated with Kanechlor 400, a commercial brand of PCBs containing 48% chlorine by weight and produced by the Kanegafuchi Chemical Industry Co. This discovery correlated well with detection of PCBs in the blood of Yusho patients. Furthermore, other batches of rice oil (produced before or after February) contained no detectable levels or trace amounts of PCBs, and consumers who used this oil had no signs of the disease. Although the exact source of the PCB contamination has never been unequivocally determined, it appears most likely that PCBs leaked into the rice oil during the final step of processing. PCBs were used in a heat transfer unit to heat the oil to 200° C in a deodorizing step. Small holes were found in a section of pipe through which the Kanechlor could have leaked into the rice oil. The name "Yusho" was thus applied to the disease, as it is translated literally to mean "rice oil disease."

More recent analysis of the rice oil has brought some new evidence to light. Initially, it was reported that the PCB concentration in the rice oil was 2000 to 3000 ppm. At the time, no specific analytical methods were available for PCBs, and chlorine content was used to provide an estimate. More recently, PCBs were measured directly, and a figure of 1000 ppm was reported (Nagayama et al., 1976) suggesting that the remaining chlorine content must be associated with other chlori-

nated compounds. PCDFs were detected at a concentration of 5 ppm, a value that was 250 times what would have been expected based on levels in unused Kanechlor 400 (Nagayama et al., 1976). This discrepancy has been accounted for by the suggestion that the prolonged heating of PCBs could have produced some conversion to PCDFs (Miyata et al., 1978). The remaining chlorine content has been attributed to polychlorinated quaterphenyls (PCQs) at a concentration of 866 ppm (Kamps et al., 1978; Miyata et al., 1978). Furthermore, both PCQs (Kashimoto et al., 1981) and PCDFs (Nagayama et al., 1977) have been detected in Yusho patients. The ramification of such findings are complex. Based on these more recent findings, the symptoms of Yusho cannot be attributed solely to PCBs, but the role of these other contaminants is unclear. The symptomatology of Yusho is similar to that of occupationally exposed patients, supporting the view that PCBs were a contributing factor in Yusho but not necessarily ruling out the possibility that other chlorinated compounds may play a role in the disease.

The progress of Yusho patients is still being followed. Urabe (1979) reported on the status of 1665 Yusho patients that had been identified by 1977. Over the years 1969 to 1975 most of the mucocutaneous lesions had improved in 64% of the patients, however systemic disorders persisted or appeared later (Urabe, 1979; Kuratsune, 1980). In addition to the dermal and ocular signs described above, patients also reported general fatigue, heavy headedness and headache, numbness and swelling in the limbs, coughing, anorexia, and abdominal pain. Laboratory findings included elevated serum triglycerides and altered adrenocorticosteroid metabolism. Children born to Yusho mothers were severely affected, showing dermal and ocular signs and retarded growth development.

2). Occupational exposure

Soon after PCBs were introduced into industry (around 1930) reports on health effects began to appear involving workers employed in the production of PCBs and in the manufacture of products containing PCBs, most commonly in the electrical industry. In these early reports, chloracne and hepatic effects were the most common symptoms (Drinker et al., 1937; Greenburg et al., 1939; Meigs et al., 1954; Schwartz, 1936, 1943).

As awareness grew of the toxicity of PCBs, investigations of the health effects of PCBs in the occupational setting became more detailed and generally included analysis of environmental levels of PCBs in the workplace and PCB levels in blood, a record of medical history and physical examination of workers, and a series of laboratory tests for various biochemical parameters.

In 1976, Ouw surveyed environmental levels and health effects of workers in a capacitor plant in New South Wales, Australia in which Aroclor 1242 was used. Symptoms reported include chloracne, abnormal liver function tests, and irritation of the eyes and skin. Mean PCB levels in blood were 400 ppb.

Altered lipid metabolism has been observed in humans, as demonstrated by the detection of elevated serum triglycerides in several populations of occupationally exposed workers (Fischbein et al., 1979; Smith et al., 1978).

Detailed analysis of 326 workers in a capacitor plant is given by Fischbein et al. (1979). Employees were given a complete physical examination and evaluated for symptoms of PCB toxicity. Medical histories were taken, and 26 biochemical parameters were evaluated. A

large percentage of those studied (~50%) reported dermatological symptoms. Equally numerous (also ~50%) were those who complained of neurological problems including headache, dizziness, depression, memory loss, fatigue, nervousness, and sleep disorders. While not particularly striking, laboratory tests revealed some abnormal values for liver function and serum lipids. Determination of serum PCB levels provided a significant correlation between dermal symptoms and PCB levels and between abnormal SGOT values and PCB levels.

In a report published separately on this group of capacitor workers (Warshaw et al., 1979), ocular and upper respiratory irritation was observed in 58% of those examined. Spirometric measurements showed reduced vital capacity in 14% of a group of workers who had been identified as having no previous exposure to asbestos, textile dust, or talc. This observation correlates well with animal studies in which it was found that PCBs accumulated selectively in the mucosa of the bronchi, trachea, and larynx of mice (Brandt, 1977) and were also found to persist in lung parenchyma (Brandt et al., 1981). Furthermore, Yusho patients also exhibit symptoms of bronchitis (Kuratsune, 1980).

Finally, in addition to the United States and Japan, Italy has also seen its share of PCB-related toxicity. Moroni et al. (1981a, b) surveyed environmental levels in a capacitor plant and blood concentrations in its workers (values ranged from ~50 to 1000 ppb). Medical histories were taken and physical examinations and laboratory tests were performed on 80 workers with an average of 12 years of employment. Ten cases of chloracne and folliculitis were reported, and hepatic effects, including liver enlargement and abnormal liver enzyme values, were observed in 16 individuals. A correlation between blood levels and frequence of liver involvement was established.

Thus, it can be seen that PCB-exposure in the workplace produces many of the same symptoms that have been observed in Yusho patients as well as in animal studies. Because the role of contaminants, especially PCDFs, in the PCB-contaminated rice oil in Japan remains unclear, these data on toxicity in PCB-exposed workers provide evidence for the contribution of PCBs to the symptomatology of Yusho.

B. Neurotoxic effects of PCBs

Only a limited number of studies have appeared in the literature in which the effects of PCBs on the nervous system have been examined. A few of these have been selected and will be described here.

Studies in two nonmammalian species have approached this problem from a neurochemical angle. Fingerman and Russell (1980) detected decreased norepinephrine (NE) and dopamine (DA) content in brains of Gulf killifish that had been exposed to Aroclor 1242 (0.0004% in the water) for 3 days. Increased locomotor activity was also observed in these fish. Similarly, Heinz et al. (1980) fed Aroclor 1254 (1, 10, or 100 ppm in the diet) to ring doves for 8 weeks and found reduced levels of NE and DA in brain.

The remaining studies employed mammals and focused primarily on behavioral measures in offspring of pregnant animals that were exposed to PCBs. The rationale for in utero exposure stems from the fact that PCBs are able to cross the placenta (see Introduction - Pharmacokinetics) and affects infants born to exposed mothers (see Introduction - Yusho). A "spinning syndrome" has been observed in mice that were exposed to a tetrachlorobiphenyl in utero. The authors (Chou et al., 1979) describe this condition as "spontaneous sustained turning" that is often unidirectional and with at least 40 turns/min. Turning was modified by administering DA agonists and was completely abolished by haloperidol, a DA antagonist, thus suggesting that the syndrome involves a biochemical lesion in the dopaminergic system. Tilson et al. (1979) exposed mice to the same compound and examined these offspring at adulthood. "Spinners" displayed increased activity, and all PCB-exposed mice ("spinners" and "non-spinners") exhibited impaired motor

coordination and increased latencies in an avoidance task, evidence suggestive of a learning deficit. Alternatively the observed behavioral changes could be due to impaired ability to process sensory input. The authors conclude that these irreversible changes in behavior are due to damage during a critical period in the developing nervous system because 1) no PCBs were detected in brains of affected adult offspring exposed solely to PCBs in milk, and 3) this syndrome has not been observed in adults exposed to large doses of PCBs.

The sensitivity of the developing nervous system to PCBs has been confirmed in rhesus monkeys. Mothers were exposed to Aroclor 1248 in their diet through gestation and 3 months of nursing. The offspring were then subjected to a battery of behavioral tests. Bowman et al. (1978) reported hyperactivity and deficits in a number of discrimination learning tasks in these animals. Again, these changes were irreversible and not dependent upon sustained PCB levels, as the learning deficits persisted even after PCBs had been cleared from the body. An interesting development later appeared in these monkeys at the age of 44 months. Hypoactivity was observed in these adolescent monkeys who were hyperactive as juveniles (Bowman and Heironimus, 1981). Therefore, a delayed neurotoxic effect of much earlier exposure was observed.

The importance of pursuing these studies in experimental animals is evident from observations of human PCB exposure. As mentioned above, there is good reason for examining the effects of prenatal exposure on neurological function. Equally important are the effects on adults that were described in the preceeding section.

Thus, is can be seen that there is precedence for evaluating PCB neurotoxicity, both on clinical grounds and on the basis of a sparce literature base. The experiments to be described here were intended to expand the experimental information in this area.

C. Experimental Objectives

Although a number of neurological symptoms have been reported by humans exposed to PCBs, there is only limited data available in the literature on the central neurotoxic effedts of PCBs in experimental animals.

The experiments in this thesis were designed to verify the previously reported toxic effects of PCBs on the CNS and to determine PCBs' mechanism of action. More specifically, the objectives were 1) to determine the behavioral effects of PCBs in mice as an indication of central neurotoxic effects and to establish an orally active dose and time course of the effect of PCBs. While behavior may be a manifestation of both CNS and PNS function, experiments can be designed to rule out the contribution of peripheral function to observed effects; and 2) to evaluate the effects of PCBs on neurochemical function in the CNS, as behavioral effects of PCBs would presumably be associated with an alteration in neurochemistry. These neurochemical experiments might suggest a mechanism of action for the central effects of PCBs.

The mouse was selected for the experiments in this thesis for a number of reasons. While there has been little investigation of the neurotoxic effects of PCBs in animals, the mouse has been used extensively for evaluating other toxic effects of PCBs as well as the pharmacokinetics of PCBs, such that comparison of the results of this thesis project with the existing literature can be made. Furthermore, most of the techniques that were to be used (e.g. behavioral measures and neurochemical assays) have been worked out in the mouse.

II. GENERAL METHODOLOGY

A. Animals

Male CD-1 mice (25-29 g) were obtained from Charles River Laboratories (Wilmington, MA) and allowed free access to Purina laboratory chow and tap water. They were housed in community stainless-steel cages (measuring 25 x 45 cm, with 15 mice/cage) and allowed 1 week to adapt to the environment. Animals were maintained on a 12 hr light-dark cycle. For subchronic experiments in which mice were housed for longer periods of time (14 or 90 days), the number of mice was reduced to 4/cage.

B. General materials and methods

A commercial mixture of PCBs, Aroclor 1254 (Monsanto Chemical Company, St. Louis, MO) was employed in these experiments. It was dissolved in 95% ethanol (EtOH) for _in vitro_ experiments and was diluted in an emulphor: saline (1:8, v/v) vehicle for _in vivo_ administration. Calculations were based on an average molecular weight of 327 for Aroclor 1254. Emulphor® is a polyoxyethylated vegetable oil (GAF Corp., N.Y., N.Y.). Aroclor 1254 was administered orally to mice by gavage. Because of the limited solubility in aqueous medium, the most concentrated solution of Aroclor 1254 prepared was 25 mg/ml. Therefore, a dosing volume of 20 ml/kg body weight was employed to yield a dose of 500 mg/kg. Solutions for lower doses were prepared accordingly so that a constant dosing volume could be used throughout. Exceptions are noted in the text.

In experiments in which radioactive isotopes were used (e.g., ^{3}H-neurotransmitters, ^{14}C-PCBs, and ^{14}C-pentobarbital), quench correc-

tion was made using the external standard ratio method (Wang et al., 1975). Appropriate quench curves were determined for each radioactive isotope for use in the conversion of counts per min (cpm) to disintegrations per min (dpm). The counting fluids used were Aqueous Counting Scintillant (ACS; Amersham Searle) for aqueous samples and a TPP scintillation cocktail (toluene containing 0.4% diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene) for organic samples.

C. Statistical analysis

Appropriate statistical analyses have been applied to the data from each experiment using standard methods as described by Bruning and Kintz (1977) and Snedecor and Cochran (1967). The specific analysis employed is given for each experiment as the data are presented in each "Results" section. The term "significant" is used throughout this thesis to denote statistical significance. The level of significance is given for each comparison, but in no case is it greater than 0.05.

III. EXPERIMENT 1 - ASSESSMENT OF THE BEHAVIORAL EFFECTS OF AROCLOR
      1254

A. Introduction

As discussed in the general introduction, a number of neurological
symptoms have been reported by humans who have been exposed to PCBs.
While this clinical picture exists, there have been very few analyses
of the neurotoxic effects of PCBs in experimental animals and only
limited assessment of neurochemical effects. Because of the paucity of
preliminary data available, a number of behavioral measures were selec-
ted to screen for neurotoxicity and as a means for determining a suit-
able range of doses of Aroclor 1254 and time course of action. The use
of behavioral measures for evaluating neurotoxicity has precedence in
the literature and has been the subject of several reviews (Norton,
1978; Reiter, 1978; Tilson et al., 1980).

Behavior can be viewed as the functional output of the integration
of a number of related processes, such as motor, sensory, and cogni-
tive. Because of the complex interactions of these processes, behavior
cannot possibly be evaluated by one single test. A number of behav-
ioral tests are available, and these tests may be divided into measures
of spontaneous behavior, those requiring little or no training, and
more complex tasks, those that require more extensive training. The
former are generally simple, rapid, and inexpensive and measure rather
gross parameters, while the latter are typically more sensitive indi-
cators of subtle changes in behavior. Because of the time involved in
training and testing animals repetatively in operant procedures, the
more simple screens were chosen for these experiments, as the data they

provide are sufficient for the intended purpose. In the experiments described herein, the behavioral effects of Aroclor 1254 were evaluated by rotor rod and inverted screen tests (as indicators of motor coordination), spontaneous activity (a manifestation of a composite of behaviors - motor, sensory, and exploratory), and convulsant activity (as a measure of CNS arousal).

Because many behavioral measures (whether they are operational tasks or spontaneous, unelicited behaviors) require normal functional capacity of the neuromuscular system, it is important to determine the effect of the compound in question on neuromuscular control and coordination. Performance on the rotor rod (Kinnard et al., 1957; Durham et al., 1957) and inverted screen (Balster, 1980) were selected for this purpose.

Spontaneous motor activity is a measure of a complex summation of behaviors that are inherent to the animal's normal behavior, such as walking, rearing, gnawing, licking, sniffing, grooming, sitting, turning, eating, and drinking. Various tests have been devised for evaluating spontaneous activity, and these methods have recently been reviewed (Reiter, 1978; Robbins, 1977).

The last experiment in this chapter is an evaluation of the convulsant activity of Aroclor 1254. There are actually two facets of this approach. An experiment of this sort can be designed to detect either CNS excitatory or depressant effects, i.e. convulsant or anticonvulsant effects. Anticonvulsant activity is evaluated using one of two experimental models. Maximal electroshock has been used as a model for grand mal seizures, whereas pentylenetetrazol, a medullary stimulant, induces convulsions representative of petit mal seizures. These models are

generally good predictors of the clinical efficacy of new anticonvul-
sant agents. A review of this area has been given by Krall et al.
(1978a, b).

The converse of this situation is that a compound may have convul-
sant effects of its own. This possibility can be explored by pre-
treating with the test compound and then challenging the animal with a
subconvulsive dose of pentylenetetrazol or with subthreshold electro-
shock voltage. Potentiation of the effects of the convulsant treatment
would indicate that the compound may exhibit convulsant properties of
its own or that it may exacerbate a preexisting condition.

B. Materials and Methods

1. Effect of Aroclor 1254 on spontaneous motor activity

Spontaneous motor activity was measured in a novel environment using photocell chambers. Chambers, consisting of a rectangular plastic cage traversed longitudinally by a photobeam, were placed in an enclosure to attenuate sound and light. The photocell was connected to a digital counter such that each interruption of the beam by movements of the animal was recorded as one count. Mice were administered Aroclor 1254 or vehicle and were returned to their home cage. At the appropriate time following drug administration (test latency), two mice were placed in each plastic cage (18 x 29 cm), covered with a perforated plastic lid, and allowed a 5 min orientation period. Combined activity of the two mice in each cage was monitored during a subsequent 10 min period. Longer test periods could not be used, because an adaptation phenomenon resulted when mice remained in the chambers for longer than 10 min. Vehicle and Aroclor 1254 groups were always tested concurrently, so that statistical comparison could be made when applicable between each treatment and its own control using Student's t-test.

The time course of the effect on spontaneous activity was determined by administering vehicle or Aroclor 1254 (500 mg/kg) and testing mice 0.25, 0.5, 0.75, 1, 2, 3, 8, or 24 hr later. Assessment of a dose-response relationship was made by giving Aroclor 1254 at doses of 0.1, 1, 2, 10, 30, 100, 250, or 500 mg/kg and testing 45 min later, the time at which the greatest difference between treatment and control was observed.

Before determining the effects of subchronic dosing of Aroclor 1254 on spontaneous activity, mice were pretested in pairs on day 0 (the day previous to the first day of treatment). Two determinations were made on this day which were separated by a 75 min interval in order to simulate the protocol that was to be used during treatment. Mice were then gavaged daily for 14 days with either vehicle or Aroclor 1254 (30 or 100 mg/kg) and tested 45 or 120 min later on days 1, 3, 7, 10, and 14. Mice were also tested on day 15 which was 24 hr after the last dose. In addition, mice were weighed throughout the 2 week period.

2. Effect of Aroclor 1254 on mouse performance on the rotor rod

The rotor rod is used to measure motor coordination by evaluating the ability of a mouse to remain on a rotating wooden rod. The rod is 2.5 cm in diameter and is placed at a height of 30 cm above the counter top to deter mice from jumping off. The apparatus used in these experiments was adjusted to a speed of 7 rotations/min. Mice were allowed a 5 min training period, and only those mice which were successful in completing this prescreening task were chosen for subsequent testing. Vehicle or Aroclor 1254 (3, 100, 200, or 500 mg/kg) was administered, and mice were returned to their home cage. At a specified time thereafter (0, 5, 10, 15, 30, 60, or 120 min after treatment), mice were placed on the rotor rod, and their ability to remain on the rotating rod was determined during a 2 min test period. Data were recorded as the number of mice that fell during the test period.

3. Inverted screen test

The inverted screen is another device used for evaluating motor coordination. The test was carried out essentially as described by Balster (1980). The apparatus consists of a row of 6 wire mesh squares mounted horizontally on a metal rod. Vehicle or Aroclor 1254 (500 mg/kg) was administered, and mice were tested 45 min later. After placing one mouse on each screen, the rod was rotated 180° such that mice and screens were inverted. At the end of a 60 sec test period, the number of mice which had climbed to the top of the screen was counted.

4. Convulsive activity of Aroclor 1254

Convulsive activity was examined by determining the effects of Aroclor 1254 on pentylenetetrazol-induced convulsions. Pentylenetetrazol was administered in doses of 75 or 125 mg/kg (i.p.), and observation of animals began immediately. Mortality and the number of tonic and clonic convulsions were recorded during a 20 min period. The effect of Aroclor 1254 on pentylenetetrazol-induced convlusions was evaluated by pretreating animals with vehicle or Aroclor 1254 (500 mg/kg) 45 min or 2 hr before the convulsant.

C. Results

1. Effect of Aroclor 1254 on spontaneous motor activity

The time course of the effects of Aroclor 1254 on spontaneous activity is presented in Figure 3. Locomotor activity was decreased significantly from vehicle control between 15 min and 3 hr after dosing but had returned to control values by 8 hr. Assessment of a dose-response relationship revealed that only the highest dose of Aroclor 1254 tested (500 mg/kg) significantly decreased activity (Figure 4), however at lower doses 10, 30, or 100 mg/kg) there appeared to be a trend, though not significant, toward increased activity relative to control. This potentially biphasic response is not unprecedented. Similar biphasic effects on activity have been observed with amphetamine, a CNS stimulant. Amphetamine stimulates spontaneous activity at low doses while depressing activity at high doses (see Seiden and Dykstra, 1977). For this reason, lower doses of Aroclor 1254 were tested. With doses as low as 0.1, 1, and 2 mg/kg, there was an increase in activity, though not significant, relative to vehicle control.

The effects of subchronic dosing of Aroclor 1254 on spontaneous activity are shown in Table 1. All groups began with the same baseline level activity on day 0. A small decrease in activity was observed on this day when the animals were retested at 2 hr, but this effect was not apparent in subsequent test sessions over the 2 week period. A rather pronounced drop in activity was observed on day 1 relative to activity on day 0. This effect occurred across all groups, and therefore is not a result of Aroclor 1254 treatment, but rather is probably

Figure 3

Time course of the effect of a single oral dose of Aroclor 1254 (■ ,
500 mg/kg) and vehicle (●) on spontaneous activity. Time, on the
abscissa, refers to the latency between Aroclor 1254 administration and
the beginning of the test period. Values represent the mean ± S.E. for
determinations from 6 pairs of mice. To minimize adaptation, a sepa-
rate group of mice was tested at each time point. Significance as
evaluated by Student's t-test is indicated: *, $p < .05$; **, $p < .01$.
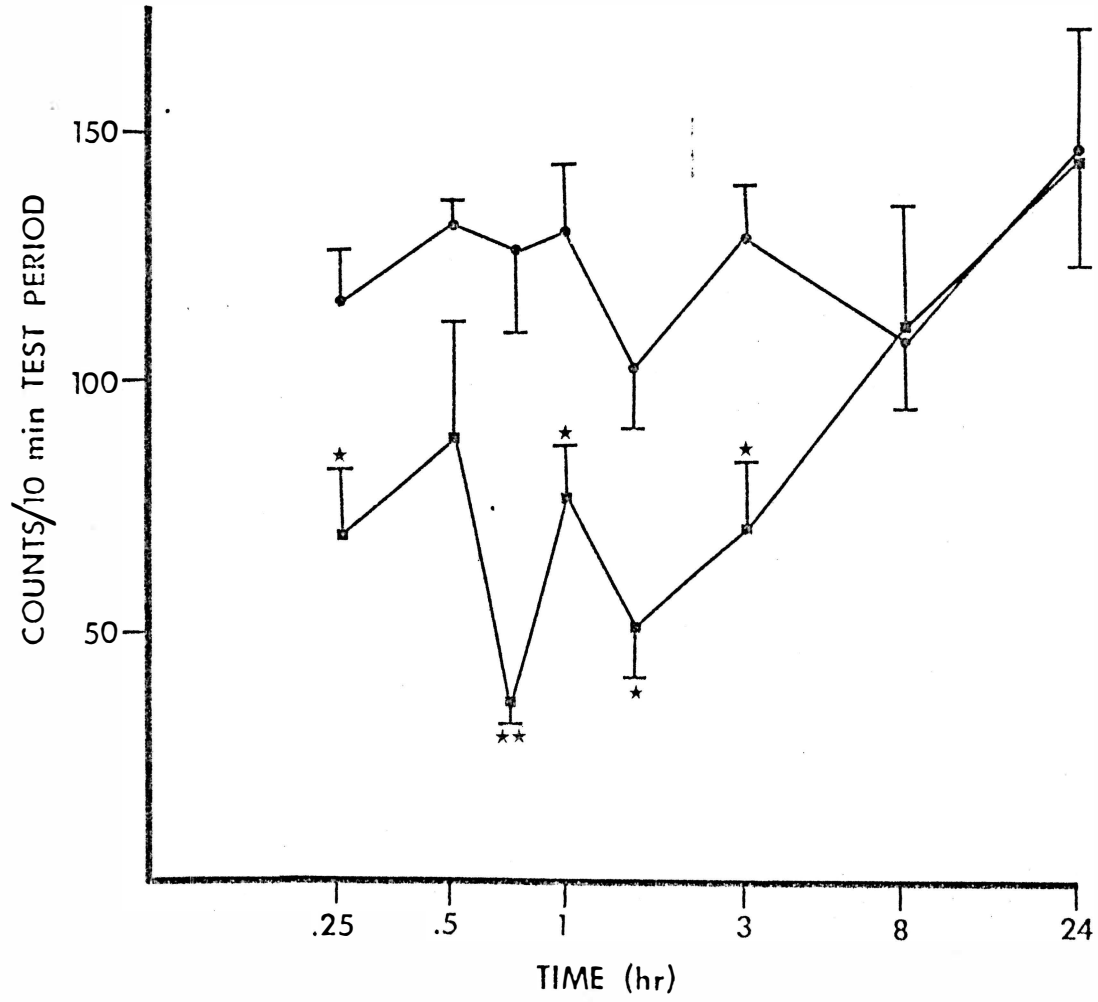
Figure 4

Dose-response relationship for the effect of single oral doses of Aroclor 1254 on spontaneous activity. Open bars, vehicle; shaded bars, Aroclor 1254. Test periods began at 45 min following vehicle or Aroclor 1254 administration. Values represent mean ± S.E. of determinations from 6 pairs of mice. The effect of Aroclor 1254 at a dose of 500 mg/kg was significant at the $p < .05$ level (Student's t-test).

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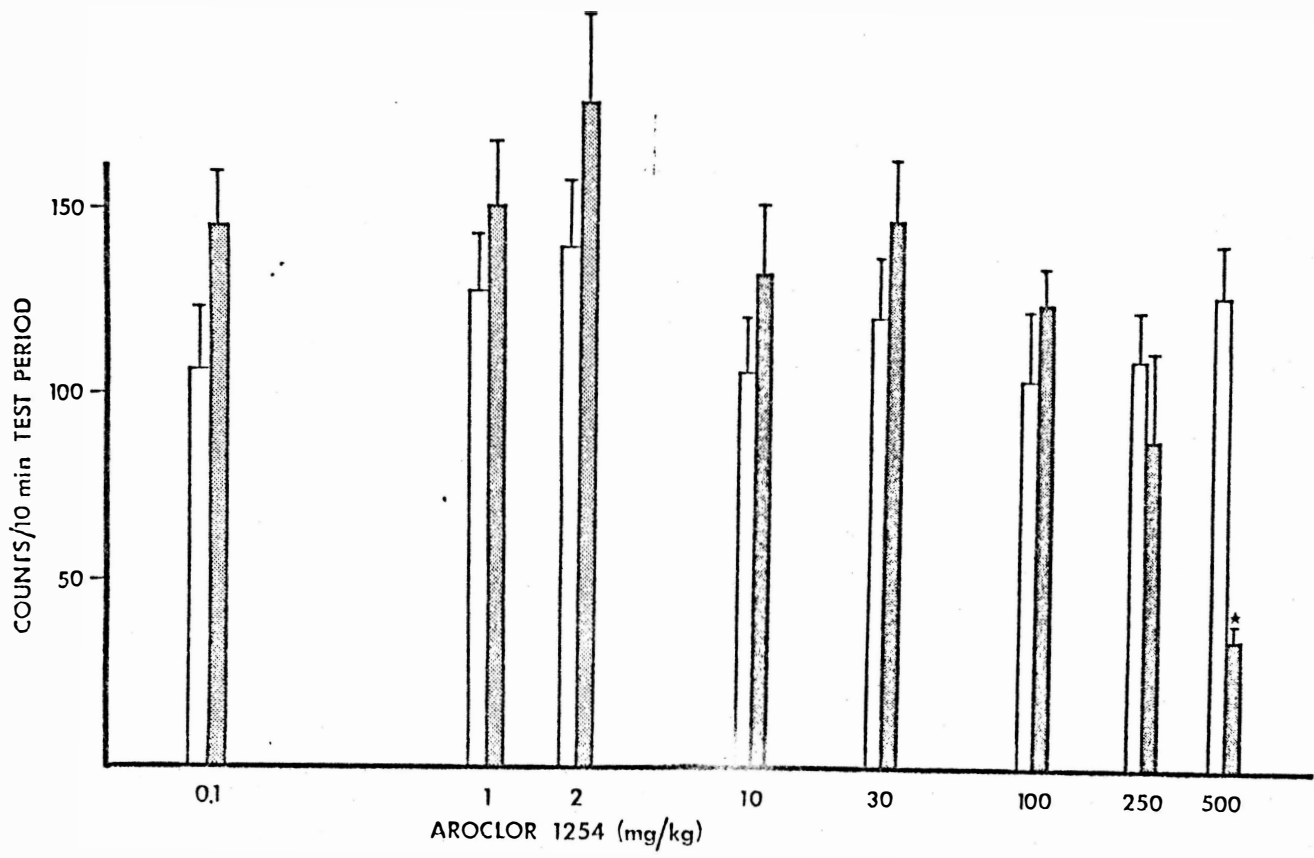
TABLE 1

Effects of 14 Day Oral Administration of Aroclor 1254 on Spontaneous Activity in Mice

Counts/10 min Test Period[a]

Day of Testing	Vehicle		Aroclor 1254 30 mg/kg		Aroclor 1254 100 mg/kg	
	45'	120'	45'	120'	45'	120'
0	205 ± 10	186 ± 11	197 ± 12	179 ± 14	206 ± 15	186 ± 12
1	147 ± 13	143 ± 20	138 ± 16	134 ± 19	146 ± 15	147 ± 16
3	151 ± 14	156 ± 17	139 ± 16	127 ± 16	139 ± 17	153 ± 19
7	138 ± 18	100 ± 17	137 ± 18	126 ± 16	122 ± 17	131 ± 18
10	136 ± 15	112 ± 16	144 ± 20	148 ± 18	105 ± 22	125 ± 24[b]
14	152 ± 17	147 ± 13	156 ± 18	162 ± 13	127 ± 34	139 ± 33[c]
15[d]	139 ± 14		162 ± 9		150 ± 26[c]	

[a] Values represent the mean ± S.E. for N = 12 pairs of mice (except as indicated) for test sessions beginning at 45 and 120 min after treatment

[b] N = 10 pairs

[c] N = 7 pairs

[d] Mice were tested at 24 hr after the last dose (day 14)

due to an adaptation phenomenon (M.J. Kallman, personal communication).
No effects of Aroclor 1254 on activity were observed relative to vehicle at either the 45 or 120 min time points, except perhaps on days 7 through 14 for the 100 mg/kg dose of Aroclor 1254 at the 45 min time point. On these days, a small reduction in activity was observed relative to days 1 through 6, but this was less apparent at the 2 hr time point. Activity remained constant on day 15, 24 hr after the last dose of vehicle or Aroclor 1254. Although animals exhibited no overt signs of toxicity, 2 pairs of mice in the high dose group had died by day 7 and a total of 5 pairs by day 14. Despite the lethality of the high dose of Aroclor 1254 (100 mg/kg), Aroclor 1254 had no significant effect on body weight compared to vehicle, and all groups gained weight over the course of 14 days (two-way analysis of variance with repeated measures; $p \leq .01$).

2. Effect of Aroclor 1254 on mouse performance on the rotor rod

The effects of Aroclor 1254 on mouse performance on the rotor rod are summarized in Table 2. The values at each time point are repeated measures on the same group of animals. At all times and doses tested, Aroclor 1254 had no effect on rotor rod performance when compared to the appropriate control.

3. Effect of Aroclor 1254 on mouse performance on the inverted screen test

Motor performance following exposure to Aroclor 1254 (500 mg/kg) was also evaluated using the screen test. Of 12 mice tested in each group, 11 or 10 of the vehicle- or Aroclor 1254-treated mice, respec-

TABLE 2

Effect of Aroclor 1254 on Rotor
Rod Performance

Treatment	Number of Falls[a] Time[b]						
	0	5	10	15	30	60	120
Vehicle[c]	0		0		0	0	
Aroclor 1254, 3 mg/kg[c]	0		0		0	0	
Aroclor 1254, 100 mg/kg[c]	0		0		0	0	
Aroclor 1254, 200 mg/kg[c]	0		0		0	0	
Vehicle[d]		0		1	1	1	1
Aroclor 1254, 500 mg/kg[d]		1		1	1	1	0

[a] Refers to the number of mice out of a total of 6 that fell off the rod during a 2 min test period

[b] Time of testing (min) after treatment

[c] Vehicle or Aroclor 1254 delivered in a volume of 10 ml/kg body weight

[d] Vehicle or Aroclor 1254 delivered in a volume of 20 ml/kg body weight

tively, were able to complete the task and climb to the top of the screen within 60 sec. No mice fell off the screen during this time. Thus, Aroclor 1254 had no effect on motor coordination as measured by this procedure.

4. Convulsive activity of Aroclor 1254

Convulsive activity of Aroclor 1254 was evaluated by examining the effects of Aroclor 1254 on pentylenetetrazol-induced seizures. The low dose of pentylenetetrazol alone (75 mg/kg) produced both tonic and clonic convulsions, whereas the high dose (125 mg/kg) produced predominantly tonic convulsions (Table 3). While vehicle-pretreatment may have had some effect on the pattern of clonic and tonic convulsions in the low dose group, the effects of Aroclor 1254-pretreatment on convulsions induced by pentylenetetrazol were no different from vehicle-pretreatment regardless of time of pretreatment.

TABLE 3

Effect of Aroclor 1254 On
Pentylenetetrazol-induced Seizures

Pretreatment	Time[b]	Pentylenetetrazol (mg/kg, i.p.)	% of Total[a]			
			Tonic[c]	Clonic[d]	Mortality[e]	No effect
-----	--	75	40	40	40	20
-----	--	125	90	10	90	--
Vehicle	45	75	20	80	20	--
		125	100	0	100	--
Aroclor 1254 500 mg/kg	45	75	10	80	10	10
		125	80	20	80	--
Vehicle	120	75	0	70	0	30
		125	80	10	70	10
Aroclor 1254 500 mg/kg	120	75	0	90	0	10
		125	90	10	90	--

[a] N = 10

[b] Time (min) between pretreatment (p.o.) and pentylenetetrazol

[c] Percent of animals displaying tonic convulsions

[d] Percent of animals displaying clonic convulsions

[e] Percent of animals that died

D. Discussion

The purpose of the experiments described here was to assess the neurobehavioral effects of Aroclor 1254. Only the highest dose of Aroclor 1254 (500 mg/kg) tested was found to be behaviorally active (spontaneous activity was depressed). It was important to determine the effects of Aroclor 1254 on motor function, since the latter is an important component of spontaneous activity. While depressed activity could be a manifestation of impaired motor coordination, this did not appear to be the case for Aroclor 1254, since acute administration of Aroclor 1254 had no effect on mouse performance on rotor rod or inverted screen.

