Prevention of DMBA-induced adrenal necrosis by pretreatment of rats with DDT.

Stephen Russell. Burgess

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L’AVONS, RECUE
PREVENTION OF DMBA-INDUCED ADRENAL NECROSIS BY PRETREATMENT OF RATS WITH DDT

by

STEPHEN RUSSELL BURGESS

A thesis presented to the University of Windsor in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Biology

Windsor, Ontario, 1981

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ABSTRACT

Pretreating rats with \( \text{p},\text{p}'-\text{DDT} \) \([1,1,1\text{-trichloro-2,2-bis(p-chlorophenyl)ethane}]\) in the diet, in concentrations as low as 10 ppm for two weeks, effectively protected the adrenals from DNBA-induced \([7,12\text{-dimethylbenz(a)anthracene} \text{-induced}]\) adrenocorticonecrosis. DDT exposure also effectively induced liver growth; increased in vivo metabolism of drugs, as evidenced by a reduction in pentobarbital anesthesia; increased hepatic microsomal AMN \([\text{aryl hydrocarbon hydroxylase}]\) activity; and, reduced \([3H]\)-DNBA uptake by the adrenals. These effects appeared to be DDT-dose-related. The organophosphate pesticide malathion, however, had no observable effect on liver growth and functioning in the rat.

Adrenal microsomal AMN activity was found to be approximately 2.5 times that in control liver. Adrenal AMN was noninducible in vivo by DDT, PB or BNF; and appeared to be similar to the cytochrome P450 form of this enzyme found in the liver. Intestinal AMN was also noninducible by DDT or PB, but highly inducible in vivo by BNF, suggesting it too is of the P450 form.

These results support the proposal that adrenal protection from DNBA by DDT pretreatment is due to the ability of
DDT to promote DMBA metabolism in the liver, before levels of 'activated' DMBA, toxic to the adrenal cells, are reached. DMBA activation to its adrenocorticolytic form probably occurs within the adrenal itself, since this organ normally contains high levels of microsomal AHR activity.
ACKNOWLEDGEMENTS

I would like to acknowledge the technical assistance of Charles Silinskas, David Keightley, and particularly that of Michelle Mason. The recommendations of Drs. J.E.J. Habowsky and G.W. Wood regarding this thesis were appreciated. I am particularly grateful to Dr. A.H. Okey for his guidance and patience in seeing this project completed.

I would like to thank family and friends who have encouraged me to see these studies through to completion.

I am deeply indebted to my wife, Linda, for her constant patience, encouragement and understanding.
DEDICATION

To Linda: for her steadfast support and encouragement to seek new horizons.
ABBREVIATIONS

1. ACTH = adrenocorticotropic hormone
2. AHH = aryl hydrocarbon hydroxylase
3. ANF = alpha-naphthoflavone
4. ANOVA = analysis of variance
5. BNF = beta-naphthoflavone
6. BP = benzo(a)pyrene
7. CRF = corticotrophin releasing hormone
8. FDA = 2,2-bis(p-chlorophenyl)acetic acid
9. DDD = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane
10. DDI = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
11. DDMS = 1-chloro-2,2-bis(p-chlorophenyl)ethane
12. DDMU = 1-chloro-2,2-bis(p-chlorophenyl)ethylene
13. DDNU = unsym-bis(p-chlorophenyl)ethylene
14. DDOH = 2,2-bis(p-chlorophenyl)ethanol
15. DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
16. DMBA = 7,12-dimethylbenz(a)anthracene
17. DNA = deoxyribonucleic acid
18. Duncan = Duncan's multiple range test
19. [3H]- = tritiated compound
20. i.p. = intraperitoneal
21. Km = Michaelis-Menten constant
22. LSM = least squares means test
23. MC = 3-methylcholanthrene
24. NADH = nicotinamide adenine dinucleotide, reduced
25. NADPH = nicotinamide adenine dinucleotide phosphate, reduced
26. 7-OHM-12-MBA = 7-hydroxymethyl-12-methylbenz-(a)anthracene
27. P-450 = cytochrome P-450
28. P1-450 = cytochrome P1-450
29. PAH = polycyclic aromatic hydrocarbon
30. PH = phenobarbital
31. PBH = polybrominated biphenyl
32. PCH = polychlorinated biphenyl
33. p.o. = per os (by mouth)
34. ppm = parts per million (i.e., mg/kg, etc.)
35. RNA = ribonucleic acid
36. SEM = standard error of the mean
37. TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin
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Chapter I

INTRODUCTION

1.1 XENOBIOTIC EXPOSURE

Our exposure to toxic and potentially toxic chemicals is steadily increasing. Exposure to a toxic compound initiates the following general events: (1) contact of the toxic material with the organism, (2) penetration of the toxin into the organism, (3) metabolism of the chemical, (4) storage of the parent compound and/or its metabolites at inert sites and their excretion, (5) transport of the parent compound and/or its metabolites to the 'target' or susceptible tissue(s), and (6) interaction of these chemicals with the 'target' tissue(s) (Eto, 1974). Qualitative and quantitative differences in these steps are reflected in the selectivity of toxicity, and hence, the ultimate effect of the agent on the organism.

Differences in xenobiotic metabolism and detoxification in different organisms probably are the most important criteria which determine the selectivity and sensitivity of observed effects of these agents. A simple example is the high selective toxicity of malathion, an organophosphate insecticide. Carboxyesterase activity is high in mammalian tissues compared to its low levels in susceptible insects.
Since this enzyme's activity is considered to be the key step in the detoxification of malathion, its activity is regarded as the main reason for malathion being so selective (Ito, 1974).

Polycyclic aromatic hydrocarbons (PAH) are a class of compounds that have received considerable study over the last thirty years. They are a common by-product of combustion of hydrocarbons and known to be polluting the environment. Interest in these chemicals has resulted from their noticeable involvement in cancer induction. Particularly, their isolation from cigarette smoke, and consequent potential involvement in lung cancer induction. Benzo(a)pyrene (BP) and benz(a)anthracene are the most abundant PAHs isolated from cigarette smoke (Surgeon General's Report, 1981); both are known to induce tumors in experimental animals. Chemical induction of cancers however, is a complex phenomenon. For example, no simple relationship exists between the chemical composition of cigarette smoke condensates and carcinogenicity. The simplest model of this relationship to date contains nine terms, including: BP concentration, nicotine concentration, levels of weak acids, condensate pH, and varied interactions among these parameters (Surgeon General's Report, 1981). Thus is the frustration of evaluating the potential toxic and carcinogenic potential of chemicals. Such predictions generally defy simple explanations. These processes obviously involve many variables, some which have been alluded to above.
As stated previously, the metabolism of xenobiotics is probably the most important factor controlling their ultimate toxic and/or carcinogenic effect(s). The smooth endoplasmic reticulum is known to contain oxidative enzymes capable of oxidizing a wide variety of endogenous and exogenous lipophilic compounds, including steroids, lipids, many drugs, and foreign organic chemicals (Harper, Rodwell and Mayes, 1979; White, Handler and Smith, 1968). Metabolism of PAHs by these monooxygenase enzymes is considered to be the primary initial metabolic route of these chemicals.

The monooxygenase-catalyzed metabolism of xenobiotics includes: aromatic and aliphatic hydroxylation, O- and N-dealkylation, N-oxide formation, desulfuration, sulfide oxidation, deesterification, and epoxidation -- summarized in Figure 1 (Eto, 1974; White, Handler and Smith, 1968). Depending on the compound, some of these reactions may be considered either 'activation' or 'detoxification' processes. In some cases these pathways may even be partially shared. They all generally lead to increasing polarity of the product.

Common to all of the oxidative reactions of the microsomal monooxygenase system, one of the atoms of molecular oxygen gets incorporated into the compound, and the other is reduced to water (Eto, 1974; Harper, Rodwell and Mayes, 1979; White, Handler and Smith, 1968). This reaction may be generalized:

$$\text{RH} + \text{NADPH} + \text{H} + \text{O}_2 \rightarrow \text{ROH} + \text{NADP} + \text{H}_2\text{O}$$
The levels of these 'mixed-function oxidases' (so named because of the diverse types of substrates) varies with species, and even within species. Generally, hepatic microsomal mixed function oxidase activity decreases in the following manner:

mammals > aves > fish

Due to the wide variety of known toxic and carcinogenic chemicals and potentiators and inhibitors of their actions, plus the complexity of variable metabolic pathways involving such compounds, simplified models are required for study. Dimethylbenz[a]anthracene (DMBA) is a PAH that has received considerable attention, since it possesses mutagenic, carcinogenic, and toxic abilities. Because of the extensive research on DMBA's interaction with organisms, it is a good model compound for evaluating the effects of simultaneous exposure to PAHs and insecticides. These latter compounds are also common environmental pollutants, and have been extensively studied for toxic effects.

In this study DDT was used as a representative organochlorine pesticide and malathion was used as a representative organophosphorous pesticide. Such studies are timely considering the increasing use of such compounds, and hence, environmental contamination. Although DDT use is restricted in North America, evaluating the effects of DDT still is relevant. DDT is still commonly used in other countries (Polischuk et al., 1977). It also has an estimated environ-
mental half-life approaching 20 years (Wolfe et al., 1977).
Possibly more important, DDT has been found to behave very
similarly to polychlorinated biphenyls (PCB) and polybromi-
nated biphenyls (PBB), compounds which are known to be
common environmental contaminants. (Ghiasuddin, Wenzler and
Nelson, 1975; Parkki, Narniemi and Vainio, 1977; Rees et al.,
1977; Dent et al., 1977). Studying DDT's effects could help
suggest what the long-term results of PCB and PBB exposure
might be.

1.2 DMBA: A MODEL COMPOUND FOR STUDYING PANS

7,12-Dimethylbenz(a)anthracene (DMBA), a polycyclic
aromatic hydrocarbon, has two interesting characteristics.
First, it is a potent inducer of mammary cancer, with a
single oral dose as low as 5 mg causing tumor formation in
rats and mice (Dao, 1962; Huggins, Grand and Fukunishi,
1964). Second, with oral doses greater than 15 mg it causes
severe adrenocortical lesions in rats (Wong, Warner and
Yang, 1962). Many compounds of related chemical structure,
such as 3-methylcholanthrene (MC) and benzo(a)pyrene (BP),
also are effective inducers of mammary cancer, but are not
adrenocorticalytic.

These carcinogens all possess strong 'K-regional' in
their ring structures which formerly were deemed necessary
for aromatics to be carcinogenic (see Figure 2) (Szent-Gyor-
gyi, Isenberg and Baird, 1960; Dewhurst, Kitchen and
Calcutt, 1972). Metabolism at the 'K-region' was believed necessary for covalent binding to cellular macromolecules, with the subsequent initiation of tumorigenesis. Possibly the adrenocortical effects of DMBA are the result of a similar process (Boyland and Sims, 1965; Allison and Dingle, 1966; Huggins, Grand and Fukunishi, 1964; Pataki et al., 1971).

Interestingly, many different compounds, when administered prior to DMBA, have been found to effectively reduce or prevent tumour formation and/or adrenal damage (Dao and Tanaka, 1963a; Huggins and Pataki, 1965; Kovacs and Somogyi, 1969). p,p'-DDT has been demonstrated to effectively inhibit DMBA-induced rat mammary cancer (Okey, 1972). This present study was undertaken to test whether p,p'-DDT might also act as an adrenal protector against DMBA-induced insult.

A second objective was to better assess the hydrocarbon metabolizing ability of the adrenal gland. The liver is known to have significant levels of microsomal enzyme systems capable of hydroxylating xenobiotics (Conney, Miller and Miller, 1957), which is thought to be necessary for their eventual detoxification and excretion (Falk, Thompson and Kotin, 1965; Laws, 1971). Previous reports on the levels of such enzymes in the adrenal however, have been conflicting (Dao and Yogo, 1964; Pelkonen, Arvela and Karki, 1971; Gielen and Nebert, 1971). Yet, considerable
hydroxylating enzyme systems must be present in the adrenal cortex for normal steroid synthesis (Juchau and Pedersen, 1973). The level of hydrocarbon hydroxylating enzyme system present in the adrenal gland thus was measured. Indication of the degree of metabolism of DMBA, either to some 'active' adrenocorticalytic metabolite or detoxification of DMBA and/or its 'active' metabolite, could thus be assessed. Since p,p'-DDT is known to increase the hepatic metabolism of hydrocarbons (Okey, 1972), its effect on adrenal metabolic systems also was assessed. Since treatments in this study were primarily given orally the hydrocarbon metabolizing ability of the small intestine and liver were assessed for their possible influences.

Organophosphate pesticides have been reported to inhibit the metabolism of hydrocarbons (Weber, Coon and Triolo, 1974). Malathion, a commonly used organophosphate insecticide, was thus used to assess if it would produce the reverse effects expected of p,p'-DDT.
1. Aromatic hydroxylation
   \[ R-\text{CH}_3 \rightarrow R-\text{CH}_2\text{-OH} \]

2. Aliphatic hydroxylation
   \[ \text{O} - \text{O-CH}_2-R \rightarrow \text{O} - \text{OH} + RCHO \]

3. Dealkylation
   \[ R-N(\text{CH}_3)_2 \rightarrow R-N(\text{CH}_3)_2 \]

4. N-oxide formation
   \[ \text{O} \]
   \[ \text{O} - \text{NH-CH}_2-R \rightarrow \text{O} - \text{NH}_2 + RCHO \]

5. N-dealkylation
   \[ \angle \text{P=S} \rightarrow \angle \text{P=O} \]
   \[ \angle \text{C=S} \rightarrow \angle \text{C=O} \]

6. Desulfuration
   \[ R-S\angle R' \rightarrow R-S\angle R' \rightarrow R-S\angle R' \]

7. Sulfide oxidation
   \[ S \]
   \[ (\text{RO})_2\text{P-O-Ar} \rightarrow (\text{RO})_2\text{P-OH} + \text{HOAr} \]

8. Deesterification
   \[ \rightarrow \]

9. Epoxidation
Figure 1: Summary of the Monoxygenase-Catalyzed Reactions
7,12-DMBA

7,12-Dimethylbenz(a)anthracene
Illustration of K-, L-, M-, and Bay-regions (DIGiovanni and Juchau, 1980).

Figure 2: Structure of 7,12-Dimethylbenz(a)anthracene
Chapter II

DMBA: A TOXIC AND CARCINOGENIC AGENT

2.1 CANCER INDUCTION BY DMBA AND ITS PREVENTION

Polycyclic aromatic hydrocarbons (PAHs) have been found to be ubiquitous in urban environments (Mench, Casagrande and Henderson, 1974). Some of these PAHs are known to be potent carcinogens in experimental animals (Huggins and Yang, 1962). DMBA is one such PAH, with single oral doses as low as 5 mg causing tumour formation in rats and mice (Dao, 1962; Huggins, Grand and Fukunishi, 1964). Repeated multiple doses of these carcinogens have been shown to be more effective in inducing cancers and leukemias than single doses (Shimkin et al., 1969; Huggins and Grand, 1973).

Many compounds when administered prior to DMBA have been found to effectively reduce or prevent tumour formation in rats and mice (Dao and Tanaka, 1963a, b; Huggins and Pataki, 1965). DDT was shown to effectively do the same (Okey, 1972; Silinsky and Okey, 1975). The necessity of metabolic 'activation' of DMBA to its carcinogenic form has long been established (Szent-Györgyi, Isenberg and Baird, 1960). Controversy over the actual 'active' carcinogenic form of DMBA, and similar PAHs, still exists (Dewhurst, Kitchen and Calcutt, 1972; Thorgeirsson and Neubert, 1977).
There is however, a growing consensus that metabolism of the PAHs in the 'target' tissue is required for initiation of tumorigenesis, and that PAH metabolism within the liver is principally a detoxification route (Okey, 1972; Thorgeirsson and Nebert, 1977). The protective effect of liver metabolism may be due to the liver being better equipped with secondary conjugating detoxifying mechanisms than are non-hepatic tissues. Thus, enhancement of general liver metabolic systems should promote the detoxification and elimination of carcinogens, with a resultant decrease in tumour formation. Examples of such have been numerous, including the use of DDT (Okey, 1972; Silinskas and Okey, 1975).

If however, initial metabolic systems are stimulated in sensitive non-hepatic tissues that do not possess adequate secondary detoxification systems, then potentiation of the carcinogenic and/or toxic effects of the compound could be expected (Thorgeirsson and Nebert, 1977). A parallel with this argument is seen in the hepatotoxicity of acetaminophen, an aspirin substitute, which is discussed in a later chapter.

The metabolism of benzo(a)pyrene (BP), a known chemical carcinogen, is thought to be representative of most PAHs including DMBA, since both compounds possess 'K' and 'bay-regions' (see Figure 2) (Thorgeirsson and Nebert, 1977). Metabolism of BP by the microsomal cytochrome P-450 monoox-
yrenease system is thought to be predominantly at the 4,5-position to form a K-region arene oxide, which gets converted by epoxide hydratase to a diol (see Figure 22). P450 however, principally acts at the 7,8-position to form a 7,8-oxide which again gets converted to a 7,8-diol. This 7,8-diol is then known to undergo conversion to a 7,8-diol-9,10-epoxide by either the P450 or P-450 systems. It currently is felt that these P450 metabolites are the principal compounds which undergo covalent bonding to cellular macromolecules, with subsequent initiation of carcinogenicity. Thus, not only the amount of primary and secondary metabolic systems present, but also the type is important. If a tissue normally possesses a primary system producing predominantly carcinogenic metabolites, or if such is induced, then this tissue will inherently be more sensitive to tumour induction, regardless of the presence of efficient secondary detoxifying systems, compared to a tissue producing only low amounts of carcinogenic, or less carcinogenic, metabolites by its primary metabolic systems.

2.2 DMBA-INDUCED ADRENOCORTICAL NECROSIS

2.2.1 Normal Adrenal Gland Architecture and Functioning

Grossly, the normal adult rat adrenal gland is a yellow-tan colour. Histologically, it is divided into a peripheral cortex, surrounded by a connective tissue capsule, and a central medulla. The cortex is further
divided into three layers. The outermost is a narrow band of cells arranged in arch-like groups called the zona glomerulosa. Adjacent is the zona fasciculata. These cells are arranged in radial columns separated from one another by blood sinuoids. This is the widest zone of the cortex. The innermost cortical layer is the zona reticularis. These cells are irregularly arranged around blood sinuoids [see Figure 3] (Delman, 1971; Ham, 1969).

The zona glomerulosa produces the mineralocorticoids desoxycorticosterone and aldosterone. These hormones maintain the electrolyte levels in extracellular body fluids by regulating their excretion by the kidney tubules. Although ACTH does not directly influence mineralocorticoid production, the normal functioning of the pituitary gland is required for normal zona glomerulosa functioning (Tan and Mulrow, 1980). Normal aldosterone production is regulated by the renin-angiotensin system and plasma potassium levels.

The zona fasciculata and zona reticularis synthesize the glucocorticoids cortisone, hydrocortisone and corticosterone. The hypothalamus, via the adenohypophysis (CRH and ACTH, respectively), regulates the functioning of these inner cortical layers. Estrogens and androgens are also produced in these cortical layers.

Cells in the cortex tend to have small round well-stained nuclei with prominent nucleoli. The cytoplasm is usually mildly acidic and can appear quite vacuolated due to
high lipid content, predominantly cholesterol stores.

Generally, the lipid content and vacuolization increases with increased cellular activity. Glomerulosa cells tend to have deeper staining cytoplasm compared to the other cortical layers (Dellman, 1971; Ham, 1969).

Medullary cells are larger with large nuclei containing prominent nucleoli. The cytoplasm is granular and generally appears mildly basophilic. Medullary cells form irregular clusters around blood sinusoids. These cells produce epinephrine and norepinephrine under autonomic nervous system control (Dellman, 1971; Ham, 1969).

2.2.2 DMBA-Induced Adrenal Lesions

With oral doses of DMBA 15 mg or greater to adult rats, gross adrenal lesions usually are not seen within the first 24 hours. Histologically, foci of cells showing pyknosis and karyorrhexis in the zonas fasciculata and reticularis are observed (Wong, Warner and Yang, 1962). Engorgement of blood vessels and blood sinusoids with blood, and inflammatory cell infiltration are sometimes observable.

Within 48 hours of DMBA challenge the adrenals grossly appear to be engorged with blood. Microscopically there is considerable coagulative necrosis and interstitial hemorrhage of the inner cortical zones. Neutrophilic infiltration is prevalent. Usually, the zona glomerulosa and medulla show congestion but no cellular damage. The necro-
sis involves progressively larger portions of the cortex, being somewhat dependant on DMHA dosage.

By 72 hours the most severe damage is evident. Karyolysis is diffuse, with only eosinophilic granular cellular debris remaining, but with the underlying reticular framework preserved. The zona glomerulosa is usually heavily congested, with neutrophilia and sometimes small foci of cellular damage. Infrequently some animals show medullary damage which is similar to that described for the cortex, though this is seen only with high DMHA doses (Hudson, Greengard and Lisco, 1967).

If the animal survives, by day 5 regeneration of the cortex begins. This proceeds from the zona glomerulosa towards the medulla. The necrotic tissue is now composed of cellular debris, inflammatory cells, and old hemorrhage. By day 7, there is increasing regeneration of the zona fasciculata. Dystrophic calcification is beginning in the necrotic areas. The regenerated cortex is separated from the necrotic area by a developing band of granulation tissue. In 15 to 21 days the necrotic area is well encapsulated and the cortex is nearly completely regenerated. Patches of granulation tissue, frequently showing dystrophic calcification, usually remain permanently (Wong, Warner and Yang, 1962).

Throughout these changes the cells in the zona glomerulosa become more darkly stained and the cytoplasm more acidophilic. Medullary cells also show more darkly stained
nuclei, their cytoplasm becoming more basophilic. These changes are transient [see Figures 4, 5 and 6].

Adrenal necrosis induced by DMBA is generally preceded by general toxicity and bone marrow destruction (Harris, 1969). Leukopenia, thrombocytopenia, and bone marrow aplasia are common observances, their severity being somewhat dose-dependent (Gillett, Mitchell and Brodie, 1974). Very high doses of DMBA (> 100 mg orally) can result in extensive hemorrhage and necrosis in mesenteric lymph nodes, bone marrow, and the gastrointestinal tract (Duo, 1964). Death within 48 hours usually occurs with such doses.

2.2.3 Adrenocorticolyltic Agents

Though DMBA was the first PAA observed to have an adrenocorticolyltic effect, structural analogs of it have also been shown to produce this effect (Pataki and Huggins, 1967), these being:

a) 7-hydroxymethyl-12-methylbenz(a)anthracene
   (7-OH-12-MBA)

b) 7-(1-hydroxyethyl)-12-methylbenz(a)anthracene
c) 7-(2-hydroxyethyl)-12-methylbenz(a)anthracene
d) 7-(1-hydroxypropyl)-12-methylbenz(a)anthracene
e) 7-methoxymethyl-12-methylbenz(a)anthracene
f) 7-formyl-12-methylbenz(a)anthracene
g) 7-chloromethyl-12-methylbenz(a)anthracene
Several common characteristics of these adrenocorticolytic agents are: they have only two substituents; they have a methyl group at carbon-12; and, they have an hydroxyalkyl or chloroalkyl group at carbon-7, or a potential hydroxyalkyl group at this position.

2.2.4 Adrenal Gland Maturation & Adrenocorticolysis

Susceptibility to the adrenocorticolytic effect of DMBA, or its effective structural analogs, has been shown to be dependant on the age and state of functioning of the adrenal gland (Morii and Huggins, 1962). Rats under the age of 27 days show no DMBA-induced adrenal damage. 35 to 45 day old animals show a progressive susceptibility to adrenal insult. Over the age of 45 days all rats show adrenal damage when challenged with a single oral dose of 30 mg DMBA.

Administration of ACTH to 25 day old rats however, results in their susceptibility to DMBA-induced adrenal damage (Morii and Huggins, 1962). Similarly, removal of the pituitary in adult animals inhibits DMBA-induced adrenal insult; susceptibility being restored by ACTH therapy.

There is a progressive increase in the corticosterone content of the adrenal glands of rats with age up to 50 days. It is suggested that the DMBA-induced damage is correlated with the concentration of corticosterone in the adrenal cortex, and hence the ACTH levels (Morii and
Huggins, 1962; Dao and Tanaka, 1963a, b). In support of this, metopirone, which inhibits 11-betahydroxylation in the adrenal cortex (blocking synthesis of cortisol and corticosterone), protects the adrenals from DMBA and 7-OHM-12-MBA-induced damage. Adrenal damage appears to be inflicted only if there is active corticosteroid synthesis (Wheatley et al., 1966).

Jellink and Goudy (1966) have proposed that DMBA damages the adrenal due to its steric similarity to corticosterone, and that 7-OHM-12-MBA is even more similar.

2.2.5 Active Adrenocorticolysis Agents

 Hoyland, Sims and Huggins (1965) showed 7-OHM-12-MBA, a metabolite of DMBA, to be approximately six times as effective as DMBA in inducing adrenocortical damage. 7-Hydroxymethylbenz(a)anthracene and 12-hydroxymethyl-7-methylbenz(a)anthracene, both were inactive with regard to this. Allison and Dingle (1966) also found that the 7-OHM-12-MBA derivative stimulated in vitro release of enzymes from isolated adrenal lysosomes. It had only marginal effect on lysosomes isolated from other tissues. DMBA had similar but reduced effects. 12-Hydroxymethyl-7-methylbenz(a)anthracene and methylcholanthrene were ineffective in this regard.

Rats with impaired liver function -- by carbon tetrachloride treatment or partial heptectomy -- are protected
from the adrenal damaging effects of DMBA (Wheatley et al., 1966). Yet, similarly treated rats were not protected from the adrenocorticoletic effects of 7-OHN-12-MBA. This further substantiated that a metabolite of DMBA was the actual adrenal-damaging compound. This also suggested that conversion of DMBA to its adrenocorticoletic 'active' form does not occur primarily within the adrenal.

Pataki et al., (1971) tested several derivatives of benz(a)anthracene. They concluded that 7-OHN-12-MBA was the active adrenocorticoletic agent formed in vivo from DMBA.