Whereas acute Aroclor 1254 administration altered spontaneous activity, no effect on spontaneous activity was observed either during or immediately after a 14 day regimen of Aroclor 1254 administration. PCBs are highly lipophilic compounds and readily accumulate in tissue (see Introduction - Pharmacokinetics). Therefore, it was anticipated that the 14 day treatment would have some effect on spontaneous activity. Several explanations can be offered for this lack of effect. During the course of treatment, no change in spontaneous activity was observed, but this could easily be attributed to the presence of sub-threshold doses (30 and 100 mg/kg) as observed in the acute study. Although one might expect to see an accumulation of Aroclor 1254 after 14 days of exposure, accumulation may have occurred, but not to a level sufficient to alter spontaneous activity. Additionally, accumulation may have been offset by a metabolic effect, inasmuch as PCBs are potent inducers of the liver microsomal enzymes. Enzyme induction could easily have occurred given the doses and duration of exposure (see

Introduction - Toxicology). Because PCBs are also metabolized by this same enzyme system, they could be inducing their own metabolism, thereby enhancing clearance and precluding tissue accumulation. An additional explanation for the lack of effect is that pharmacodynamic or behavioral tolerance (as distinctions from the metabolic tolerance described above) may have developed to the behavioral effect of Aroclor 1254.

Assessment of the convulsant properties of Aroclor 1254 was chosen for two reasons, one being the antithesis of the other. On the one hand, the effects of Aroclor 1254 on spontaneous activity would suggest that Aroclor 1254 may be exerting a CNS depressant effect. If so, one might expect to detect anticonvulsant properties of Aroclor 1254 as is the case for phenobarbital, a general CNS depressant. On the other hand, convulsive properties have been reported for other classes of chlorinated hydrocarbons (Joy, 1976). The protocol used in the experiments described here was designed with these two possibilities in mind. Prudent selection of pentylenetetrazol doses affords the feasability of detecting either convulsant or anticonvulsant properties of the test compound. The lower dose given alone produced only clonic convulsions, whereas both tonic and clonic convulsions were observed with the higher dose. Thus, a stimulant or convulsant effect of a test drug would be observed as an exacerbation of the effects of the lower pentylenetetrazol dose, and a depressant effect would appear as an antagonism of the convulsant effects of the higher pentylenetetrazol dose. However, Aroclor 1254 did not alter the convulsant activity of either dose of pentylenetetrazol.

Thus, spontaneous activity was the only behavioral measure altered by Aroclor 1254. To reiterate, this small sampling of simple behavioral tasks was chosen to screen for central neurotoxicity of Aroclor 1254. The possibility that Aroclor 1254 may alter performance in other behavioral measures cannot be ruled out.

IV. EXPERIMENT 2 - A COMPARISON OF THE EFFECTS OF ACUTE AND SUBCHRONIC

ADMINISTRATION OF AROCLOR 1254 ON PENTOBARBITAL IN-

DUCED SLEEP TIME AND ^{14}C-PENTOBARBITAL DISPOSITION

A. Introduction

The observation that acutely administered Aroclor 1254 depressed

spontaneous motor activity without impairing motor coordination (see

Experiment 1) suggested that Aroclor 1254 may be having a direct depres-

sant effect on the central nervous system (CNS). It was thus of inter-

est to examine the effects of Aroclor 1254 in some other system capable

of predicting CNS depression. It is well known that many of the CNS

depressants (e.g., barbiturates and alcohol) have additive effects

(Goodman and Gilman, 1980). Sleep time is a response to barbiturates

that can easily be quantitated. If the reduction in sleep time is a

manifestation of CNS depression, one might expect to see a synergistic,

or at best additive, effect of Aroclor 1254 and pentobarbital which

would be manifested as an increase in pentobarbital-induced sleep time.

If so, it would be interesting to establish the mechanism of this

interaction. Aroclor 1254 could alter the responsiveness of the CNS to

pentobarbital, the interaction could simply be one of additive effects,

or it could be a pharmacokinetic effect, i.e. altered metabolism and/or

disposition of pentobarbital.

In contrast to the aforementioned hypothesis that acutely adminis-

tered Aroclor 1254 could enhance pentobarbital-induced sleep time, it

has been clearly established that chronically administered PCBs induce

microsomal enzymes and enhance drug metabolism (Bruckner et al., 1977;

Goldstein, 1979; Grant et al., 1974; Litterst et al., 1972). As a

consequence of this enzyme induction, chronically administered PCBs enhance pentobarbital metabolism (Chu et al., 1977; Stella and Chu, 1980) and reduce pentobarbital-induced sleep time (Sanders and Kirkpatrick, 1975; Sanders et al., 1974; Villeneuve et al., 1972; Zepp et al., 1974). Thus, if the effects of chronically administered Aroclor 1254 on pentobarbital-induced sleep time are of metabolic origin, it would be interesting to pursue the etiology of an acute Aroclor 1254/pentobarbital interaction. This reasoning prompted the experiments to be described herein in which a comparison was made between the effects of acute and subchronic Aroclor 1254 administration on pentobarbital-induced sleep time and on pentobarbital disposition.

B. Materials and Methods

1. Materials

5-[ring-2-^{14}C]-Ethyl-5-(1-methylbutyl) barbituric acid (^{14}C-pento-barbital; 52 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Its radiochemical purity was established by thin layer chromatography (TLC) on silica gel plates (Analtech, Newark, DE) using a chloroform:acetone (9:1) solvent system (Cochin and Daly, 1963) and visualizing with iodine vapors. Sodium pentobarbital (Nembutal$^{®}$) was purchased from Abbott Laboratories (North Chicago, IL) and diluted with the same vehicle [95% ethanol:propylene glycol:H_2O (1:4:5)] in which it was supplied.

2. Pentobarbital-induced sleep time

Mice were pretreated with Aroclor 1254 or vehicle by oral gavage. Some experiments also contained a naive group which received no treatment prior to pentobarbital. Mice then received a 25 mg/kg dose of pentobarbital (i.p.), and sleep times were recorded for each mouse. Pentobarbital-induced sleep time is defined as the time interval between loss and regaining of the righting reflex.

A time course of effects of Aroclor 1254-pretreatment on sleep time was determined by treating mice with vehicle or Aroclor 1254 (500 mg/kg) at 0, 0.75, 2, 4, 8, or 24 hr before pentobarbital. Once the pretreatment time for maximum effect had been determined, a dose-response relationship for Aroclor 1254 was then established by administering vehicle or Aroclor 1254 in doses of 5, 10, 25, 50, 100, 250 or 500 mg/kg 2 hr prior to pentobarbital.

The effects of subchronic treatment of Aroclor 1254 on sleep time were determined following oral administration of vehicle or Aroclor 1254 (30 or 100 mg/kg) for 14 successive days. Mice in each treatment group were administered pentobarbital (25 mg/kg, i.p.) either 45 min or 24 hr after receiving vehicle or Aroclor 1254 on day 14. Sleep times were then recorded for all groups.

3. Disposition of ^{14}C-pentobarbital

a. Validation of the extraction method

The extraction method of Brodie et al. (1953) reportedly separates pentobarbital from its metabolites. The major metabolites of pentobarbital are the pentobarbital alcohol and pentobarbital carboxylic acid (Brodie et al., 1953; Cooper and Brodie, 1957; Maynert and van Dyke, 1950), the structures of which are shown in Figure 5. These metabolites are much more water soluble than the parent compound, and therefore can readily be separated from pentobarbital by using an extraction system that capitalizes on differences in solubiltiy of the compounds to be separated. The method of Brodie et al. (1953) employs this concept. Their method involves detection of pentobarbital by a spectrophotometric method. A modification of their method was employed for these studies. If ^{14}C-labelled pentobarbital is administered to animals, and if in fact, parent compound can be selectively extracted into the organic phase of this extraction system, then pentobarbital can be quantitated by counting an aliquot of the organic phase. It was thus necessary to demonstrate both qualitative and quantitative validation of this application of the Brodie method, i.e., that all of the radioactivity in the organic phase is, in fact, pentobarbital and that the

Pentobarbital
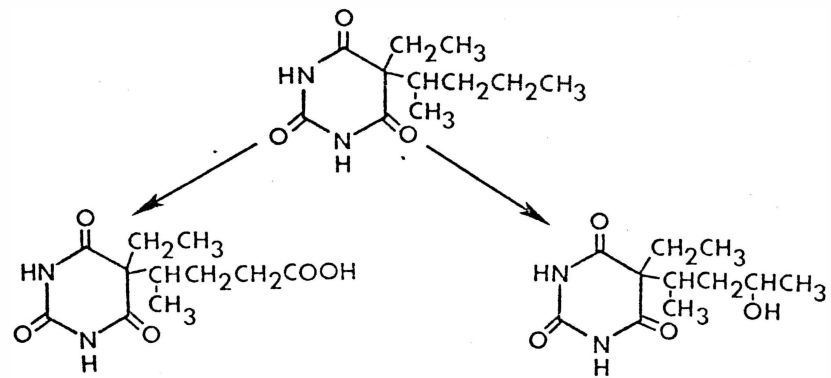
Pentobarbital carboxylic
acid

Pentobarbital alcohol

Figure 5 Metabolism of pentobarbital

method can efficiently extract pentobarbital from tissue samples. These two criteria were verified as follows.

In order to determine the extraction efficiency of the system, plasma, liver and brain were taken from untreated mice. Brain and liver were homogenized in 3 volumes of ice cold 0.1 N HCl using a Brinkman Instruments polytron (Rexdale, Ont., Canada). Extraction of pentobarbital is improved in acidified medium, because pentobarbital, as the free acid, is less soluble in aqueous medium and more likely to partition into the organic phase. An aliquot of plasma (50 μl) or tissue homogenate (1.5 ml) was buffered with 1.8 or 1.5 ml, respectively, of 1 M NaH_2PO_4 (pH 5.5). Each tissue sample was then spiked with 5 μl ^{14}C-pentobarbital (~5 nCi), swirled rapidly to mix, and 15 ml of petroleum ether containing 1.5% isoamyl alcohol was added immediately to prevent any possible metabolism of the pentobarbital. Samples were shaken for 30 min and centrifuged. Aliquots of the aqueous and organic phases were counted in 10 ml of ACS or TPP, respectively. Of the total radioactivity added, < 10% was found in the aqueous layer and between 90 and 95% in the organic layer for all 3 tissues. Thus, the solvent system is capable of efficiently extracting ^{14}C-pentobarbital from tissues.

Pentobarbital has reportedly been separated from its metabolites in the TLC system described by Cochin and Daly (1963). Because this TLC system was to be used to establish the presumptive identity of unknown radioactive compounds in the extraction samples, it was necessary to confirm the chromatographic behavior of known standards in this system. The ideal choice of standards for this particular experiment would have been pentobarbital and its major metabolites.

Because these metabolites are not readily available commercially, authentic samples of pentobarbital and thiopental, a less polar barbiturate than pentobarbital, were chromatographed in the same TLC system. The resulting Rf values, 0.51 and 0.78 for pentobarbital and thiopental, respectively, were consistent with those reported by Cochin and Daly (1963). According to Cochin and Daly (1963), polar metabolites migrate more slowly than pentobarbital and would appear as spots closer to the origin.

In order to address the qualitative aspect of the extraction method 3 mice were injected with ^{14}C-pentobarbital (80 µCi/25mg/kg). Mice were sacrificed 90 min later, and plasma, brain, and liver were taken. This time point was chosen because it was anticipated that a large proportion of the pentobarbital would already have been metabolized. High tissue levels of metabolites would thus maximize the challenge to the extraction system. Tissues were extracted as described above, an aliquot of the organic phase was counted in 10 ml TPP, and the remainder was evaporated to dryness under a stream of nitrogen. The dried samples of the organic phase were then reconstituted in a small volume of chloroform, and aliquots were subjected to analysis by TLC as described in "Materials." Pentobarbital and thiopental were used as reference standards. The resolved spots of unknown sample were visualized with iodine, and bands of absorbent were scraped from the plate. To these samples 1 ml of 50% methanol was added, samples were sonicated for 30 min to solubilize radiolabelled material, 10 ml of ACS was added, and the samples were counted. In all three tissues, >90% of the radioactivity in an aliquot of the organic phase co-chromatographed with the pentobarbital standard indicating that all of the radioactiv-

ity in the organic phase was associated with parent compound and that, apparently, no metabolites were present in this phase. This observation thus validates the application of the method of Brodie et al. (1953) for quantitating levels of ^{14}C-pentobarbital in tissue samples.

b. <u>Effects of Aroclor 1254 on disposition of ^{14}C-pentobarbital</u>

Tissue levels of ^{14}C-pentobarbital and metabolites were determined by the above mentioned modification of the method of Brodie <u>et al</u>. (1953). In general, Aroclor 1254 or vehicle was administered orally to mice 2 hr prior to ^{14}C-pentobarbital (80 μCi/25 mg/kg, i.p.). Mice were decapitated, and trunk blood was collected from the cervical wound and centrifuged at 1000 x <u>g</u> for 20 min to obtain plasma. Brain and liver were removed and homogenized as described previously. Aliquots of plasma and tissue homogenates (50 μl) were taken to determine total radioactivity. In order to extract parent compound (pentobarbital), an aliquot of plasma (200 μl) or tissue homogenate (1.5 ml), was buffered with 1.8 or 1.5 ml, respectively, of 1 M NaH_2PO_4 buffer (pH 5.5) before the addition of 15 ml of petroleum ether containing 1.5% isoamyl alcohol. Samples were shaken for 30 min and centrifuged. A 6 ml aliquot of the organic phase was counted in 10 ml of TPP. Pentobarbital (in the organic phase) was then calculated from the specific activity of the starting material and expressed as nmol per g of tissue or ml of plasma. Levels of metabolites were calculated as the difference between total radioactivity and that associated with parent compound.

More specifically, three experiments were designed to compliment the sleep time studies. In the first experiment, vehicle or Aroclor 1254 (500 mg/kg) was given 2 hr before ^{14}C-pentobarbital, and mice were sacrificed 0.25, 0.5, 1, 1.5, 2, or 3 hr after ^{14}C-pentobarbital. The second experiment involved adminstration of vehicle or varying doses of Aroclor 1254 (5, 10, 25, 50, 100, 250, or 500 mg/kg) 2 hr prior to ^{14}C-pentobarbital and sacrificing mice 2 hr after ^{14}C-pentobarbital. For the third experiment, vehicle or Aroclor 1254 (30 mg/kg) were ad-

ministered for 14 days, and mice were weighed every other day. As in the sleep time study, animals were divided into two groups on day 14. Group A received ^{14}C-pentobarbital 45 min after vehicle or Aroclor 1254, and group B received ^{14}C-pentobarbital 24 hr after vehicle or Aroclor 1254. Mice were sacrificed at 30, 60, or 90 min after ^{14}C-pentobarbital.

C. Results

1. Effects of Aroclor 1254 on pentobarbital-induced sleep time

Aroclor 1254, given at various times prior to pentobarbital, enhanced sleep time relative to vehicle pretreament. As shown in Figure 6, co-administration of Aroclor 1254 and pentobarbital (at a pretreatment time of zero hr) produced a modest increase in sleep time when compared to vehicle-pretreated mice ($p < .05$). The effect of Aroclor 1254 on pentobarbital-induced sleep time was enhanced as pretreatment time was increased until a maximum was seen at 2 hr. Aroclor 1254 still had significant effects at the 4 and 8 hr pretreatment times. By 24 hr, Aroclor 1254 produced a small but significant decrease in sleep time compared to vehicle-pretreatment. Not shown in Figure 6 is the sleep time for naive animals (51 ± 2, $N = 10$). Analysis of variance using a 2-factor factorial design resulted in a significant effect of Aroclor 1254 and of time of Aroclor 1254-pretreatment. A significant interaction between Aroclor 1254-treatment and pretreatment time was also found, i.e., the effect of Aroclor 1254 on sleep time depends on the time interval between Aroclor 1254 and pentobarbital administration.

Based on the above time course, a 2 hr pretreatment time was chosen in order to examine a dose-response relationship for Aroclor 1254 (Figure 7). Sleep time for naive animals ($N = 30$) was 54 ± 4 (data not shown). Sleep time was increased in a dose-dependent fashion relative to vehicle- or Aroclor 1254-pretreatment up to an Aroclor 1254 dose of 25 mg/kg, at which point sleep time increased only slightly with larger doses of Aroclor 1254. The effect of a dose of 500 mg/kg

Figure 6

Pretreatment time course of the effect of Aroclor 1254 on pentobarbi-
tal-induced sleep time. Mice were pretreated orally with either ve-
hicle (⊙—⊙) or Aroclor 1254 (▨-▨; 500 mg/kg). Values are given as
mean ± S.E. for groups of 10 mice each. A 2 x 6 factorial analysis of
the data revealed significant effects of Aroclor 1254-pretreatment,
time of pretreatment, and an interaction of treatment and time
($p < .001$). Sleep time following Aroclor 1254-pretreatment was signifi-
cantly different from that following vehicle-pretreatment at all pre-
treatment times ($p < .001$) except at zero time which significance
was established at the $p < .05$ level (Student's t-test).

SLEEP TIME (min)

Aroclor 1254 Pretreatment Time (hr)

62
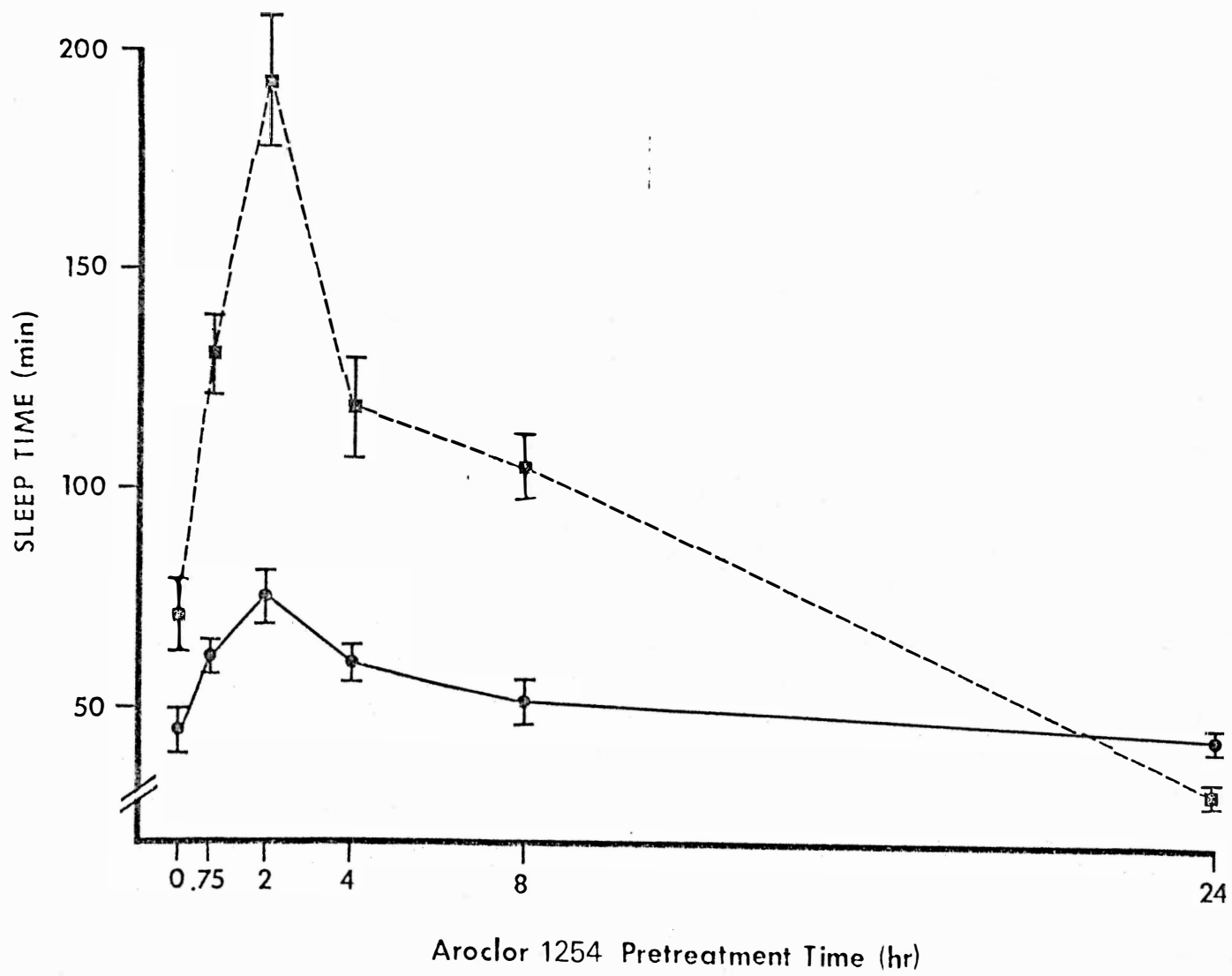
Figure 7

Dose-response curve for the effect of Aroclor 1254 on pentobarbital-induced sleep time. Vehicle (V) or Aroclor 1254 was administered orally 2 hr before pentobarbital. Values represent mean ± S.E. of groups of 20 to 40 mice. All but the 5 mg/kg dose of Aroclor 1254 were significantly different from control ($p < .01$) as determined by analysis of variance and Dunnett's t-test.
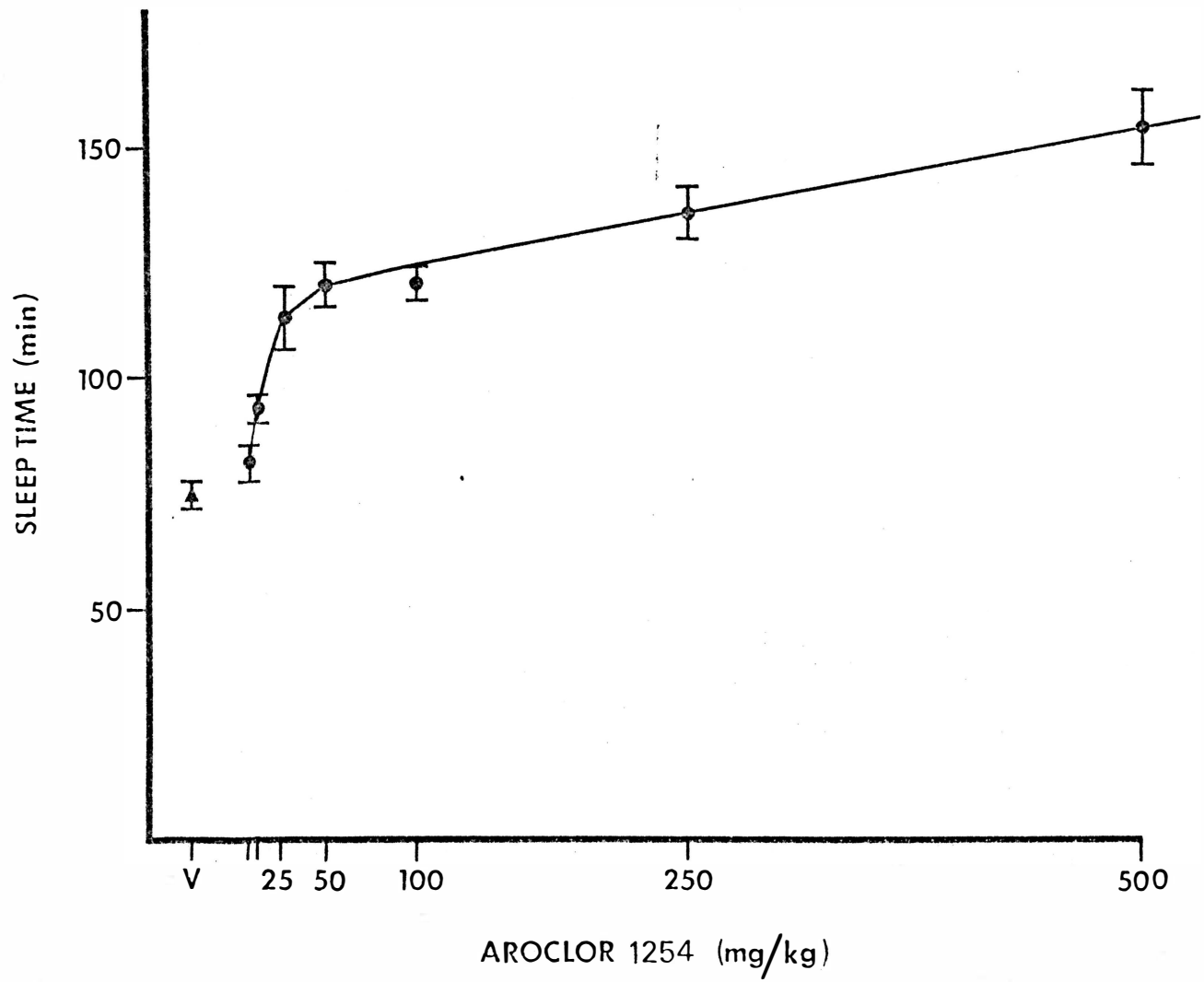
was significantly greater than the effects of doses of 5, 10, 25, 50, or 100 (p < .01) but not 250 mg/kg as determined by analysis of variance and Duncan's multiple range test.

Figure 8 illustrates the results of the subchronic study. In contrast to the acute studies, pentobarbital-induced sleep time was reduced in animals treated with Aroclor 1254 for 14 days. This effect was dependent upon the dose of Aroclor 1254 as well as the time interval between the last dose of Aroclor 1254 (day 14) and administration of pentobarbital. The degree of suppression of sleep time was greater for the 24 hr than the 45 min time interval. Significance was established for the effect of Aroclor 1254 and of the time interval between Aroclor 1254 and pentobarbital administration but not for an interaction between the two factors as evaluated by analysis of variance using a 2-factor factorial design. Thus, the effect of varying doses of Aroclor 1254 does not depend upon the latency between Aroclor 1254 and pentobarbital administration. In addition, the effect observed at various time intervals does not depend upon the dose of Aroclor 1254. As in the 2 previous studies, sleep time for naive animals (63 ± 2, N = 10) was less than vehicle. Sleep time is reported for a smaller number of mice in the group receiving the higher dose of Aroclor 1254 due to the lethal effects of this dose. A total of 10 mice of the original 25 in this group had died by day 14. Despite the lethal effects of the high dose, mice exhibited no overt signs of toxicity. All groups gained weight over the course of 14 days, and Aroclor 1254 had no significant effect on this weight gain when compared to controls (two-way analysis of variance with repeated measures), indicating that mice were apparently in good health.

Figure 8

Pentobarbital-induced sleep time following 14 day administration of vehicle (V) or Aroclor 1254. Shaded bars, pentobarbital given 45 min after the last dose of V or Aroclor 1254. Open bars, pentobarbital given 24 hr after V or Aroclor 1254. Values represent mean ± S.E. Numbers over S.E. bars indicate number of mice per group. Significant effects of Aroclor 1254-pretreatment (p < .001) and time of administration of pentobarbital (p < .05) were revealed by a 2 x 3 factorial analysis of the data, however there was no significant interaction between treatment and time.
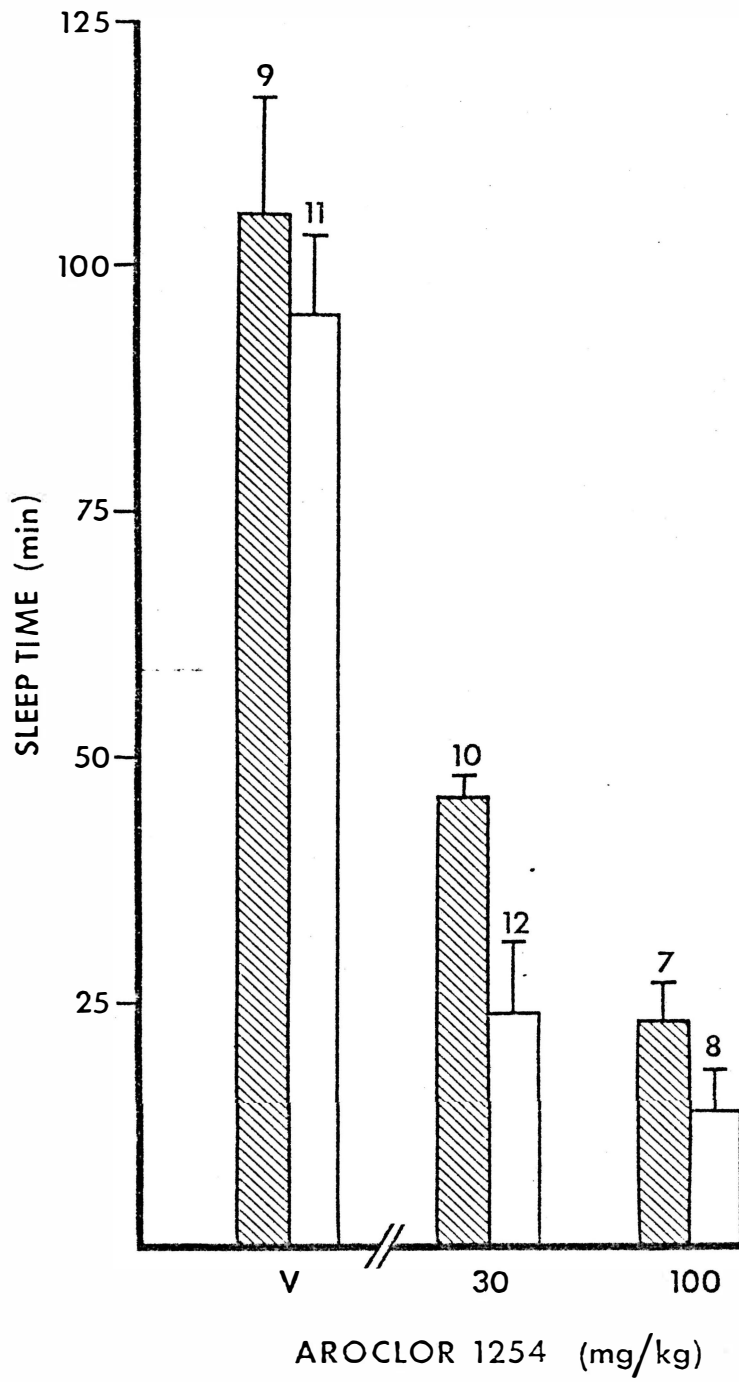
2. Effect of Aroclor 1254 on disposition of ^{14}C-pentobarbital

Having established that Aroclor 1254 alters pentobarbital-induced sleep time, the question of how this was occurring remained unanswered. One possible explanation could be that Aroclor 1254 alters the pharmacokinetics of pentobarbital. Therefore, a design similar to the sleep time studies was chosen to examine the disposition of ^{14}C-pentobarbital following adminstration of Aroclor 1254. The first experiment was executed to determine the effect of Aroclor 1254- or vehicle-pretreatment on the time course of levels of parent compound (pentobarbital) and metabolites in brain, liver, and plasma (Figure 9). In all three tissues, levels of pentobarbital were higher in the Aroclor 1254-pretreated groups than in the vehicle-pretreated groups. Levels of metabolites were lower in liver and plasma in Aroclor 1254-pretreated groups. No detectable levels of metabolites were found in brain, i.e., all of the radioactivity was associated with the parent compound.

A dose-response curve for Aroclor 1254-pretreatment was then generated. Aroclor 1254 increased pentobarbital levels and decreased metabolite levels dose-responsively in all three tissues relative to the vehicle control, and this effect began to level off with Aroclor 1254 doses of 100 mg/kg and higher (Figure 10).

For the pharmacokinetic compliment of the subchronic study, mice were exposed to vehicle or Aroclor 1254 for 14 days. Mice showed no overt signs of toxicity. As in the previous 14 day study, all mice gained weight over the 14 day period, and Aroclor 1254 had no effect on body weight as compared to vehicle (two-way analysis of variance with repeated measures), indicating that all mice were apparently in good health. Levels of pentobarbital and metabolites in brain, liver and

Figure 9

Time course of levels of pentobarbital and metabolites in brain, liver, and plasma of mice which received Aroclor 1254 (500 mg/kg) or vehicle 2 hr before ^{14}C-pentobarbital. Values given as mean ± S.E. for determinations from 5 mice. Where S.E. bars are not shown, the values lie within the bounds of the symbol. Abscissa, time of sacrifice following pentobarbital administration. Solid lines, pentobarbital levels. Dashed line, metabolite levels. ▲, Aroclor 1254-pretreated mice. ◉, vehicle-pretreated mice.
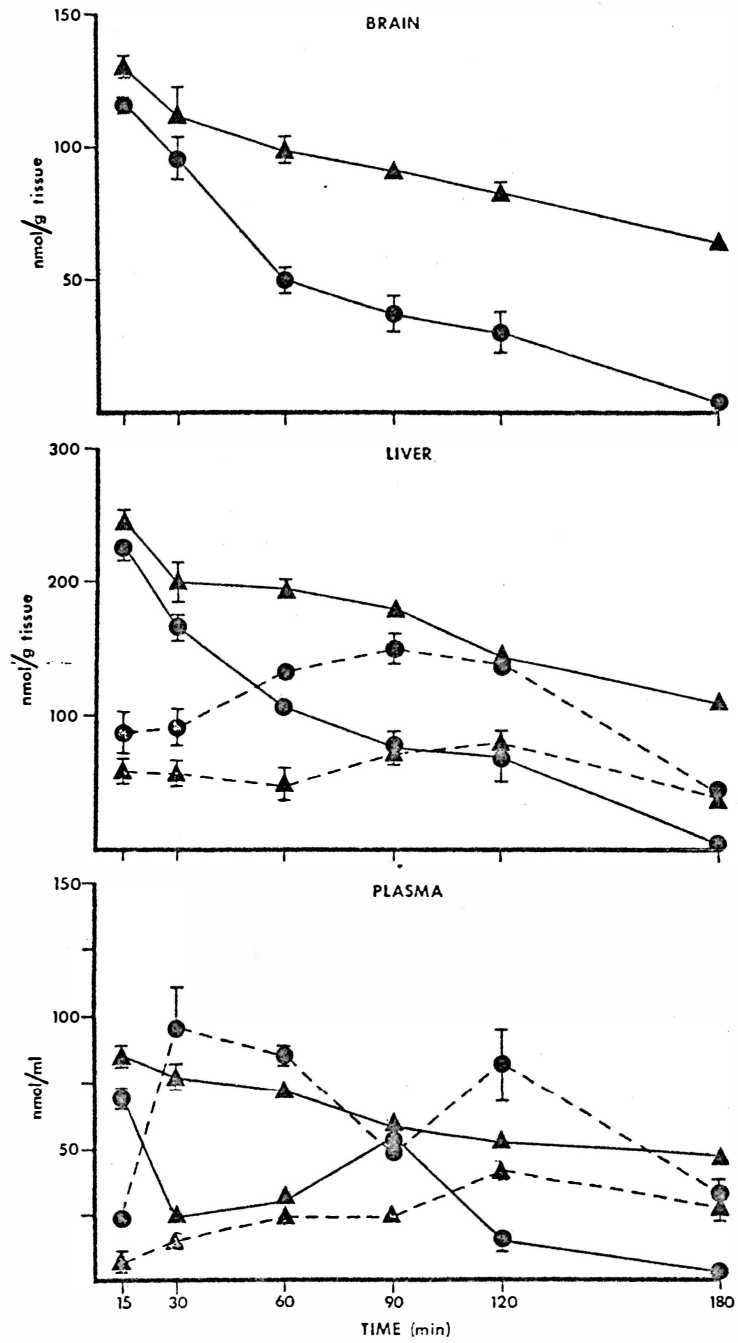
Figure 10

Effects of varying doses of Aroclor 1254 on tissue levels of pento-
barbital and metabolites. Aroclor 1254 or vehicle (V) was administered
2 hr prior to ^{14}C-pentobarbital, and mice were sacrificed 2 hr after
^{14}C-pentobarbital. Values are given as mean ± S.E. for determinations
from 5 mice (for all except V and 50 mg/kg where N = 10). Where S.E.
bars are not shown, the values lie within the bounds of the symbol.
● , pentobarbital levels. ■ , metabolite levels.