Active corticosteroid synthesis in the adrenal gland is thought to be a prerequisite to DMBA-induced damage. Yet, cortisol therapy enhances adrenal damage (Somogyi and Kovacs, 1970). These same authors (1971) further showed that stressing rats, either by restraint or by forced muscular exercise, increased the adrenocorticoletic effects of both DMBA and 7-OHN-12-MBA.

Thus, adrenal damage by DMBA likely is due to its metabolism to some 'active' intermediate. This could possibly be 7-OHN-12-MEA or a subsequent metabolite of this compound. This 'activation' of DMBA possibly occurs outside the adrenals, or at least the initial stages of 'activation' might. And, the adrenals must be functionally mature and capable of normal corticosteroid synthesis to be susceptible to this damage. It has been suggested (Wong, Warner and Yang, 1962) that the steric resemblance between DMBA and
hydroxycorticosteroids indicates that they may act at the same cellular sites, but with DMBA resulting in adrenocorticolyis. It is conceivable that the genome receptor sites regulating corticosteroid synthesis, or an enzyme in the corticosteroid synthetic pathway, could be affected by DMBA, or its 'active' metabolite, resulting in the eventual death of the adrenocortical cell. To date, the possibility of target tissues possessing receptor sites for compounds such as DMBA has not been ruled out.

2.3 PREVENTION OF DMBA-INDUCED ADRENAL INSULT

Pretreatment of rats with many compounds, prior to an adrenocorticolytic dose of DMBA, has been shown to protect the adrenals from injury. 3-Methylcholanthrene, benzo(a)anthracene, benzo(a)pyrene, anthracene, phenanthrene, low DMBA doses (Dao and Tanaka, 1963a, b); Sudan III and similar azo dyes (Huggins and Pataki, 1965); metopirone, an inhibitor of 11-betahydroxylation in the adrenal cortex (Wheatley et al., 1966); spironolactone, a steroid lactone (Kovacs and Somogyi, 1969a,b); ethylestrenol, an anabolic steroid (Somogyi and Kovacs, 1970); SC-11927, pregnenolone-16-alpha-carbonitrile, dexamethasone acetate, betamethasone acetate, sodium phenobarbital (Somogyi et al., 1971); and, cyproterone acetate, a potent catatonic steroid (Szabo, Lazar and Kovacs, 1973), have all been confirmed as adrenal protecting agents against DMBA-induced injury. With the
exception of metopirone, they have all been shown to be effective inducers of hepatic microsomal hydroxylating enzymes. These works have led to the general conclusion that increasing the hepatic hydroxylating enzymes results in increased metabolic inactivation of DMBA before it, or its active metabolite, can inflict adrenal damage.

Aryl hydrocarbon hydroxylase (AHH) is one of the microsomal hydroxylating enzymes used as an indicator of hepatic oxidative activity (Thorpe et al., 1977). This enzyme, using benzo(a)pyrene as a substrate, results in ring hydroxylation. The activity of this enzyme will be discussed in detail in a later chapter. It is hoped that these discussions -- along with the experimental data to be presented regarding the adrenal protective ability of DDT against DMBA-induced adrenal damage -- will help elucidate the complex metabolism and interactions of aromatic hydrocarbons within the body. By contrast, impairment of liver functioning, by carbon tetrachloride treatment or induction of fatty liver, prolongs DMBA retention within the body and aggravates DMBA-induced adrenal damage (Huggins and Pataki, 1965).
The regions of the normal rat adrenal gland are shown. cap: capsule; c: cortex; m: medulla; z: zona glomerulosa; f: zona fasciculata; r: zona reticularis. Hematoxilin and Eosin, x 90.

Figure 3: Normal Rat Adrenal Gland
Foci of DMBA-induced toxic lesions in the rat adrenal gland are evident. 'cap': capsule; 'g': zona glomerulosa; 'f': zona fasciculata; 'r': zona reticularis; 'm': medulla; 'nod': subcapsular nodule of cortical cells; 'ct': connective tissue; 1: foci of fasciculata, cells showing early toxic changes to DMBA; 2: foci of fasciculata and reticularis cells showing more advanced toxic changes to DMBA. Toxic changes include progressive swelling of cytoplasm, loss of cytoplasmic vacuolization, and increasing cytoplasmic eosinophilia. Note the loss of normal adrenal gland architecture as the lesions become progressively more severe. Hematoxylin and Eosin, x 90.

Figure 4: DMBA-Induced Adrenocortical Lesions
A region of DMBA-induced hemorrhagic necrosis in the cortex of the rat adrenal gland is shown under higher magnification. f: normal zona fasciculata cells; i: cells showing toxic injury to DMBA, swollen cytoplasm, loss of cytoplasmic vacuolization, and increased cytoplasmic eosinophilia; h: hemorrhage, resulting from blood congestion replacing destroyed cortical cells. Arrow indicates an example of a dead cortical cell undergoing lysis, containing a pyknotic nucleus. Hematoxylin and Eosin, x 200.

Figure 5: DMBA-Induced Adrenocortical Hemorrhagic Necrosis
Rat adrenal gland showing recent and old DMBA-induced lesions. f: zona fasciculata; m: medulla; s: dilated blood sinusoid; h: recent DMBA-induced hemorrhagic lesion; scar: old DMBA-induced cortical lesion which has reorganized to form a connective tissue scar, displaying dystrophic calcification (dys). Hematoxylin and Eosin, x 90.

Figure 6: Recent & Old DMBA-Induced Adrenal Lesions
Chapter III
EFFECTS OF EXPOSURE TO DMBA PLUS EITHER DDT OR MALATHION

Piperonyl derivatives act as synergists for most insecticides, including DDT. They inhibit enzymatic detoxification therefore causing a retention and accumulation of the toxic insecticide, or its derivatives (Falk, Thompson and Kotin, 1965). These synergists may also prevent the detoxification of organic carcinogens. They have been shown to interfere with the elimination of benzo(a)pyrene, decreasing its detoxification and biliary excretion.

Thus, any compound which impairs liver function or inhibits hepatic enzymatic detoxification systems, may potentiate the carcinogenicity of chemical carcinogens by increasing their retention within the body. In support of this, hepatic damage by carbon tetrachloride was shown to increase the retention of DMBA and increase the number of tumor-bearing rats (Falk, Thompson and Kotin, 1965).

Possibly, the reverse also is true. Anything that would potentiate hepatic enzymatic metabolism of chemical carcinogens would decrease their tumour induction ability.

The hepatic microsomal mixed-function hydroxylating enzyme system hydroxylates or dealkylates many different substances. Aflatoxin (a carcinogen) is converted to
nontoxic metabolites by this system. Thus the toxicity of aflatoxin can be reduced by treating rats with DDT or PB (Jukes, 1970). Similarly, mice receiving DDT therapy showed reduced incidence and increased latent period of tumor development with MC-induced ependynoma (Laws, 1971). Again, increased hepatic detoxification of the carcinogen was thought to be responsible. In support of this, Okey (1972) showed that pretreatment of rats with DDT inhibited DMBA-induced mammary tumors. Uptake of labelled DMBA by mammary tissues was also reduced by DDT treatment. The mammary tissue from these rats showed more labelled compounds associated with DMBA metabolites, compared to non-DDT-treated animals.

The aspect of altering specific effects of a toxic chemical, DMBA, by parallel treatment with either a potential promoter or antagonist will be presented. Malathion, an organophosphate insecticide, represents a possible promoter of DMBA toxic effects. DDT, an organochlorine insecticide, represents a possible antagonist of the toxic effects of DMBA. The proposed means by which these compounds act, as either antagonist or promoter of DMBA action, will be presented. In vivo studies are presented which examine the effects of these compounds on hepatic functioning and, their effect on a particular toxic effect of DMBA -- adrenocortical necrosis.
3.1 DDT AND MALATHION

3.1.1 The Chemistry and Prevalence of DDT

DDT \( [1,1-(2,2,2\text{-trichloroethylidene})\text{-bis}(4\text{-chloro-}
benzene)] \) was first synthesized by the German chemist
Zeldler in 1874. It was patented as an insecticide in
Germany in 1934 (DuBois and Coiling, 1959). But its first
real use was by J. F. Geigy in Switzerland in 1939 to
control moths and lice (Ormsbee, 1945). Of the 45 possible
isomers, \( p,p^\prime \)-DDT is the most active insecticidal form. It
is insoluble in water, moderately soluble in mineral and
vegetable oils, and readily soluble in organic solvents
(Conley, 1951). It has been used commercially: to control
plant and livestock pests, against disease-carrying insects
(mosquitoes, ticks, fleas, lice, etc.), filth and nuisance
insects (flies, roaches, bedbugs, beetles, etc.) and,
therapeutically as a pediculicide and scabicide.

Over the last forty years an estimated \( 2.8 \times 10^{+9} \) kg
of DDT has been dumped into the biosphere. North American
agricultural soils are estimated to contain approximately
1.5 pounds of DDT per acre \( [0.85 \text{ kg/hectare}] \) (Wolfe et
al., 1977).

The stability of DDT in the environment has been the
subject of much controversy. Many have felt DDT to be a
very persistent chemical, with a half-life approaching
twenty years (Peckall and Lincor, 1970; Saschenbrecker,
1976; Wolfe et al., 1977). In sandy loam soil DDT was shown
to maintain its pesticide activity over a 48-week period (Eto, 1974), whereas, 90 percent of DDT was reported to have decomposed in sea water within 90 days (Jukes, 1972). An extremely short half-life of 7 hours was reported for DDT in sewage sludge (Jensen, Gothe and Kindstedt, 1972; Albone, et al., 1972). Obviously, persistence depends upon many complex factors and it must therefore be evaluated cautiously.

Though the level of DDT residues in North America is known to be decreasing (Nes et al., 1977; Kutz et al., 1977), due to government restrictions on its use, its levels in Third World populations are increasing (Polishuk et al., 1977).

More disturbing are the increasing levels of DDT-structurally related halogenated compounds, such as polychlorinated biphenyls (PCB) and polybrominated biphenyls (PBB). In a recent sampling of animal fats from slaughterhouses across Canada, over 90 percent of the samples showed minimal detectable levels of DDT and its metabolites. The residues also declined over the four year study. However, the levels of PCBs increased over the same period (Saschenbrecker, 1976). Fish have been found contaminated with PCBs (Nes et al., 1977; McDermott-Ehrlich et al., 1977; Sims et al., 1975; Sims et al., 1977) some with levels exceeding 100 ppm in their flesh (Spagnoli and Skinner, 1977). Increased human exposure and retention of these compounds has been

Thus, continued studies on the effects of DDT in the environment are still warranted. In some locations it shows very long-lasting potency. It is still widely used worldwide. More relevant to North Americans it may serve as a model compound for predicting the potential effects of PCBs and similar compounds contaminating the environment. More regarding this latter statement follows.

3.1.2 Metabolism of DDT

DDT absorption from the mammalian gut is slow. The majority of ingested DDT is excreted, unchanged, in the feces. The main urinary excretory products are DDA and its esters (see Figure 7). Biliary excretion is significant but of unknown form. DDT and its metabolites are found in the milk of lactating animals. Body fat is the main storage depot for DDT and its metabolites (Ormsbee, 1945). Though all tissues take up DDT, their uptake is in direct proportion to their lipid content, due to the high lipid solubility of DDT (Morgan and Roan, 1971). In most species excretion of stored DDT is very slow, with approximate half-lives of 3 or more months (Conley, 1951; Backstrom, Hansson and Ullberg, 1965; Morgan and Roan, 1971).
Generally, in humans, DDE comprises an average of 60 percent of total body DDT (Hayes et al., 1958; Morgan and Roan, 1971; Tomatis et al., 1971). The metabolism of DDT is as shown in Figure 7, the result of several studies (Peterson and Robinson, 1964; Hayes, 1965; Datta, Laugh and Klein, 1964; Datta, 1970). Tomatis et al., (1974) confirmed the conversion of DDT to DDA. However, they found the conversion of DDF through to DDNU to primarily occur in the liver. The final conversion to DDA principally took place in the kidney.

3.1.3 **Toxic Effects of DDT**

Though it is a peripheral nervous system poison in insects, DDT acts principally as a central nervous system toxin in mammals and higher animals (Conley, 1951; Ormsbee, 1945). Muscular tremors become increasingly more intense until a convulsive state is reached, with death occurring from central depression and respiratory arrest. It is postulated that DDT interferes with the recalcification process of neurons (Conley, 1951).

The minimum lethal dose for DDT is approximately 50 mg/kg for most species, with the median lethal dose being considerably higher: 150-250 mg/kg for mice and rats; 150-300 mg/kg for guinea pigs and rabbits; > 200 mg/kg for monkeys; > 300 mg/kg for cows and horses; 1000 mg/kg for sheep and goats; 3000 mg/kg for chickens; speculated at
500 mg/kg for humans (Conley, 1951). Compared to other chlorinated hydrocarbons commonly used in agriculture and public health DDT is significantly less toxic.

Acute toxicity of technical DDT (comprised of both p,p'- and o,p'-isomers, approximately 80:20 respectively) is due almost exclusively to the p,p'-DDT isomer (Okey and Page, 1974). Treatment for acute DDT poisoning is mainly symptomatic and supportive, with barbiturate therapy used as the usual medical treatment (Dubois and Gelling, 1959).