BRAIN

LIVER

PLASMA

nmol/g tissue

nmol/g tissue

nmol/ml

PCBs (mg/kg)
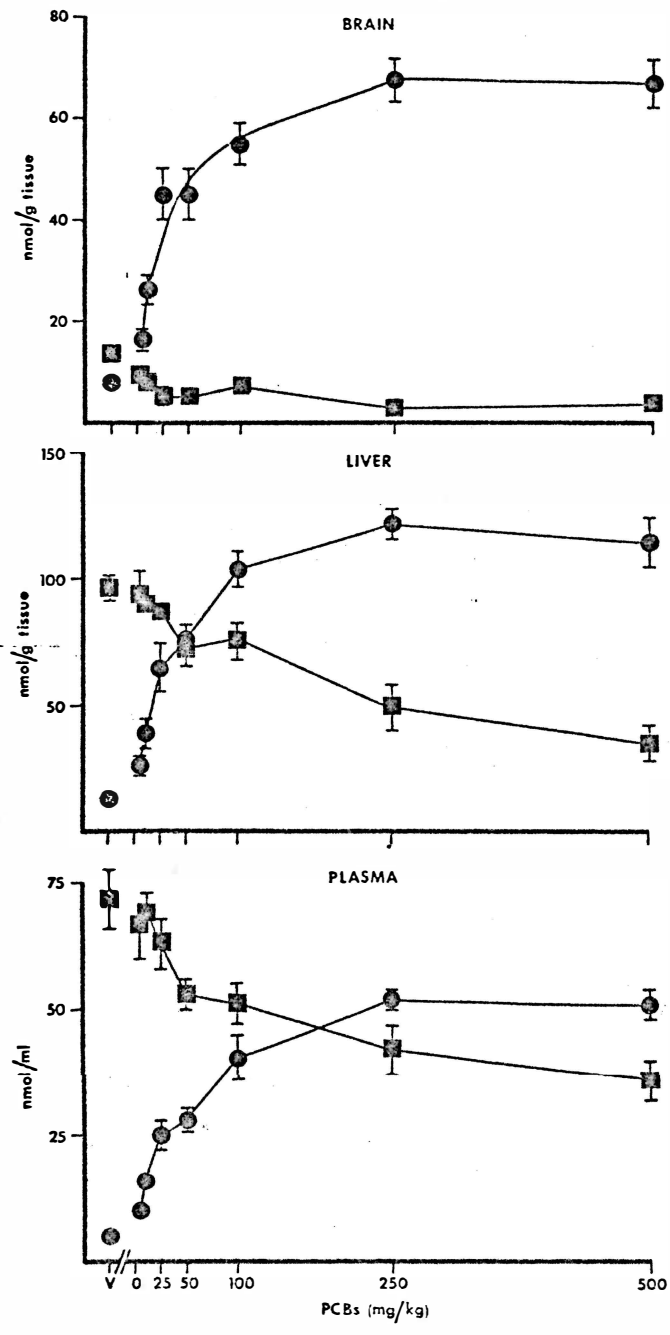
plasma are shown in Figure 11. In all tissues from vehicle-pretreated mice, levels of pentobarbital declined in a linear fashion (correlation coefficients ranged from .9929 to .9991). The slopes of these lines were approximately equal when comparing the 45 min vs. 24 hr latency for pentobarbital levels in all three tissues (brain, -0.967 vs. -1.02; plasma, -0.817 vs. -0.70; liver, -1.95 vs. -2.05). Aroclor 1254-pretreatment reduced the levels of pentobarbital in all three tissues relative to levels from vehicle-pretreated mice. Metabolite levels were generally higher in Aroclor 1254-pretreated mice except in some cases at the later time points (i.e., 60 or 90 min after ^{14}C-pentobarbital), when levels dropped below those of vehicle-pretreated mice. In contrast to the vehicle-pretreated groups, there was a difference in the pattern of pentobarbital disposition when comparing groups A and B from Aroclor 1254-pretreated mice. Pentobarbital levels were lower initially in tissues from group B, declined more rapidly, and reached a lower level by 90 min than those in group A. The slopes of these lines were -0.417 and -0.343 in plasma, -0.567 and -0.490 in brain, and -1.32 and -1.04 in liver for the 45 min and 24 hr latencies, respectively (correlations coefficients ranged from .9393 to .9997).

One additional observation was made in this experiment. Liver weight was determined at the time of sacrifice, and as may be expected, liver enlargement was observed in the Aroclor 1254-treated groups. Liver wet weight/body weight ratios were 0.048 ± 0.001 and 0.058 ± 0.001 for vehicle-treated mice (groups A and B, respectively) and 0.071 ± 0.002 and 0.082 ± 0.002 for Aroclor 1254-treated mice (groups A and B, respectively) for groups of 15 mice each.

Figure 11

Effects of 14 day administration of vehicle or Aroclor 1254 (30 mg/kg)
on tissue levels of pentobarbital and metabolites. ^{14}C-pentobarbital
was administered 45 min (left panel; group A) or 24 hr (right panel;
group B) after the last dose of vehicle or Aroclor 1254. Mice were
sacrificed 30, 60, or 90 min after ^{14}C-pentobarbital. Values are given
as mean ± S.E. for determinations from 5 mice. Where S.E. bars are not
shown, values lie within the bounds of the symbol. Abscissa, time of
sacrifice following pentobarbital administration. Solid lines, pento-
barbital levels. Dashed lines, metabolite levels. ▲, Aroclor 1254-
pretreated mice. ⬤ , vehicle-pretreated mice.

BRAIN

45 min   24 hr

nmol/g tissue

LIVER

nmol/g tissue

PLASMA

nmol/ml

TIME (min)
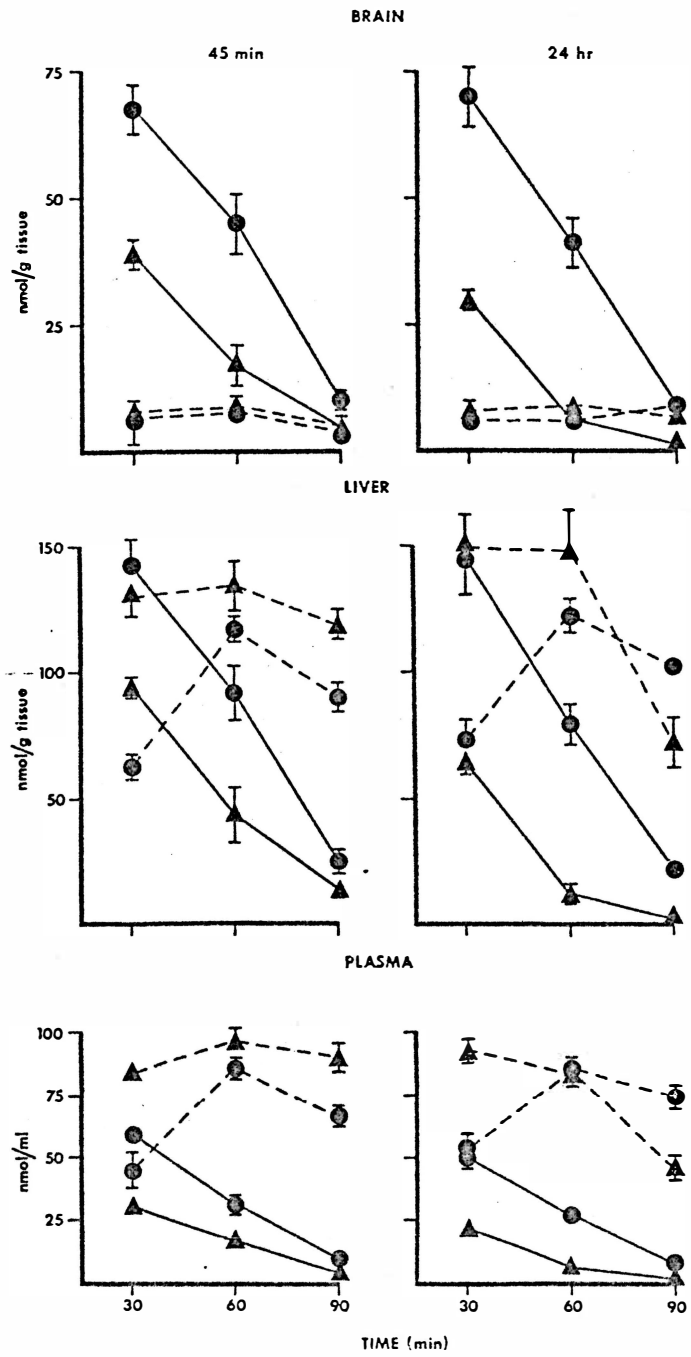
D. Discussion

As discussed in the previous chapter, reduction of spontaneous activity by Aroclor 1254 without motor impairment would suggest that Aroclor 1254 may be exerting a CNS depressant effect. In order to pursue this finding, studies were undertaken to determine whether Aroclor 1254 would augment the central effects of pentobarbital, a known CNS depressant. An interaction with other CNS depressants might provide some insight into the mechanism of action of Aroclor 1254.

Initial experiments involved the use of a high dose of Aroclor 1254, 500 mg/kg, since this is a behaviorally active dose that is known to depress spontaneous locomotor activity as demonstrated in the previous chapter. The results of these experiments showed that acutely administered Aroclor 1254 potentiated the effect of pentobarbital on the CNS as evidenced by a prolonged sleep time. The reason for the observed difference in sleep time between naive and vehicle-treated animals is not known. Certainly, it would be preferrable to see no difference in effects between naive and vehicle-treated animals, or to be able to explain the effects of vehicle. However, PCBs are insoluble in aqueous solutions and therefore, must be prepared in a suitable vehicle. Treatment of animals with PCBs necessarily involves concommitant exposure to vehicle. The limitations of this methodological problem can only be minimized by comparing all Aroclor 1254 groups to a vehicle control.

Aroclor 1254 enhancement of pentobarbital-induced sleep time could be attributed to the following concepts: 1) Aroclor 1254 may alter CNS responsiveness to pentobarbital or 2) Aroclor 1254 may have a direct depressant effect on brain that is additive with the effect of pento-

barbital, or 3) the pharmacokinetics of pentobarbital, in particular distribution to the brain, may be modified by Aroclor 1254. Evidence for the latter is provided by the disposition studies reported here. It appears that prolonged sleep time produced by Aroclor 1254 can be ascribed to increased levels of pentobarbital in brain. Sharom and Mellors (1980) have shown that membrane perturbation by PCBs is the result of a nonspecific physical toxicity. Thus, it was initially proposed that Aroclor 1254 increased levels of pentobarbital in brain by altering brain permeability. The data, however, do not support this proposed mechanism, but rather, they point to a pharmacokinetic effect. This conclusion is evident when comparing the results shown in Figure 7 with those in Figure 10. Increased sleep time as well as increased pentobarbital levels in brain were observed as the dose of Aroclor 1254 was increased to 50 mg/kg. The patterns of sleep time and pentobarbital brain levels were similar in that little increase in either was found following higher doses of Aroclor 1254. Inasmuch as reduced levels of pentobarbital metabolites were found in liver and plasma of Aroclor 1254-pretreated mice, it is likely that Aroclor 1254 is inhibiting the metabolism of pentobarbital, perhaps by competing for common metabolic sites in the liver. Support for this hypothesis can be inferred from the work of Schmoldt et al. (1977) who reported that PCBs caused competitive inhibition of aminopyrine demethylation in vitro by rat liver microsomal monoxygenases. Consistent with their findings that higher concentrations of PCBs produced no further inhibition of metabolism, a similar finding was also observed here, i.e., higher doses of Aroclor 1254 produced no further increase or decrease of pentobarbital or metabolite levels, respectively. Nevertheless, one

cannot rule out the possibility that alterations produced by Aroclor 1254 in pentobarbital's absorption, distribution, or elimination may contribute to the observed changes in disposition of pentobarbital and its metabolites.

The effect of Aroclor 1254 on pentobarbital-induced sleep time is dependent upon the frequency of exposure to Aroclor 1254. The data reported herein demonstrate that acute administration of Aroclor 1254 enhanced pentobarbital's actions, while chronic administration of PCBs has been reported to reduce pentobarbital-induced sleep time in rats (Chu et al., 1977; Villeneuve et al., 1972), rabbits (Zepp et al., 1974), and mice (Sanders and Kirkpatrick, 1975; Sanders et al., 1974). Potentiation of pentobarbital sleep time has also been observed by Cecil and her colleagues (1975) who reported increased sleep times in Japanese quail pretreated with a single 100 mg/kg dose of a mixture of PCBs (Aroclors 1221, 1232, 1242, 1248, 1254, 1260, 1262, and 1268) and decreased sleep times after 3 days of ad libitum feeding of 300 ppm of PCBs. However, these investigators were not able to demonstrate dose-responsiveness, nor did they pursue the etiology of these two different responses. By contrast, the results from the acute and subchronic studies reported herein revealed a dose-response relationship between Aroclor 1254 and pentobarbital-induced sleep time. Furthermore, these data may suggest an explanation for this biphasic response. The observation that chronic Aroclor 1254 treatment reduced pentobarbital-induced sleep time is not unexpected. This reduction of sleep time is probably a reflection of induction of pentobarbital-metabolizing enzymes following 14 day administration of Aroclor 1254, inasmuch as an inductive effect of PCBs on microsomal enzymes has been well esta-

blished in the literature (Bruckner et al., 1974, 1977; Litterst et al., 1972; Litterst and van Loon, 1974). On the other hand, the graded response with respect to the interval between the last Aroclor 1254 dose and administration of pentobarbital is rather curious. The reduction in pentobarbital sleep time seen in mice 24 hr after the last Aroclor 1254 treatment was partially reversed in animals who received pentobarbital only 45 min after the last Aroclor 1254 treatment. At this latter time point (45 min), it is conceivable that existing levels of Aroclor 1254 in the liver compete with pentobarbital for the already induced enzymes, thereby partially reversing the inductive effect. Results of the disposition of ^{14}C-pentobarbital following the subchronic Aroclor 1254 regimen further substantiate this claim. Successive 14 day treatments with Aroclor 1254 reduced pentobarbital levels in a pattern paralleling the effect of Aroclor 1254 on sleep time, i.e., levels were lower and sleep time was shorter in the group that received pentobarbital 24 hr after Aroclor 1254 compared to those that received pentobarbital 45 min after Aroclor 1254 in which levels and sleep time were intermediate between the "24 hr/Aroclor 1254" group and vehicle-pretreated mice. The observed liver enlargement in the Aroclor 1254-treated mice lends further support, though not all unexpected, for a metabolic role in the Aroclor 1254-pentobarbital interaction. This finding is consistent with previous reports in the literature (Allen et al., 1976; Bruckner et al., 1974; Grant et al., 1974).

The alternative explanation of this Aroclor 1254-pentobarbital interaction, i.e., that Aroclor 1254 may alter CNS responsiveness, is unlikely in view of the disposition studies. At a time when mice awakened from pentobarbital anesthesia (at the 3 hr time point for

Aroclor 1254-pretreatment and at 45 to 60 min for vehicle-pretreatment, in Figure 9), equivalent levels of pentobarbital were found in brain of both vehicle- and Aroclor 1254-pretreated mice. Therefore, the prolonged sleep times in Aroclor 1254-pretreated mice apparently result from higher levels of pentobarbital in brain and not from a change in sensitivity to the depressant effects of pentobarbital.

Thus, it has been demonstrated that acutely administered Aroclor 1254 enhanced pentobarbital-induced sleep time while subacute administration reduced sleep time. These observations can be attributed to alterations in the pharmacokinetics of pentobarbital. Whereas prolonged Aroclor 1254 exposure appears to increase pentobarbital metabolism by inducing liver microsomal enzymes, a single oral dose of Aroclor 1254 may be depressing pentobarbital metabolism by competing for metabolic sites in the liver. Although Aroclor 1254 appears to exert a depressant effect upon the central nervous system, it apparently does not alter brain sensitivity to pentobarbital.

More important, however, are the implications suggested by these data. Alterations in pentobarbital-induced sleep time and pentobarbital disposition were observed in mice with doses of Aroclor 1254 (5 or 10 mg/kg) that were much lower than those that directly altered behavior (500 mg/kg). These observations may reflect alterations in liver metabolism. If so, low level exposure to Aroclor 1254 might alter biotransformation of other drugs, environmental contaminants, or endogenous substrates that are metabolized by the liver. Such an effect could produce adverse reactions to drug therapy or changes in normal physiological function that depend on liver metabolism.

V.   EXPERIMENT 3 - NEUROCHEMICAL EFFECTS OF AROCLOR 1254

Neurochemistry was chosen as a means of evaluating Aroclor 1254 neurotoxicity for several reasons. Ideally, one would like to correlate observed behavioral changes with alterations in neurochemistry. This is not an easy task, but there is certainly precedence in the literature for the role of neurotransmitters in behavior. For example, serotonin has been implicated in the regulation of sleep, sexual behavior, and aggressive behavior (see Chase and Murphy, 1973), and there is certainly ample evidence that a striatal dopamine deficiency accounts for the motor dysfunction associated with Parkonsonism (see review by Hornykiewicz, 1973). Similarly, neurochemical imbalances have been proposed as causative factors in the behavioral manifestations of schizophrenia (Kety, 1959; Matthysse, 1978) and affective disorders (Schildkraut, 1965, 1978). On a more fundamental level, however, the biochemical basis for the actions of therapeutically useful CNS agents and drugs of abuse has been explored in great detail over the years. One might distinguish between pharmacology and toxicology solely on the basis of dose, as suggested by Paracelsus (1493 - 1541) - "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy." Thus, if neurochemical effects of pharmacologically active CNS agents can be detected, one might also expect that it is feasible to determine neurochemical changes resulting from exposure to a toxic compound for which neurological symptoms have been reported. Thus, based on the behavioral effects of Aroclor 1254 reported here and on the neurological symptoms reported by humans who have been occupationally exposed to PCBs, an evaluation of the effects of Aroclor 1254 on neurochemistry was undertaken.

Because of the diversity of neurochemical parameters evaluated, this chapter is divided into two sections. Part A includes data on neurotransmitter uptake and release and calcium (Ca^{++}) flux in the nerve ending, whereas Part B includes data on nerve terminal ATPases. The effect of Aroclor 1254 within each area will be discussed separately, after which an overall discussion (in General Discussion) will attempt to unify these concepts.

Part A - Effects of Aroclor 1254 on neurotransmitter uptake and release and Ca^{++} flux

I. Introduction

Transmission of electrical impulses within the CNS is essential for CNS function and is dependent upon neurotransmitter function. Any changes in the processes associated with neurotransmission may result in an observable CNS effect. Sites at which pharmacological or toxicological agents may act are depicted in Figure 12. To describe these briefly, neurotransmitters are synthesized and stored within the nerve ending. Release is initiated by a depolarization-induced influx of Ca^{++} and a subsequent increase in intracellular Ca^{++} concentration, $[Ca^{++}]_i$ (Rubin, 1974). Upon release from the nerve ending, the neurotransmitter diffuses across the synaptic cleft to the postsynaptic membrane where it can bind to specific receptors and elicit a response.

Neurotransmission is a temporally discrete event, and therefore, some regulatory system must be present for the fine tuning of this phasic on-off process. Several mechanisms contribute in this regulatory scheme. 1) Remembering that Ca^{++} influx appears to be the trigger for neurosecretion, there must exist some mechanism for the regulation

Figure 12

Schematic model of the biochemical events involved in neuro-

transmission.
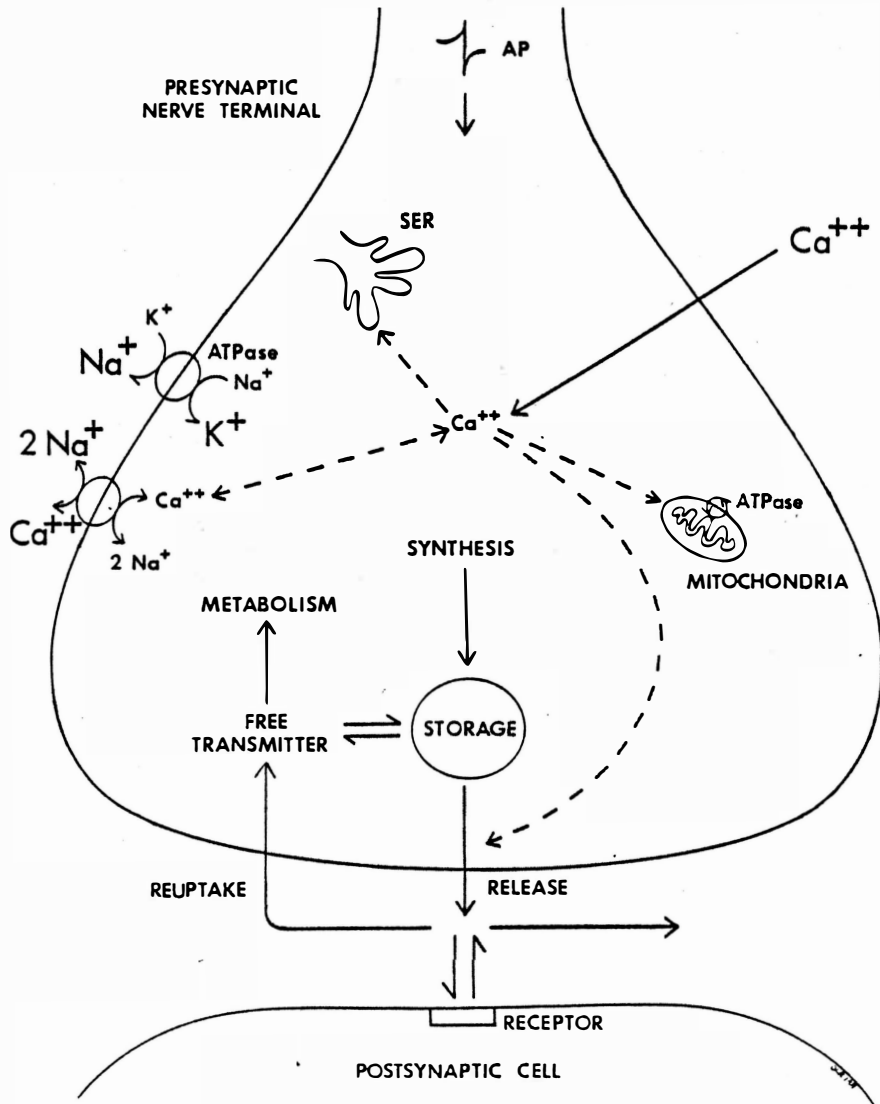
of $[Ca^{++}]_i$ and the termination of transmitter release. 2) An addition-
al mode of regulation of release involves presynaptic receptors on the
nerve terminal membrane (see reviews by Langer, 1977; Starke, 1981;
Westfall, 1977). These receptors are believed to be sensitive to
concentrations of transmitter in the synapse, in that high concentra-
tions inhibit release and low concentrations enhance release. 3) Once
a neurotransmitter has been released, there must be some means for
terminating its action at the postsynaptic site. In the case of the
biogenic amines, a high-affinity active-transport mechanism accounts
for the termination of transmission by re-uptake of the neurotrans-
mitter into the presynaptic neuron, thereby removing it from the synap-
tic cleft and also providing a means for conservation of t: nsmitter,
i.e., this released transmitter can be taken up and reused (Iversen,
1971, 1973). The neurotransmitters, norepinephrine (NE), dopamine
(DA), and serotonin, (5-HT), are taken up by synaptosomes in vitro
(Coyle and Snyder, 1969; Kuhar et al., 1972) as is tryptophan (TP), the
5-HT precursor (Kuhar et at., 1972)..Metabolism is the sole means of
terminating the action of ACh (Eccles et al., 1942; Katz and Thesleff,
1957). Acetylcholine, itself, is not taken up into the presynaptic
terminal, however there is a re-uptake mechanism for Ch (Haga and Noda,
1973; Yamamura and Snyder, 1973), a product of ACh metabolism as well
as its synthetic precursor. 4) Finally, both synaptosomal and mitochon-
drial ATPases may play a role in neurotransmitter re-uptake and release
(see Experiment 3 - Part B).

As mentioned previously, increased $[Ca^{++}]_i$ triggers neurotrans-
mitter release, but following release, $[Ca^{++}]_i$ must be returned to
pre-existing low levels, on the order of $\sim 10^{-7}$ M or less (free cyto-

plasmic concentration). There are a number of different mechanisms involved in this process. As the membrane repolarizes, Ca^{++} probably diffuses away from the active region of the terminal (where secretion occurs). Free cytoplasmic $[Ca^{++}]$ can then be reduced by a number of buffering mechanisms. Calcium may bind to intracellular anions such as ATP and citrate or to cytoplasmic Ca^{++}-binding proteins (Baker and Schlaepfer, 1978). Calcium can also be sequestered by intracellular organelles. Mitochondria have the capacity to accumulate large quantities of Ca^{++} by a respiration-dependent transport process (see Lehninger et al., 1967). However, it appears that nonmitochondrial storage sites are probably the major site of intracellular Ca^{++} sequestration. Evidence has recently been presented for the existence of an ATP-dependent nonmitochondrial Ca^{++}-buffering site (Blaustein et al., 1978a), for involvement of a Ca^{++}/Mg^{++}-ATPase in the sequestration mechanism (Blaustein et al., 1978b), and for identification of smooth endoplasmic reticulum (SER) as the organelle in which Ca^{++} is stored (Blaustein et al., 1980; McGraw et al., 1980). Although SER may play a more important role acutely in buffering $[Ca^{++}]_i$, mitochondria may still represent an important storage site from which Ca^{++} can be released. Finally, free cytoplasmic Ca^{++} can be extruded from the nerve terminal by a Na^+/Ca^{++}-exchange mechanism (Blaustein and Ector, 1976; Blaustein and Hodgkin, 1969; Blaustein and Oborn, 1975).

This discussion of the neurochemistry of the nerve ending may be summarized by considering the sites at which a drug or toxic compound may interact to alter neurotransmission, as seen in Figure 12. Some of these potential interactions are 1) inhibition of synthesis, 2) interference with storage, 3) enhanced or reduced release, 4) inhibition of

re-uptake, 5) agonist or antagonist effects at the postsynaptic receptor, 6) inhibition of metabolism, 7) enhanced or depressed Na^+/K^+-ATPase activity, and 8) alteration of $[Ca^{++}]_i$ by any of a number of mechanisms.

The objective of the experiments to be described here was to determine the effect of Aroclor 1254 on neurochemical function.

B. <u>Methods</u>

1. <u>Tissue preparation</u>

Synaptosomes and mitochondria were obtained by subcellular frac-
tionation of mouse brain following the method of McGovern et al. (1973).
Mice were decapitated, and the brains were removed rapidly and homoge-
nized in 0.32 M sucrose containing 5.0 mM HEPES (N-2-hydroxyethyl-N-2-
ethane-sulfonic acid) that had been adjusted to pH 7.4 with 1.0 M Tris
(2-amino-2-hydroxymethylpropane-1,3-diol). Crude mitochondrial pellets
were prepared by centrifugation (18,000 x g for 20 min) of the 1000 x g
(10 min) supernatant of the homogenate. The resulting pellets were
subsequently subfractionated on a discontinuous Ficoll floatation
gradient which was centrifuged at 82,500 x g (at r_{av} = 11.8 cm) for 90
min. Synaptosomes (banding at the 7.5% - 16% Ficoll interface) and
mitochondrial pellets were recovered and resuspended in appropriate
media. The buffered salt solution (BSS) for synaptosomes consisted of
120 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10
mM glucose, and 20 mM HEPES which was adjusted to pH 7.4 with 1.0 mM
Tris. Mitochondrial buffer (pH 7.4) contained 250 mM sucrose, 50 mM
KCl, 1.0 mM NaH_2PO_4, 0.2 mM $CaCl_2$, and 2.0 mM HEPES. This purified
fraction of synaptosomes was employed for all assays except the synap-
tosomal neurotransmitter and precursor uptake assay in which a crude
synaptosomal preparation was used (see below). Protein content of
tissue samples was determined by the method of Bradford (1976). The
standard calibration for protein determination is given in the Appendix
(Figure 34).

2. Synaptosomal ^{3}H-neurotransmitter and precursor uptake

Uptake of radiolabeled neurotransmitters and their precursors in a crude synaptosomal preparation was assessed according to the method of Coyle and Snyder (1969). 3,4-{ethyl-1-^{3}H(N)}-dihydroxyphenylethylamine tartrate (^{3}H-DA, 16.6 Ci/mmol), 5-{1,2,-^{3}H(N)}-hydroxytryptamine bi-noxylate (^{3}H-5-HT, 27.4 Ci/mmol) and {methyl-^{3}H}-choline (^{3}H-Ch, 80 Ci/mmol) were purchased from New England Nuclear (Boston, MA). 1-{ethyl-1,2-^{3}H(N)}-norepinephrine (^{3}H-NE, 35 Ci/mmol) was purchased from Amersham Searle. ^{3}H-tryptophan (^{3}H-TP, 5.6 Ci/mmol) was obtained from Schwarz/Mann. Mouse forebrain was homogenized in 0.32 M sucrose and centrifuged at 1000 x $\underline{g}$ for 10 min. The resulting supernatant yielded a crude synaptosomal suspension. Aliquots of the suspension were preincubated at 37° C for 5 min in the presence of vehicle or various concentrations of test drug (10^{-7} to 10^{-4} M Aroclor 1254 or 10^{-8} to 10^{-4} M imipramine) in a modified Krebs-Henseleit (1932) bicarbonate medium (K-H buffer). The K-H buffer (pH 7.4) was modified to contain Ca^{++} (1.3 mM), glucose (11 mM), ethylenediamine tetraacetic acid (0.13 mM), and ascorbic acid (1.1 mM). Pargyline (125 μM) was also added in appropriate experiments to inhibit monoamine oxidase. Uptake was then initiated by the addition of radiolabeled transmitter or precursor (0.1 μCi). Final concentrations of these compounds were 25 nM ^{3}H-5-HT, 50 nM ^{3}H-DA, 10 μM ^{3}H-TP and 0.5 μM ^{3}H-Ch. Incubations proceeded for an additional 5 min. The reaction was then terminated by addition of ice cold 0.9% NaCl and centrifugation at 20,000 x $\underline{g}$ for 20 min. Pellets were washed once with cold K-H buffer, recentrifuged, and solubilized in 0.5 N NaOH by heating at 100° C for 30 min. Digestates were neu-tralized with 0.5 N HCl and counted in 10 ml of ACS. Samples were

corrected for nonspecific uptake by measuring uptake of ^{3}H-NE, ^{3}H-DA, and ^{3}H-5-HT at 2° C and of ^{3}H-TP and ^{3}H-Ch in the presence of excess substrate.

To determine the effects of in vivo exposure to Aroclor 1254 on neurotransmitter and precursor re-uptake, mice were given vehicle or a single dose of Aroclor 1254 (30 or 300 mg/kg). Mice were sacrificed 45 min later, and a crude synaptosomal fraction was prepared from each brain for uptake assays. On a separate day, mice were given vehicle or a dose of 500 mg/kg of Aroclor 1254, and uptake was deter ned.

In a second experiment, the effect of subchronic Aroclor 1254-treatment on uptake was determined in 4 groups of mice. The naive group received no treatment but was handled in the same manner as all other groups over the 90 day period. The remaining three groups of mice received vehicle or Aroclor 1254 (0.3 or 3 mg/kg) by oral gavage for 90 consecutive days. These doses of Aroclor 1254, 0.3 and 3 mg/kg, correspond to 3,000 and 30,000 times, respectively, the estimated average daily human consumption of PCBs in drinking water. On day 91, 24 hr after the last dose, mice were sacrificed and uptake of neurotransmitters and precursors was determined.

3. Synaptosomal ^{3}H-neurotransmitter release

Tritiated neurotransmitter or precursor was incubated with the synaptosomal suspension at 37° C in order to label neurotransmitter pools. Concentrations of ^{3}H-neurotransmitters were always less than the K_m's for high affinity uptake (~10^{-7} M). Incubations with ^{3}H-DA (6 x 10^{-8} M) and ^{3}H-5-HT (3.6 x 10^{-8} M) proceeded for 10 min. Synaptosomes were loaded with ^{3}H-NE during a 30 min incubation in the presence

of 1.0 mM ascorbic acid; final concentration of ^{3}H-NE was 2.9 x 10^{-8} M. ^{3}H-Ch, the precursor used to label ACh stores, was incubated with synaptosomes (at a final concentration of 8 x 10^{-8} M) for 30 min. Uptake was terminated by addition of ice cold BSS. The labeled synaptosomes were centrifuged at 18,000 x g for 10 min, washed once with ice cold BSS, and resuspended in BSS to yield a final protein concentration of 0.5 mg/ml. Aliquots of the synaptosomal suspension were incubated with vehicle or various concentrations of test drug (10^{-7} to 10^{-3} M Aroclor 1254 or positive control) for 2 min. Spontaneous neurotransmitter release was then initiated by the addition of prewarmed (37° C) BSS (containing 5 mM KCl). Depolarization-evoked release was initiated by addition of prewarmed Na-free BSS containing high [K^+] to yield a final concentration of 60 mM KCl. Incubation was continued for 10 min, following which release was terminated by addition of Mg^{++}, Ca^{++}-free BSS containing 3.0 mM EGTA (ethyleneglycol-bis-{β-aminoethyl ether}-N-N'-tetraacetic acid). Suspensions were centrifuged immediately at 18,000 x g for 10 min. Aliquots of supernatant were counted in 10 ml of ACS for determination of the amount of ^{3}H-neurotransmitter released.

4. Synaptosomal $^{45}Ca^{++}$ uptake

The method described by Blaustein and Weisman (1970) was used to assess synaptosomal $^{45}Ca^{++}$ uptake. Synaptosomes were prepared as described above, and aliquots were preincubated in the presence of vehicle or various concentrations of test drug (10^{-6} to 10^{-4} M Aroclor 1254 or positive control) for 2 min at 37° C. Uptake was then initiated by adding prewarmed BSS containing 2.0 μCi $^{45}Ca^{++}$ ($^{45}CaCl_2$, New England Nuclear, 15-20 Ci/mmol). Final K^+ concentrations of 5 mM KCl

and 60 mM KCl were used to simulate spontaneous and depolarization-
evoked influx, respectively. Following a 10 min incubation, influx was
terminated by addition of ice cold BSS-EGTA and immediate centrifuga-
tion at 18,000 x $\underline{g}$ for 10 min. Synaptosomal pellets were washed once
with Ca^{++}-free BSS, solubilized as described for neurotransmitter up-
take studies, and counted in 10 ml ACS to determine synaptosomal $^{45}Ca^{++}$
content.