Controversy has raged over the carcinogenicity of DDT. Jukes (1970) stated that high oral doses of DDT produced fine degeneration in liver lobules, with focal necrosis and centrilobular necrosis. These changes were reversible when DDT was removed. Low doses of DDT did not bring about any liver necrosis. Rather, changes to hepatocytes by DDT were seen as similar to those caused by phenobarbital, pyrethrum, and ethanol. Jukes further stated that earlier experiments implicating DDT as a carcinogen were poorly designed and did not distinguish between DDT magnifying an existing condition or predisposition and a new phenomenon. Further, no cases of human cancer occurred in DDT-factory workers exposed to high levels, for prolonged times.

In support of this, a 21-month study employing human prison volunteers ingesting technical grade DDT or p,p'-DDT, in doses up to 35 mg/kg/day, was undertaken (Hayes, Dale and Pirkle, 1971). This dose was estimated to be over 500 times
the average intake of all DDT-related compounds by the
general population, and more than 1200 times the average
p,p'-DDT intake. No evidence of injury to anyone was
observed.

Similar studies of individuals occupationally exposed
to high levels of DDT (average daily intake of 18 mg/man),
some for up to 20 years, showed no abnormal health or
clinical symptoms. This study represented over 1300
man-years of exposure at high levels without above normal
numbers of cases of cancer developing, a statistically
improbable event (Laws, Curley and Biros, 1967; Laws, 1971;
Morgan and Roan, 1974).

The controversy over DDT led Delichmann (1972) to
strongly criticize the laws governing the testing of carci-
nogens. According to laws in force at that time, tumours
occurring in any animal species could presumably be applied
directly to man, even though man and the test animal might
metabolize the compound differently. He strongly stated
that these laws had

"... become an excuse for oncologists to use
inappropriate and unrealistically high levels of
compounds in testing for carcinogenic potential."

Delichmann then went further to state that thirty years of
high use in the world has shown no specific adverse effects
of DDT on humans. He then raises an interesting idea.

"... perhaps some of the cancers that plague us in
the highly industrialized countries are partially
related to the constantly increasing lifespan
which provides the longer latent period between
exposure to a carcinogen and the appearance of
cancer. Perhaps this precarcinogenic process is stimulated by endogenous metabolites which were abundant during active growth. Perhaps the bacterial flora of the bowel plays a much greater role than we are considering. We know that the flora is influenced considerably by dietary carbohydrates, fats, roughage, and certain drugs, and it is not difficult to visualize that the biological effects of certain partially metabolized endogenous compounds could be altered materially by them, and in such a way that they support the production of certain cancers, which need not be confined to the digestive tract.

DDT has also been blamed for causing thin-shelled eggs in wild birds due to environmental contamination, specifically by inhibiting the enzyme carbonic anhydrase. It was shown, however, that at levels several times those found in the environment, that DDT did not inhibit carbonic anhydrase activity (Dvorcich, Istin and Maren, 1971). Large doses of DDT caused only slight inhibition of this enzyme's activity.

Thus, the fate of DDT in the environment and its possible toxic effects are confusing and controversial. Such confusion has led individuals to claim that the ban on DDT use in North America is too severe, with the benefits of DDT use outweighing its possible harmful effects (Jukes, 1972; versus, Wurster, 1973). It does appear to be a safer compound than was earlier believed, and much less toxic than many chemicals that have replaced it in use.
3.1.4 **Toxic Effect of Malathion**

Malathion exerts its insecticidal action through acting as an inhibitor of cholinesterase. However, it is a poor inhibitor of this enzyme until it is converted to its o xo-analog. This occurs in vivo by oxidative desulfuration of the thiophosphoryl group (see Figure 8). This transfer occurs in the microsomal mixed-function oxidase system (Eto, 1974). Thus, the activation and degradation of this insecticide occurs through the microsomal mixed-function oxidase system (Eto, 1974).

3.1.5 **Chemistry, Prevalence, and Toxicity of Malathion**

Malathion [S-1,2-di(ethoxycarbonyl)-ethyl]-dimethylphosphorothioiclorothionate] was first introduced as an insecticide in 1950 by the American Cyanamid Company. It was acknowledged as the first organophosphorous insecticide with selective toxicity (Eto, 1974). Low mammalian toxicity--acute oral LD₅₀ in rats is 1375 mg/kg--led to its widespread use. Rats fed 1000 ppm for 2 years showed normal growth (Eto, 1974).

Even under conditions where DDT shows long stability, malathion generally loses pesticidal action within 2-4 weeks in soils (Eto, 1974). Thus, it is not considered to be an accumulative insecticide.

The high selective toxicity of malathion is attributed to the carboxy ester group, which is readily hydrolyzed by
mammalian carboxylesterase. This esterase activity is low in susceptible insects. Bioactivation -- formation of its o xo-analog -- occurs faster in insects than mammals (Eto, 1974).

3.1.6 Effects of DDT on Hepatic Tissues

Technical grade DDT in high doses in the diet of rats and dogs was shown to advance puberty in females. Rats also had longer reproductive life-spans with greater numbers of successful pregnancies (Ottoboni, 1972). These effects were attributed to the α, p'-DDT isomer (Clement and Okey, 1972; Heinrichs et al., 1971), but only at high doses. At low doses both the α, p' and p, p'-DDT isomers showed antiestrogenic effects. Low DDT doses also reduced the estrogenic response to injected estradiol (Clement and Okey, 1972; Levin, Welch and Conney, 1968; Bitmann et al., 1968; Fahim et al., 1970). Similarly, the uptake of testosterone by the male prostate gland was decreased by DDT treatment of rats (Smith et al., 1972).

Increases in liver weight, hepatocyte smooth endoplasmic reticulum (Hart and Fouts, 1963; Davis, Morris and Tinsley, 1973), as well as the rate of amino acid incorporation into microsomal proteins, total liver protein levels, and hepatocyte synthesis of RNA and NADPH (Sanchez, 1967), are observed following DDT exposure. These changes were blocked by puromycin (Morello, 1965) and actinomycin D.
(Kacew and Singhal, 1973). These changes all suggest that DDT induces microsomal protein synthesis in the liver.

DDT treatment was also shown to increase the hepatic levels of several microsomal epoxidases, and these increases were dose dependent (Gillett, Chan and Terriere, 1966; Gillett, 1968). Similar increases in hepatic microsomal 0-demethylation enzyme activity by DDT treatment was reported (Hoffman et al., 1970). Fetal rat hepatocytes in cell culture showed similar, dose-dependent, enzyme induction (Gieben and Nebert, 1971). As little as 1 ppm DDT in the diet has been shown to induce hepatic microsomal hydroxylating enzyme activity in rats. Maximum induction is reached within three weeks of DDT treatment (Morello, 1965).

Thus, the antisteroidal effects of DDT are due to its ability to induce the hepatic microsomal enzyme system to metabolize these hormones faster, resulting in their faster turnover (Clement and Okey, 1972; Fahim et al., 1970). In fact, the DDT-metabolizing ability of hepatic microsomes can be stimulated by pretreatment of rats with DDT (Morello, 1985). Thus DDT can stimulate its own metabolism. Specifically, DDT treatment elevates hepatic cytochrome P-450, and general microsomal metabolizing ability, in many species (Sell and Davison, 1973; Davison and Sell, 1972). This enzyme system induced by DDT has broad substrate metabolizing ability. The in vivo measurement of drug metabolizing ability is considered to be an indicator of this enzyme
system's level of activity (Clement and Okey, 1972). Measurement of several of these parameters, and their correlation to specific metabolizing ability of rat tissues follows.

It is thought by some that environmental contamination with DDT could lead to increased turnover of hormones in man, due to their increased metabolism. It was speculated that this could result in abnormal physiological and behavior functioning (Street, Mayer and Wagstaff, 1969). This, however, is unlikely. The threshold level of DDT in the diet for detectable enzyme induction is well above the estimated ingestion rate of DDT in the North American human population (Morelló, 1965; Saschenbrecker, 1976; Wes et al., 1977; Kutz et al., 1977).

There may be significance in the apparent replacement of DDT by PCBs and PBBS as environmental contaminants. Both of these chemicals have been shown to behave similarly to DDT in inducing hepatic microsomal oxidative enzymes (Ghasseledin, Menzer and Nelson, 1975; Parkki, Marniemi and Vainio, 1972; Roes et al., 1977; Dent et al., 1977). Induction of these enzymes has been shown to lower both insulin (Berdanier and Dennis, 1977) and testosterone levels (Street, Mayer and Wagstaff, 1969). This and potentially more serious consequences of such contaminants, particularly ingested concomitantly, must be considered as potential threats to human health.
3.1.7 Effects of Malathion on Hepatic Tissue

Microsomal mixed-function oxidases both activate and degrade organophosphate insecticides. The resistance of some houseflies to poisoning by organophosphates and DDT is thought to be due to the high microsomal oxidation of these compounds. Piperonyl butoxide, a mixed-function oxidase inhibitor, reduces the metabolism of these insecticides by the microsomes, and promotes insecticide toxicity in these same flies (Eto, 1974).

Organophosphates can thus act as inhibitors of microsomal mixed-function oxidases, probably acting as alternate substrates (Eto, 1974). In support of this, malathion is known to inhibit steroid hydroxylation in vitro (Welch, Levin and Conney, 1967), and the toxicity of organophosphates to rats is decreased by pretreatment with phenobarbital, benzo(a)pyrene, dieldrin, chlordane, DDT, and several steroids (Eto, 1974).

In order to act as an effective inhibitor the alternate substrate must have a lower turnover and higher affinity (Km) with the microsomal mixed-function oxidase system than does the natural substrate. It is postulated that this enzyme has different binding sites for different substrates, and that allosteric effects may be involved for the interaction of the enzyme and alternate substrate (Eto, 1974). Specific characterization of these effects for malathion have not yet been done. However, it is established that
malathion prolongs barbiturate sleeping-time in mice and inhibits hexobarbital metabolism in vivo and in vitro (Conney et al., 1967; Welch, Levin and Conney, 1967).

3.2 PREVENTION OF DMBA-INDUCED ADRENOCORTICAL NECROSIS

3.2.1 Effects of DDT and Malathion on the Liver

3.2.1.1 Methods

The gross effects of dietary pesticide treatments were monitored in several experimental groups of animals. In all cases, rats, age 36 days, were randomized into experimental groups and caged separately in opaque polypropylene cages with woodchip bedding. Experimental diets were assigned to respective groups, fed ad libitum and with free access to tap water, and maintained until sacrifice. Body weights were recorded regularly. Experimental diets consisted of: 10 ppm DDT (equivalent to 10 ug/kg feed); 100 ppm DDT; 250 ppm malathion; and a control diet. Diets were formulated in powdered rat chow as described in Appendix C.

At age 51 days, after two weeks on the experimental diets, pentobarbital sleep-time test was performed on a group of female Wistar rats. Sodium pentobarbital was given i.p. at a dose of 35 mg/kg body weight. Sleep-time was determined as the time from the loss of the righting-reflex to regaining it. After recovery the animals were returned to their respective diets. At age 57 days these same animals then received 1.0 ml of corn oil containing 15 mg
DNBA p.o. Three days following, they were sacrificed by cervical dislocation, tissues removed, blotted dry, and weighed.

A second group of female Sprague-Dawley rats were similarly placed on the experimental diets for 2 weeks. Then at age 50 days each animal received a single oral dose of corn oil or corn oil containing 15 mg DNBA. Three days following they were sacrificed and tissues examined.

A third group of male Sprague-Dawley rats, age 40 days, were placed on the experimental diets for two weeks. Each then received 1.0 ml of corn oil or corn oil containing 30 mg DNBA p.o. Again, three days following they were sacrificed and tissues examined.

A fourth group of female Sprague-Dawley rats, age 36 days, included a 50 ppm DDT diet. One treatment group on the control diet received daily injections of phenobarbital (80 mg/kg body weight, in saline, i.p.) for 5 days prior to sacrifice. A second treatment group on the control diet received beta-naphthoflavone (80 mg/kg body weight, in corn oil, i.p.) at 48 and 24 hours prior to sacrifice. At age 51 days all animals were sacrificed and tissues excised.

Statistical interpretation of treatment effects was done by analysis of variance (ANOVA), least square means (LSM), and Duncan's multiple range test (Duncan) (SAS, 1979).
3.2.1.2 Results

In all cases weight gain by rats on the different diets was similar (Figure 9). Though food intake was not directly monitored, its consumption appeared similar between treatment diets. The times of the secondary treatments -- sleep-time test and DMBA dosage -- are indicated in Figure 9. In all experimental groups, a relative decline in body weight following DMBA exposure was observed. A parallel reduction in food intake also was seen with these animals. By 48 hours after DMBA treatment, food intake appeared normal. There were no significant differences between treatment group body weights in any of the studies.

The sleep-time test showed a significant dose-related reduction following DDT treatment (see Figure 10). Malathion exposure had no effect on sleep-time.

Figures 11, 12, 13 and 14 show the effects of treatments on liver size in the different experimental groups. At low pesticide levels the combined effects of 10 ppm DDT and DMBA appear additive (see Figures 12 and 13). Levels of at least 50 ppm DDT were required in these studies to show a significant increase in liver size (see Figure 14). At higher DDT levels no additive increase by DMBA was observable (see Figure 13). Short-term DMBA treatment alone could, in some cases (see Figures 12 and 13), stimulate liver growth. DMBA caused a similar significant increase in liver growth when given in parallel with malathion (see
Figure 12). Malathion by itself had no stimulatory or inhibitory effects on liver growth (see Figures 11 and 12). The response of liver growth to DDT appears to be dose-related (Figure 14). Phenobarbital treatment resulted in significant liver growth equivalent to the 100 ppm DDT dosage (Figure 14). This latter figure also shows that beta-naphthoflavone produced liver growth equivalent to that of phenobarbital and 100 ppm DDT.