5. Mitochondrial $^{45}Ca^{++}$ uptake

The technique of Reed and Bygrave (1975) was modified for studying
$^{45}Ca^{++}$ uptake in brain mitochondria. Mitochondria were isolated and
resuspended in mitochondrial buffer, as described above, to yield a
protein concentration of 0.7 mg/ml. Preincubation of aliquots of
mitochondrial suspensions proceeded for 1 min at 30° C in the presence
of vehicle or various concentrations of test drug (10^{-7} to 10^{-3} M
Aroclor 1254 or positive control). Uptake was initiated by adding
prewarmed buffer containing 1.0 μCi $^{45}Ca^{++}$. Uptake was assessed under
basal conditions (assay medium containing no mitochondrial substrate)
or in the presence of 2.0 mM succinate or 800 μM ATP. The reaction was
terminated by adding ice cold Ca^{++}-free basal mitochondrial buffer
containing 1.0 mM EGTA. Suspensions were centrifuged immediately at
18,000 x $\underline{g}$ for 4 min. The mitochondrial pellets were washed once with
EGTA buffer before the $^{45}Ca^{++}$ content was measured as described for
synaptosomal $^{45}Ca^{++}$ uptake studies.

C. Results

1. Synaptosomal ^{3}H-neurotransmitter and precursor uptake

Evaluation of the effects of Aroclor 1254 on neurochemical parameters began with an assessment of synaptosomal neurotransmitter and precursor uptake.

Addition of Aroclor 1254 to the incubation media of isolated synaptosomes produced a concentration-dependent inhibition of neurotransmitter (NE, DA, and 5-HT; Figure 13) and precursor uptake (Figure 14). These results can be summarized and expressed as IC_{50} values for Aroclor 1254 as calculated from the means of replicate experiments and defined as the concentration of Aroclor 1254 that inhibited uptake by 50% relative to control. IC_{50} values for Aroclor 1254 for inhibition of NE, DA, 5-HT, and Ch uptake were 36.3, 33.6, 47.9, 92.2 μM, respectively. An IC_{50} value for inhibition of TP uptake was not calculated, as uptake was inhibited only 23% at a concentration of 10^{-4} M Aroclor 1254. From these data, it appears that the neurotransmitters were more sensitive to this inhibitory effect than the precursors.

The tricyclic antidepressant, imipramine, was used as a positive control in these studies. As expected, imipramine inhibited neurotransmitter uptake in a dose-dependent fashion (Figure 15). There was little effect on uptake of the precursors, Ch and TP; 10^{-4} M imipramine produced only 28 and 24% inhibition, respectively, of Ch and TP uptake. IC_{50} values for imipramine, calculated as described above, for inhibition of NE, DA, and 5-HT uptake were 10.7, 21.4, and 0.13 μM, respectively, indicating that catecholamine uptake was 2 orders of magnitude less sensitive to the effects of imipramine than was 5-HT uptake.

Figure 13

Concentration-dependent inhibition of mouse brain synaptosomal uptake of ^{3}H-neurotransmitters by Aroclor 1254. DA, ^{3}H-DA (▲). 5-HT, ^{3}H-5-HT uptake (●). NE, ^{3}H-NE uptake (▣). S, saline control. E, EtOH vehicle for Aroclor 1254. Values are expressed as mean ± S.E. of data from 3 experiments; samples were replicated 6 times within each experiment. Where S.E. bars are not shown, values lie within the bounds of the symbol. Least squares linear regression analysis yielded correlation coefficients of -0.9042, -0.9735, and -0.8414, respectively, for DA, 5-HT, and NE.
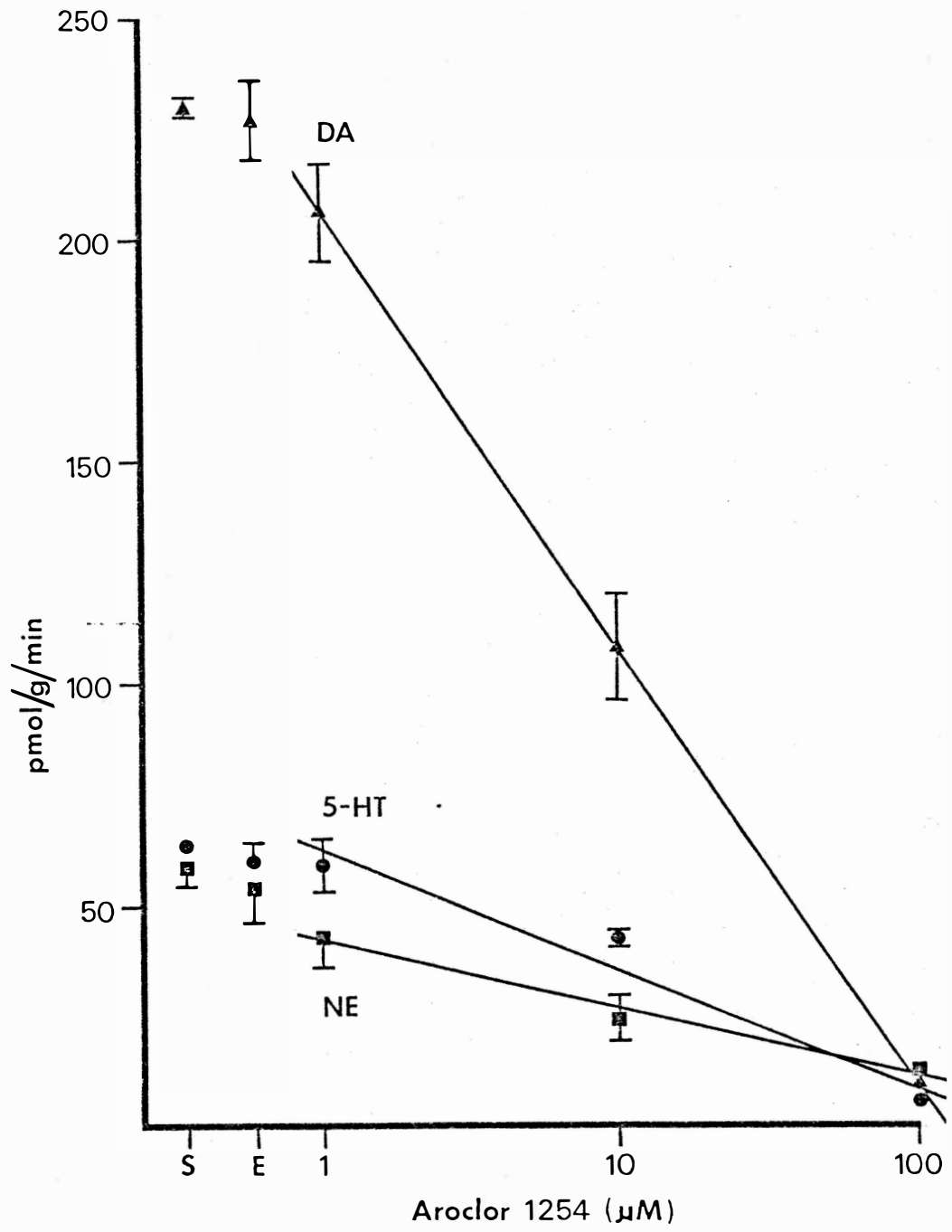
Figure 14

Concentration-dependent inhibition of mouse brain synaptosomal uptake of ^{3}H-precursors by Aroclor 1254. Ch, ^{3}H-Ch uptake (▲). TP, ^{3}H-TP uptake (●). S, saline control. E, EtOH vehicle for Aroclor 1254. Values are expressed as mean ± S.E. of data from 3 experiments; samples were replicated 6 times within each experiment. Least squares linear regression analysis yielded correlation coefficients of -0.9686 and -0.9362, respectively, for Ch and TP.
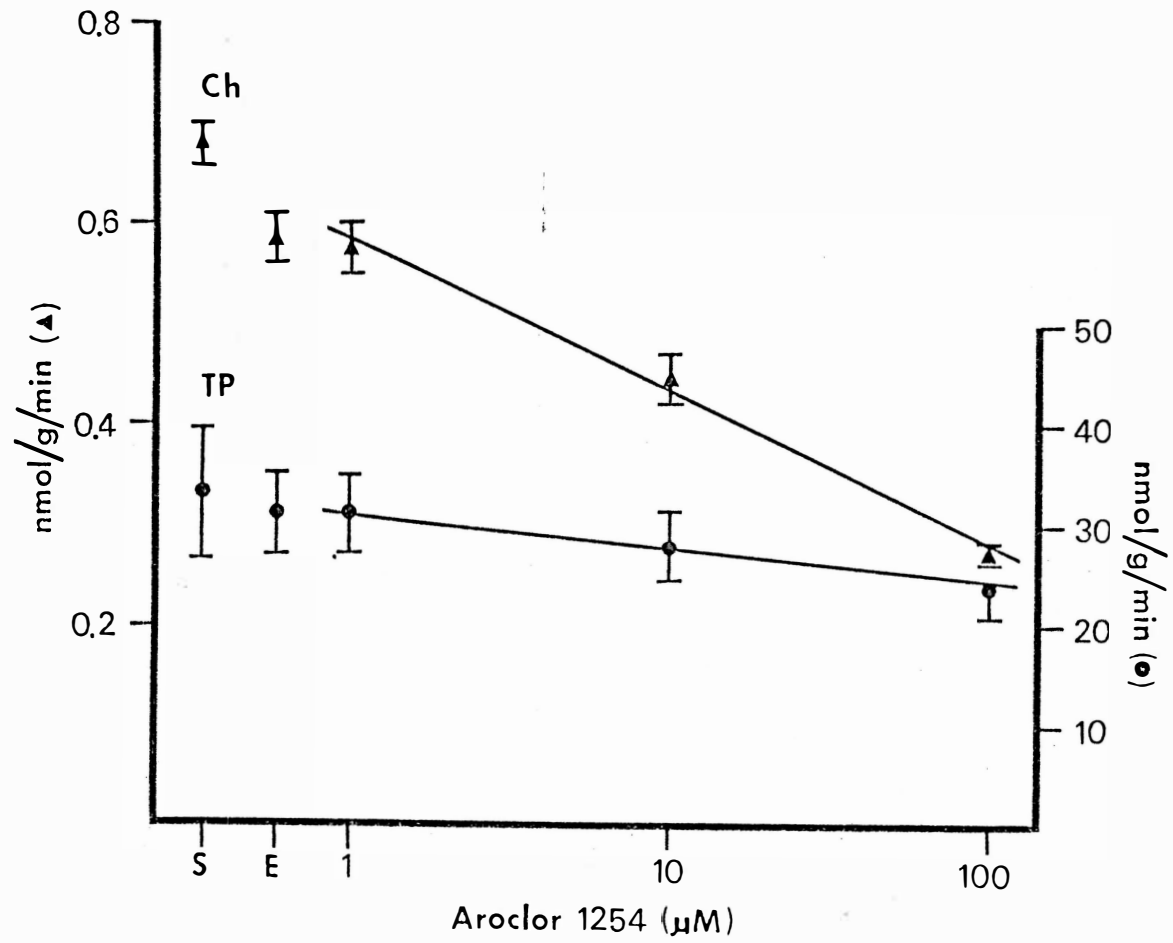
Figure 15

Effect of the tricyclic antidepressant, imipramine, on uptake of ^{3}H-neurotransmitters by mouse brain synaptosomes. S, saline control. NE, ^{3}H-NE uptake (▲). Values represent mean ± S.E. (N = 6 replicates) of data from one experiment. The experiment was repeated twice with similar results. DA, ^{3}H-DA uptake (▨). Values represent mean ± S.E. (N = 6 replicates) of data from one experiment. The experiment was repeated 3 times with similar results. 5-HT, ^{3}H-5-HT uptake (●). Values represent mean ± S.E. of data from 4 experiments; samples were replicated 6 times within each experiment. Where S.E. values are not shown, values lie within the bounds of the symbol. Least squares linear regression analysis yielded correlation coefficients of -0.9617, -0.9642, and -0.8339, respectively, for NE, DA, and 5-HT.
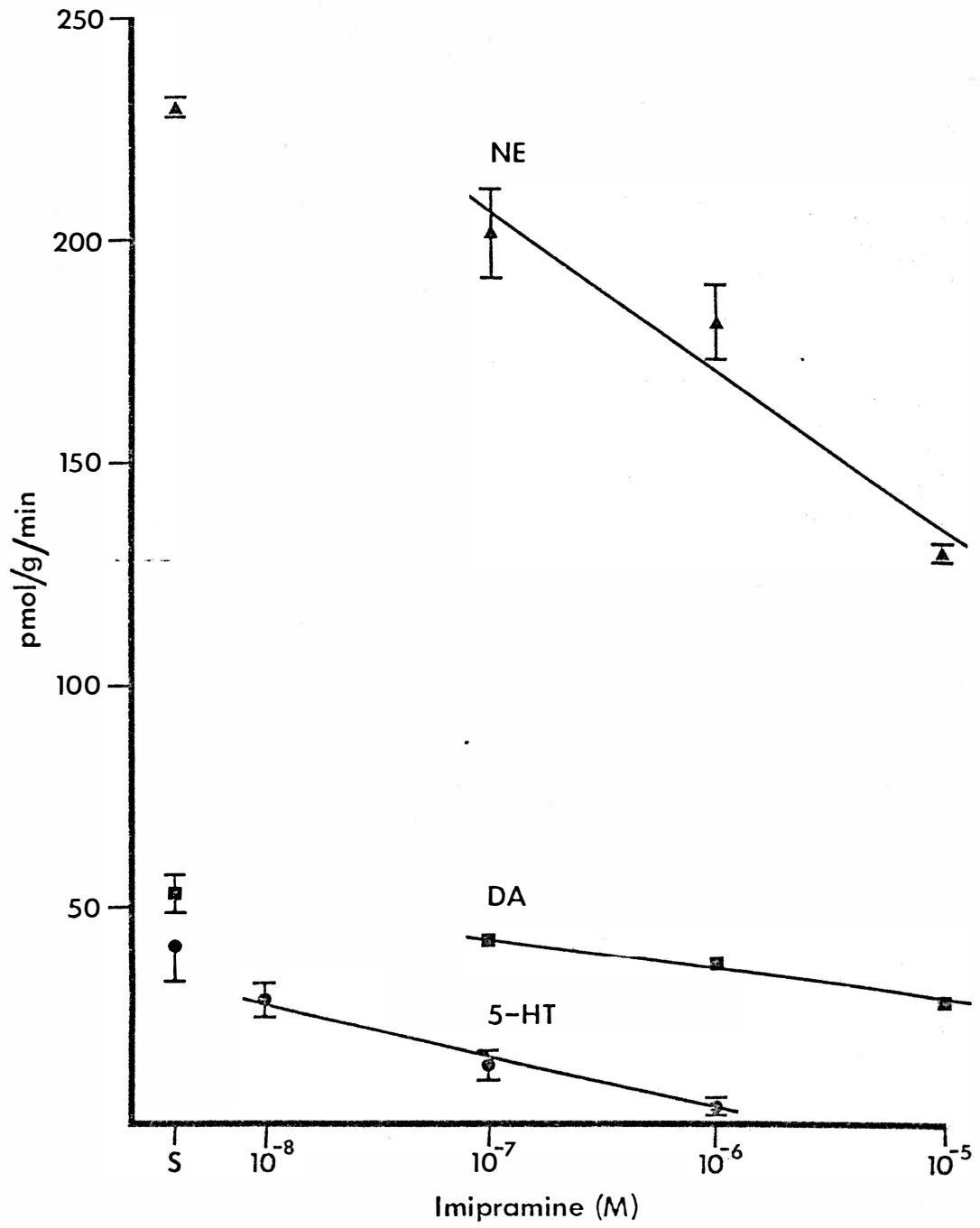
The next two experiments were executed to determine whether effects on uptake could be demonstrated following in vivo exposure to Aroclor 1254. The first experiment involved the acute administration of vehicle or Aroclor 1254. As seen in Table 4, uptake into synaptosomes from Aroclor 1254-treated mice was no different from that in appropriate controls. The value for 5-HT uptake following vehicle administration was higher on the second day than on the first. Still, Aroclor 1254 (500 mg/kg) had no effect on 5-HT uptake when compared to its paired control (vehicle on the same day).

The second experiment was a 90-day study involving 4 groups of mice. Uptake was unaltered by 90-day Aroclor 1254-treatment as compared to the vehicle, nor was there any difference between the naive and vehicle-treated mice (Table 5).

2. Synaptosomal ^{3}H-neurotransmitter release

To further examine the neurochemical effect of Aroclor 1254 that was observed in vitro, neurotransmitter release from isolated synaptosomes was evaluated in the presence of Aroclor 1254. It was found that Aroclor 1254 enhanced the spontaneous release (in the presence of 5 mM KCl, i.e., resting state) of ^{3}H-NE, ^{3}H-DA, ^{3}H-5HT and ^{3}H-ACh (Figures 16, 17, 18, and 19) in a concentration-dependent manner. Summarizing these results, EC_{50} values (calculated from replicate experiments and defined as the concentration of Aroclor 1254 that enhanced release by a value of 50% of control) are 34.2, 31.0, 61.2, and 86.1 μM for NE, DA, 5-HT, and ACh release, respectively. These data indicate that the four neurotransmitters examined are equally sensitive to the effects of Aroclor 1254. By contrast, Aroclor 1254 was without effect on potassium-

TABLE 4

Neurotransmitter Uptake by Mouse Brain Synaptosomes
Following Acute Oral Administration of Aroclor 1254

$pmol/g/min^a$

Treatment	Norepinephrine	Dopamine	Serotonin
Vehicle[b]	47 ± 1	110 ± 11	43 ± 3
Aroclor 1254[b], 30 mg/kg	47 ± 4	104 ± 18	42 ± 5
Aroclor 1254[b], 300 mg/kg	49 ± 3	94 ± 5	43 ± 2
Vehicle	45 ± 4[c]	95 ± 6[d]	74 ± 8[d]
Aroclor 1254[d], 500 mg/kg	47 ± 6	91 ± 3	64 ± 4

[a] Mean ± S.E.

[b] N = 3 mice

[c] N = 5 mice

[d] N = 6 mice

TABLE 5

The Effects of 90 Daily Gavages of Aroclor 1254 on the Uptake Of Neurotransmitters and Precursors By Mouse Brain Synaptosomes

pmole/g/min[a]

Treatment	Serotonin	Dopamine	Norepinephrine	Choline	Tryptophan
Naive	49 ± 2	60 ± 1	22 ± 3	457 ± 41	26,400 ± 3400
Vehicle	46 ± 2	65 ± 5	24 ± 2	456 ± 55	29,400 ± 2800
Aroclor 1254, 0.3 mg/kg	46 ± 3	61 ± 3	24 ± 2	449 ± 19	26,000 ± 2500
Aroclor 1254, 3.0 mg/kg	46 ± 2	63 ± 4	27 ± 3	434 ± 56	26,900 ± 2500

[a] Values represent the mean ± S.E. for determinations from 6 mice

Figure 16

Concentration-dependent enhancement of basal release of ^{3}H-NE from mouse brain synaptosomes by Aroclor 1254. C, control (▨). E, EtOH vehicle (▲) for Aroclor 1254 (◕) and A23187 (10^{-6}; ◆). Values represent mean ± S.E. of data from 4 experiments; each sample was replicated 4 times within each experiment. Closed symbols, basal release (in the presence of 5 mM KCl). Open symbols, depolarization-evoked release (in the presence of 60 mM KCl). Least squares linear regression analysis of the Aroclor 1254 concentration-effect relation-ship yielded a correlation coefficient of 0.9694.
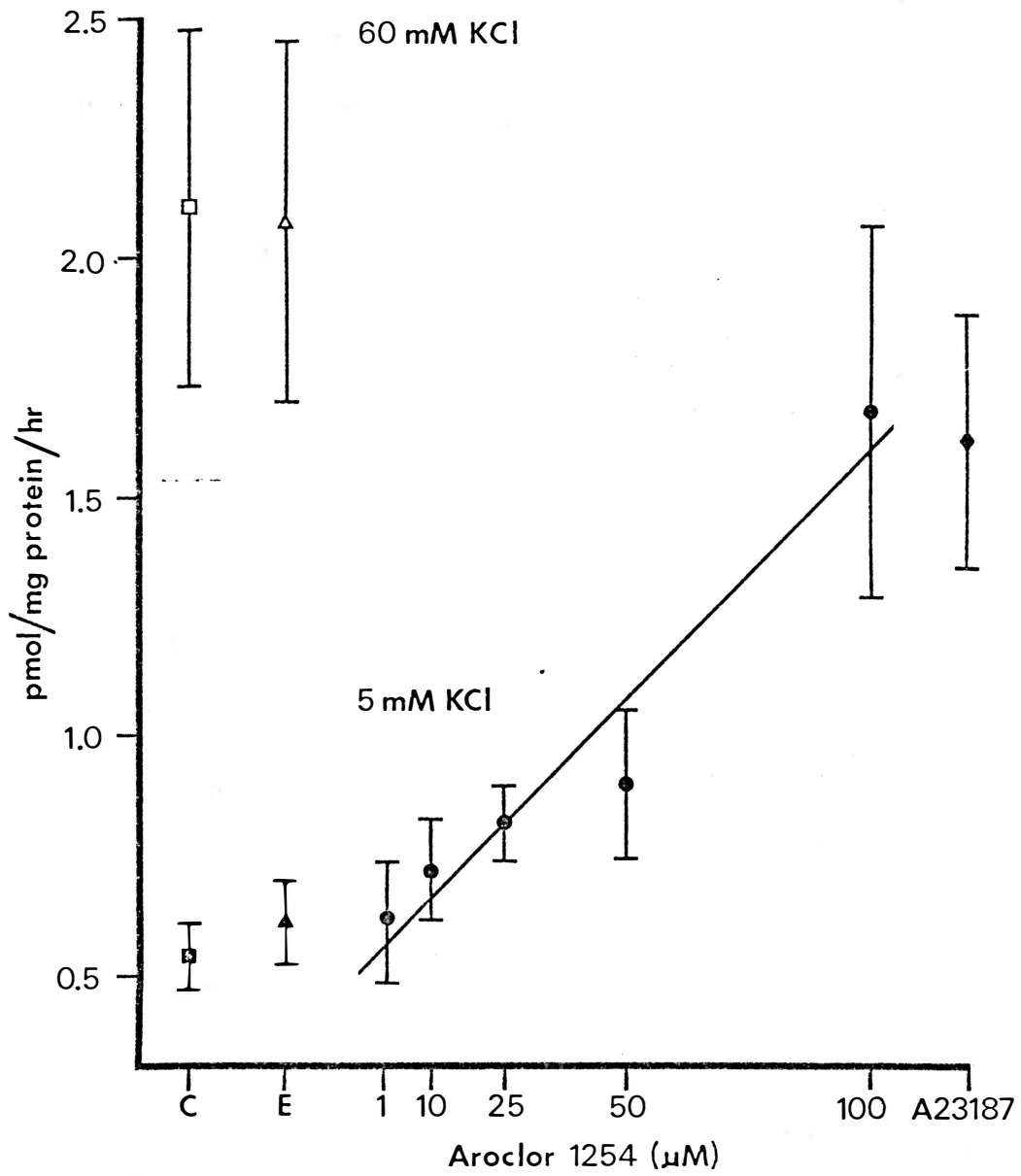
Figure 17

Concentration-dependent enhancement of basal release of ^{3}H-DA from

mouse brain synaptosomes by Aroclor 1254. Aroclor 1254 (⬤). K,

kepone (10^{-5} M; ▧). A23187 (10^{-6} M; ▲). Values represent mean ±

S.E. of data from 4 experiments; samples were replicated 4 times within

each experiment. Ordinate, effects are expressed as percent of release

observed in the presence of EtOH vehicle (EtOH had no effect of its own

compared to control). Values for absolute release (pmol equivalents of

transmitter/mg protein/hr) were therefore normalized to paired EtOH

vehicle controls (except for the 60 mM KCl value which is expressed as

percent of the 5 mM KCl value). No S.E. bars are shown for the kepone

point, because this corresponds to results of only one experiment. Ab-

solute values of basal dopamine release under control conditions were

approximately 1 pmol/mg protein/hr. Least squares linear regression

analysis of the Aroclor 1254 concentration-effect relationship yielded

a correlation coefficient of 0.9979.
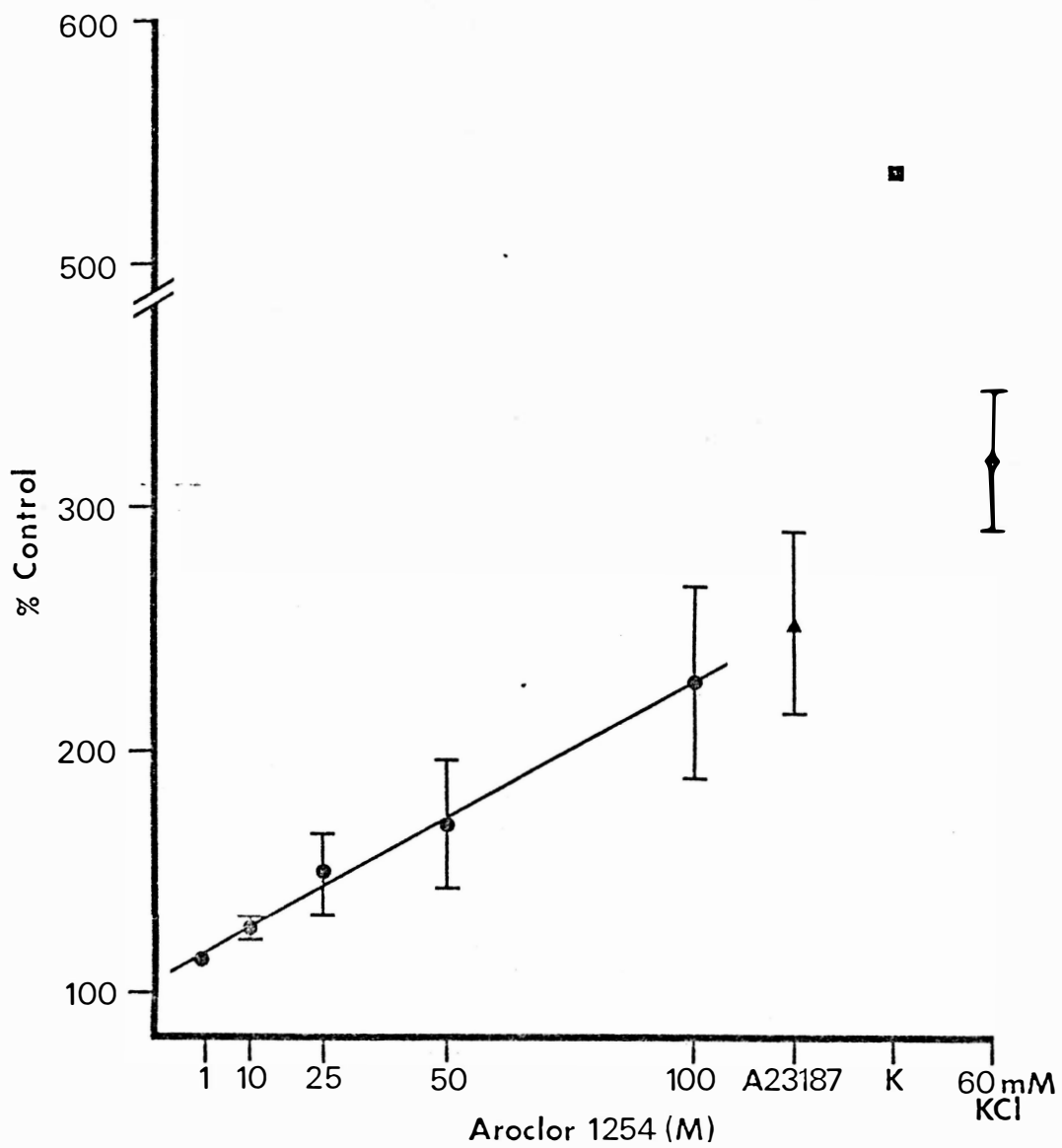
Figure 18

Concentration-dependent enhancement of basal and depolarization-evoked

release of ^{3}H-5-HT from mouse brain synaptosomes by Aroclor 1254.

C (■), control. E (▲), EtOH vehicle for Aroclor 1254 (●) and 10^{-6} M

A23187 (♦). Values represent mean ± S.E. (N = 4 replicates) of data

from 1 experiment; the experiment was repeated twice with similar

results. Closed symbols, basal release (in the presence of 5 mM KCl).

Open symbols, depolarization-evoked release (in the presence of 60 mM

KCl). Least squares linear regression analysis of the Aroclor 1254

concentration-effect relationships yielded correlation coefficients of

0.7725 and 0.9742 for the 5 and 60 mM KCl conditions, respectively.
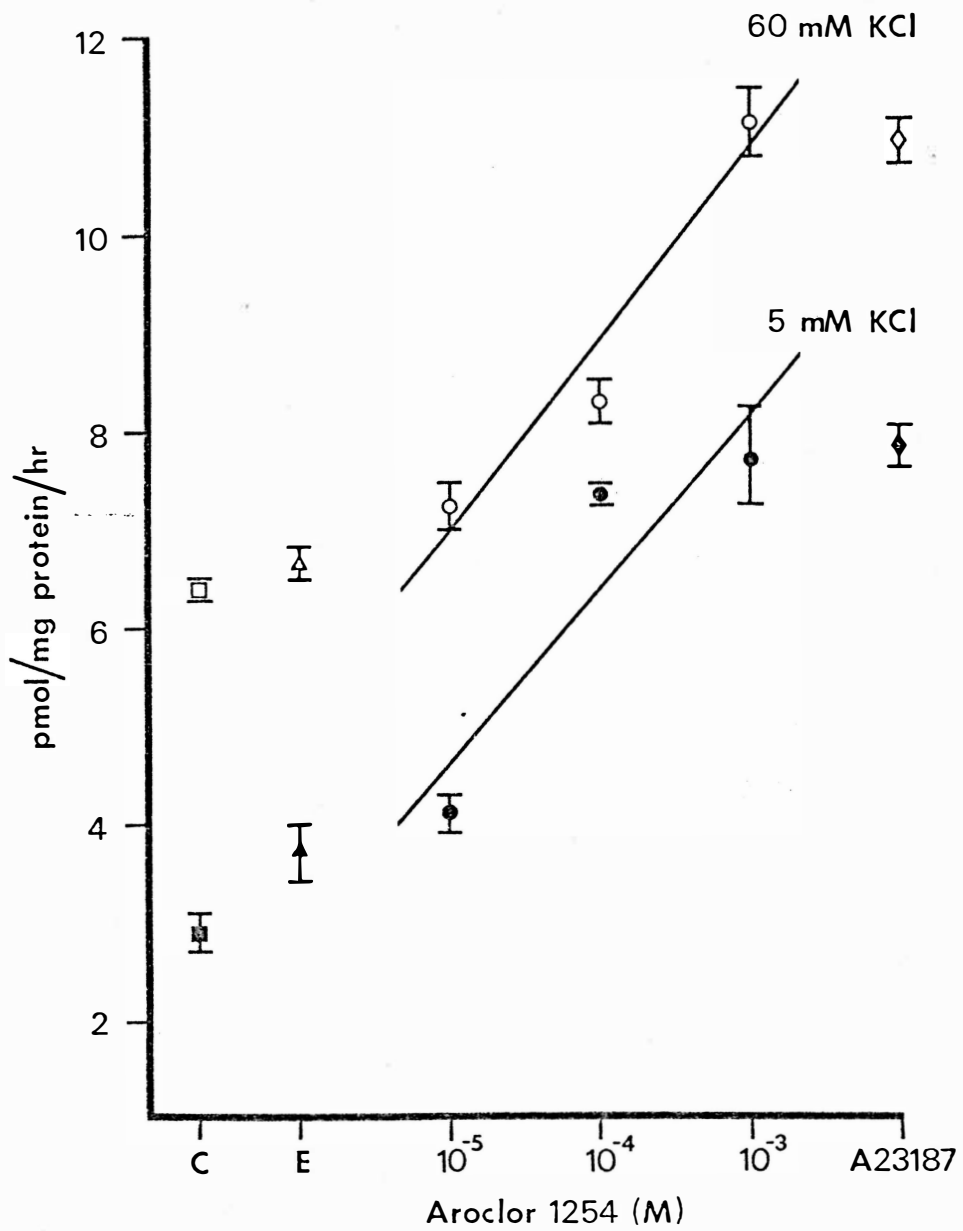
Figure 19

Concentration-dependent enhancement of basal release of ^{3}H-ACh from mouse brain synaptosomes by Aroclor 1254. C (■), control. E (▲), EtOH vehicle for Aroclor 1254 (●) and 10^{-6} M A23187 (◆). Values represent mean ± S.E. (N = 4 replicates) of data from 1 experiment; the experiment was repeated twice with similar results. Closed symbols, basal release (in the presence of 5 mM KCl). Open symbols, depolarization-evoked release (in the presence of 60 mM KCl). Least squares linear regression analysis of the Aroclor 1254 concentration-effect relationship yielded a correlation coefficient of 0.9621.
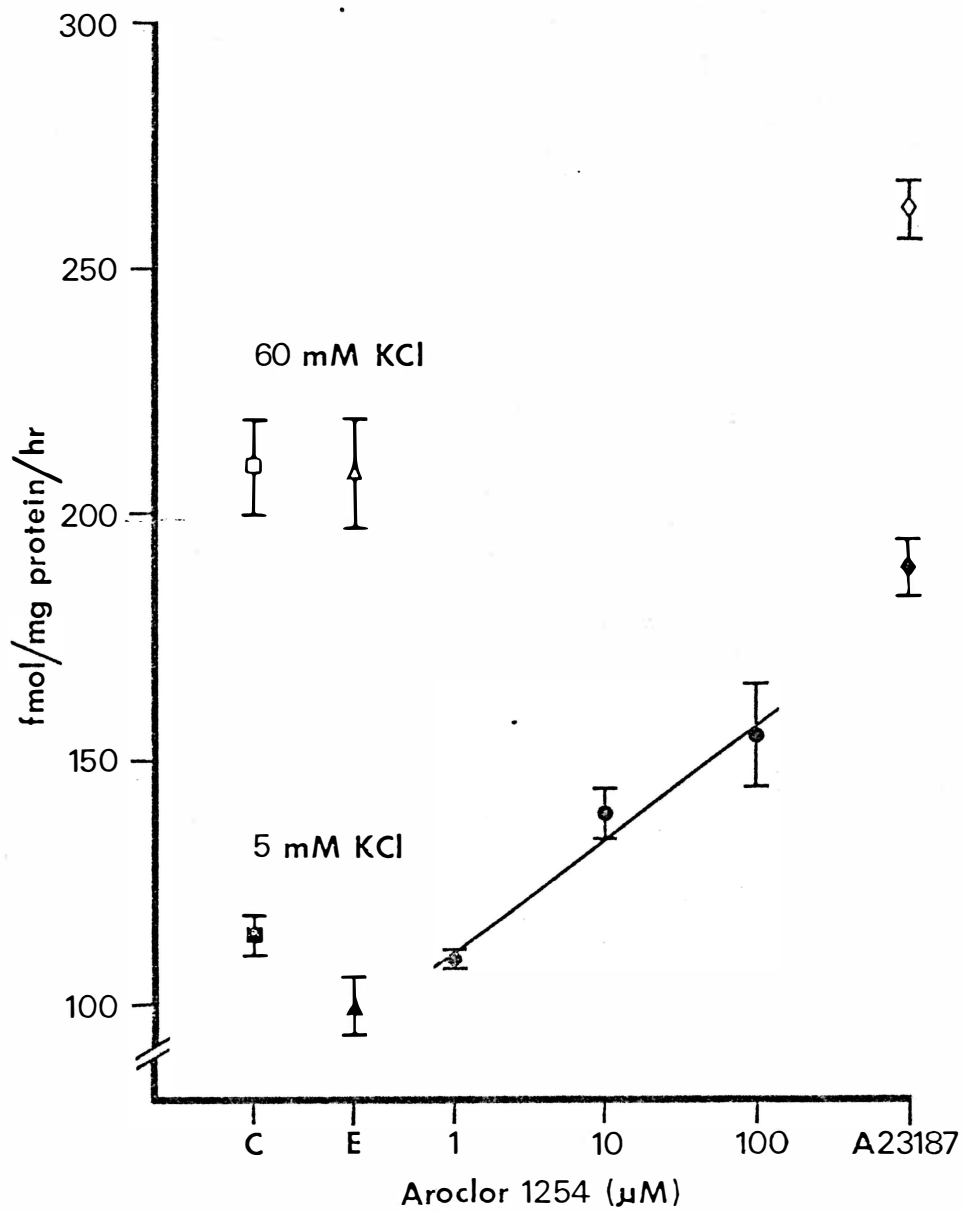
induced release (i.e., depolarization-evoked release in the presence of 60 mM KCl) of ^{3}H-NE, ^{3}H-DA, or ^{3}H-ACh. However, larger concentrations of Aroclor 1254 did enhance depolarization-evoked release of 5-HT (Figure 18) yielding an EC_{50} value of 7.46 x 10^{-4} M Aroclor 1254. It should be noted that ACh release measured by the method described here is not a specific measure, i.e. the total radioactivity released from the synaptosome may be a reflection of the release of ^{3}H-Ch plus ^{3}H-ACh. This limitation of the technique will be addressed in the Discussion.

In order to validate the method used for the release assay, a number of positive controls were included in each experiment. Chlordecone (Kepone$^®$, Life Science Products Co., Hopewell, VA), another chlorinated hydrocarbon, has been reported to enhance neurotransmitter release from rat brain synaptosomes (End, 1978). Similar results were found using mouse brain synaptosomes. Chlordecone (10^{-5} M) produced a 2-, 5-, and 8-fold increase in spontaneous release of ^{3}H-5-HT, ^{3}H-DA, and ^{3}H-NE, respectively, and a 1.5- to 2-fold enhancement of depolarization-evoked (60 mM KCl) release of these neurotransmitters.

The calcium ionophore, A23187, has also been shown to enhance neurotransmitter release by virtue of its ability to transport Ca^{++} across membranes. The result is an increased availability of intracellular Ca^{++} for neurosecretion (Colburn et al., 1976; Holz, 1975). As with chlordecone, A23187 (10^{-6} M) enhanced both spontaneous and potassium-induced transmitter release (2- to 3-fold and 1- to 1.5-fold, respectively). Selected examples of this effect of ionophore and of chlordecone are shown in Figures 16, 17, 18, and 19. The EtOH vehicle used for Aroclor 1254, chlordecone, and A23187 had no effect on release (final concentration of EtOH was 17 µM).

3. Synaptosomal $^{45}Ca^{++}$ uptake

The requirement of Ca^{++} for neurotransmitter release prompted $^{45}Ca^{++}$ flux studies in isolated mouse brain synaptosomes and mitochondria. Calcium influx in both resting and depolarized states was simulated by measuring $^{45}Ca^{++}$ uptake by synaptosomes in vitro. In the presence of 60 mM KCl, $^{45}Ca^{++}$ uptake was enhanced 2-fold from resting levels. Chlordecone (10^{-5} M) significantly inhibited both basal and potassium-stimulated $^{45}Ca^{++}$ uptake (Figure 20). Resting uptake of $^{45}Ca^{++}$ was enhanced only 20% by 10^{-4} M Aroclor 1254 and potassium-stimulated uptake was unaffected.

4. Mitochondrial $^{45}Ca^{++}$ uptake

Energy-dependent $^{45}Ca^{++}$ transport in mitochondria was examined in the presence or absence of substrate (succinate or ATP). Basal uptake of $^{45}Ca^{++}$ was stimulated 2.5- and 7.5-fold in the presence of ATP (800 μM) and succinate (2 mM), respectively (Table 6). The EtOH vehicle had no effect on $^{45}Ca^{++}$ uptake. Two mitochondrial inhibitors were used as positive controls. Both succinate and ATP-stimulated $^{45}Ca^{++}$ uptake were inhibited (~50%) by 10^{-5} M 2,4-dinitrophenol, a mitochondrial uncoupler, whereas only the ATP-stimulated $^{45}Ca^{++}$ uptake was inhibited (~50%) by the Mg^{++}-ATPase inhibitor, oligomycin, at a concentration of 10^{-6} M. Basal and succinate-supported $^{45}Ca^{++}$ uptake were not altered by Aroclor 1254 in concentrations up to 10^{-4} M. However, Aroclor 1254 enhanced ATP-supported $^{45}Ca^{++}$ uptake (Figure 21). Linear regression analysis of the dose-response curve yielded an EC_{50} value of 2.8 x 10^{-4} M.

Figure 20

Effect of Aroclor 1254 on synaptosomal $^{45}Ca^{++}$ uptake measured in the presence of 5 mM or 60 mM KCl. Values represent mean ± S.E. (N = 4 replicates) of data from one experiment; the experiment was repeated twice with similar results. Analysis of variance and Dunnett's t-test revealed significance as *, $p < .05$; **, $p < .005$.
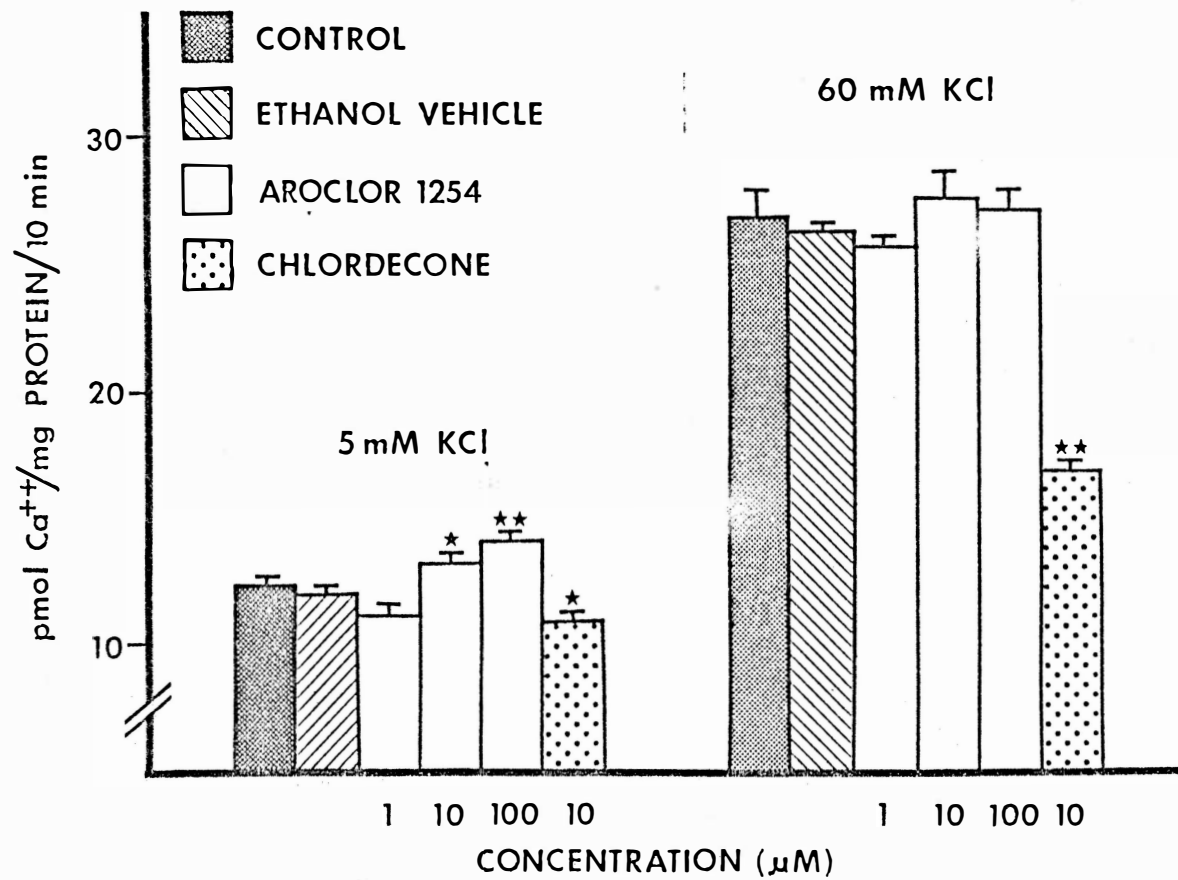
TABLE 6

Uptake Of $^{45}Ca^{++}$ By Mitochondria
Isolated From Mouse Brain

pmol Ca^{++}/mg protein/10 min

	Basal	800 μM ATP	2 mM Succinate
Control	9.3 ± 0.2	22.8 ± 1.1	69.7 ± 3.3
Ethanol	8.9 ± 0.2	17.9 ± 0.7	52.0 ± 1.9

Figure 21

Effect of Aroclor 1254 on mitochondrial $^{45}Ca^{++}$ uptake in the presence

of 800 μM ATP. C, control. E, EtOH vehicle. Values represent mean ±

S.E. (N = 6 replicates) of data from one experiment; the experiment was

repeated 3 times with similar results. Analysis of variance and

Dunnett's t-test revealed significance as *, p < .01 compared to EtOH

control.
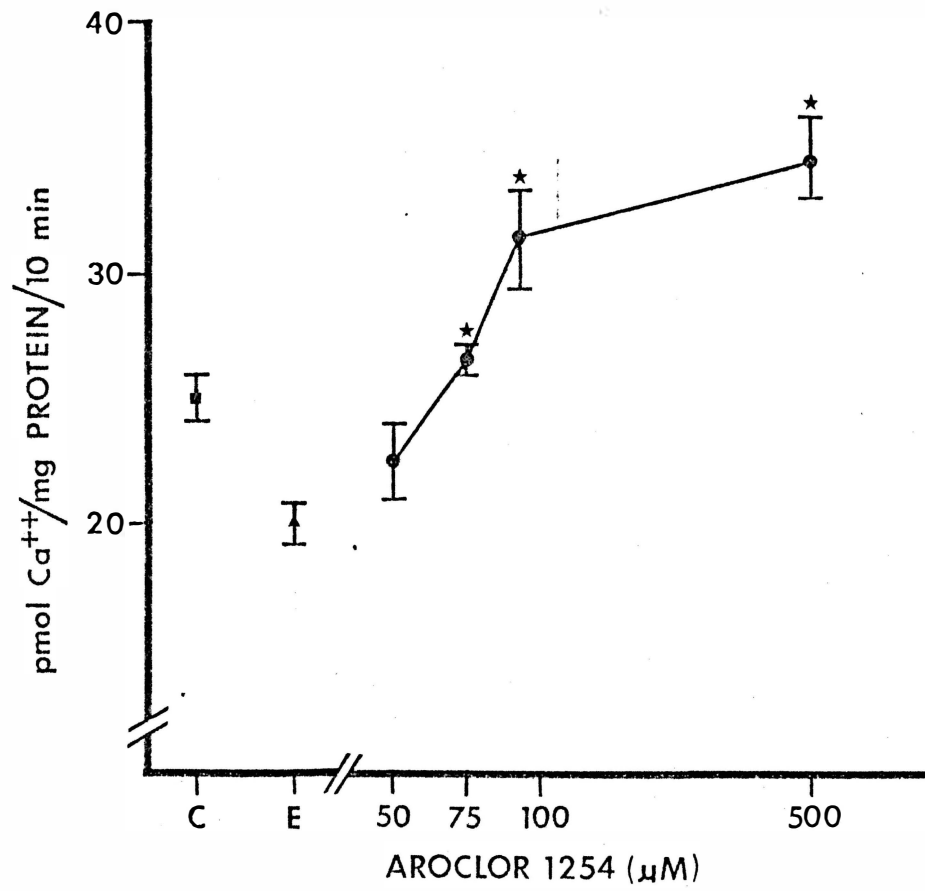
D. <u>Discussion</u>

The neurochemical data presented here indicate that Aroclor 1254 alters neurotransmitter function <u>in</u> <u>vitro</u>, but the data do not yet point to a specific site of action. Aroclor 1254 produced both a concentration-dependent inhibition of neurotransmitter uptake and enhancement of neurotransmitter release. This may be interpreted as a manifestation of the same event, i.e., increased release is simply a measure of an increase in net flux of neurotransmitter produced by an inhibition of re-uptake. This interpretation is plausible in light of the similarity in concentrations required to produce these effects. Furthermore, these effects on uptake and release were common to all neurotransmitters evaluated suggesting that Aroclor 1254 may act non-specifically at a site which would indirectly alter neurotransmitter function. It is also possible that these observed biochemical changes are due in part to a physical toxicity produced by Aroclor 1254 as suggested by Sharom and Mellors (1980), i.e., the physical properties of PCBs may contribute to membrane perturbation.

The stimulatory effect of Aroclor 1254 on NE, DA, and ACh release was observed only for basal and not for depolarization-evoked release. Thus, whatever the molecular mechanism of this effect, it is not addi-tive with the effect of depolarization on release. One might propose that the system is maximally stimulated by 60 mM KCl and that no more transmitter is available for release. These two stimuli - Aroclor 1254 and 60 mM KCl - may effect different pools of transmitter, however, there is certainly available transmitter in the nerve ending, because other compounds, such as chlordecone and the ionophore, A23187, are capable of enhancing depolarization-evoked release as demonstrated

herein. The CNS stimulant, amphetamine, also enhances depolorization-evoked release of neurotransmitter (End, 1979; Holmes and Rutledge, 1976).

In contrast to NE, DA, and ACh, 5-HT was the only neurotransmitter for which Aroclor 1254 enhanced both basal and potassium-induced release. It is impossible from the available data to account for this difference, and little significance has been accorded this observation in view of the high concentrations of Aroclor 1254 required. Furthermore, no difference was observed in sensitivity of NE, DA, or 5-HT uptake mechanisms to Aroclor 1254, which might diminish one's expectation of finding a selective effect of Aroclor 1254 on 5-HT release. Still and all, one must not discount the possibility that Aroclor 1254 alters serotonergic function in a manner not observed for the other neurotransmitters.

As mentioned in the Results section, the limitation of the technique for measuring ACh release must be addressed. Acetylcholine release was not measured directly, but rather total radioactivity released from synaptosomes preloaded with ^{3}H-Ch was determined. The contribution to this total activity might consist of released ^{3}H-ACh (synthesized from ^{3}H-Ch within the synaptosome), nonspecifically released ^{3}H-Ch, and ^{3}H-Ch resulting from the enzymatic hydrolysis of ^{3}H-ACh. One solution to this problem would be to measure directly the contributions of each of these components to the total radioactivity. This is certainly feasible from a methodological point of view, but analysis of each individual sample would prove to be a monumental task. The alternative solution is to develop a method which guarantees the identity of released radioactivity as ^{3}H-ACh. This approach is one

that has received a great deal of attention in the literature (e.g., see Haga, 1971; Marchbanks and Israel, 1971; Richter, 1976; Richter and Marchbanks, 1971), and while this approach merits consideration, it was not pursued on the following grounds. The effect of Aroclor 1254 on neurotransmitter release was probably a nonspecific effect, in that all neurotransmitters were affected to approximately the same degree. Therefore, it was felt that the time spent on development and validation of a new method (particularly one whose technical difficulties are so frequently referred to in the literature) was not warranted. Rather, the data must be interpreted accordingly. Perhaps, then, a better representation of the results would use a more appropriate terminology, such as, "Aroclor 1254 enhanced the release of radioactivity from synaptosomes preloaded with the ACh synthetic precursor, ^{3}H-Ch."

The sensitivity of these in vitro systems to external stimuli was validated by the use of positive controls. Inhibition of uptake by imipramine is consistent with previous findings that the serotonergic system is more sensitive to the effects of antidepressant parent compounds than the catecholaminergic system (Shaskan and Snyder, 1970). Both chlordecone and the ionophore, A23187, enhanced neurotransmitter release as expected. Furthermore, the EC_{50}'s of these positive controls were only about 10-fold lower than those of Aroclor 1254 in the uptake and release systems suggesting that what may appear as rather high concentrations of Aroclor 1254 may be appropriate to these in vitro systems.

Calcium, because it plays a pivotal role in neuronal function, presented itself as a candidate for study. Although Aroclor 1254 did alter Ca^{++} metabolism, the relevance of this change to neurotransmitter

function is uncertain. Whereas Aroclor 1254 altered neurotransmitter release and uptake, a 10-fold higher concentration of Aroclor 1254 was required to observe only a modest enhancement of synaptosomal Ca^{++} influx. No change in depolarization-evoked synaptosomal Ca^{++} uptake was observed with Aroclor 1254, implying that the stimulatory effect of Aroclor 1254 on depolarization-evoked 5-HT release occured via a mechanism other than enhanced Ca^{++} influx. In addition, mitochondrial Ca^{++} uptake (in the presence of ATP) was stimulated by Aroclor 1254, an effect that would oppose an increase in $[Ca^{++}]_i$ produced by stimulation of synaptosomal Ca^{++} influx. It would appear from these data that this effect on neurosecretion is not Ca^{++}-mediated, but that a nonspecific membrane effect might be proposed in view of the highly lipophilic nature of PCBs. One cannot, however, rule out the possibility that Aroclor 1254 increased $[Ca^{++}]_i$ by an alternate method such as release from or inhibition of sequestration into intracellular organelles.

It is possible that the effect of Aroclor 1254 on neurotransmitter uptake and release is a reflection of Na^+/K^+-ATPase inhibition. It has been suggested that Na^+/K^+-ATPase may play a regulatory role in neurotransmitter uptake (Bogdanski et al., 1968; Prakash et al., 1973; Tissari et al., 1969) and release (Gilbert et al., 1975). Recent reports indicate that PCBs inhibit ATPase activity in a variety of mammalian tissues (La Rocca and Carlson, 1979; Narbonne et al., 1978). Such an effect would be consistent with the findings reported here for Aroclor 1254's effects on neurotransmitter systems. This possibility will be explored in part B of this chapter.

Although the effects of Aroclor 1254 on neurotransmitter uptake and release in vitro are concentration-dependent, the question of

specificity and relevance of these changes lies open to speculation in view of the high concentrations employed. The data to be presented in a subsequent chapter, dealing with disposition of ^{14}C-PCBs, may help to justify these concentrations. In addition, Chang-Tsui and Ho (1979, 1980) have reported inhibitory effects of chlordecone, another chlorinated hydrocarbon, on synaptosomal neurotransmitter uptake at similarly high concentrations.

Finally, some mention should be made of the lack of neurochemical effects in synaptosomes isolated from mice that had been exposed to Aroclor 1254 in vivo. Without preempting the results that will be discussed in part B of this chapter, similar negative findings were found for ATPase activity following in vivo exposure. Therefore, this issue will be handled as a whole in the General Discussion.

In summary, the data in this section point to a neurochemical effect of Aroclor 1254 in vitro for which an explanation cannot yet be advanced. Further investigation of this response, to be presented in subsequent chapters, may help to illuminate this issue.

PART B - EFFECT OF AROCLOR 1254 ON SYNAPTOSOMAL AND MITOCHONDRIAL
   ATPases

I. Introduction

   Adenosine 5'-triphosphatases (ATPases) can be defined generally as
a group of enzymes that catalyze the hydrolysis of adenosine 5'-tri-
phosphate (ATP) to yield adenosine disphosphate (ADP) and inorganic
phosphate (P_i) with the liberation of metabolic energy. Individually,
the enzymes can be defined on the basis of their ionic requirements,
e.g., Na^+/K^+-activated, Mg^{++}-dependent ATPase and Mg^{++}/Ca^{++}-dependent
ATPase. The physiological roles subserved by these enzymes are as di-
verse as their distribution throughout the body.

   Within the brain, a number of different ATPases have been loca-
lized in a variety of subcellular fractions. The Na^+/K^+-ATPase is
found in the plasma membrane of neurons (Stahl and Broderson, 1976),
glia (Grisar et al., 1979), myelin (Reiss et al., 1981), intact synap-
tosomes (Grisar et al., 1979), and synaptic membranes (Hosie, 1965).
Germain and Proulx (1965) identified a Mg^{++}/Ca^{++}-ATPase in synaptic
vesicles from rat brain and suggested that it may play a role in trans-
mitter storage and release. A Mg^{++}-ATPase has been localized in synap-
tic vesicles (Hosie, 1965) and chromaffin granules (Konings and de
Potter, 1980), and its role in catecholamine uptake has been suggested
(Njus and Radda, 1978).

   Another Mg^{++}/Ca^{++}-ATPase has been characterized in microsomes from
pig brain (Nakamaru et al., 1967) and bovine brain (Saermark and
Vilhardt, 1979). Nakamaru (1971) suggested that the enzyme may play a
role in regulation of $[Ca^{++}]_i$ by one or both of the following mecha-

nisms: 1) Ca^{++} sequestration by the smooth endoplasmic reticulum and 2) extrusion of Ca^{++} from the cell by a Ca^{++} pump. Robinson and Lust (1968) have also demonstrated such a relationship between Ca^{++} accumulation and Mg^{++}/Ca^{++}-ATPase activity in rat brain microsomes, and a similar function was suggested for a fraction of isolated vesicles from calf, rabbit and rat brain resembling sarcoplasmic reticulum (Blitz et al., 1977). Evidence for Nakamaru's second proposal comes from the work of DiPolo and Beaugé (1977) who suggested that an ATP-driven pump (separate from the Na^+/Ca^{++} exchange mechanism) may contribute to the physiological control of $[Ca^{++}]_i$ in squid axon.

Last, but not least, mitochondria contain a Mg^{++}-stimulated ATPase (Hosie, 1965) that functions in the coupling events of oxidative phosphorylation (Pullman et al., 1960).

The role of the Na^+/K^+-ATPase in the active transport of Na^+ and K^+ across the cell membrane was first suggested by Skou's discovery of a Na^+/K^+-ATPase in crab nerve (1957). The metabolic energy derived from ATP hydrolysis is converted into mechanical energy required to pump Na^+ out of the cell against an electrochemical gradient. This extrusion of Na^+ is coupled with transport of K^+ into the cell. In this manner, the cell is able to maintain osmotic equilibrium by maintaining a low intracellular Na^+ concentration ($[Na^+]_i$) and high intracellular K^+ concentration ($[K^+]_i$). This system is also referred to as the sodium pump. Additional details may be found in two recent reviews (Schwartz et al., 1975; Sweadner and Goldin, 1980).

Maintaining osmotic equilibrium is particularly important in excitable cells, such as in myocardium and neurons. In the neuron, an action potential is generated by a stimulus and is propagated along the axon to the nerve terminal. Depolarization of the excitable neuronal

cell membrane is coupled with Na^+ influx and K^+ efflux. The Na^+/K^+-ATPase plays a key role in restoring the normal intracellular ion concentrations and membrane resting potential (Guyton, 1979).

In addition, another function of the Na^+/K^+-ATPase is its proposed role in neurotransmission. Although it is well accepted that Ca^{++} is required for neurotransmitter release (Rubin, 1970) and that release probably (though perhaps not always) occurs through a Ca^{++}-dependent exocytotic mechanism (Kelly et al., 1979), the exact mechanism through which Ca^{++} induces release is unknown. It has been suggested (Gilbert et al., 1975; Paton et al., 1971; Vizi, 1972) that the Na^+/K^+-ATPase may play a role in triggering neurotransmitter release. The evidence for this hypothesis was reviewed by Vizi (1978), and more recent reports shed further light on this matter. An increase in neurotransmitter release occurs in the presence of agents or conditions that inhibit the ATPases, whereas release is depressed concommittant with enzyme stimulation. For example, Meyer and Cooper (1981) demonstrated that a number of agents that inhibited the ATPase (e.g., ouabain and parachloromercuribenzene) also produced an enhancement of ACh release, an effect which was shown to be Ca^{++}-independent. By contrast, a Ca^{++}-dependent mechanism was involved in the enzyme inhibition and increased ACh release produced by veratridine, electrical stimulation, and the Ca^{++} ionophore, A23187, all of which induce Ca^{++} influx. Since it is known that increased $[Ca^{++}]_i$ inhibits the Na^+/K^+-ATPase (Skou, 1957, 1960), it is possible that this enzyme inhibition is involved in triggering neurotransmitter release.

The converse of this situation, that enzyme stimulation reduces neurotransmitter release, is explained in terms of feedback regulation

of release. A large body of evidence has accumulated for the existence of presynaptic receptors functioning in a feedback regulatory mechanism for release (Starke, 1981). It is possible that Na^+/K^+-ATPase may fit into this concept, as suggested by the observation of Gilbert et al. (1975) that NE stimulates ATPase activity and that the effect is blocked by phentolamine, a specific α-adrenoreceptor antagonist. Similar results were reported by Ádám-Vizi et al. (1980), Desaiah and Ho (1977), Logan and Donovan (1976), and Wu and Phillis (1979).

A number of toxic compounds, particularly the chlorinated hydrocarbon insecticides, have been shown to inhibit ATPases in a variety of tissues including brain. Koch (1969) was one of the first to demonstrate this ATPase inhibition in a rabbit brain subcellular fraction by chlordane, lindane, aldrin, dieldrin, and DDT. This short communication was followed shortly therafter with a more complete analysis (Koch, 1969/1970). In general, the Mg^{++}-ATPases were more sensitive to these agents than the Na^+/K^+-ATPases. Two pesticides that have received a lot of publicity are DDT and chlordecone. Each of these produce neurotoxic effects whic might be attributed to their inhibitory effects on ATPases both centrally (Cutcomp et al., 1971; Desaiah and Koch, 1975; Jackson and Gardner, 1978a, b) and peripherally (Ghiassudin and Matsumura, 1981).

PCBs, although not used as a pesticide, may be included in this group of chlorinated hydrocarbons. An inhibitory effect of four commercial PCB mixtures (Aroclors 1221, 1254, 1268, and 5460) on fish ATPases from brain, kidney, liver, and muscle was first demonstrated by Yap et al. (1971). This same group then evaluated, in more detail, the inhibitory effects of these PCB mixtures on Mg^{++}-ATPases from fish (Desaiah

et al., 1972). Experiments described in both papers involved in vitro exposure. The effects of PCBs on ATPases from rat have been reported by two groups. Narbonne and colleagues (1978) exposed rats to a French PCB mixture (Phenocolor DP_2) added to the diet in concentrations of 10, 100, or 500 ppm over a period of 30, 30, or 8 days, respectively, and assayed ATPase activity in microsomal fractions of liver, kidney, and brain. The results were tissue-, enzyme-, and dose-dependent. For example, liver ATPases were inhibited in a dose-dependent manner, kidney Mg^{++}-ATPase was stimulated, kidney Na^+/K^+-ATPase was inhibited at the low dose but stimulated at the high dose, brain Na^+/K^+-ATPase was stimulated only at the high dose, and brain Mg^{++}-ATPase was inhibited only at the lower two doses. In a somewhat different protocol, LaRocca and Carlson (1979) evaluated the in vitro effects of six commercial PCB mixtures (Aroclors 1221, 1242, 1248, 1254, 1260, and 1262) and numerous chlorinated biphenyl congeners on ATPase activity in homogenates of kidney, heart, or brain. In general, they demonstrated increasing inhibitory effects with increasing chlorination of the biphenyl ring.

The experiments involving ATPases that appear in this thesis were initiated for several reasons. Despite the presence of the aforementioned reports on the effects of PCBs on ATPases, it was felt that it would be prudent to conduct these ATPase experiments using the same PCB mixture which has been used throughout this project (Aroclor 1254), in the same species (the mouse), and with the same tissue preparation that has been used for the other neurochemical experiments (mitochondria and synaptosomes), in an effort to correlate the effects of Aroclor 1254 on ATPases with the other neurochemical effects of Aroclor 1254 on Ca^{++}

flux and neurotransmitter function, and to investigate the role of ATPases in these neurochemical events. Furthermore, these experiments include both in vitro and in vivo exposures to Aroclor 1254. And finally, the effects of Aroclor 1254 on each individual ATPase enzyme present in the tissue, rather than total ATPase activity, were examined.

Also included here are data pertaining to the ATPase methodology. Although this spectrophotometric method has been applied to ATPase determination in a number of tissues, it was necessary to validate the response of the enzymes to positive controls (as was done for previous neurochemical experiments) and to demonstrate that experimental conditions were applicable to determination of the activity of the enzymes in the mouse brain synaptosomal and mitochondrial preparation employed herein.

The methodology used in the experiments described herein for determining ATPase activity employs a coupled enzyme ATP-regenerating system in which ATP hydrolysis is measured indirectly by continuously monitoring the oxidation of NADH in a recording spectrophotometer. The reaction sequence is as follows:

$$1) \quad \text{ATP} \xrightarrow{\quad \text{ATPase} \quad} \text{ADP} + \text{P}_i$$

$$2) \quad \text{ADP} + \text{PEP} \xrightarrow{\quad \text{PK} \quad} \text{pyruvate} + \text{ATP}$$

$$3) \quad \text{Pyruvate} + \text{NADH} \xrightarrow{\quad \text{LDH} \quad} \text{lactate} + \text{NAD}^+$$
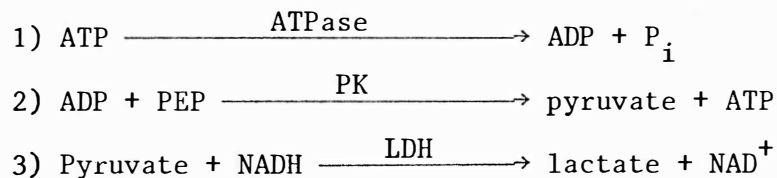
If all reagents, except the enzyme preparation, are added in excess, the first reaction, i.e. the hydroysis of ATP, is rate-limiting. Because the coupled system displays stoichiometry, the rate of NADH oxidation is directly proportional to the ATPase activity.

Classically, ATPase assays involve only the first reaction, and liberated inorganic phosphate (P_i) is quantitated colorimetrically by the method of Fiske and Subbarow (1925). This method has several limitations, in particular, 1) it is time consuming and 2) accumulation of ADP exerts a feedback inhibition on the ATPase enzyme. As an alternative to this method, the coupled enzyme assay has been used. Bucher and Pfleiderer (1955) were the first to characterize a system in which pyruvate kinase (PK) and lactate dehydrogenase (LDH) were coupled (reactions 2 and 3 above) to measure PK activity. Shortly thereafter, Pullman et al. (1960) applied this system to the measurement of ATPase activity. Validation of the method has been reported by Fritz and Hamrick (1966) and Scharschmidt et al. (1979).

There are numerous advantages to the coupled enzyme ATP-regenerating method. As mentioned previously, one of the major drawbacks of the colorimetric assay is the inhibition by ADP. The coupled system circumvents this problem by continually removing ADP and converting it to ATP (reaction 2). The result is not only the removal of ADP but also the maintenance of a constant substrate concentration, i.e. ATP, thus providing suitable conditions for kinetic analysis. Other features of this system include rapidity, reproducibility, and the ability to maintain constant temperature and record continuously over time. Less protein is also required for this assay (as little as 8 to 10 μg) than the conventional assay (~100 μg), a factor which may be important when sample size is limited. Furthermore, immediate results are available with this assay allowing the experimenter to make adjustments in the protocol as needed rather than having to wait until the end of the experiment for results. There is a disadvantage, however, and that is

that Mg^{++}-ATPase cannot be measured directly. Ordinarily, its activity could be differentiated from the Na^+/K^+-ATPase activity simply by removing K^+, however K^+ is required for PK activity and thus cannot be removed from the incubation medium. Consequently, Mg^{++}-ATPase is measured indirectly as the remaining activity when the Na^+/K^+-ATPase is specifically inhibited by ouabain.

B. <u>Materials and methods</u>

1. <u>Materials</u>

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO): disodium adenosine triphosphate (Na$_2$ATP) purchased as the vanadium-free salt prepared from equine muscle; β-nicotinamide adenine dinucleotide, reduced form (NADH), grade III (~98% purity), in pre-weighed vials, as the disodium salt; phospho(enol)pyruvate (PEP) as the tri(cyclohexylammonium) salt; ouabain (G-strophanthin); oligomycin (15% A, 85% B); pyruvate kinase (PK), type III, from rabbit muscle; lactate dehydrogenase (LDH), type, XI, from rabbit muscle; imidazole, grade III; 3,4-dihydroxyphenethylamine (DA); (-)-arterenol [(-)-norepinephrine (NE)].

2. <u>Tissue preparation</u>

Synaptosomes and mitochondria were prepared essentially according to the method of McGovern <u>et al</u>. (1973) as described in previous neuro-chemical experiments but with the following modification. Whole brain was homogenized in 0.32 M sucrose containing 10 mM imidazole and 1 mM EDTA (pH 7.5). Centrifugation then proceeded as described in previous neurochemical experiments, except that the Ficoll solutions which were used in constructing a discontinuous gradient for the subfraction step were prepared with the sucrose/EDTA/imidazole buffer instead of just 0.32 M sucrose. The purified synaptosomal and mitochondrial fractions were then resuspended in the sucrose/EDTA/imidazole buffer to yield a final protein concentration of 1 and 2 mg/kg, respectively.

3. ATPase assay

A coupled enzyme, ATP-regenerating system was used to measure ATPase activity by a recording spectrophotometric method. A modification of the methods of Desaiah and Ho (1977) and Schwartz et al. (1969) was used. Buffer was prepared fresh daily containing 20 mM imidazole, 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 4.5 mM Na_2ATP, 2.5 mM PEP, 0.2 mM NADH, 9 units of PK, and 12 units of LDH and was adjusted to pH 7.2. Other alterations in the buffer were made as noted.