3.2.1.3 Discussion

The comparable growth curves among treatment groups indicated that the pesticide treatments were palatable and did not influence food intake. The transient drop in weight 24 hours after DWHA treatment showed this chemical treatment to be very stressful.

The increased liver weights and drug metabolizing ability, is evidenced by the reduced phenobarbital anesthesia, in DDT treated rats; follows earlier reports (Okey, 1972). Both effects were DDT dose-related. The effect of combined DDT, DWHA exposure on the liver appears to be additive. 100 ppm DDT in the diet was as effective as the phenobarbital in inducing liver growth and almost as effective as beta-naphthoflavone. Though 10 ppm DDT in the diet did not effect gross changes in the liver size, it is significant that this dosage effectively increases drug metabolizing ability. These effects agree with information
previously reported (Okey, 1972; Clement and Okey, 1972; Morello, 1965; Hart and Fouts, 1963).

By comparison, malathion was ineffective at producing noticable changes in liver growth. More interesting, it showed no effect on drug metabolizing ability of treated animals, which contradicts previous reports (Eto, 1974; Conney et al., 1967; Welch, Levin and Conney, 1967). Perhaps the dosage was insufficient or the animals had adapted to its effects on metabolism.

3.2.2 Insecticide Effects on DMBA-Induced Adrenal Insult

3.2.2.1 Methods

Rats were placed on experimental diets for at least two weeks, these containing 10 ppm DDT, 100 ppm DDT, 250 ppm malathion, or control diet (see above for further details). Animals were then given 1.0 ml corn oil containing DMBA or plain corn oil by mouth. They were sacrificed by cervical dislocation 1 to 4 days later. Livers and adrenals were dissected out, examined grossly for lesions, and weighed. The right adrenals were placed in iced 0.15 M NaCl for later hemoglobin determination. The left adrenals were fixed in Bouin's fixative, paraffin embedded, stained with Hematoxilin and Eosin, and serial sections (10-12 microns) examined histologically for lesions. An index of gross adrenal hemorrhage was made as the glands were removed:

(0 to 3, indicating increased severity of visible hemorrhage) x (1 or 2, indicating uni- or bilateral involvement).
The hemoglobin content of the adrenals was measured by the method of Huggins and Pataki (1965). Each gland was homogenized in the cold in 3.0 ml of 0.15 M NaCl using a 3 ml ground-glass homogenizer. The homogenate was centrifuged at 11,000 x g for 15 minutes. 2.0 ml of the supernatant was diluted with 1.0 ml water. The absorbance of this solution was read on a Hausch & Lamb Spec-20: absorbance at 600 nm was subtracted from the absorbance at 416 nm to correct for nonspecific turbidity (see Appendix A). The pigment expressed as hemoglobin was calculated from measurements made in parallel to a reference standard of beef-blood hemoglobin (crystallized, dialized and lyophilized) diluted with water.

Statistical interpretation of treatment effects was done by analysis of variance, least square means, and Duncan's multiple range test (SAS, 1979).

3.2.2.2 Results

The results of the gross and microscopic adrenal lesions correlated well (see Tables 1 and 2) among the treatment groups. Gross hemorrhage was most severe in animals given DMBA on the control and malathion diets. Malathion did not significantly alter the incidence or severity of adrenal lesions. DDT pretreatment reduced the incidence and severity of these lesions significantly.
Histological lesions were similar to those previously reported [see Figures 3, 4, 5 and 6] (Wong, Warner and Yang, 1962; Harris, 1969; Dao, 1964). When necrosis was present it was coagulative and usually accompanied by neutrophilic infiltration. The zona reticularis showed the most severe damage, which progressively included larger portions of the zona fasciculata in severely damaged adrenals. Neither the zona glomerulosa nor the medulla showed necrosis in any animals. In severely damaged adrenals there were accumulations of neutrophils at the glomerulosa-fasciculata junction and the reticularis-medulla junction, with encroachment of inflammatory cells into the medulla.

Necrotic cells, depending on their degree of damage, appeared swollen with pyknotic or ghosted nuclei and acidophilic cytoplasm. Foci of liquefaction were present in some organs. Islands and bridges of spared cortical tissue were observed in some severely damaged glands. The undamaged cells in the cortex and medulla of adrenals with lesions were more eosinophilic than normal, with sinusoids dilated with blood [see Figures 4, 5 and 6].

No pesticide-induced changes in adrenal architecture were observed.

Adrenal weights (Figures 15 and 16) and adrenal hemoglobin content (Figures 17, 18 and 19) of the treatment groups parallel the gross and microscopic observations. Animals on the control and malathion diets that also
received DMBA showed elevated adrenal weight and hemoglobin levels. DDT pretreatment significantly prevented these DMBA-induced elevations. Though not statistically significant, this DDT pretreatment effect appeared dose-related. This inhibition by DDT of DMBA-induced adrenal changes was effective at both 15 and 30 mg DMBA dosage.

Combined DMBA-malathion treatment did increase the weight and hemoglobin content above that for DMBA-control animals; however, these increases were not statistically significant. Malathion alone had no effect on adrenal size. DDT, however, significantly increased adrenal gland size (see Figure 15).

Table 3 and Figure 20 show the results for adrenal gross appearance, weight, and hemoglobin content monitored at 1, 2, 3 and 4 days following DMBA exposure. These observations were similar to the above. DMBA-induced increases in all of these parameters were seen first 48 hours following its ingestion, reaching maximum levels at 4 days. DDT pretreatment effectively prevented these DMBA-induced elevations.

3.2.2.3 Discussion

A level of 10 ppm DDT in the diet proved effective in preventing DMBA-induced adrenal lesions, even at dosages of 30 mg of the latter. These results are similar to those of Turusov and Chemerin (1976) though they used much higher
doses of DDT. Similar adrenal protection has been demonstrated using a variety of compounds: methylicholanthrene, benz(a)anthracene, benzo(a)pyrene, anthracene, phenanthrene, low levels of DMBA (Dao and Tonaka, 1963a); some azo dyes (Huggins and Pataki, 1965); spironolactone and similar steroids (Kovacs and Somogyi, 1969a,b; Somogyi and Kovacs, 1970; Szabo, Lazar and Kovacs, 1973); and phenobarbital (Somogyi et al., 1971). All these compounds, besides protecting the adrenals from DMBA-induced insult, have also been demonstrated to be effective inducers of liver growth and hepatocyte microsomal hydroxylating enzyme activity. The effects of all of these compounds has been shown to be dose-related.

DDT in the diet resulted in a slight, but significant, increase in adrenal weight. However, no lesions or structural difference in these glands were observed from such exposure. This rules out the protective role of DDT against DMBA-induced damage as being due to adrenal atrophy, such as is caused by o,p'-DDD, a structural analog of DDT. Whether this increase in adrenal size is a direct or indirect effect is unknown. It has not been reported previously. Berdanier and Dennis (1977) showed that DDT treatment could result in altered carbohydrate and insulin metabolism. The hepatic microsomal enzyme system it induces is also responsible for o,p'-DDD is used therapeutically to treat Cushing's Syndrome in humans and dogs. It produces atrophy of the adrenal cortical layers, zona fasciculata and zona reticularis (Kelly and Darke, 1976; Spearman and Little, 1978).
metabolizing endogenous hormones. Possibly an increased adrenal activity has resulted indirectly from DDT treatment. A higher activity may be required to replace a more rapidly turning over corticosteroid pool. This is consistent with observations on the effects of chronic phenobarbital exposure on adrenal glands (Rivera-Cal locals et al., 1978). Phenobarbital was shown to induce adrenal hyperplasia which was concluded as evidence for increased cellular activity, specifically hormone biosynthesis and secretion. Hyperplasia was not evidenced however, in these experiments by DDT pretreatment.

The adrenal protecting ability of DDT was shown to be effective against doses of DMBA up to 30 mg. Such levels normally produce 100 percent adrenal insult, with a high mortality rate, and 100 percent tumour induction. Thus, the level and/or activity of the DDT-induced enzyme system has a large substrate capacity.

Evidence at this point is circumstantial that DDT-protection against DMBA-induced adrenal injury is the result of increased hepatic metabolism and clearance of DMBA before it can inflict injury. Additional evidence will be presented to further this view.

It is interesting that malathion, predicted earlier to aggravate DMBA-induced adrenal lesions, appears to have little effect in this regard.
3.3 Effect of Pesticide on [3H]-DMBA uptake by the Adrenal

Within hours of exposure, lipid-rich tissues can be observed taking up significant amounts of DMBA ingested orally. Adipose, ovarian, liver, and mammary tissues all readily take up DMBA, with the adrenals having particularly high levels (Flesher, 1967; Flesher and Sydnor, 1968; Jull and Jellinck, 1968). This high adrenal uptake is likely due to its high lipid content and efficient blood perfusion.

Since DDT effectively protects the adrenals from DMBA-induced injury, possibly through increased hepatic metabolism with resultant increased excretion, a reduced adrenal exposure to DMBA might be expected. DDT pretreatment of rats, as low as 10 ppm in the diet, was shown to effectively reduce total [3H]-DMBA uptake by mammary tissue as well as reduce the incidence of mammary tumour induction by the same (Okey, 1972). Similarly, stimulation of hepatic metabolism by 3-methylcholanthrene reduces mammary uptake of [3H]-DMBA (Levin and Conney, 1967).

Verifying that DDT pretreatment would produce a similar drop in adrenal uptake of DMBA might help explain the adrenal protecting ability of this pesticide.

3.3.1 Methods

Female Sprague-Dawley rats, age 36 days, were placed on treatment diets as described earlier: 10 ppm DDT; 100 ppm DDT; 250 ppm malathion; and, control. After 2 weeks each
animal received 50 uCi of generally labelled [3H]-DMBA in 15 
mu carrier DMBA in 1.0 ml corn oil p.o. 24 hours later the 
animals were sacrificed, adrenals removed, cleaned of 
extraneous tissue, weighed, and digested individually in 0.5 
ml of NCS Tissue Solubilizer at 50 C for 3 hours. Scintil-
lation cocktail was added to each vial and the radioactivity 
determined. Total DMBA content of the adrenals was 
expressed as moles DMBA per kg adrenal tissue.

The purity of the [3H]-DMBA was verified, prior to use, 
by TLC (see Appendix B). Quench correction and counting 
efficiencies were determined by the external standard ratio 
method. 1 dpm was equivalent to 5.266 x 10^-7 moles DMBA. 
Statistical interpretation of treatment effects was done by 
analysis of variance, least squares means, and Duncan's 
multiple range test (SAS, 1979).

3.3.2 Results

Figure 21 shows that DDT pretreatment produces a 
dose-related decrease in adrenal uptake of [3H]-DMBA. 
However, the reduction is significant only at the higher DDT 
level (100 ppm). Malathion did not affect adrenal exposure 
to DMBA.
3.3.3 Discussion

Though DDT pretreatment can significantly reduce DMBA uptake by the adrenals, the magnitude of this reduction does not appear to account for the effective adrenal protection afforded by DDT. Possibly, the DMBA is metabolized more rapidly to a non-adrenal damaging form by DDT pretreatment. In support of this, Ckey (1972) found DDT treatment resulted in mammary tissues having a lower ratio of unmetabolized DMBA to metabolized DMEA compared to control animals. These DMBA metabolites are generally considered less carcinogenic, and possibly less-adrenocortico lytic than the parent form.

It is also possible that DDT alters the microsomal enzyme system in DMBA 'target' tissues as well as in the liver. If such were the case then 'susceptible' tissue metabolism of DMBA would be expected to be altered which would conceivably alter the susceptibility of that tissue to the toxic effects. This possible effect will be examined further.
1. DDA = 2,2-bis(p-chlorophenyl)acetic acid
2. DDD = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane
3. DDE = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
4. DDNS = 1-chloro-2,2-bis(p-chlorophenyl)ethane
5. DDNU = 1-chloro-2,2-bis(p-chlorophenyl)ethylene
6. DDNU = unsym-bis(p-chlorophenyl)ethylene
7. DDOH = 2,2-bis(p-chlorophenyl)ethanol
8. DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane


Figure 7: Metabolism of DDT in Mammals
\[
(\text{CH}_3\text{O})_2\text{PSCHCHCO}_2\text{C}_2\text{H}_5
\]

\[
\text{CH}_2\text{CO}_2\text{C}_2\text{H}_5
\]

Malathion

microsomal mixed-function oxidase system

\[
O
\]

\[
(\text{CH}_3\text{O})_2\text{PSCHCHCO}_2\text{C}_2\text{H}_5
\]

\[
\text{CH}_2\text{CO}_2\text{C}_2\text{H}_5
\]

"active" anticholinesterase oxo-analog
Metabolism of malathion to its 'active' anticholinesterase oxo-derivative (Eto, 1974).

Figure 8: Bioactivation of Malathion
Female Wistar Rats

Mean Body Weight (Grams)

Age (days)

Control
10 ppm DDT
100 ppm DDT
Malathion

DMBA
Sleep-Time Test
Group means for body weight are shown (N = 5). Female Wistar rats were maintained on respective diets from age 36 days until sacrifice. At age 51 days a sodium pentobarbital sleep-time test was performed. At age 57 days all animals received a single oral dose of 15 mg DMBA. No significant difference in growth among dietary treatments was observed (ANOVA).

Figure 9: Weight Gain of Treatment Animals
Female Wistar Rats

SLEEP TIME (minutes)

Control

10 ppm DDT

100 ppm DDT α

250 ppm Malathion

ANOVA significant; p < 0.01

a: significant from control, p < 0.05

b: " " " " p < 0.001
Effect of dietary pesticide treatment on sodium pentobarbital sleep-time. Sleep-time was determined as the time from the loss of the righting reflex to regaining it after being injected i.p. with sodium pentobarbital (35 mg/kg body weight). This test was performed at age 51 days on female Wistar rats following 2 weeks of feeding on respective diets, as indicated. Displayed are the group means ($N = 5$) and standard error of the mean. A significant difference among experimental groups was shown ($p < 0.01$, ANOVA). Both DDT doses significantly reduced sleep-time as compared to control animals (LSM, Duncan).

**Figure 10: Sleep-Time**
Female Wistar Rats

LIVER WEIGHT (g/100 g body weight)

ANOVA: $p < 0.01$

$^a$ significant from DMBA only, $p < 0.05$
Female Wistar rats, age 36 days, were maintained on respective diets for 3 weeks; 10 ppm DDT, 100 ppm DDT, 250 ppm malathion, or control. After 2 weeks on these diets a pentobarbital sleep-time test was performed. All animals received 15 mg DMBA orally 3 days prior to sacrifice. Group means and standard error of the mean are plotted; the number of animals per treatment group is shown in parentheses. A significant difference in liver size between treatment groups existed ($p < 0.01$, ANOVA). Only the 100 ppm DDT treatment resulted in significantly enlarged livers compared to control ($0.05$, LSW and Duncan).

Figure 11: Liver Weight
Female Sprague-Dawley Rats

LIVER WEIGHT (g/100 g body weight)

ANOVA, p < 0.001

- a: significant from Control; p < 0.05
- b: p < 0.01
- c: p < 0.001
- d: DMBA only; p < 0.05
- e: 10 ppm DDT + DMBA, p < 0.05
- f: Malathion, p < 0.05
Female Sprague-Dawley rats, age 36 days, were maintained on respective diets for 2 weeks; 10 ppm DDT, 100 ppm DDT, 250 ppm malathion, or control. 15 mg DMBA was given orally 3 days prior to sacrifice as indicated (+ DMBA). Group means and standard error of the mean are plotted; the number of animals in each treatment group is shown in parentheses. A significant difference in liver size among treatments was observed \( p < 0.001 \), ANOVA. Groups significantly different from control, etc., are as indicated (LSW and Duncan).

Figure 12: Liver Weight
ANOVA: $p < 0.001$

a: significant from Control, $p < 0.01$

b: 10 ppm DDT + DMBA, $p < 0.01$
Male Sprague-Dawley rats, age 36 days, were maintained on respective diets for 2 weeks; 10 ppm DDT, 100 ppm DDT, or control. 30 mg DMBA was given orally 3 days prior to sacrifice as indicated (+ DMBA). Group means and standard error of the mean are plotted; the number of animals in each treatment group is shown in parentheses. A significant difference among treatments was observed (p < 0.001, ANOVA). Groups significantly different from control, etc., are as indicated (LSM and Duncan).

Figure 13: Liver Weight
Female Sprague-Dawley Rats

ANOVA, p < 0.0005

a Significant from control, p < 0.01
Female Sprague-Dawley rats, age 36 days, were maintained on respective diets for 2 weeks; 10 ppm DDT, 50 ppm DDT, 100 ppm DDT, or control. One treatment group on the control diet received daily injections of phenobarbital (80 mg/kg, i.p.) for 5 days prior to sacrifice. A second treatment group on the control diet received injections of beta-naphthoflavone (80 mg/kg, i.p.) at 48 and 24 hours prior to sacrifice. Group means and standard error of the mean for liver weights, corrected for body size, are shown; the number of animals per treatment group is shown in parentheses. A significant difference among treatments was observed (p < 0.0005, ANOVA). Groups showing significantly enlarged livers compared to control are as indicated (LSN and Duncan).

Figure 14: Liver Weight
TABLE 1

Summary of Gross and Microscopic Adrenal Lesions

<table>
<thead>
<tr>
<th>Treatment Group (N)</th>
<th>Gross Index (+/- SEM)*</th>
<th>Microscopic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNBA only (6)</td>
<td>3.0 (1.3)</td>
<td>0 +1 +2 +3</td>
</tr>
<tr>
<td>10 ppm DDT + DNBA (5)</td>
<td>2.0 (1.3)</td>
<td>1 2 1 1</td>
</tr>
<tr>
<td>100 ppm DDT + DNBA (5)</td>
<td>0</td>
<td>5 - - -</td>
</tr>
<tr>
<td>Malathion + DNBA (5)</td>
<td>3.4 (1.2)</td>
<td>2 1 2</td>
</tr>
</tbody>
</table>

Female Wistar rats were on respective diets for 3 weeks; 10 ppm DDT, 100 ppm DDT, 250 ppm malathion, or control. After 2 weeks on these diets a pentobarbital sleep-time test was performed. All animals received 15 mg DNBA orally 3 days prior to sacrifice. A visual rating of gross adrenal hemorrhage was made as the glands were dissected:

(0 to 3, indicating increasing degree of visible hemorrhage) x (1 or 2, indicating if one or both adrenals were involved)

A significant difference in gross hemorrhage among treatments was demonstrated ($p < 0.05$, ANOVA). Only the 100 ppm DDT treatment showed a significant reduction from control ($p < 0.01$, LSW and Duncan). The microscopic appearance of adrenocortical damage was graded on a scale from 0 to +3:

- 0 = no damage
- +1 = small foci of necrosis
- +2 = moderate damage with large regions of necrosis in the cortex
- +3 = severe necrosis and/or hemorrhage covering extensive areas of the cortex

Shown are the number of animals in each treatment group listed according to the severity of adrenal lesions observed. Descriptions of lesions observed are as described in text.

* Standard Error of the Mean
### TABLE 2

Summary of Gross and Microscopic Adrenal Lesions

<table>
<thead>
<tr>
<th>Treatment Group (N)</th>
<th>Gross Index (SEM)*</th>
<th>Microscopic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (21)</td>
<td>0</td>
<td>21 - - - -</td>
</tr>
<tr>
<td>DMBA only (21)</td>
<td>1.8 (0.6)</td>
<td>5 9 2 5</td>
</tr>
<tr>
<td>10 ppm DDT + DMBA (21)</td>
<td>0.4 (0.1)</td>
<td>20 - - 1</td>
</tr>
<tr>
<td>100 ppm DDT (10)</td>
<td>0</td>
<td>10 - - -</td>
</tr>
<tr>
<td>100 ppm DDT + DMBA (21)</td>
<td>0</td>
<td>21 - - -</td>
</tr>
<tr>
<td>250 ppm Malathion (10)</td>
<td>0</td>
<td>10 - - -</td>
</tr>
<tr>
<td>Malathion + DMBA (22)</td>
<td>2.2 (0.5)</td>
<td>9 5 2 6</td>
</tr>
</tbody>
</table>

Female Sprague-Dawley rats were on respective diets for 2 weeks; 10 ppm DDT, 100 ppm DDT, 250 ppm malathion, or control. Animals received 15 mg DMBA orally 3 days prior to sacrifice, as indicated. A visual rating of gross adrenal hemorrhage was made as the glands were dissected:(0 to 3, indicating increasing degree of visible hemorrhage) x (1 or 2, indicating if one or both adrenals were involved).

A significant difference in gross hemorrhage among treatments was demonstrated (p < 0.05, ANOVA). The microscopic appearance of adrenocortical damage was graded on a scale from 0 to +3:

- 0 = no damage
- +1 = small foci of necrosis
- +2 = moderate damage with large regions of necrosis in the cortex
- +3 = severe necrosis and/or hemorrhage covering extensive areas of the cortex

Shown are the number of animals in each treatment group listed according to the severity of adrenal lesions observed. Descriptions of lesions observed are as described in text.

* Standard Error of the Mean
Female Sprague-Dawley Rats

ANOVA, p < 0.01

a, significant from Control, p < 0.05

b, p < 0.01

Pairwise Adrenal Weight (mg/100 body weight)

Control (21)  DMBA only (21)  10 ppm DDT + DMBA (21)  100 ppm DDT (10)  100 ppm DDT + DMBA (21)  Malathion (10)  Malathion + DMBA (22)
Female Sprague-Dawley rats, age 36 days, were placed on respective diets for 2 weeks; 10 ppm DDT, 100 ppm DDT, 250 ppm malathion, or control. Animals were then given 15 mg DMBA orally, as indicated (+ DMBA), and sacrificed 3 days later. Adrenals were removed, cleaned of extraneous tissues, and weighed paired. Paired adrenal weights were expressed in mg/100 g body weight. Displayed are the mean and standard error of the mean; the group sizes are shown in parentheses. A significant difference among adrenal gland size was demonstrated ($p < 0.001$, ANOVA). Significant differences between groups are as indicated (LSM and Duncan).

Figure 15: Pesticide and DMBA Effects on Adrenal Size
Male Sprague-Dawley Rats

ANOVA; p < 0.05
a: significant from Control; p < 0.05
Male Sprague-Dawley rats, age 36 days, were placed on respective diets for 2 weeks; 10 ppm DDT, 100 ppm DDT, or control. Animals were then given 30 mg DWBA orally, as indicated (± DWBA). Adrenals were removed, cleaned of extraneous tissues, and weighed paired. Paired adrenal weights were expressed in mg/100 g body weight. Displayed are the mean and standard error of the mean; the group sizes are shown in parentheses. A significant difference among treatments was demonstrated (p < 0.05, ANOVA). Differences between treatments are as indicated (LSM and Duncan).

Figure 16: Pesticide and DWBA Effects on Adrenal Size
Female Sprague-Dawley Rats

ANOVA, p < 0.001
a: significant from Control, p < 0.01
Female Sprague-Dawley rats, age 36 days, were placed on respective diets for 2 weeks: 10 ppm DDT, 100 ppm DDT, 250 ppm malathion, or control. Animals then received 15 mg DMBA orally as indicated (+ DMBA), and were sacrificed 3 days later. The right adrenal glands were removed and their hemoglobin content determined (see text). Displayed are the mean and standard error of the mean; the number of animals in each treatment group is shown in parentheses. A significant difference among treatments was demonstrated ($p < 0.001$, ANOVA). Groups differing significantly from control are indicated (LSM, Duncan).

Figure 17: Adrenal Hemoglobin Content
Female Wistar rats, age 36 days, were on respective diets for 3 weeks; 10 ppm DDT, 100 ppm DDT, 250 ppm malathion, or control. After 2 weeks a pentobarbital sleep-time test was performed. After 2 weeks on their respective diets each animal received 15 mg DMBA orally, and were sacrificed 3 days later. The right adrenals were removed and their hemoglobin content determined (see text). Displayed are the mean and standard error of the mean; the number of animals in each treatment group is shown in parentheses. No significant difference among treatments was demonstrated (ANOVA), due to large intragroup variances. However, the 100 ppm DDT pretreatment showed an obvious reduced hemoglobin level.

Figure 18: Adrenal Hemoglobin Content
ANOVA, p < 0.001

a, significant from Control, p < 0.01

B: 30 mg DMBA
Male Sprague-Dawley rats, age 36 days, were on respective diets for 2 weeks: 10 ppm DDT, 100 ppm DDT, and control. Animals then received 3 mg DMBA as indicated (+ DMBA), and were sacrificed 3 days later. The right adrenals were removed, and their hemoglobin content measured (see text). Displayed are the mean and standard error of the mean; the number of animals per treatment group is shown in parentheses. A significant difference among treatments was demonstrated ($p < 0.001$, ANOVA). The DMBA treatment group on the control diet showed significantly increased hemoglobin levels compared to all other treatments ($p < 0.001$, LSm and Duncan).

Figure 18: Adrenal Hemoglobin Content
Female Sprague-Dawley Rats

ug HEMOGLOBIN per ADRENAL GLAND

DMBA only

DAYS FOLLOWING DMBA EXPOSURE

1 2 3 4

Control

DDT

DDT + DMBA
Female Sprague-Dawley rats, age 36 days, were on respective diets for 2 weeks; 100 ppm DDT or control. Each animal then received 15 mg DMBA orally, as indicated (+ DMBA). On days 1, 2, 3 and 4 following DMBA treatment, 3 animals per group were sacrificed, and the hemoglobin content in the adrenals determined (see text). Shown are the mean and standard error of the mean for each treatment. A significant difference among the treatment groups was demonstrated on days 2, 3 and 4 (p < 0.01, ANOVA).