The reaction mixture (3 ml) was preincubated in a cuvette for 5 min at 37° C. The cuvettes were maintained at constant temperature (37 ± 0.5° C) by means of a temperature controlled water bath (Lauda/Brinkmann B-2 Circulator; Messgerate-Werk Lauda, West Germany). The reaction was then initiated by the addition of 20 to 40 μl of synaptosomal or mitochondrial supension containing 8 to 10 μg of protein. For in vitro experiments, test compounds (e.g., vehicle or Aroclor 1254) were also added at this time, the contents of the cuvette were mixed by inversion of the cuvette, and the incubation proceeded for an additional 10 min. ATPase activity was measured over the next 10 min by monitoring oxidation of NADH as observed by the change in absorbance at a wavelength of 340 nm in an AMINCO DW-2 UV-VIS recording spectrophotometer (American Instrument Co., Silver Spring, MD). The instrument was calibrated according to the procedure in AMINCO's Operator's Manual. Briefly, this procedure involved setting the operating conditions on the spectrophotometer, locating the zero position for the amplifiers, and calibrating the absorbance range against the recorder axis. Specific activity was calculated from the change in absorbance over the 10 min period, because it lay in the linear portion of the curve (see Appendix, Figure 35).

4. <u>Effect of in vivo exposure to Aroclor 1254 on ATPase activity</u>

ATPase activity was determined following acute or subchronic exposure to vehicle or Aroclor 1254. In the first experiment, mice were treated orally with vehicle or Aroclor 1254 (50, 100, 250, or 500 mg/kg) and were sacrificed 45 min later. Control mice were untreated and were sacrificed along with the treated groups. Synaptosomes and mitochondria were isolated for the ATPase assay. In the second experiment, vehicle or Aroclor 1254 (3, 10, or 30 mg/kg) was administered daily for 14 days. Previous subchronic experiments (e.g. spontaneous activity and pentobarbital-induced sleep time studies) involved the use of doses of 30 and 100 mg/kg of Aroclor 1254. Lower doses were chosen for this ATPase study because of the lethality of the 100 mg/kg dose. Control mice, receiving no treatment, were also carried through the 14 days. Twenty-four hours after the last treatment, mice were sacrificed, and synaptosomes and mitochondria were isolated for the ATPase assays.

C. Results

1. Assay conditions and validation of the method

a. Dependence of ATPase activity on protein concentration

Determination of ATPase activity using the coupled enzyme ATP-regenerating system relies on the stoichiometry of the three coupled reactions and the assurance that the first reaction, hydrolysis of ATP, is the rate-limiting step. All other factors being in excess, the quantity of added ATPase should govern the observed activity, actually the rate of NADH oxidation. Stated differently, there should be a linear relationship between concentration of protein (i.e. the amount of added protein) and the rate of NADH oxidation (i.e. change in absorbance (ΔA) per unit time). And because specific activity (S.A.) of the ATPases is given as $\mu mol\ P_i$ released per mg protein per hr, S.A. should remain constant. Both of these conditions have been met experimentally.

As seen in Figures 22 and 23, addition of increasing quantities of synaptosomal or mitochondrial protein produced a linear increase in the rate of NADH oxidation reported as ΔA/min. Specific activity was then calculated from these values for each protein concentration and plotted on the same graph. While synaptosomal ATPase activity remained constant over the range of protein concentrations tested, this was not the case for the mitochondrial enzyme preparation. Activity remained constant with addition of between 2 and 10 μg of protein but dropped off dramatically (by almost one half) with increased protein (20 to 40 μg). Elucidation of the etiology of this diminution was not pursued, and therefore, in order to avoid any undefined inhibitory effects, subsequent experiments were performed using additions of mitochondrial

Figure 22

Dependence of synaptosomal ATPases on protein concentration. Points represent the mean ± S.E. of values from 4 separate experiments; samples were run in triplicate within each experiment. Ordinate: left axis, $\Delta A/min$, change in absorbance ($\bullet$); right axis, specific activity given as micromoles P_i released/mg protein/hr ($\blacktriangle$). Least square linear regression analysis of the relationship between synaptosomal protein and $\Delta A/min$ yielded a correlation coefficient of 0.994.
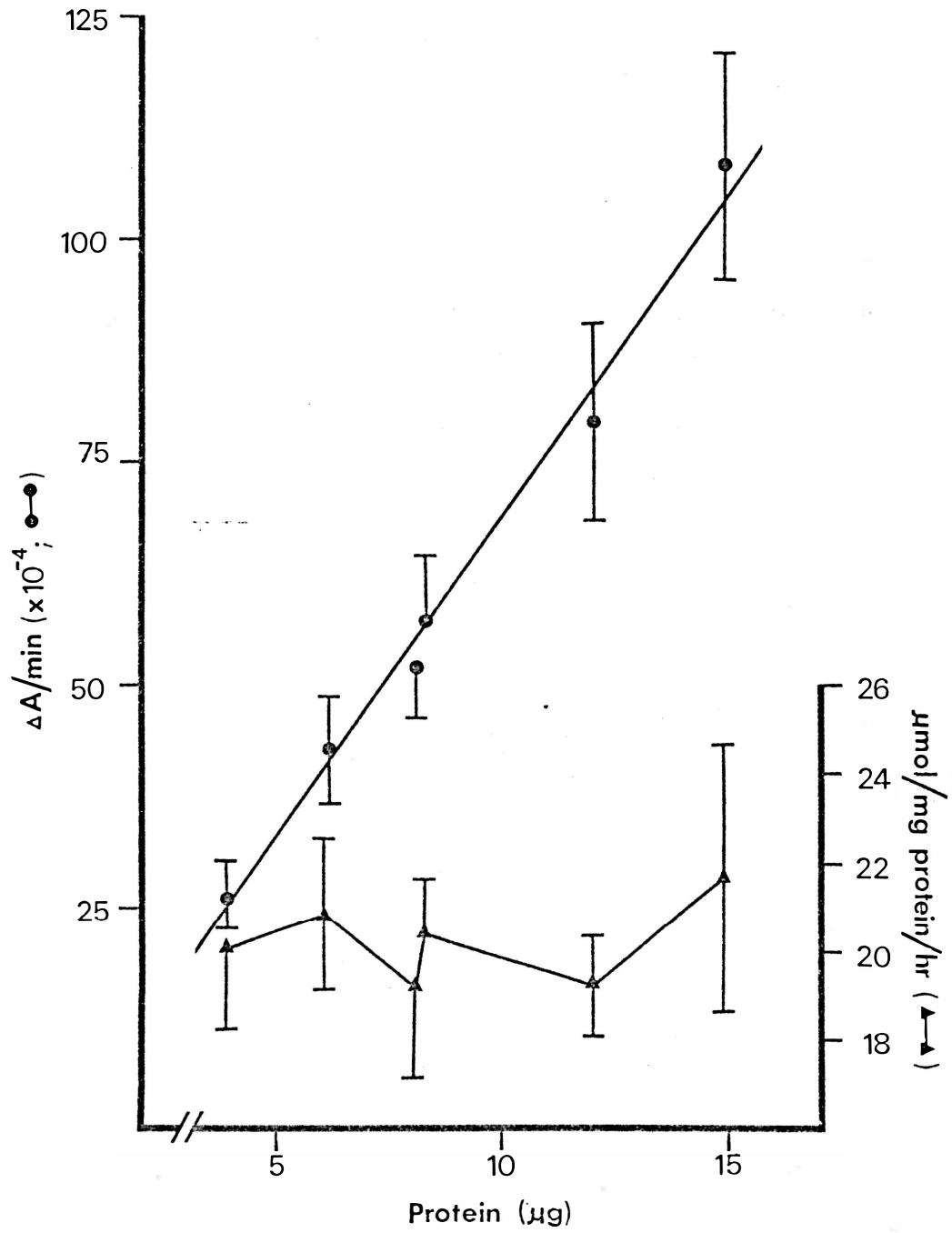
Figure 23

Dependence of mitochondrial ATPases on protein concentration. Points

represent the mean ± S.E. of values from 4 separate experiments; sam-

ples were run in triplicate within each experiment. Ordinate: left

axis, ΔA/min, change in absorbance per min (●); right axis, S.A.,

specific activity (▲). Least squares linear regression analysis of

the relationship between added mitochondrial protein and ΔA/min yielded

a correlation coefficient of 0.982.
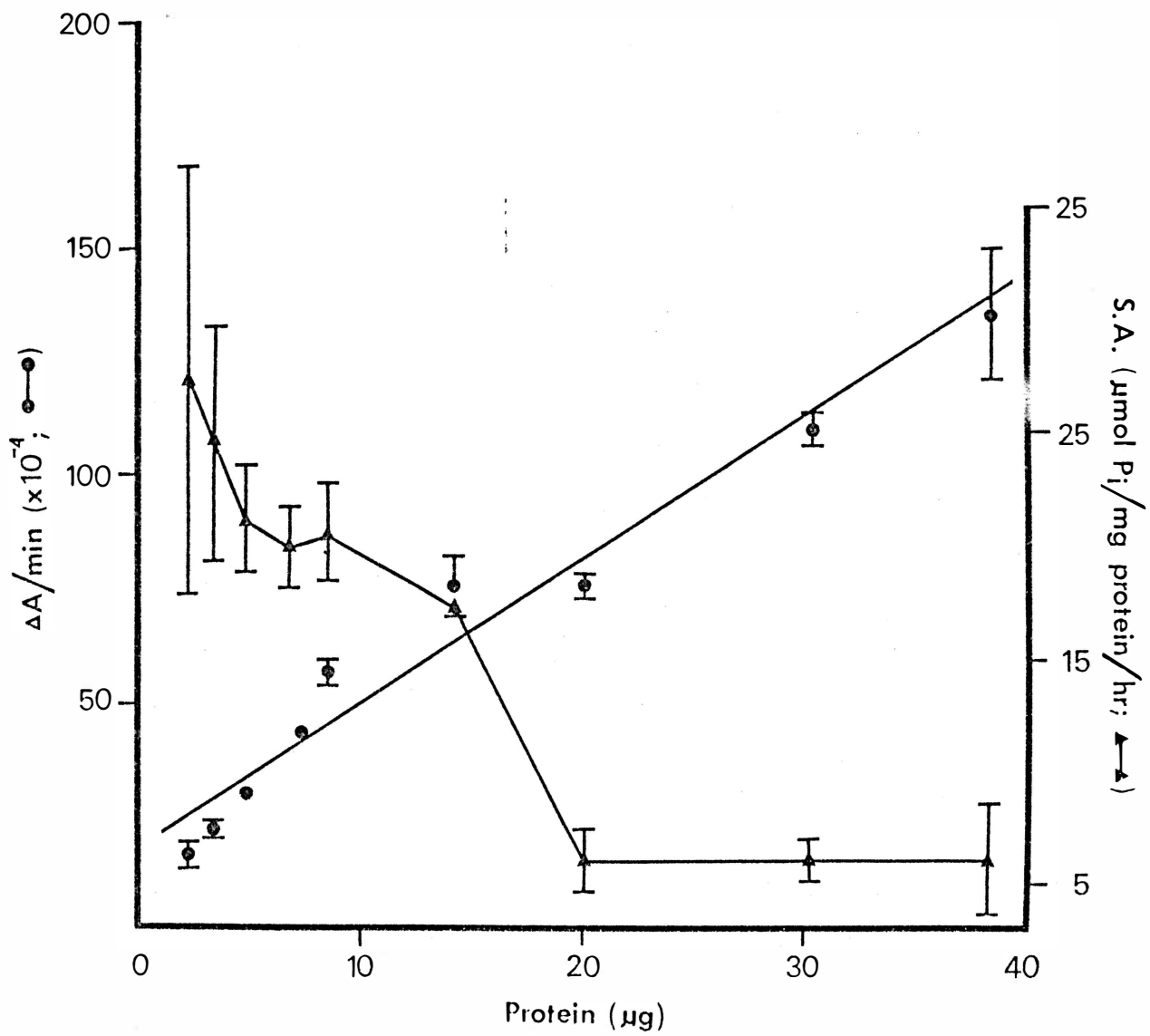
protein in the lower range of the curve where activity remeained constant (usually 8 to 10 µg). For experiments with the synaptosomal preparation, the data revealed that the entire range of added protein was suitable. Although not shown in these figures, addition of larger quantities of protein, e.g. greater than 30 µg, produced so rapid a rate of NADH oxidation that one or more of the reagents were depleted, i.e. NADH oxidation ceased within 10 min. Therefore, experiments typically involved the addition of protein in the middle range of the curve, between 8 and 10 µg of synaptosomal protein.

b.   Effect of ATP concentration on synaptosomal ATPase activity

As mentioned previously, determination of ATPase activity by this coupled enzyme system requires that all components be in excess except the added enzyme, the rate-limiting component. Thus, it was important to determine a saturation curve for ATP, i.e. the range of concentrations at which ATP would be in excess.

Medium was prepared containing various concentrations of ATP, and the usual protocol was employed to determine total synaptosomal ATPase activity at each ATP concentration. The results of these experiments can be illustrated in two ways. The inset in Figure 24 shows that the *enzyme displays saturation kinetics, and that the rate of ATP hydrolysis* remains constant at concentrations of ATP greater than 2 mM. A Lineweaver-Burk (double reciprocal) plot of this data (shown in the larger portion of the figure) is another method for illustrating that the enzyme follows Michaelis-Menten kinetics. Mathematical analysis yields a K_m of 8.98×10^{-5} M and V_{max} of 13.46. Based on these data, a concentration of 4.5 mM ATP was chosen for further studies which was a

Figure 24

Effect of ATP concentration of total synaptosomal ATPase activity

a.   Lineweaver-Burk plot. S.A., specific activity. Points represent reciprocal of mean of values from 2 to 4 experiments; samples were run in triplicate within each experiment. Least squares linear regression analysis yielded a correlation coefficient of 0.980. V_{max} = 13.46 and K_m = 8.98 x 10^{-5} M.

b.   Inset: saturation curve for ATP. Points represent mean ± S.E. of values from 2 to 4 experiments; samples were run in triplicate within each experiment.
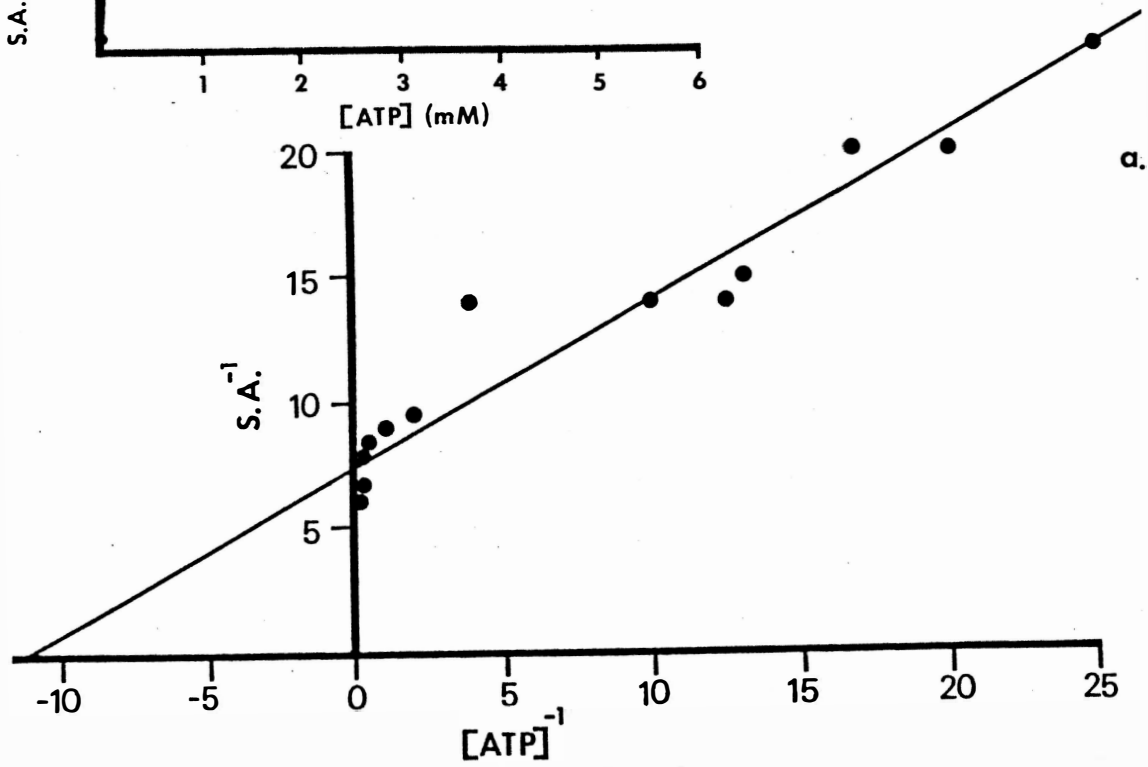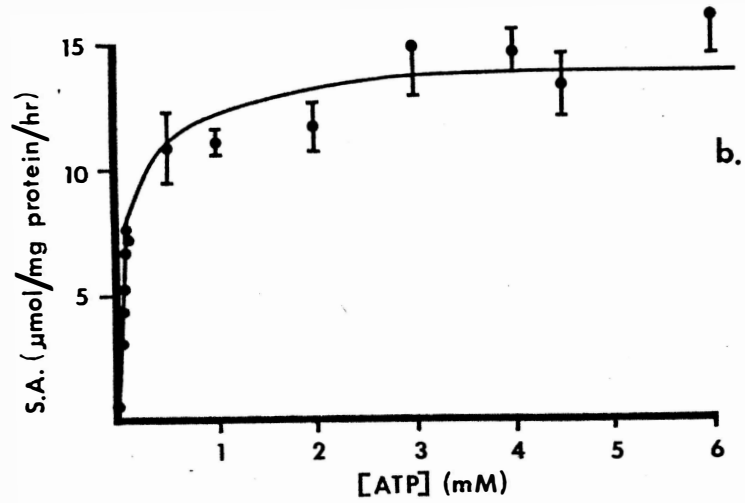
saturation concentration of ATP and the same one employed in the protocol of Desaiah and Ho (1977).

c. ATPase sensitivity to ouabain and oligomycin

The various ATPase enzymes found in a tissue preparation can be defined on the basis of ionic requirements and response to pharmacological manipulations. Under the conditions applied as stated in "Materials and Methods," synaptosomal ATPase activity consists of the combined activity of two enzymes: 1) a Na^+- and K^+-dependent enzyme, the Na^+/K^+-ATPase and 2) a Mg^{++}-dependent enzyme, the Mg^{++}-ATPase. The buffer contains Na^+, K^+, and Mg^{++} and provides suitable ionic conditions for both enzymes. While Mg^{++}-ATPase activity could be measured by removing K^+ (thereby removing Na^+/K^+-ATPase activity), this is not feasible in this system because pyruvate kinase, one of the enzymes in the regenerating system, requires K^+. The alternative is to measure total synaptosomal ATPase with all components present in the buffer, then add ouabain, a cardiac glycoside and a specific inhibitor of the Na^+/K^+-ATPase (Schwartz et al., 1975), and measure the remaining Mg^{++}-ATPase activity. The activity of Na^+/K^+-ATPase activity is obtained by subtraction.

The mitochondrial Mg^{++}-ATPase is also a composite of several enzymes. These enzymes have been difined on the basis of their sensitivity to oligomycin, an agent that uncouples oxidative phosphorylation by inhibiting mitochondrial Mg^{++}-ATPase (Lardy et al., 1958). These enzymes are termed oligomycin-sensitive and oligomycin-insensitive Mg^{++}-ATPases. Because these pharmacological tools were to be employed in determining the contribution of the various enzyme components to the

total activity, it was important to find the concentration of each that would completely suppress enzyme activity. Therefore, activity was monitored following addition of increasing concentrations of ouabain to the synaptosomal preparation and of oligomycin to the mitochondrial preparation. These results are illustrated in Figures 25 and 26, respectively. In the absence of either inhibitor, total activity was measured. With the addition of ouabain, a concentration-dependent decrease in synaptosomal ATPase activity, i.e. Na^+/K^+-ATPase activity, was observed. Maximal inhibition was observed with 10^{-4} M ouabain, the remaining activity being that of the Mg^{++}-ATPase enzyme. A concentration of 10^{-3} M ouabain was chosen for all further experiments, as this concentration is employed commonly throughout the literature (e.g., see Desaiah and Ho, 1977).

The data for oligomycin were not as clear cut. A biphasic curve was produced. Addition of 10^{-9}, 10^{-8}, and 10^{-7} M oligomycin resulted in a concentration-dependent inhibition of activity, presumably inhibition of the oligomycin-sensitive Mg^{++}-ATPase. However, as the concentration was increased to 1, 2.5, 5, and 10 µM oligomycin, activity returned to control values. This curious observation was unexpected and is presently unexplained. Although an intriguing finding, it was not pursued, since the intent of the experiment was to find the concentration of oligomycin that showed maximal inhibitory activity, and for this purpose, a concentration of 10^{-7} M oligomycin was chosen for further experiments.

Figure 25

Concentration-dependent effect of ouabain on mouse brain synaptosomal

activity. C, control. S.A., specific activity. Points represent mean

± S.E. of values from 3 experiments; samples were run in triplicate

within each experiment.

Figure 26

Effect of increasing concentrations of oligomycin on mouse brain mito-
chondrial ATPase activity. C, control. S.A., specific activity.

Points represent mean ± S.E. of values from 3 experiments; samples were

run in triplicate within each experiment.
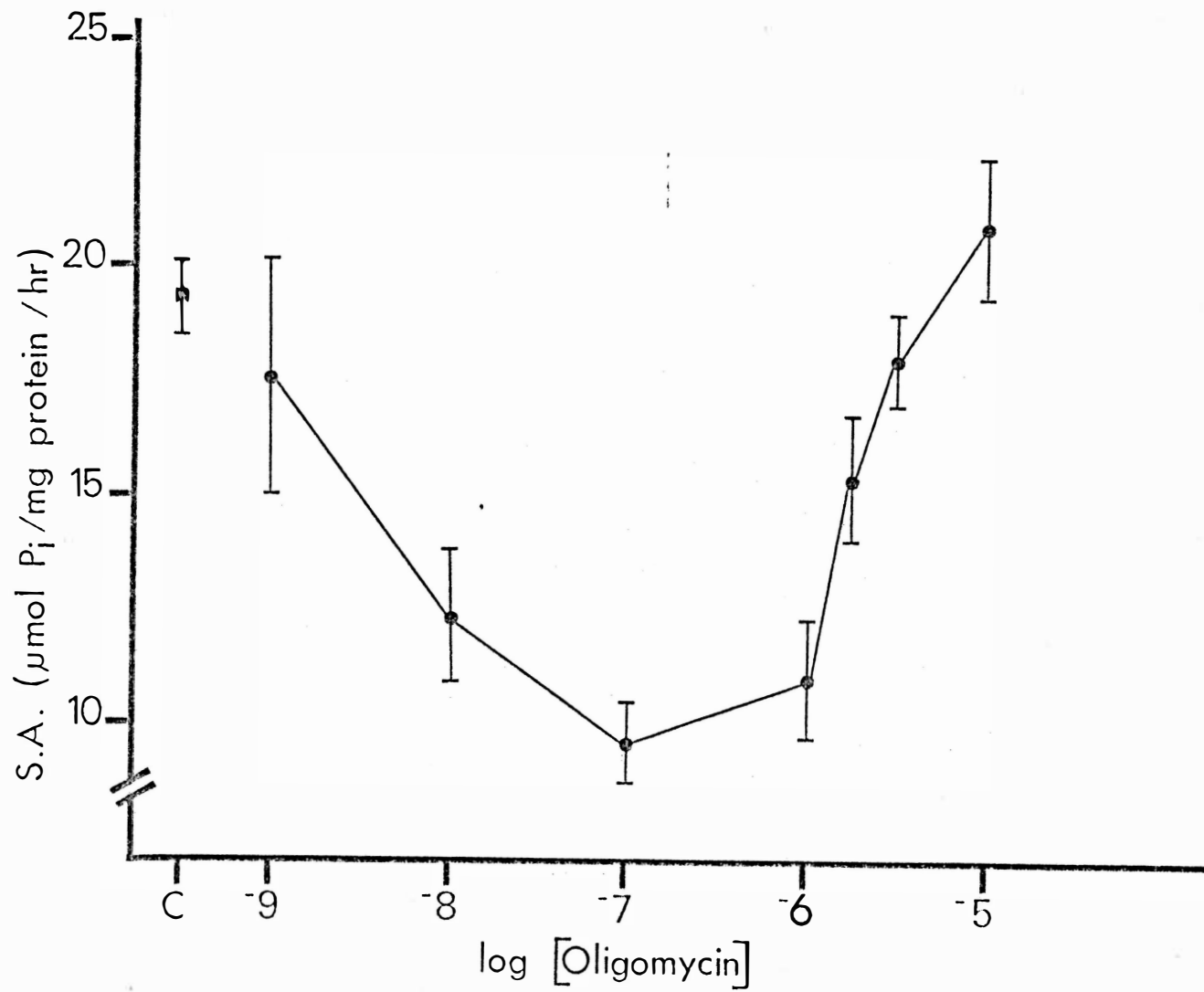
d. Response of synaptosomal ATPases to catecholamines

As a test for establishing the sensitivity of this mouse brain preparation of ATPases, the response to known pharmacological agents was examined. As discussed in the introduction, it has been postulated that synaptosomal ATPases may be involved in the processes of neurotransmitter release and re-uptake and that a variety of neurotransmitters stimulate these enzymes. Consequently, NE and DA were chosen as positive controls. NE and DA stimulated both the Na^+/K^+- and Mg^{++}-ATPases in the synaptosomal preparation in a concentration-dependent fashion (Figures 27 and 28). Maximal stimulation of Na^+/K^+-ATPase was achieved with 10^{-5} M NE; higher concentrations (10^{-4} and 10^{-3} M) produced no further increase in activity. This enzyme appeared equally sensitive to the effects of DA, however no plateau effect was observed. Furthermore, stimulation of as much as 180% of control could be produced with DA, while activity leveled off at about 160% of control in the presence of 10^{-5} to 10^{-3} M NE. The Mg^{++}-ATPase was also stimulated by NE and DA but to a lesser extent than the Na^+/K^+-ATPase. These results agree favorably with those of Desaiah and Ho (1977), and as intended they serve the function of verifying the enzymatic sensitivity of this synaptosomal preparation.

2. Effect of Aroclor 1254 on synaptosomal and mitochondrial ATPases

Having established optimal conditions for the enzymes that were to be examined, the effects of Aroclor 1254 on ATPases could be assessed. Aroclor 1254 was prepared in an EtOH vehicle for in vitro experiments. EtOH alone produced no effect on synaptosomal or mitochondrial ATPases provided that a concentration of about 6 µM (corresponding to a 10 µl

Figure 27

Stimulation of synaptosomal ATPases by NE. C, control. S.A., specific

activity. Total activity is the activity measured in the absence of

ouabain, Mg^{++}-ATPase is the activity in the presence of 1 mM ouabain,

and Na^{+}/K^{+}-ATPase is determined by subtraction. Points represent the

mean ± S.E. of replicate samples (N = 3). The experiment was repeated

twice with similar results.
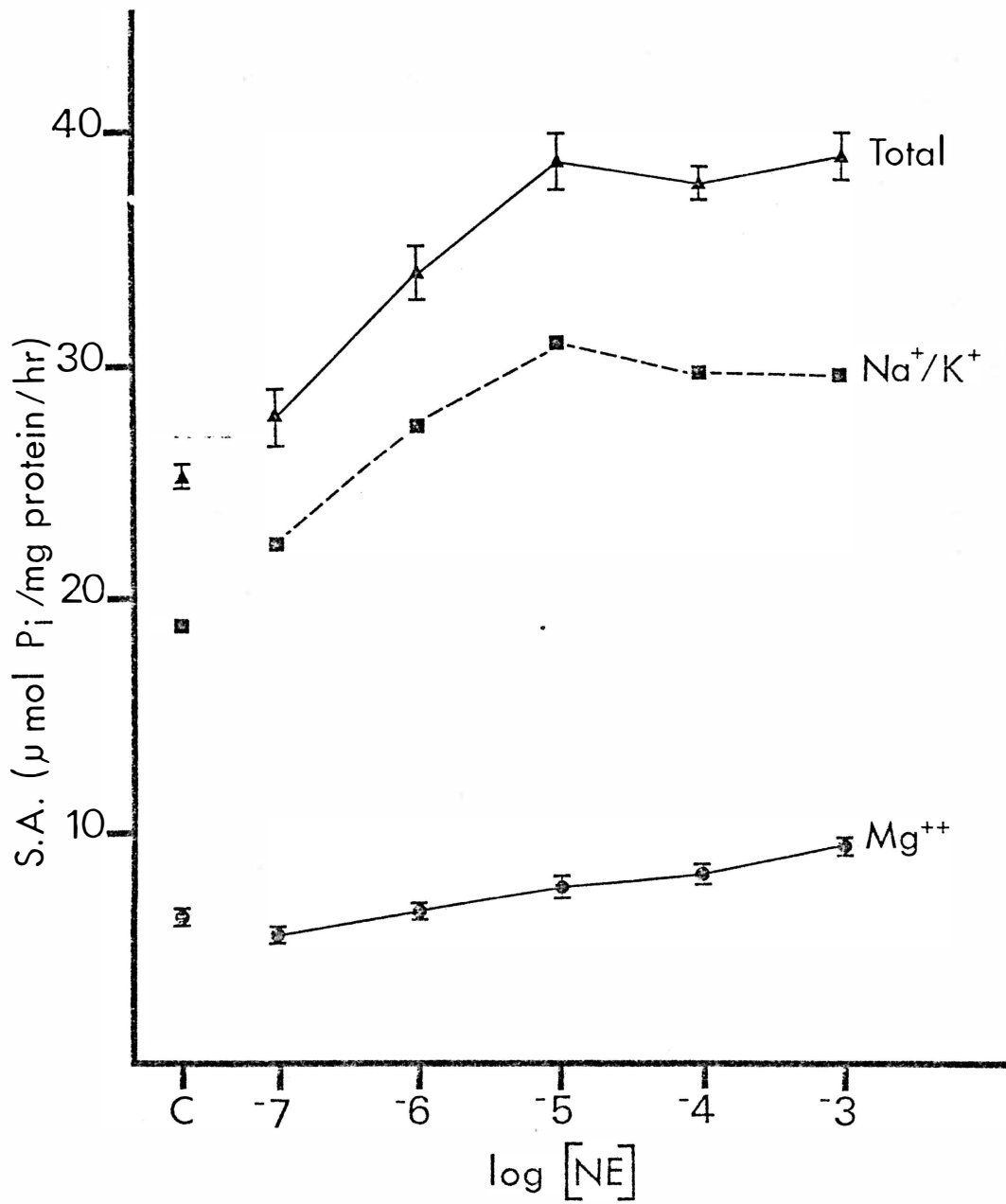
Figure 28

Stimulation of synaptosomal ATPases by DA. C, control. S.A., specific

activity. Total activity is the activity measured in the absence of

ouabain, Mg^{++}-ATPase is the activity in the presence of 1 mM ouabain,

and Na^+/K^+-ATPase activity is determined by subtraction. Points repre-

sent the mean ± S.E. of replicate samples (N = 3). The experiment was

repeated twice with similar results.
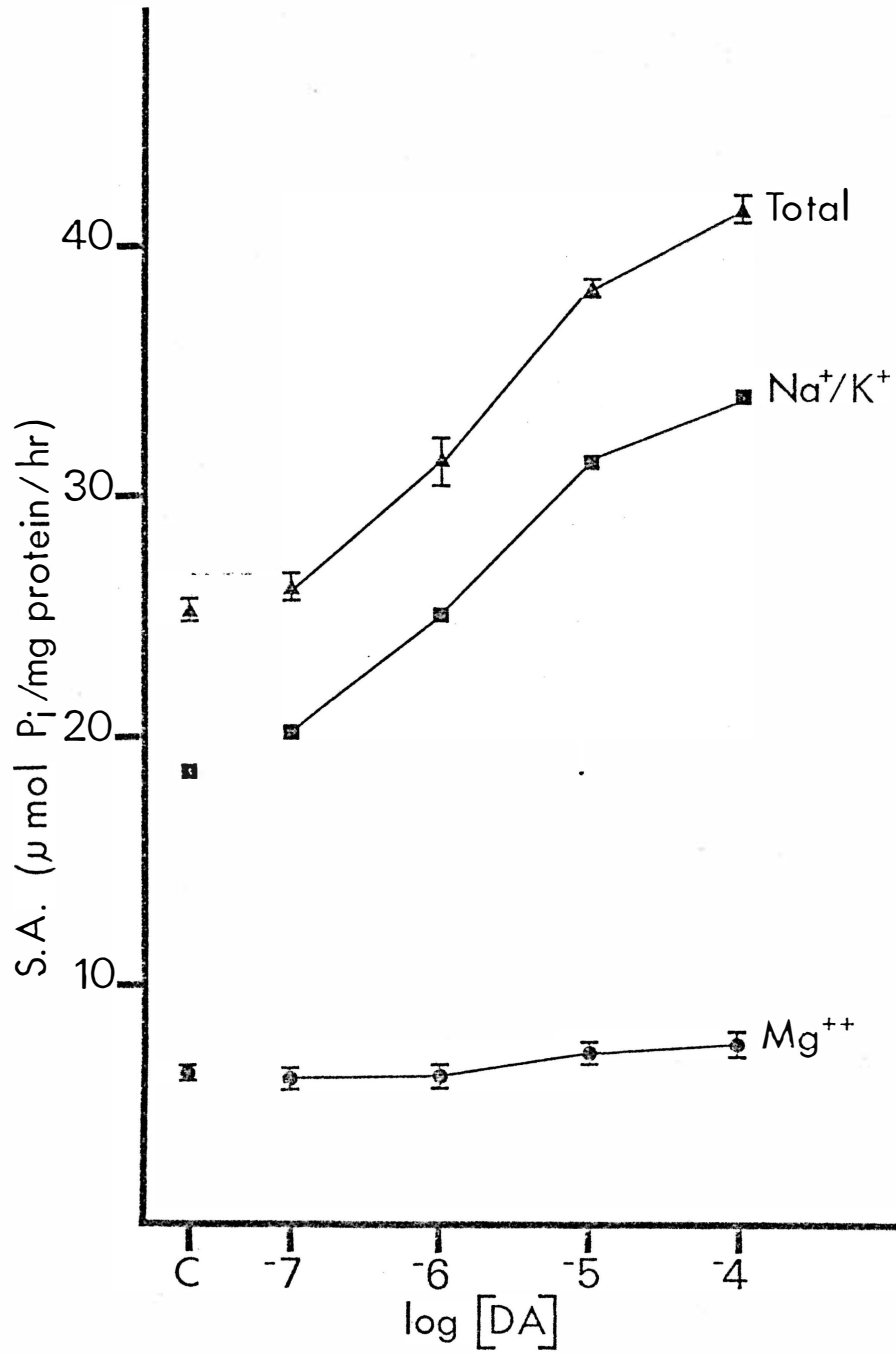
aliquot in a 3 ml assay volume) was not exceeded (Table 7). However, a three-fold increase in EtOH concentration (30 μl in 3 ml assay volume) produced a 26% decrease in mitochondrial activity, and a 12% increase in synaptosomal activity was observed with ~9 μM EtOH (15 μl in 3 ml assay volume). Thus, Aroclor 1254 (or any other test compounds that were dissolved in EtOH) could be delivered in a volume of 10 μl of EtOH.

Aroclor 1254 had an inhibitory effect on synaptosomal and mito-chondrial ATPases (Figures 29 and 30, respectively). Several observa-tions can be made about these data. Referring to Figure 29, it can be seen that the Na^+/K^+-ATPase contributes a much greater portion of the activity to the total synaptosomal activity than dose the Mg^{++}-ATPase. By contrast, the mitochondrial Mg^{++}-ATPase activity is divided equally between the oligomycin-sensitive and insensitive enzymes (Figure 30). Other investigators have reported similar findings (Desaiah and Ho, 1977; Desaiah et al, 1972). As for the Aroclor 1254 effect, the mito-chondrial enzymes were an order of magnitude more sensitive to this inhibitory effect than were the synaptosomal enzymes, as shown by the IC_{50} values given in Figures 29 and 30 (approximately 7 μM for mito-chondria and 100 μM for synaptosomes). However, while mitochondrial ATPase activities decreased linearly up to 5 or 10 μM Aroclor 1254, there was no further depression of activity at higher concentrations.

The next several experiments were designed to evaluate the effect of orally administered Aroclor 1254 on mouse brain ATPases. In the first experiment, mice were treated acutely with vehicle or Aroclor 1254 (Tables 8 and 9). Analysis of variance revealed that acute oral administration of Aroclor 1254 had no effect on any of the enzymes

TABLE 7

Effect Of EtOH on Mitochondrial and
Synaptosomal ATPase Activity

% Control[a]

μl EtOH	Mitochondria	Synaptosomes
3	90 ± 3[b]	——
5	95 ± 4	108 ± 2
10	97 ± 3	105 ± 3[c]
15	94 ± 1	112 ± 2
30	74 ± 2	——

[a] Values represent mean ± S.E. for determinations from 3 separate
tissue preparations or as otherwise indicated. Data are expressed
as percent of activity of paired control (in the absence of EtOH)

[b] N = 5

[c] N = 6

Figure 29

In vitro effect of Aroclor 1254 on synaptosomal ATPases. C, control. EtOH, vehicle for Aroclor 1254. Total activity is the ATPase activity in the absence of ouabain, Mg^{++}-ATPase is the activity in the presence of 1 mM ouabain, and Na^{+}/K^{+}-ATPase activity is determined by subtraction. Points represent the mean ± S.E. of values from 3 experim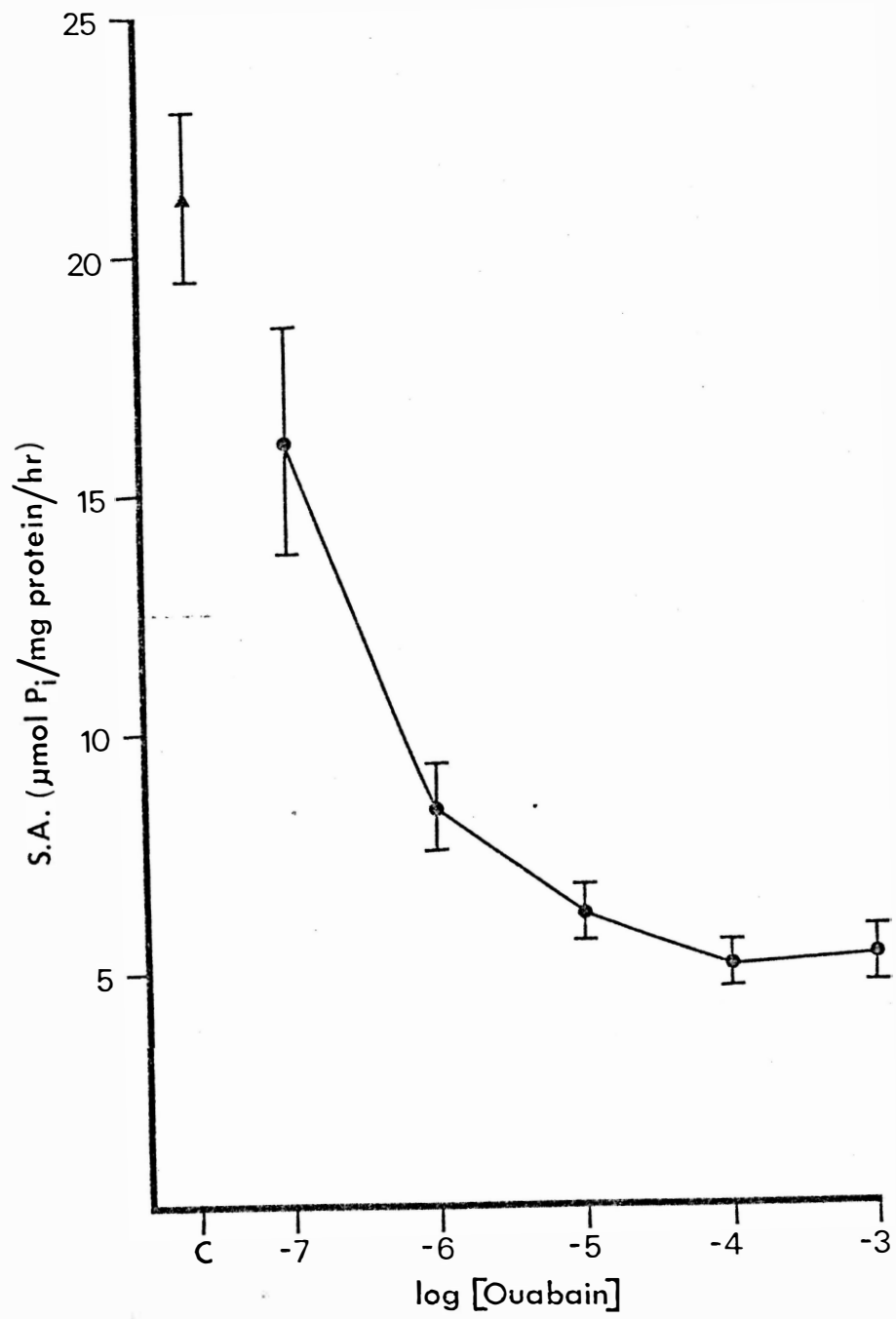ents; samples were run in triplicate within each experiment. Where S.E. bars are not shown, the values lie within the bounds of the symbols. Least squares linear regression analysis yielded correlation coefficients of 0.7467 and 0.7089 and IC_{50} values of 1.07 and 1.24×10^{-4} M for Mg^{++}- and Na^{+}/K^{+}-ATPases, respectively.
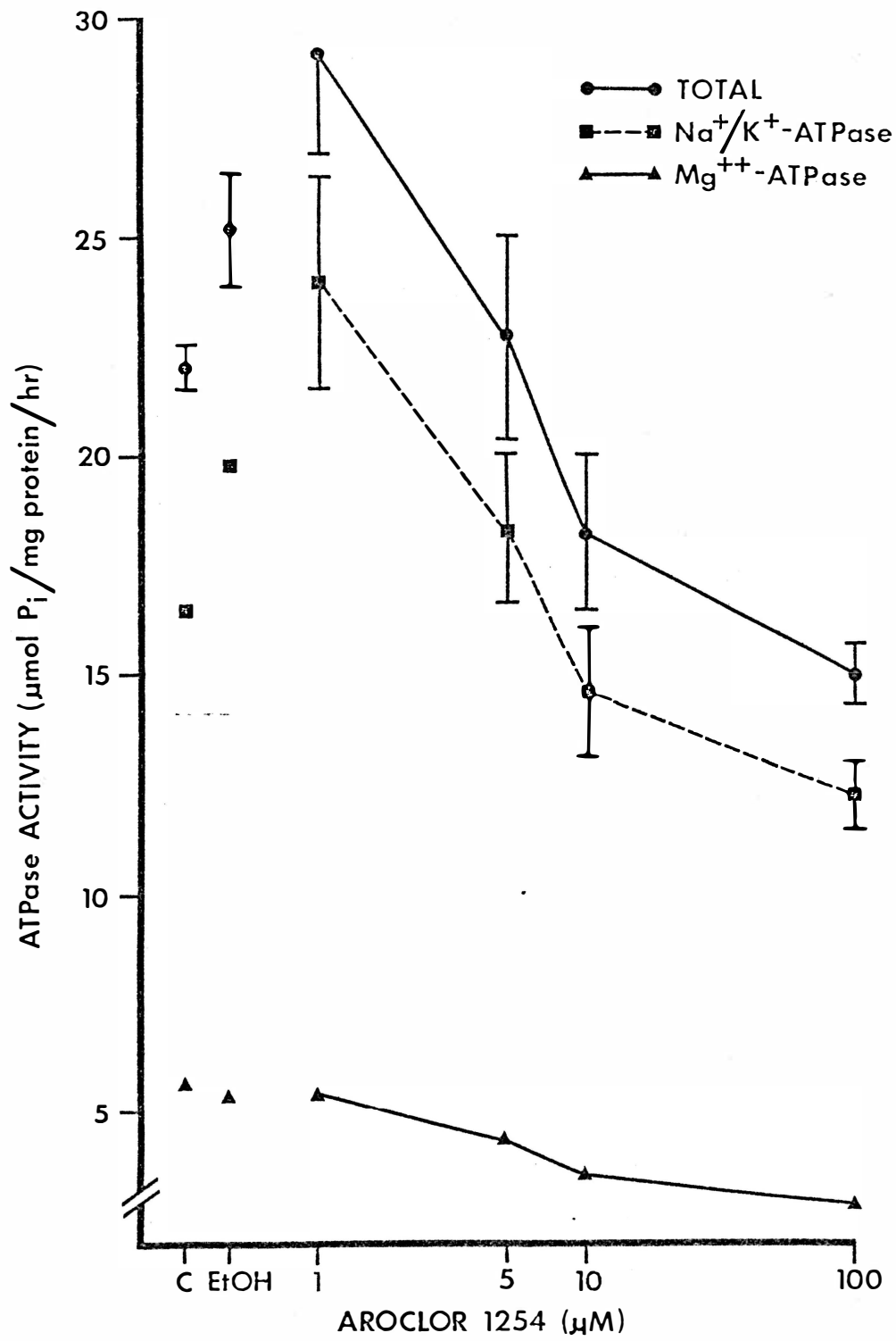
Figure 30

In vitro effect of Aroclor 1254 on mitochondrial ATPases. C, control. EtOH, vehicle for Aroclor 1254. Total activity is the activity in the absence of ouabain, Mg^{++}-ATPase is the activity in the presence of 1 mM ouabain, and Na^+/K^+-ATPase activity is determined by subtraction. Points represent mean ± S.E. of values from 3 experiments; samples were run in triplicate within each experiment. Where S.E. bars are not shown, the values lie within the bounds of the symbols. Least squares linear regression analysis of the linear portion of the curve yielded correlation coefficients of 0.9354 and 0.8410 and IC_{50} values of 7.64 and 7.27 μM for oligomycin-sensitive and insensitive Mg^{++}-ATPases, respectively.
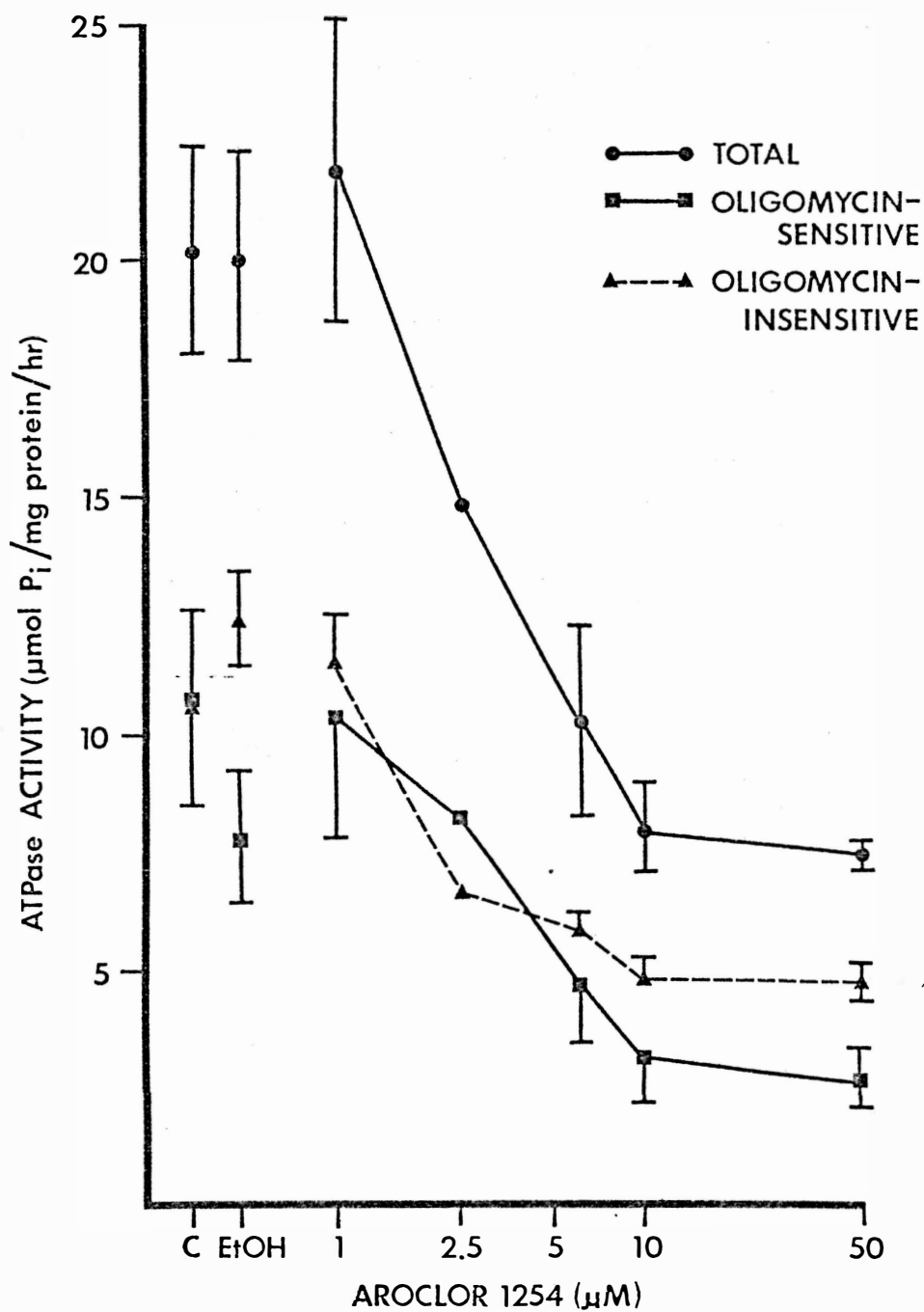
examined. The second experiment involved a subchronic dosing regimen.
Following the 14 day regimen, Aroclor 1254 had no effect on mitochon-
drial or synaptosomal ATPases (Tables 10 and 11, respectively) as
established by analysis of variance.

TABLE 8

Activity Of Mouse Brain Synaptosomal ATPases
Following Acute Oral Administration of Aroclor 1254

μmol P_i/mg protein/hr[b]

Treatment[a]	Total	Na^+/K^+-ATPase	Mg^{++}-ATPase
Control	24.9 ± 1.5	18.5 ± 1.0	6.4 ± 0.6
Vehicle	29.4 ± 2.2	23.1 ± 1.9	6.3 ± 0.7
Aroclor 1254			
50 mg/kg	26.8 ± 7.7	20.8 ± 6.4	6.0 ± 1.3
100 mg/kg	28.7 ± 7.0	22.4 ± 5.9	6.3 ± 1.0
250 mg/kg	32.7 ± 7.7	26.8 ± 6.9	6.0 ± 0.8
500 mg/kg	32.8 ± 4.6	26.1 ± 3.6	6.7 ± 1.1

[a] Mice sacrificed 45 min after treatment

[b] Values represent mean ± S.E. of 3-4 groups of 4 mice each;
tissue samples from each group were run in triplicate

TABLE 9

Activity Of Mouse Brain Mitochondrial ATPases
Following Acute Oral Administration of Aroclor 1254

μmol P_i/mg protein/hr[b]

Treatment[a]	Total	Oligomycin-Sensitive Mg^{++}-ATPase	Oligomycin-Insensitive Mg^{++}-ATPase
Control	16.7 ± 1.2	5.5 ± 0.3	11.2 ± 1.3
Vehicle	18.1 ± 1.1	6.6 ± 0.8	11.5 ± 1.9
Aroclor 1254			
50 mg/kg	21.6 ± 1.8	8.6 ± 0.7	13.0 ± 1.1
100 mg/kg	21.1 ± 1.1	7.1 ± 1.4	14.0 ± 2.6
250 mg/kg	22.6 ± 0.7	7.2 ± 0.9	15.4 ± 0.2
500 mg/kg	16.1 ± 1.1	6.1 ± 0.7	10.0 ± 1.1

[a] Mice sacrificed 45 min after treatment

[b] Values represent mean ± S.E. of 3-4 groups of 4 mice each; tissue samples from each group were run in triplicate

TABLE 10

Effect Of 14 Day Oral Administration Of Aroclor 1254
On Mouse Brain Synaptosomal ATPase Activity

μmol P_i/mg protein/hr[b]

Treatment[a]	Total	Na^+/K^+-ATPase	Mg^{++}-ATPase
Control	23.8 ± 2.3	18.2 ± 2.1	5.6 ± 0.1
Vehicle	25.2 ± 3.2	20.4 ± 2.7	4.8 ± 0.7
Aroclor 1254 3 mg/kg	22.6 ± 1.9	17.8 ± 1.8	4.8 ± 0.2
10 mg/kg	23.8 ± 1.6	18.4 ± 2.3	5.4 ± 0.7
30 mg/kg	29.2 ± 1.8	23.3 ± 1.2	5.9 ± 0.6

[a] Mice sacrificed 24 hr after last treatment

[b] Values represent mean ± S.E. of 3 groups of 4 mice each;
tissue samples from each group were run in triplicate

TABLE 11

Effect of 14 Day Oral Administration Of Aroclor 1254
On Mouse Brain Mitochondrial ATPase Activity

μmol P_i/mg protein/hr[b]

Treatment[a]	Total	Oligomycin-Sensitive Mg^{++}-ATPase	Olyigomycin-Insensitive Mg^{++}-ATPase
Control	19.6 ± 1.8	6.7 ± 1.4	12.9 ± 0.8
Vehicle	18.8 ± 0.6	5.3 ± 1.4	13.5 ± 1.1
Aroclor 1254			
3 mg/kg	20.4 ± 2.2	6.9 ± 0.9	13.5 ± 1.6
10 mg/kg	20.6 ± 0.9	6.8 ± 0.4	13.8 ± 1.0
30 mg/kg	20.0 ± 2.0	8.2 ± 1.6	11.8 ± 0.5

[a] Mice sacrificed 24 hr after last treatment

[b] Values represent mean ± S.E. of 3 groups of 4 mice each;
tissue samples from each group were run in triplicate

D. Discussion

The recording spectrophotometric method for determination of ATPase activity using the coupled enzyme, ATP-regenerating system is one that has been in use for many years and has been validated for numerous tissue preparations. A thorough and exhaustive characterization of the system and optimization of assay conditions was therefore unwarranted. Rather, the purpose of these methodological pursuits was to verify selected parameters and to insure that the application of this method to the measurement of ATPase activity in mouse brain synaptosomes and mitochondria was justified.

The experiments described herein lend support for the assumptions inherent in this assay system, namely that stoichiometry is maintained (so that the rate of ATP hydrolysis is directly proportional to the assay end point, i.e. the rate of NADH oxidation) and that the ATPase enzyme is the rate-limiting step. While optimal conditions for only a few parameters were determined, the results of these experiments were sufficient for the intended purpose. The two key experiments were 1) the response to varying concentrations of ATP and 2) the response to varying quantities of added protein. The interpretations of these results, i.e. 1) that the enzyme displayed classical Michaelis-Menten kinetics, 2) that the substrate, ATP, is present in saturating concentrations, and 3) that the added ATPase is, in fact, the rate-limiting component, were central to validation of the method. In addition, the experimentally determined K_m and V_{max} for this system are similar to those reported by Desaiah and Ho (1977) for mouse brain synaptosomal ATPases isolated and assayed by a similar method.

In order to examine the components of this composite enzyme system, it was necessary to establish sensitivity to classical inhibitors. Two pharmacological probes were chosen for this purpose. Ouabain is a specific inhibitor of the Na^+/K^+-ATPase. The maximally effective inhibitory concentration (1 mM) found in these experiments is consistent with literature values (e.g., see Desaiah and Ho, 1977; Schwartz et al., 1975). Likewise, oligomycin inhibited mitochondrial Mg^{++}-ATPase activity as anticipated. The biphasic response to oligomycin, however, was quite unexpected (activity began to return to control levels at concentrations larger than 10^{-7} M), as Lardy et al. (1965) reported a concentration-dependent inhibition of the enzyme with concentrations of about 10^{-7} to 10^{-5} M. This discrepancy is unexplained at present. While it is an interesting observation, no further investigation of its etiology was pursued as such an endeavor was beyond the scope of this project. Nevertheless, the goal of these experiments was achieved, i.e. a concentration of oligomycin was found that could be used to selectively inhibit what is nominally defined as the oligomycin-sensitive component of the mitochondrial Mg^{++}-ATPase.

Finally, the ATPase system was selected as a means of evaluating neurochemical toxicity because of its proposed role in neurotransmission. Therefore, as a positive control for the sensitivity of the preparation to a challenge representative of its neurochemical role, DA and NE were tested for their effect on synaptosomal ATPases. Consistent with previous reports in the literature, catecholamines produced a concentration-dependent increase in synaptosomal Na^+/K^+- and Mg^{++}-ATPases.

Appropriate responses to the positive controls was convincing evidence that the assay was functioning properly. Therefore, evaluation of the effects of Aroclor 1254 on mouse brain ATPases was undertaken. Addition of Aroclor 1254 in vitro to isolated mitochondria and synaptosomes produced a concentration-dependent inhibition of ATPase activity. The mitochondrial ATPases were more sensitive to this inhibitory effect than the synaptosomal ATPases. Desaiah et al. (1972) also observed similar inhibitory effects on ATPases in fish brain homogenates and reported IC_{50} values of 6 and 5.2 ppm of Aroclor 1254 for inhibition of what they defined as oligomycin-sensitive and insensitive Mg^{++}-ATPases. While these results are qualitatively consistent with those reported here (i.e. Mg^{++}-ATPases were inhibited in a concentration-dependent fashion and the oligomycin-sensitive and insensitive ATPases were equally sensitive), it is impossible to compare ATPase activities of their crude tissue homogenate preparations with those of the purified preparation used here.

The in vivo results are not as easily explained. Neither acute nor 14 day administration of various doses of Aroclor 1254 had any effect on ATPases examined. These results conflict with those of Narbonne et al. (1978) who showed a 33% increase of Na^+/K^+-ATPase from brain homogenates of rats that had received 500 ppm of Phenoclor DP_6 in their diet for 8 days, but no effect was observed with 10 or 100 ppm in the diet for 30 days. These investigators did, however, show a significant decrease in Mg^{++}-ATPase in brain homogenates from rats that had received 10 or 100 ppm of PCBs in the diet for 30 days but not in those on the 500 ppm regimen for 8 days. No explanation was offered by the authors for their findings.

The disparity between previous results and those reported here can be accounted for by several factors. It is certainly possible that a species difference exists, since Narbonne et al (1978) used rats and the experiments reported herein involved mice. There were also two different commercial PCB mixtures used; Narbonne et al. (1978) used a French product (Phenoclor) while the experiments reported herein involved a U.S. product (Aroclor). Phenoclor DP_6 is similar in composition to Aroclor 1260 (IARC, 1978). Numerous investigators have reported varying sensitivities to PCBs of differing chlorine content in a variety of systems (see Introduction), a factor which could account for the differences encountered here. Furthermore, the tissue preparations used by Narbonne et al. (1978) were not the same as those used here. Several other factors that could be included here are 1) the use of different vehicles for PCBs and 2) the slightly different protocols used for administering PCBs, i.e. although both studies administered PCBs orally, one was by gavage and the other by dietary administration. This latter point is one that has often been subject to criticism and question, i.e. a more constant delivery of the compound in the diet over the course of the day may have entirely different effects than a bolus of compound by oral gavage.

Any one or a combination of these factors mentioned here could account for the observed differences in the in vivo effects of PCBs, but more importantly, the experiments described here show in vitro but not in vivo effects of Aroclor 1254 on ATPases. Similar findings were described in the previous section (Neurochemical Effects - Part A), i.e. inhibitory effects of Aroclor 1254 on neurotransmitter uptake were found in vitro but not in vivo. Because of this similar response, this topic will be addressed in the "General Discussion" that follows.

In summary, the experiments reported in this chapter have provided evidence for validation of the coupled enzyme, ATP-regenerating system method for determining ATPase activity in mouse brain synaptosomes and mitochondria. It has also been demonstrated that Aroclor 1254 produced a concentration-dependent inhibition of these ATPases in vitro, but not in vivo, which is consistent with the previously demonstrated effects on other neurochemical events. An overall interpretation of the effect of Aroclor 1254 on all of the neurochemical parameters examined is reserved for the "General Discussion."

VI. EXPERIMENT 4 - DISPOSITION OF ^{14}C-PCBs <u>IN</u> <u>VITRO</u> AND <u>IN</u> <u>VIVO</u>

A. <u>Introduction</u>

The disposition of PCBs has been reported using a variety of different commercial preparations and individual congeners, in different species, and using various routes and duration of exposure (see Introduction - Pharmacokinetics). However, the results of the behavioral and neurochemical experiments reported here prompted a more detailed analysis of Aroclor 1254 disposition with experiments designed to mimic the conditions employed for the behavioral and neurochemical experiments. The reasons for initiating this project are as follows. Aroclor 1254 was found to depress spontaneous activity. Neurochemical function was altered by Aroclor 1254 added <u>in</u> <u>vitro</u> to isolated synaptosomes and mitochondria, but <u>in</u> <u>vivo</u> administration had no effect on neurotransmitter uptake or on nerve terminal ATPases. It was thus of interest to determine levels of Aroclor 1254 in brain at times when spontaneous activity was measured and when neurochemical parameters were evaluated. Because of the disparity between <u>in</u> <u>vitro</u> and <u>in</u> <u>vivo</u> effects of Aroclor 1254 on neurochemistry, experiments were performed to compare the levels found in subcellular organelles from brains of animals that had been exposed to a behaviorally active dose of Aroclor 1254 to levels taken up by isolated synaptosomes and mitochondria <u>in</u> <u>vitro</u>. It was felt that this series of experiments would aid in providing a unifying interpretation of the results of the behavioral and neurochemical experiments.

B. Methods

1. Materials

A uniformly labelled (U-^{14}C) mixture of PCBs with 50% chlorine by weight (^{14}C-PCBs, 37 mCi/mmol; Amersham Searle) was employed for the disposition studies. This particular radiolabelled product was selected because its chemical composition is a close approximation to that of Aroclor 1254, i.e. the chlorine contents are 50 and 54% (w/w), respectively, for the ^{14}C-PCBs and Aroclor 1254. Aroclor 1254, itself, is not commercially available in a radiolabelled form. The experiments described here involve in vitro and in vivo exposure to a mixture of Aroclor 1254 and the radiolabelled product, to which the general term "^{14}C-PCBs" is applied.

2. In vitro uptake of ^{14}C-PCBs by isolated synaptosomes and mitochondria

Synaptosomes and mitochondria were isolated by the method of McGovern et al. (1973) as described previously and resuspended in appropriate media to yield a tissue concentration of 0.5 to 1.0 mg protein/ml. Aliquots (1.0 ml) of the tissue suspension were preincubated (2 min for synaptosomes and 1 min for mitochondria in order to simulate conditions in previous neurochemical experiments) in the presence of unlabelled Aroclor 1254 (10^{-6} to 10^{-3} M) spiked with 0.5 µCi of ^{14}C-PCBs. Incubation temperatures, as in the neurochemical experiments, were 37° C for synaptosomes and 30° C for mitochondria. The assay then proceeded as for mitochondrial ^{45}Ca^{++} uptake (basal conditions) or synaptosomal neurotransmitter release (in the presence of 5 or 60 mM KCl). Uptake was terminated by the addition of ice cold buffer followed by centrifugation at 18,000 x g for 4 min. The resulting pellets were

washed once and resuspended in distilled water. Distilled water was used for resuspension rather than NaOH (which was used in the neuro-chemical experiments) to circumvent the problem of dissolving bound ^{14}C-PCBs from the walls of the test tube. Suspensions were then trans-ferred to scintillation vials containing 10 ml of ACS, and the amount of bound ^{14}C-PCBs was determined by LSC.

2. Disposition of ^{14}C-PCBs in vivo

The distribution of ^{14}C-PCBs in vivo was evaluated by measuring radioactivity in tissue following an oral dose of 500 mg/kg. Because of the complexity of the commercial mixture of PCBs and their metabo-lism, only total radioactivity was determined and not the level of in-dividual components or metabolites. Data are therefore expressed as pmol equivalents of PCBs as calculated from total radioactivity.

a. Time course of levels of ^{14}C-PCBs in brain, liver, and plasma

Mice were administered ^{14}C-PCBs (70 µCi/500 mg/kg, p.o.) and de-capitated 0.25, 0.5, 0.75, 1, 2, 4, or 8 hr later. Trunk blood was collected from the cervical wound in heparinized tubes and centrifuged to obtain plasma. Aliquots (50 µl) of plasma were counted directly in 10 ml of ACS. Whole brain was removed and a mid-saggital cut was made. A piece of liver (~200 mg) and one half brain were dried overnight and then oxidized in a Packard Sample Oxidizer. Radioactivity was counted as ^{14}CO$_2$ in Carbosorb and Permafluor V (a CO$_2$ absorber and scintilla-tion cocktail, respectively, supplied by Packard Instruments Co., Downers Grove, IL).

b. Distribution of ^{14}C-PCBs in brain regions

As in the above study, mice received a dose of ^{14}C-PCBs (70 µCi/500 mg/kg, p.o.) and were sacrificed 45 or 2 hr later. Plasma was obtained and aliquots (50 µl) were counted in ACS. Brain was removed, placed in a plexiglass holder, and microwave-irradiated for 7 sec (1.2 kW, 2.450 GHz, General Medical Engineering, Peabody, MA). The brain was then dissected into six regions by the method of Glowinski and Iversen (1966). After drying overnight, brain regions were oxidized, and radio-activity was counted.

c. Distribution of ^{14}C-PCBs in subcellular fractions of whole brain

Mice were administered ^{14}C-PCBs (140 µCi/500 mg/kg, p.o.) 45 or 120 min before sacrificing. Plasma was obtained and counted as de-scribed above. Subcellular fractionation of whole brain was done by the combined methods of deRobertis et al. (1962) and Mulé et al. (1967) to yield the fractions shown in Figure 31. Aliquots of each fraction were taken for protein determination and for counting in ACS.

Figure 31

Centrifugation scheme for the subcellular fractionation of whole brain. CM fractions: A, myelin fragments; B, small fragments of nerve endings; C, cholinergic nerve endings; D, non-cholinergic nerve endings; E, free mitochondria. CN fractions: N_1, large myelin fragments; N_2, nuclei, myelin fragments, and mitochondria; N_3, whole cells, tissue debris, and blood vessels.

WHOLE BRAIN

HOMOGENATE

(10 VOL. 0.32M SUCROSE WITH 10 uM Ca^{++})

900 x g x 10 MIN

(TWO WASHINGS)

CRUDE NUCLEAR (CN)   11,500 x g x 20 MIN

RESUSPEND   (ONE WASH)

SEPARATE ON DISCONTINUOUS   105,000 x g x 30 MIN   CRUDE MITOCHONDRIAL (CM)

SUCROSE GRADIENT   MICROSOMES   SUPERNATANT   HOMOGENIZE

(50,000 x g x 120 MIN)   SEPARATE ON DISCONTINUOUS

SUCROSE GRADIENT

(50,000 x g x 120 MIN)

Adapted from Mule et al. (1967)

C. Results

1. In vitro uptake of ^{14}C-PCBs by isolated synaptosomes and mitochondria

Synaptosomes and mitochondria were exposed to ^{14}C-PCBs in the same manner as they were handled for previously described neurochemical experiments, so that the quantity of ^{14}C-PCBs actually taken up by the organelles could be determined. As shown in Table 12, increasing concentrations of ^{14}C-PCBs in the medium produced a parallel increase in uptake by synaptosomes and mitochondria. Uptake by synaptosomes was examined under two conditions, in the resting state (in the presence of 5 mM KCl) and in the potassium-induced depolarized state (in the presence of 60 mM KCl). Levels of ^{14}C-PCBs were somewhat higher in the depolarized synaptosomes. A 2 x 4 factorial analysis of variance of the synaptosomal levels showed a significant effect of ^{14}C-PCB concentration ($p < .01$), but no significant interaction between the two factors. In other words, a parallel increase in ^{14}C-PCB uptake was observed with increasing ^{14}C-PCB concentration under both KCl conditions, and levels were significantly higher in the 60 mM KCl group than in the 5 mM KCl group.