Figure 20: Adrenal Hemoglobin Content: Time Course
Female Sprague-Dawley rats, aged 36 days, were placed on respective diets for 2 weeks: 10 ppm DDT, 100 ppm DDT, 250 ppm malathion, or control. Each animal received 15 mg DMBA containing 5 µCi of generally-labelled [3H]-DMBA p.o. 24 hours later the adrenals were removed, weighed, digested individually in NCS Tissue Solubilizer, and radioactivity measured using a scintillation counter. 1 cpm was equivalent to $5.266 \times 10^{-7}$ moles of total DMBA (0.135 ng). Shown are the mean and standard error of the mean for each treatment; the number of animals in each group is shown in parentheses. A significant difference in [3H]-DMBA uptake among the treatments was demonstrated ($p < 0.05$, ANOVA). Though both DDT pretreatments reduced the [3H]-DMBA levels, only the 100 ppm DDT pretreatment produced a statistically significant reduction ($p < 0.01$, LSW and Duncan).

Figure 21: Adrenal Uptake of [3H]-DMBA
### TABLE 3

**Time Course: Indices of Adrenal Hemorrhage**

(a) Paired Adrenal Weights (mg/100 g body weight)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>day 1</th>
<th>day 2</th>
<th>day 3</th>
<th>day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>42.2</td>
<td>39.5</td>
<td>42.4</td>
<td>46.7</td>
</tr>
<tr>
<td>DMBA</td>
<td>41.4</td>
<td>49.2</td>
<td>61.2</td>
<td>68.6</td>
</tr>
<tr>
<td>100 ppm DDT + DMBA</td>
<td>43.7</td>
<td>47.3</td>
<td>47.2</td>
<td>50.0</td>
</tr>
<tr>
<td>100 ppm DDT</td>
<td>49.4</td>
<td>50.9</td>
<td>44.3</td>
<td>55.9</td>
</tr>
</tbody>
</table>

(b) Index of Gross Adrenal Hemorrhage (+/- SEM)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>day 1</th>
<th>day 2</th>
<th>day 3</th>
<th>day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMBA</td>
<td>0</td>
<td>2.7(0.5)</td>
<td>3.4(0.7)</td>
<td>4.0(1.1)</td>
</tr>
<tr>
<td>100 ppm DDT + DMBA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100 ppm DDT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Female Sprague-Dawley rats, age 36 days, were placed on respective diets for 2 weeks. Each animal then received 15 mg DMBA orally, as indicated. On days 1, 2, 3 and 4 following DMBA treatment, 3 animals per group were sacrificed, adrenals removed, and examined. Displayed are the group means with the standard error of the mean (SEM) indicated in parentheses. The severity of gross adrenal hemorrhage was determined as described in Table 1. Paired adrenal weights are expressed in mg per 100 g body weight.
Chapter IV

ARYL HYDROCARBON HYDROXYLASE INDUCTION

4.1 INTRODUCTION TO AHH

It has been over twenty years since hydroxylase activity in rat liver homogenates was reported (Conney, Miller and Miller, 1957). It was described as a microsomal enzyme system, NADH, NADPH and oxygen dependent, and heat and trypsin labile. It metabolized benz(a)pyrene (BP) to monohydroxy derivatives, and hence was initially called benzopyrene hydroxylase. Due to the many hydrocarbons known to be metabolized by this enzyme system it is now referred to as aryl hydrocarbon hydroxylase (AHH) enzyme system (Thorgeirsson and Nebert, 1977).

A key aspect of this enzyme system was its inducibility by treating rats with BP or other 'methylcholanthrene-type' inducers. This induction could be blocked by parallel treatment with ethionine or puromycin, indicating protein synthesis was necessary for induction.

Quickly, many other compounds were found to effectively induce AHH activity in livers in vivo: naphthalene, anthracene (Arcom, Conney, and Buu-hoi, 1961); phenobarbital; DDT (Hart and Fouts, 1963; Sanchez, 1967); 3-methylcholanthrene (Conney and Gilman, 1963; Dao and Yogo, 1964); flavones,
beta-naphthoflavone (Wattenburg, Page and Leong, 1968a,b); cannabis resin, delta-9-THC (Witschi and Saint Francois, 1972); 5,5-diphenyl-2-thiohydantoïn (Dalton, Kamm and Bartl, 1972); polychlorinated biphenyls (Litterest et al., 1972); spironolactone, ethylestrenol, pregnenolone-16-alpha-carbonitrile, dexamethasone acetate, betamethasone acetate (Somogyi et al., 1971); with 2,3,7,8-tetrachlorodibenzo-p-dioxin (Kouri et al., 1974) being the most potent inducer yet found. Many of these same compounds were also found to effectively stimulate AHR activity in vitro in liver cell cultures (Wattenburg, Leong and Gailbraith, 1968; Gielen and Nebert, 1971; Gielen and Nebert, 1972; Benedict et al., 1973). Both in vivo and in vitro common characteristics were found. With stimulation there was increased amino acid incorporation into microsomal proteins which paralleled increased protein synthesis. This protein synthesis was the result of DNA-directed RNA synthesis.

This microsomal enzyme system normally metabolizes endogenous steroids. However, foreign compounds and xenobiotics may also be normally metabolized by this same enzyme system to more polar metabolites. This would function as a detoxification mechanism aiding in the excretion of metabolites from the body (Kuntzman, 1964).

AHR activity has also been found in many other body tissues. Adrenal (Dao and Yogo, 1964), mammary gland (Pysh
and Okey, 1973; Fush and Okey, 1979), small intestine (Dao and Varella, 1966), lung (Wattenburg, Leong and Gailbraith, 1968), fetal liver and adrenals (Pelkonen, Arvela and Karki, 1971; Gieson and Nebert, 1971), human foreskins (Levin et al., 1972), and lymphocytes (Kouri et al., 1974) have all been demonstrated to have varying degrees of inducibility. The functioning of such an enzyme system in these non-hepatic tissues, however, is not clear.

4.2 AHr CHARACTERISTICS

Hydrophobic xenobiotics would remain in the body for extended periods of time if it were not for their metabolism to more polar metabolites. These polar metabolites would then be available for conjugation, thus allowing their excretion from the body in bile, urine, etc. One of the initial metabolic enzyme systems described to date is AHr, a cytochrome P-450-mediated monooxygenase (Thorpeissson and Nebert, 1977). This enzyme system is known to be membrane-bound in hepatic microsomes. It is capable of metabolizing a very large variety of compounds, such as: polycyclic aromatic hydrocarbons (i.e., benzo(a)pyrene, 3-methylcholanthrene, and DMBA), halogenated hydrocarbons (i.e., DDT, PCB, and PBB), nitrosamines, epoxides, many drugs (i.e., hexobarbital and phenobarbital), endogenous and exogenous steroids and biogenic amines, to name just a few.
However, this same enzyme system may also be detrimental to the cell or organism. Many xenobiotics are known to require partial metabolism to some 'active' intermediate before they can inflict their toxic or mutagenic effect. Examples are BP and DMBA (Sims et al., 1974) both of which are inactive as toxic and carcinogenic agents until metabolized. Further metabolism of these 'reactive' intermediates is required to eliminate their detrimental effects. The first step in 'activation' of such compounds is their hydroxylation by the microsomal AHH enzyme system. Thus, stimulation of AHH activity without a parallel increase in the 'detoxification' enzymes would be detrimental to the organism (Thorpe and Neubert, 1977). An analogy of this latter aspect is seen with the antipyretic drug acetaminophen, used as an aspirin substitute. In acute acetaminophen poisonings severe, and potentially fatal, liver necrosis occurs. This is the result of formation of an 'active' intermediate, by the cytochrome P-450 system, which binds to cellular macromolecules initiating cell death (Levy et al., 1975; Nison et al., 1980; Nelson, Montes and Goldstein, 1980; Fernández, Calder and Ham, 1980). Normally, this 'active' intermediate is inactivated by its conjugation with glutathione, sulfate or glucuronide. However, if liver stores of these conjugating compounds, notably glutathione, are rapidly depleted then the 'active' metabolite is free to bind to macromolecules. The toxicity of acetamino-
pheh is increased in cases where cytochrome P-450 and microsomal hydroxylase activity have been increased by prior phenobarbital exposure. This is due to the more rapid formation of the 'active' acetaminophen intermediate which would deplete liver glutathione stores and exceed the latter's synthesis capacity (Thorgeirsson and Nebert, 1977; Levy et al., 1975; Hinson et al., 1980; Nelson, Montes and Goldstein, 1980; Fernando, Calder and Ham, 1980). There is, therefore, a complex interaction between 'activating' and 'detoxifying' enzymes which determines what effect, if any, exogenous agents will have on the cell.

Current research indicates there are at least two forms of AHH present in the liver and other tissues. The difference appears to be the type of cytochrome P-450 present in the cell (Nebert et al., 1975). The AHH enzyme form present in livers of phenobarbital, DDT, PCB, and untreated rats primarily utilizes cytochrome P-450. Animals treated with MC, and similar PAH, contain predominantly cytochrome P1-450 in liver microsomes. The different forms of this enzyme system appear responsible for the generation of different ratios of metabolites from the same substrate or parent molecule (Thorgeirsson and Nebert, 1977). Therefore, the form of this enzyme present may influence the toxicity and/or carcinogenicity of a compound due to the difference in metabolites formed by each (see Figure 22).

2 Cytochrome P-450 and P1-450 (P-448) refer to absorption maxima of the carbon monoxide-reduced cytochrome pigments.
A simple in vitro test to distinguish the form of AHH present was developed using 7,9-naphthoflavone (alpha-naphthoflavone -- ANF) (Goujon, Nebert and Gielens, 1972; Ryan, West and Levin, 1975). ANF, when added to the incubation medium, will selectively inhibit the P1-450 form of AHH with little of the P-450 form affected.

4.3 PRODUCTS OF THE AHH SYSTEM

Thorgeirsson and Nebert (1977) suggest it is the ratio of P1-450 to P-450 in the tissues that controls the toxicity and/or carcinogenicity of potential xenobiotics. They state that the P1-450 levels are generally much higher than the P-450 levels in non-hepatic tissues. The importance of this lies in the different metabolites produced by the two enzyme systems (see Figure 22). Metabolism of BP by P-450 results primarily in the formation of K-region arene oxides, which get subsequently converted to diols by epoxide hydrolase. P1-450, on the other hand, primarily metabolizes BP to its 7,8-oxide, which also gets converted to a diol by epoxide hydrolase. The 7,8-diol can then be readily converted to a 7,8-diol-9,10-epoxide which binds more readily to DNA than do the K-region epoxides formed by P-450. The adrenal toxicity of DMBA may also be analogous to this proposed mechanism for BP carcinogenicity.
4.4 ADRENAL AMH

Dao and Yogo (1964) were the first to report AMH activity in the adrenal. They reported its levels to be substantially lower than that of rat liver, and to be depressed by treating rats with MC. Later investigators substantiated AMH presence in the adrenal glands, but with conflicting results regarding the level of its activity (Dao and Varella, 1966; Pelkonen, Arvela and Karki, 1971; Hrycry and O'Brien, 1972; Juchau and Pedersen, 1973). There was agreement on the fact that it was non-inducible, by either phenobarbital or MC-related compounds.

The primary cytochrome pigment in human fetal adrenal glands had a reduced carbon monoxide absorption peak between 446 to 448 nm, similar to the hepatic P450 cytochrome (Juchau and Pedersen, 1973). Others have identified two cytochrome P450 forms in the adrenal cortex: a mitochondrial form, mainly involved in side chain cleavage and 11-beta-hydroxylation of steroids; and, a microsomal form, which catalyzes the 21-hydroxylation during steroid synthesis (Honeck, 1978). The adrenal microsomal P450 appears to be analogous to the hepatic P450 microsomal form.
4.5 DDT AND HEPATIC AH

Hart and Pouts (1963) were the first to report on the ability of DDT to induce liver growth, hepatocyte smooth endoplasmic reticulum proliferation, and increased AH activity with increased drug metabolism. DDT was found to be metabolized by this newly-induced enzyme (Morello, 1965; Sanchez, 1967; Welch, Levin and Conney, 1967). A parallel increase in hepatocyte microsomal epoxidase activity by DDT, and its metabolite CDE, treatment was demonstrated (Gillett, Chan and Terriec, 1966). It was also shown to be effective in vitro at stimulating liver cell culture AH activity (Gieien and Vebert, 1971). Both in vivo and in vitro the level of AH induction by DDT was dose-dependent (Gillett, 1968); with 2 to 3 ppm in the diet considered the minimum exposure required to show a measurable increase in AH levels (Hoffman et al., 1970).

As with all AH inductions, DDT-induced increases were transitory and required synthesis of microsomal protein (Davis, Morris and Tinsley, 1973). Increased levels of P-450 in livers of birds has demonstrated the ubiquity of DDT's capability for enzyme stimulation (Davison and Sell, 1972; Sell and Davison, 1973).
4.6 MALATHION-AND HEPATIC AHU

Malathion is an organophosphate insecticide. It, and similar organophosphates, have been shown to inhibit steroid hydroxylation (Welch, Levin and Conney, 1967) as well as other hydroxylating enzymes (Conney et al., 1967; Ieland and Smith, 1972). Specific inhibition of BP metabolism in rats (Weber, Coon and Triolo, 1974) was shown to be due to the insecticide covalently binding to the hepatic microsomal hydroxylase enzyme catalytic site (Stevens, 1974).