2. Disposition of ^{14}C-PCBs in brain, liver, and plasma

a. Time course of levels of ^{14}C-PCBs in brain, liver, and plasma

The time course of the brain and plasma levels of radioactivity following a single oral dose of ^{14}C-PCBs is shown in Figure 32. Both brain and plasm levels increased between 15 min and 2 hr. Maximum levels were attained in 2 and 4 hr for plasma and brain, respectively. Levels remained constant in plasma, but those in brain began to decline by 8 hr. It is important to note that these levels correspond to total

TABLE 12

Uptake of ^{14}C-PCBs by Isolated
Synaptosomes and Mitochondria

pmol equivalents/mg protein^a

PCBs[b]	Synaptosomes		Mitochondria
	5mM KCl	60mM KCl	
10^{-6}	14 ± 1	19 ± 1	20 ± 1
10^{-5}	186 ± 15	264 ± 7	221 ± 6
10^{-4}	3281 ± 182	3811 ± 340	3538 ± 254
10^{-3}	27,304 ± 1073	29,896 ± 752	29,198 ± 911

[a] Values represent mean ± S.E. (N = 6)

[b] Molar concentration

Figure 32

Time course of levels of radioactivity in brain and plasma following a single oral dose of ^{14}C-PCBs (500 mg/kg). Abscissa, time after administration of ^{14}C-PCBs. Ordinate: left axis, brain levels (—◐—); right axis, plasma levels (—◈—). Values represent mean ± S.E. for determinations from 3 mice.
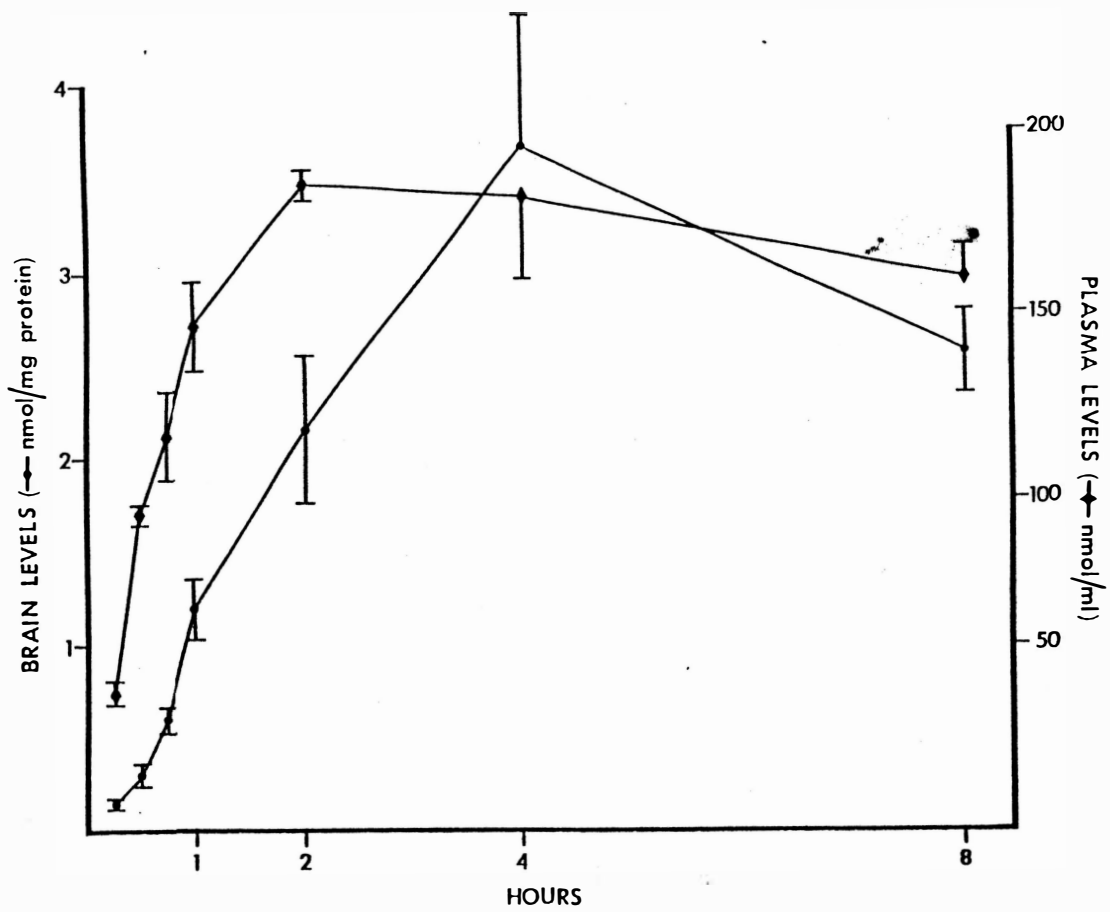
Page 178 intentionally left blank

Figure 33

Time course of levels of radioactivity in liver and brain following a single oral dose of ^{14}C-PCBs (500 mg/kg). Abscissa, time after administration of ^{14}C-PCBs. Values represent mean ± S.E. for determinations from 3 mice. Where no S.E. bars are shown, the values lie within the bounds of the symbol.
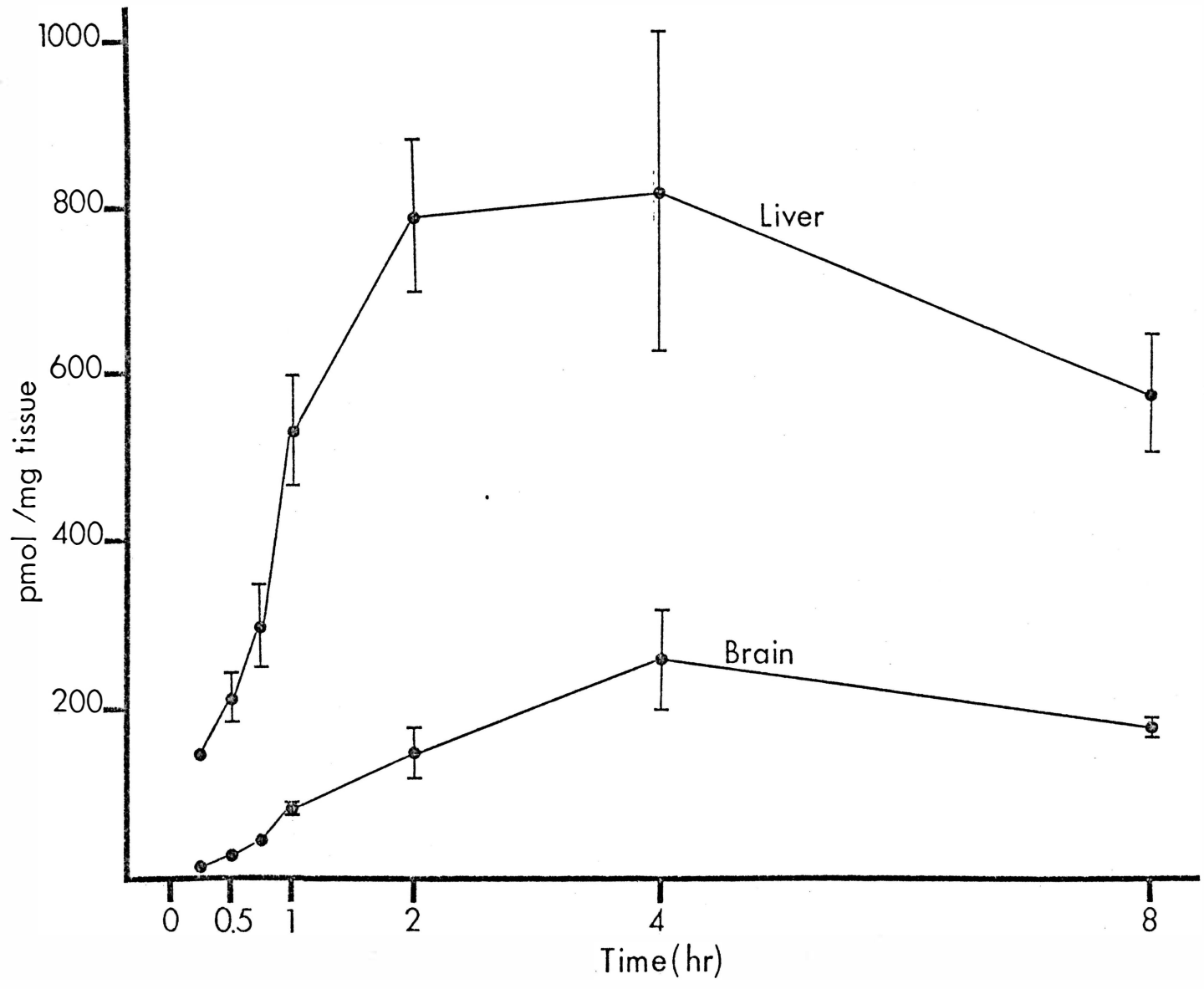
activity and may reflect the sum of parent compounds and metabolites.

A similar pattern of levels of radioactivity in liver is illustrated by Figure 33. As was seen in brain and plasma, levels increased between 15 min and 2 hr at which point they leveled off. Like brain, levels in liver began to decline by 8 hr. Also shown in this figure are brain levels plotted in units of pmol/mg tissue recalculated from the values in Figure 32 (which were expressed as pmol/mg protein for purposes of comparison to the in vitro data) in order to compare brain levels to liver levels. This comparison, while reiterating the pattern of levels observed over time, also points out another important feature, i.e. levels in liver increased more rapidly than those in brain and the maximum levels achieved in liver were higher (about 4-fold) than in brain.

b. Distribution of ^{14}C-PCBs in brain regions

From the above results and from previous studies, two time points were chosen for brain region and subcellular distribution studies. At both 45 and 120 min, Aroclor 1254 produced a significant decrease in spontaneous locomoter activity with the greatest difference between treatment and control noted at 45 min after treatment (see Experiment 1). Furthermore, maximal enhancement of pentobarbital-induced sleep time was produced by Aroclor 1254 given 2 hr prior to pentobarbital (see Experiment 2).

The distribution of ^{14}C-PCBs in brain regions following a single oral dose is given in Table 13. As expected, levels were higher at 2 hr than at 45 min, however none of the brain regions specifically accumulated ^{14}C-PCBs, i.e. radioactivity was distributed uniformly throughout all six brain regions. Plasma levels from these animals

TABLE 13

Distribution of ^{14}C-PCBs in
Mouse Brain Regions

pmol/mg protein[a]

Brain Region	45 min[b]	120 min[c]
Cerebellum	900 ± 80	2,023 ± 351
Medulla-Pons	943 ± 105	2,370 ± 646
Midbrain	958 ± 131	2,211 ± 119
Hippocampus	941 ± 90	1,682 ± 204
Striatum	720 ± 51	1,478 ± 245
Cortex	856 ± 29	1,989 ± 309

[a] Mean ± S.E.

[b] N = 4

[c] N = 3

were 30 ± 2 and 66 ± 6 nmol/ml for the 45 min and 2 hr time points, respectively.

c. Distribution of ^{14}C-PCBs in subcellular fractions of whole brain

Subcellular distribution was determined 45 or 120 min after administration of ^{14}C-PCBs (Tables 14, 15, and 16). Of particular interest are the levels found in mitochondria (fraction E) and synaptosomes (fractions C and D), since they are comparable to levels taken up by isolated synaptosomes and mitochondria in vitro (Table 12). The significance of these findings will be discussed below. It might also be pointed out that the largest proportion of total radioactivity was found in myelin, synaptosomes, and microsomes (25, 21, and 27% of total radioactivity, respectively, at 45 min and 32, 21, and 31% at 2 hr). Although levels in the crude nuclear subfractions (Table 16) appear rather high, they are artificially inflated by the calculation of these values which involves dividing total pmol equivalents in the subfraction by the protein content, which for these subfractions is quite low. In actuality, less than 10% of the total radioactivity in brain was associated with the combined crude nuclear fraction. Plasma levels for these groups of animals were 46 ± 7 and 98 ± 8 nmol/ml for the 45 min and 2 hr time points, respectively.

TABLE 14

Distribution of ^{14}C-PCBs in Subcellular
Fractions of Mouse Brain

pmol/mg protein[a]

Fraction	45 min.	120 min.
Homogenate	557 ± 143	1,640 ± 247
Crude Nuclear	201 ± 34	774 ± 39
Crude Mitochondrial	601 ± 88	1,991 ± 128
Microsomal	897 ± 104	2,049 ± 273
Supernatant	47 ± 16	192 ± 24

[a] Mean ± S.E. for determinations from 3 mice

TABLE 15

Distribution of ^{14}C-PCBs in Crude Mitochondrial
Subfractions of Mouse Brain

	pmol/mg protein[a]	
Fraction	45 min.	120 min.
A - Myelin	2,530 ± 276	5,563 ± 241
B - Membranes	1,267 ± 70	3,078 ± 478
C - Cholinergic	876 ± 118	2,712 ± 672
D - Non-Cholinergic	778 ± 148	2,109 ± 227
E - Free Mitochondria	416 ± 63	814 ± 130

[a] Mean ± S.E. for determinations from 3 mice

TABLE 16

Distribution of ^{14}C-PCBs in Crude Nuclear
Subfractions of Mouse Brain

	pmol/mg protein[a]	
Fraction[b]	45 min.	120 min.
N_1	1,981 ± 230	3,060 ± 366
N_2	1,230 ± 143	2,202 ± 485
N_3	775 ± 73	1,700 ± 157

[b] Refer to Figure 31 for description of these subfractions

D. Discussion

The results of the study of the disposition of ^{14}C-PCBs revealed several important factors regarding ^{14}C-PCB absorption and distribution. Following a single oral dose, ^{14}-PCBs were readily absorbed from the GI tract as seen from the levels in plasma at 15 min and the rapid increase in levels between 15 min and 2 hr. Tissue distribution also follows a similar pattern, although the rate of entry into brain may be somewhat slower that that of liver. Because ^{14}C-PCBs were given orally, rapid distribution to the liver and the resulting high levels of ^{14}C-PCBs may be accounted for by the perfusion of the liver by the portal circulation. The slower rate of absorption by the brain may simply be a result of a first pass effect, i.e. a large proportion of ^{14}C-PCBs may have been cleared from the circulation on the first pass through the liver. In either case, it is obvious that the lipid content of both brain and liver facilitates tissue uptake of these highly lipophilic compounds.

The experiment involving brain regional distribution of ^{14}C-PCBs was chosen, in part, as a screening method. To explain further, the only behavioral effect of Aroclor 1254 administration that was detected was the depression of spontaneous activity. Thus, if ^{14}C-PCBs were distributed specifically to one or more brain regions, a direction for further studies may have been indicated. Seen from another viewpoint, a differential distribution of ^{14}C-PCBs in brain may have provided some basis for correlating the depressed spontaneous activity with a pharmacokinetic effect. However, these hypotheses were not borne out by the data, as ^{14}C-PCBs were distributed evenly throughout brain.

The distribution studies reported here have some bearing on previous experiments, particulary as concerns the behavioral studies. The time course of behavioral activity, i.e., the observed decrease in spontaneous activity, indicates a rapid onset with significant depression of spontaneous activity appearing by 15 min and remaining up to 3 hr. However, brain levels increased up to 4 hr and remained high until 8 hr. The lack of correlation between brain levels and behavioral effects is difficult to resolve, but several explanations may be offered. It is possible that maximal effects on activity occurred with initial concentrations of ^{14}C-PCBs in brain (i.e., those levels appearing at the early time points) or that tolerance to the effect developed rapidly such that no further depression of activity occurred despite increased brain levels. An alternative explanation may contribute to this observation as well as account for the return of activity to control values by 8 hr. Although levels of radioactivity in brain remained at a plateau at 8 hr, these values may reflect levels of inactive metabolites in addition to parent compound, since the nature of the labelled compound dose not permit differentiation of individual components but only determination of total radioactivity. If this is true, it may account for the loss of effect by 8 hr.

The correlation between ^{14}C-PCB disposition and neurochemical effects was also investigated in these experiments. Although the effects of Aroclor 1254 on neurochemistry in vitro were found to be concentration-dependent, there was some concern over the specificity and relevance of these changes in view of the high concentrations employed. The data on ^{14}C-PCB disposition may help to justify these

concentrations. ^{14}C-PCBs, added to the medium in concentrations that altered neurochemistry <u>in</u> <u>vitro</u>, were taken up by isolated brain mitochondria and synaptosomes. Tissue levels paralleled both concentration and effect on neurochemical parameters (e.g., decreased uptake and increased release of neurotransmitters). It was somewhat surprising to see the small, but significant, increase in synaptosomal levels of ^{14}C-PCBs in the potassium-induced depolarized state. This might be explained by considering the events that follow nerve terminal depolarization. The configurations of proteins within the lipid matrix of the nerve cell membrane are sensitive to changes in membrane potential. The molecular mechanism of ion flux across cell membranes lies in conformational shifts of these protein-lipid interactions to change the position of these ion "gates" (Hille, 1976). This change in the microenvironment of the membrane during conditions simulating depolarization (i.e., 60 mM KCl buffer) may explain the increased levels of ^{14}C-PCBs in depolarized synaptosomes. While purely conjectural, this inference may be made from the work of Sharom and Mellors (1980) who demonstrated a relationship between PCB toxicity and molar volume, a paramenter whose calculation includes the concentration of the chemical in the lipid biophase of the system. In other words, many of the biological effects of PCBs may be due to a physical toxicity, i.e. their ability to penetrate and concentrate in cell membranes, that relates to their lipid solubility. Thus, changes in membrane configuration in response to nerve terminal depolarization may improve PCB uptake by masking hydrophilic regions of the membrane. Although this may offer an explanation for the disposition data, the increased levels of ^{14}C-PCBs in depolarized synaptosomes do not correlate with the biochemical effects.

Aside from serotonin release, Aroclor 1254 had no effect on depolarization-evoked release of neurotransmitters or depolarization-induced synaptosomal $^{45}Ca^{++}$ influx. The interpretation of these biochemical observations will be addressed in the General Discussion.

The relevance of the data from the in vitro study of the uptake of ^{14}C-PCBs becomes apparent when compared to the in vivo data. An oral dose of ^{14}C-PCBs that depressed spontaneous activity yielded levels in subcellular fractions of brain that were comparable to those taken by isolated organelles incubated with ^{14}C-PCBs at concentrations (10^{-5} to 10^{-4} M) that altered neurotransmitter function in vitro. Furthermore, the time course study (Figure 33) shows that levels of ^{14}C-PCBs in brain ranged from 150 to 3000 pmol/mg protein at times when Aroclor 1254 was found to be behaviorally active, and these values were also comparable to tissue levels taken up by isolated mitochondria and synaptosomes that were incubated with ^{14}C-PCBs in vitro. These in vivo disposition studies thus offer a justification for the concentrations of Aroclor 1254 employed in the in vitro neurochemical studies.

While the above discussion explains the in vitro data, it does not account for the lack of neurochemical effects following oral administration of Aroclor 1254 either acutely or chronically. It would appear from these disposition data that orally administered ^{14}C-PCBs are able to enter the cell and accumulate in subcellular organelles at concentrations effective in altering neurochemistry in vitro. Although one of the limitations of subcellular distribution studies lies in the fact that redistribution of the labelled compound may occur during the fractionation process, the possibility remains that the results of the subcellular distribution study accurately reflect distribution of

^{14}C-PCBs <u>in</u> <u>vivo</u>, that sufficient levels of ^{14}C-PCBs occur in brain, but that compensatory mechanisma in the intact animal preclude the manifestation of any neurochemical changes that would occur <u>in</u> <u>vitro</u>. This issue will be given further attention in the General Discussion.

VII. GENERAL DISCUSSION

Because each of the previous chapters included its own discussion, this section will elaborate on previously mentioned general topics and attempt to mold what may appear at first glance to be fragments of ideas into a unified concept. For the sake of defining a framework for discussion, the following broad topics will be addressed: 1) the association between the behavioral and neurochemical data, 2) a more general overview of the interrelationships of the neurochemical parameters altered by Aroclor 1254, and 3) an explanation of the disparity of effects following in vitro vs. in vivo exposure to Aroclor 1254.

As discussed previously (see Experiment 3), there is precedence in the literature for correlating neurotransmitter function with behavior. From the results of the experiments described herein, an association between the neurochemical alterations produced by Aroclor 1254 in vitro and the depression of spontaneous locomotor activity may be suggested. A review of the involvement of neurotransmitters in spontaneous loco- motor activity is given by Seiden and Dykstra (1977). To summarize briefly, DA appears to be the primary neurotransmitter involved in regulating locomotor activity, but NE probably plays a modulatory role. In general, any manipulation that depletes catecholamines in brain will also reduce spontaneous activity. The opposite effect, stimulation of activity, is best exemplified by amphetamine. Its stimulant effects are probably accounted for by release of catecholamines. Thus, it is possible that the slight stimulation of activity observed with low doses of Aroclor 1254 may be due to neurotransmitter release. Converse- ly, the profound depression of activity observed following exposure to the 500 mg/kg dose of Aroclor 1254 is somewhat paradoxical if one were

to use the above argument of neurotransmitter release. Nevertheless, if higher doses of Aroclor 1254 deplete neurotransmitter stores by prolonged release, this could account for suppression of activity.

Looking now at the neurochemical data alone, several interpretations can be drawn. As discussed in the introduction to Experiment 3, ATPases have been implicated in a regulatory role in neurotransmitter release and reuptake. Taking this into account, all of the neurochemical effects produced by Aroclor 1254 in vitro, i.e. inhibition of $Na^+K/^+$-ATPase, stimulation of neurotransmitter release, and inhibition of neurotransmitter re-uptake, might be drawn into one picture. As discussed previously, agents that inhibit the Na^+/K^+-ATPase also produce an increase in neurotransmitter release (see Vizi, 1978). Likewise, inhibition of the Na^+/K^+-ATPase has been linked to an inhibition of neurotransmitter re-uptake (Bogdanski et al., 1968; Prakash et al., 1973; Tissari et al., 1969). The effects of Aroclor 1254 on neurotransmitter release and re-uptake could be drawn together by implicating the inhibitory effect of Aroclor 1254 on synaptosomal Na^+/K^+-ATPase, as suggested by the results of the above mentioned literature reports. If so, this would explain the lack of Ca^{++}-dependence in the enhanced neurotransmitter release that is suggested (but not necessarily proven) by the results of the Ca^{++} studies reported here and would be consistent with the suggestion of Vizi (1978) that the Na^+/K^+-ATPase plays a role in release that follows the depolarization-evoked influx of Ca^{++}.

The effects of Aroclor 1254 on Ca^{++} flux might also be associated with ATPase inhibiton. Inhibition of the Na^+/K^+-ATPase results in accumulation of Na^+ within the nerve ending, since it then cannot be

pumped out. This increase in $[Na]_i$ could trigger the Na^+/Ca^+-exchange mechanism, which is sensitive to $[Na]_i$ (Baker, 1972), and thereby stimulate Ca^{++} influx. Only a modest increase in synaptosomal Ca^{++} influx was observed with Aroclor 1254. Although this might be accounted for by ATPase inhibition, the small degree of change may be due to the tremendous capacity of nerves to regulate their $[Ca^{++}]_i$ (Blaustein et al., 1980).

From another viewpoint, the observed increase in neurotransmitter release may, in fact, be due to increased intracellular $[Ca^{++}]$, but not necessarily from an extracellular source. The previous discussion pointed to a Ca^{++}-independent mechanism based on the small degree of synaptosomal Ca^{++} influx, the lack of correlation of concentrations with those that increased transmitter release, and stimulation of mitochondrial Ca^{++} uptake, an effect that would oppose any increase in synaptosomal Ca^{++} influx and would reduce $[Ca^{++}]_i$. The alternative is that release could occur by a Ca^{++}-dependent mechanism and that the source of this Ca^{++} may be intrasynaptosomal. As mentioned previously, Ca^{++} buffering is a complex process and involves a number of intracellular organelles. It is possible that Ca^{++} may be released from an intracellular depot upon exposure of the synaptosomes to Aroclor 1254 and that this elevation of $[Ca^{++}]_i$ triggers neurotransmitter release.

Thus, each of the observed effects of Aroclor 1254 on neurochemistry might be accounted for by considering the complex interrelationships of these parameters within the nerve ending. But an alternate view may also be true. This foregoing discussion may simply be an academic exercise, and an explanation of the effects of Aroclor 1254 on neurochemical function could be relegated to the result of a nonspeci-

fic physical membrane perturbation as described by Sharom and Mellors (1980).

Suggesting a neurochemical mechanism for the behavioral effects of Aroclor 1254 is hampered to some degree by the lack of observable effects following in vivo exposure to Aroclor 1254. The possibility cannot be dismissed that other measures of neurochemical activity not yet examined (e.g., neurotransmitter turnover, alterations in number or affinity of neurotransmitter receptors, neurotransmitter synthesis, etc.) might be altered by Aroclor 1254 or that neurochemical function was altered but not detected by the methods employed here, as indicated by the disparity between the in vitro and in vivo effects of Aroclor 1254 on neurochemical function. A number of explanations could be offered for this disparity, one of which is that the levels of Aroclor 1254 in vitro were much higher than those achieved in vivo. Results of the ^{14}C-PCB disposition studies can probably eliminate this possiblity, as concentrations of ^{14}C-PCBs that were sufficient to alter neurochemical function in vitro were found in subcellular fractions of brains from mice that had been exposed to a behaviorally active dose of ^{14}C-PCBs.

Alternatively, consider the means by which normal function is maintained in the CNS. The brain has a tremendous capacity to maintain homeostasis, and it is possible that alterations in biochemical, physiological, or morphological parameters could occur without observable functional change. There are two means by which the CNS could compensate for alterations produced by an insult of any kind, including a neurotoxic agent. The first of these, tolerance, can be defined generally as the process or processes by which the brain is able to compensate for an induced change (e.g., a drug effect or toxicolo-

logical effect) and retain normal function, in other words, an adaptive phenomenon. Applied specifically to drug effects, tolerance refers to the phenomenon in which increasing doses of a drug must be given in order to obtain the effect produced by the original dose. A variety of mechanisms may contribute to the development of tolerance, e.g., increased metabolism; reduced receptor number, affinity, or sensitivity; and compensatory feedback mechanisms. Some of these mechanisms, as they relate to adaptive function in neurochemical systems, will be discussed in more detail. The second method by which the CNS can maintain homeostasis involves the presence of structural redundancy. In the CNS, there may be more neurons than actually required in a particular tract, or another pathway altogether may exist that is able to serve as an alternate route for transferring information that would normally be carried by the impaired pathway.

Thus, the adaptive capabilites of the CNS, i.e., its tremendous plasticity, may account for the lack of observable effects on neurochemistry following oral administration of Aroclor 1254. This may also explain the loss of the behavioral effect at a time when ^{14}C-PCB levels were still at a plateau in brain (see Figures 3 and 38), i.e., tolerance may have developed to the effect of Aroclor 1254 on spontaneous activity.

The validity of suggesting an adaptive phenomenon as an explanation for the lack of in vivo effects of Aroclor 1254 might be further supported by an example from the literature of compensatory mechanisms. The neurotoxin, 6-hydroxydopamine (6-OHDA), is an agent that selectively destroys catecholaminergic neurons. Despite a well established literature on the pharmacology of 6-OHDA (see reviews by Kostrzewa and

Jacobowitz (1974) and Theonen and Tranzer (1973)), Zigmond and Stricker (1979) observed little change in a variety of behavioral measures in 6-OHDA-treated rats. Nevertheless, they were able to explain this lack of functional impairment on the basis of several observed neurochemical changes that appear to be homeostatic mechanisms. For example, they report increased catecholamine (CA) turnover, increased tyrosine hydroxylase activity (the rate-limiting step in CA synthesis), and increased CA receptors. It is conceivable that similar compensatory mechanisms may occur in the face of a neurotoxic insult produced by Aroclor 1254 that mask any effect on neurochemistry as observed in the in vitro studies. Additional experiments may reveal these effects.

In conclusion, the experiments described herein have demonstrated that neurotoxic effects of Aroclor 1254 occur in mice. While it is premature to suggest a mechanism for the neurotoxic effects, the alterations in neurochemical function, as observed in vitro, may be contributing factors. In any case, sensitivity of the in vitro measures of neurochemical function to Aroclor 1254 has been demonstrated, suggesting that these measures may be useful for assessing neurotoxicity of other environmental contaminants. Further evaluation may be required to determine whether adverse effects on neurochemical function occur following in vivo exposure to Aroclor 1254.

VIII. SUMMARY

1. Acute oral administration of Aroclor 1254 at a dose of 500

mg/kg resulted in depression of spontaneous locomotor activity

for up to 3 hr. Subchronic (14 day exposure did not produce

this effect. Neither was there any alteration in other be-

havioral tests - rotor rod, inverted screen, or pentylenetetrazol-

induced convulsions. The effect on spontaneous activity

suggests a general CNS depression.

2. The above results prompted further examination of this suspec-

ted CNS depression. Acutely administered Aroclor 1254 (at

doses of 5 to 500 mg/kg) produced a dose-dependent increase in

pentobarbital-induced sleep time. With a dose of 500 mg/kg,

the peak effect was observed with an Aroclor 1254-pretreatment

time of 2 hr. Conversely, subchronic administration of Aroclor

1254 resulted in reduced pentobarbital-induced sleep time, but

this effect was dependent upon the latency between the last

dose of Aroclor 1254 and administration of pentobarbital.

The suppression in sleep time with a 24 hr latency was partial-

ly reversed when measured at only a 45 min latency.

Studies of the disposition of ^{14}C-pentobarbital following

Aroclor 1254 administration revealed an alteration in pentobar-

bital pharmacokinetics by Aroclor 1254 rather than a change in

CNS sensitivity to pentobarbital or an additive depressant

effect of the two compounds. The results suggest that acutely

administered Aroclor 1254 reduced pentobarbital metabolism,

while the subchronic studies confirm previous reports in the

literature that PCBs induce the liver mixed-function oxidase system and enhance pentobarbital metabolism.

3. Aroclor 1254 altered neurochemical function $\underline{in}$ $\underline{vitro}$ at concentrations of ~10^{-5} to 10^{-4} M, but no change in neurotransmitter uptake or ATPase activity could be demonstrated in synaptosomes or mitochondria isolated from brains of mice that had been exposed to acute or subchronic doses of Aroclor 1254. The following neurochemical changes were observed $\underline{in}$ $\underline{vitro}$: enhancement of neurotransmitter release, inhibition of neurotransmitter and precursor uptake, a modest increase in synaptosomal $^{45}Ca^{++}$ influx, an increase in ATP-supported mitochondrial $^{45}Ca^{++}$ uptake, and inhibition of synaptosomal and mitochondrial ATPases. The sensitivity of each these neurochemical systems to external stimuli was validated by the use of positive controls.

4. Studies of the disposition of ^{14}C-PCBs revealed the following. Following an oral dose of ^{14}C-PCBs, levels of ^{14}C-PCBs in brain, liver, and plasma increased up to about 2 hr and remained constant up to 8 hr, an observation that was inconsistant with behavioral effects. No difference in brain regional distribution of ^{14}C-PCBs was found. Levels of ^{14}C-PCBs found in synaptosomes and mitochondria from subfractionated brains of mice that were exposed to a behaviorally active dose of PCBs compared favorably with levels taken up by isolated synaptosomes and mitochondria that had been incubated with concentrations of ^{14}C-PCBs that altered neurotransmitter function $\underline{in}$ $\underline{vitro}$.

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X. APPENDIX

Figure 34

Standard calibration curve for the determination of protein concentration in tissue samples. Bovine serum albumin (BSA) was used as a reference standard, and absorbance of the Coomasie Brilliant Blue G-protein complex was measured at 595 nm. Points represent mean ± S.E. of values from 5 representative experiments. Least squares linear regression analysis yielded a correlation coefficient of 0.9931.
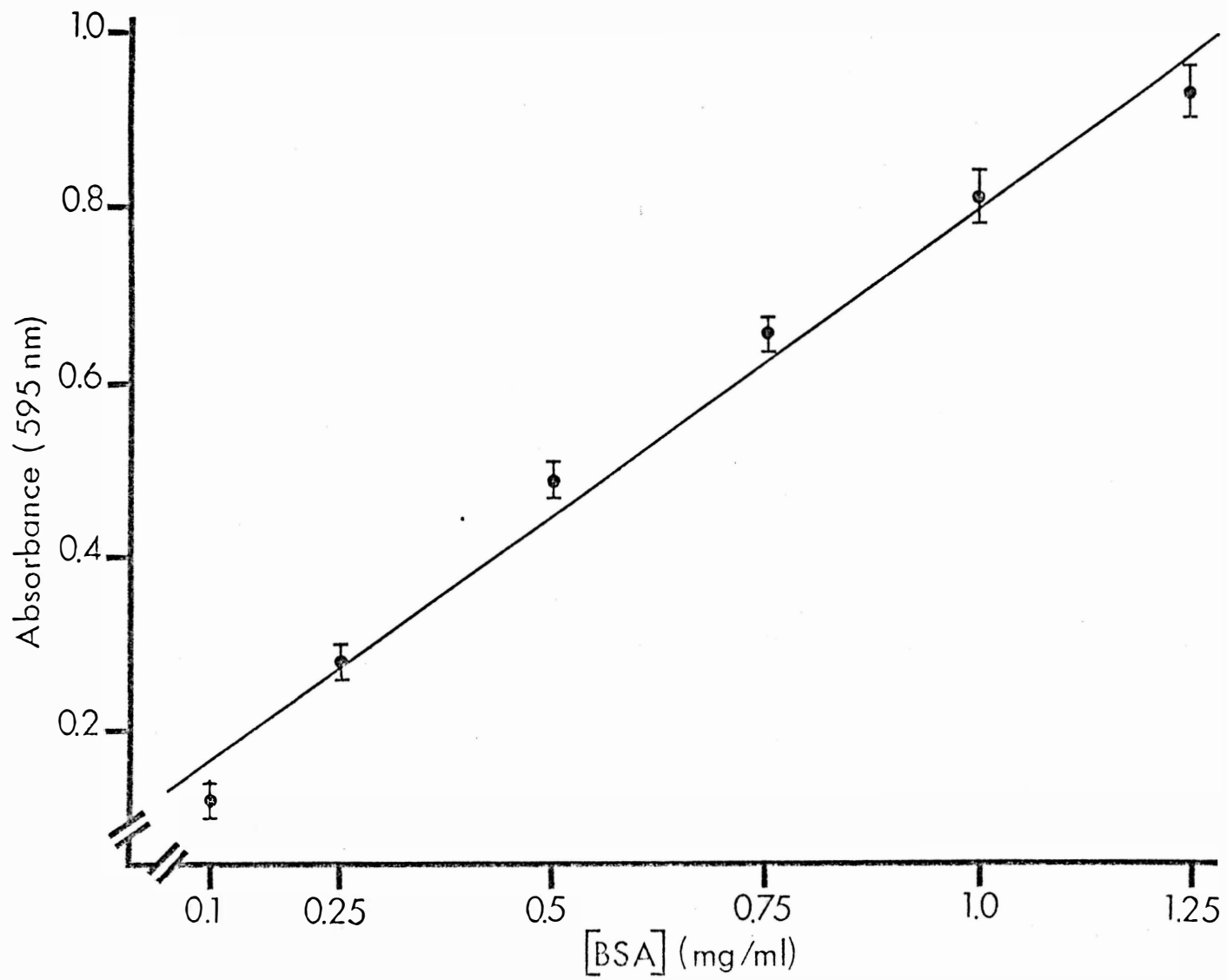
X. APPENDIX

Figure 35

Recording spectrophotometric method for the determination of ATPase

activity. The plot shown here is a reproduction of an actual tracing

from a control sample. Change in absorbance measured at 340 nm is

plotted over time and represents the disappearance of NADH, i.e. NADH

oxidation, as shown in the biochemical reactions in the upper right

corner of the figure. ATPase activity, being proportional stiochio-

metrically to the rate of NADH oxidation, is calculated, as shown, from

the slope of the line. This calculation is based on Beer's Law:

$$A = \varepsilon bc$$

where A = Absorbance

ε = Molar absorptivity

b = Sample path length   .

c = Concentration

SPECTROPHOTOMETRIC ASSAY FOR ATPase ACTIVITY

$$ATP \xrightarrow{\text{ATPase}} ADP + P_i$$

$$ADP + \text{phosphoenolpyruvate} \xrightarrow{\text{PK}} \text{pyruvate} + ATP$$

$$\text{Pyruvate} + NADH + H^+ \xrightarrow{\text{LDH}} \text{lactate} + NAD^+$$

$$\text{ACTIVITY} (\mu\text{mole } P_i/\text{mg protein}/\text{hr})$$

$$= \frac{\text{slope (hr}^{-1})}{\left(\epsilon_{mM}^{NADH} = 6.22 \frac{cm^2}{\mu mol}\right)\left(1 \text{ cm}\right)} \times \frac{\text{vol (ml)}}{\text{protein (mg)}}$$