Due to this inhibition of hepatic detoxification by malathion, it was tested to see if it would potentiate DMBA actions. This might occur by it decreasing the clearance of DMBA from the body, thereby increasing tissue exposure to higher and more prolonged levels of the carcinogen/toxin.

4.7 EXAMINATION-OF TISSUE AHU

4.7.1 Methods

4.7.1.1 Preparation of Microsomes

Paired adrenal glands, liver and small intestine were placed in 1.0 ml, 10.0 ml and 10.0 ml, respectively, of iced 0.25 M KH2PO4 buffer, pH 7.25, upon dissection, and stored at -20°C. The tissues were homogenized using a pre-chilled Polytron PT-10 homogenizer at near maximum speed for two 15-second pulses. Tissue homogenates were centrifuged at 11,000 x g in a Sorval refrigerated centrifuge for 15 minutes. The supernatant was transferred to pre-chilled
ultacentrifuge tubes (WSE or Beckman) and recentrifuged at
105,000 x g for 1 hour. The microsomal pellet was resus-
pended using 1.0 ml, 2.0 ml, and 5.0 ml of iced 0.25 M
glycerolphosphate buffer, pH 7.25, for the adrenals, small
intestine, and liver respectively. A 15 second pulse with
the Polytron PT-10 homogenizer ensured complete resus-
pension. These microsomal suspensions were stored at -20 C
until enzyme assay. Protein concentrations were determined
by the method of Lowry et al., (1951), with BSA as the
reference standard.

4.7.1.2 AHH Assay System

AHH activity in tissue microsomal preparations was
measured essentially by the method of Nebert and Gelboin
(1968). 0.9 ml of the buffer-cofactor pool (see Table 4)
was added to 25 ml Erlenmeyer flasks and placed on ice. 100
ul of the appropriate microsomal sample was added to each
flask, except blanks. Each sample was done in duplicate.

When testing the in vitro effects of ANF on AHH, ANF was
added in methanol to give a final concentration of 10 uM in
the reaction vessel. The remaining procedure was performed
in a dimly lit room. The reaction was initiated by adding
50 ul of 2 mM benzo(a)pyrene (final concentration = 100 uM)
in methanol to each flask and incubating at 37 C in a water
bath with mechanical shaker. The adrenal assays were
incubated for 60 minutes, liver for 10 minutes, and small
intestine for 15 minutes. The reaction was stopped by rapidly adding 4.25 ml of iced acetone:hexane (1:3.25) to each flask and then placing on ice, covering each flask with a glass marble. 100 ul of microsomal preparation was then added to the blanks. All flasks were re-incubated at 37 C for 10 minutes to extract the NAP metabolites into the acetone:hexane layer. Flask contents were transferred to separate 13 ml glass-stoppered tubes, vortexed, and 1.0 ml of the upper acetone:hexane layer transferred to a 6 ml extraction tube containing 3.0 ml of 1 M NaOH. These tubes were mixed by inversion for 30 seconds. The bottom NaOH layer was transferred to a disposable glass tube and the fluorescence determined. Excitation at 396 nm and emission at 522 nm, on a Turner Model 430 spectrofluorometer, were used. The average fluorescence per sample was determined and the blank value subtracted.

AHH activity was expressed as picomoles 'product' formed per mg microsomal protein per minute. 'Product' refers to the alkali-extractable metabolites of BP that are spectrophotofluorometrically; 3-hydroxybenzo(a)pyrene, extracted under conditions identical with those of samples, was used as a standard, and was a generous gift from Dr. H.V. Gelboin, National Cancer Institute, Bethesda, Maryland to Dr. W.T. Allaben.

The effects of varying incubation time, microsomal protein concentration, ANF, heating of microsomes at 100 C,
and eliminating cofactors MgCl₂, NADH and NADPH from the reaction mixture, on adrenal AHH activity were investigated.

4.7.1.3 Animal Pretreatment

The effect of pretreating animals with DDT, PB or BNF on adrenal, liver and small intestine AHH levels was evaluated. Female Sprague-Dawley rats, age 36 days, were placed on treatment diets for 2 weeks as described previously. The diets contained 10 ppm, 50 ppm or 100 ppm p,p'-DDT or were control. One group of animals on the control diet received daily injections of phenobarbital (80 mg/kg body weight, i.p., in saline) for 5 days prior to sacrifice. A second group on the control diet received BNF (80 mg/kg body weight, i.p., in corn oil) injections at 48 and 24 hours prior to sacrifice. Animals were sacrificed by cervical dislocation, adrenals, liver and small intestines removed, cleaned of extraneous tissue, weighed, placed in iced 0.25 M KH₂PO₄ buffer, pH 7.25, and then frozen at -20°C until further processing.

4.7.2 Results

4.7.2.1 Characteristics of Adrenal AHH

Adrenal AHH is heat labile, and NADH and NADPH dependant for full activity (see Table 5). The enzyme is inhibited approximately 40 percent by ANF in vitro. Removal of MgCl₂ from the assay mixture had no effect — which may be
due to trace contamination of glassware with sufficient
divalent cations to promote full enzyme activity, since
acid-rinsed glassware was not used for these assays. The
quantity of product formed is directly related to both
incubation time (see Figure 23) and microsomal protein
concentration (see Figure 24).

4.7.2.2 Induction of AHH

Adrenal AHH was refractory to induction by all
pretreatments: DDT, PB or HNF (see Table 6, Figure 25).
Small intestine AHH was inducible by HNF but not by DDT or
PB. Liver AHH however, was inducible by all three
compounds. Liver AHH induction by DDT was dose-related;
with a 1.6, 3.2 and 3.4 fold increase by 10, 50 and 100 ppm
DDT pretreatment respectively.

Surprisingly, the basal level of AHH activity in the
adrenal microsomes is about 2.5 times that of the liver (see
Table 6, Figure 25). The small intestine by comparison has
normally less than 15 percent the AHH activity of the liver.

While the HNF pretreatment did not affect adrenal AHH
activity, it resulted in a 38-fold increase in liver AHH
level. By comparison, PB and 100 ppm DDT effected only a
3.4-fold increase in liver AHH above basal levels. Even
though small intestinal AHH was highly inducible by HNF,
these induced levels were still only approximately 16
percent of the levels achieved in induced livers.
4.7.3 Discussion

Contrary to previous reports (Dao and Yogo, 1964; Dao and Varela, 1966; Pelkonen, Arvela and Karki, 1971) AHH activity was found to be approximately 2.5 times higher in adrenal microsomes than in the liver of normal rats. As found by others (Hrycay and O'Hrien, 1972; Juchau and Pedersen, 1973) however, it was non-inducible and inhibited in vitro by ANF (Guenthner, Menard and Nebert, 1979). This suggests that adrenal AHH is similar to the hepatic PI-450 form of the enzyme, again as suggested before (Juchau and Pedersen, 1973). Its lack of inducibility indicates that the genome controlling its functioning is insensitive to stimulation by xenobiotics.

Thus, the adrenal appears to have sufficient hydroxylation activity to 'activate' DMBA to its ultimate adrenocorticolytic form. However, protection of the adrenals by pretreatment with DDT, or other adrenal-protecting compounds, probably is not due to increased or altered metabolism of DMBA in the adrenals. This is seen in the lack of induction of adrenal AHH and its continued functioning at normal basal levels regardless of pretreatment. Possibly, since both DDT and DMBA are substrates for the AHH system, DDT competitively inhibits DMBA metabolism within the adrenal thus reducing the adrenals' direct exposure to the 'active' metabolite. This does not seem likely however, since it does not account for the increased rate of metabo-
lism and clearance of DMBA, and its metabolites, by DDT pretreatment (Okey, 1972); though these changes were small by comparison to the magnitude of prevention of DMBA-induced mammary cancer reported by this author. Neither, does it account for the reduced [3H]-DMBA levels found in the adrenals of animals pretreated with DDT; again, even though these reductions were small by comparison to the magnitude of adrenal protection afforded by DDT pretreatment against DMBA insult. The source of adrenal protection by DDT pretreatment must lie elsewhere.

Similar to the adrenals, the small intestine is refractory to AHH induction by DDT or PB. Thus, adrenal protection from DMBA by pretreatment with these compounds is not due to increased intestinal breakdown of DME. The intestines also probably have little functional role in metabolizing ingested xenobiotics. This is seen in the very low basal levels of microsomal AHH in the intestines, approximately 1/8 and 1/20 the level normally found in the liver and adrenal respectively. However, intestinal AHH is readily inducible by AHH, showing over a 50-fold increase above its basal levels. Thus, continued ingestion of compounds similar to BNF could induce intestinal AHH levels sufficient to affect the pharmacokinetics and/or ultimate effects of these compounds. The stimulation of intestinal AHH by BNF suggests it is similar to the hepatic P1-450 form.
Induction of hepatic AHt by DDT has been well established (Hart and Fouts, 1963; Sanchez, 1967; Welch, Levin and Conney, 1967; Gielen and Nebert, 1971; Gillett, 1968; Hoffman et al., 1970; Davis, Norris and Tinsley, 1973; Davison and Sell, 1972; Sell and Davison, 1973). Levels of 10 ppm DDT in the diet were shown to increase liver AHt levels over 1.5 times normal. The parallel increase in epoxide hydrolase activity by DDT treatment is known (Gillett, Chan and Terriere, 1966). It thus appears that these increases could significantly increase the rate of metabolism to nontoxic forms, and, hence, excretion, of DMBA. This seems to be the most likely means by which DDT protects the adrenals from DMBA insult. In support of this, adrenal uptake of [3H]-DMBA was reduced by DDT pretreatment. Though these reductions did not seem dramatic enough to account for the significant adrenal protection observed, similar studies by Okey (1972) with rat mammary glands showed both a reduction in mammary uptake of [3H]-DMBA by DDT pretreatment and, increased formation of DMBA metabolites considered to be noncarcinogenic. It seems probable that a similar system is operating with the adrenals. Not only does DDT appear to stimulate increased DMBA conversion to nont-adrenocortico-lytic forms, but the types of metabolites formed are probably inherently less toxic to the adrenals. Such a proposal is supported by Thorgeirsson and Nebert (1977), though they were concerned with tumor induction by PAHs. These authors
suggested that if the AHH system is induced in target tissues without a parallel increase in accessory detoxifying enzymes that this would be detrimental to the organism. However, if these accessory detoxifying systems were also stimulated, then an overall benefit would result due to increased clearance and hence reduced exposure to the toxin/carcinogen. From this it also seems probable that tissues inherently high in AHH activity but low in the secondary detoxifying enzymes would be most susceptible to the toxic and/or carcinogenic effects of compounds like DMBA. The adrenals seem to fit this proposal.
**TABLE 4**

**ABH Assay Buffer-Cofactor Pool**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M Tris buffer, pH 7.25</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>0.1 M MgCl₂</td>
<td>0.03 ml</td>
</tr>
<tr>
<td>water</td>
<td>0.62 ml</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.3 mg</td>
</tr>
<tr>
<td>NADH</td>
<td>0.3 mg</td>
</tr>
<tr>
<td>bovine serum albumin</td>
<td>0.7 mg</td>
</tr>
</tbody>
</table>

The above contents were added to each reaction flask. To this, 100 ul of microsomal preparation was added, and the reaction initiated by adding 50 ul of 2 mM benzo(a)pyrene in methanol (final concentration of BP = 0.1 mM).
<table>
<thead>
<tr>
<th>Reaction System</th>
<th>µmoles 3-CH-BP produced/mg protein/minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>32.2</td>
</tr>
<tr>
<td>Microsomes heated at 100 °C</td>
<td>0</td>
</tr>
<tr>
<td>10 μM ANF added</td>
<td>19.0</td>
</tr>
<tr>
<td>NADH removed</td>
<td>16.6</td>
</tr>
<tr>
<td>NADPH removed</td>
<td>3.3</td>
</tr>
<tr>
<td>MgCl₂ removed</td>
<td>30.8</td>
</tr>
</tbody>
</table>

The effect of altering the reaction mixture is demonstrated. The effect of removing NADH, NADPH, or MgCl₂ from the reaction mixture is shown, as well as prior heating of microsomes at 100 °C for 10 minutes. ANF was added to monitor its inhibitory effects. Microsomes from untreated rats had a protein concentration of 1.9 mg/ml. All assays were performed as described in the text.
Proposed model showing the primary metabolic routes of BP by the P-450 and P1-450 forms of hepatic AHH. BP is shown with its carbon atoms labelled 1 through 12. The K-region arene oxide is formed predominantly by P-450. P1-450 predominantly results in 7,8-oxide formation. Epoxide hydratase is involved in secondary hydroxylation reactions for both AHH forms (from: Thorgeirsson and Nebert, 1977).

Figure 22: P-450 and P1-450 Metabolism of BP
Variation of incubation time on the AHH activity of two adrenal microsomal preparations from untreated rats are shown. Activity is expressed as pmoles 3-hydroxybenzo(a)pyrene produced per mg microsomal protein per minute. The straight lines represent linear regression fits of the data points up to 60 minutes incubation. Regression data summary: line "a"; slope = 1.718, y-intercept = 5.64, correlation coefficient = 0.9947; line "b"; slope = 0.511, y-intercept = -0.15, correlation coefficient = 0.9884. Incubation time of 60 minutes was used routinely for all other adrenal AHH assays.

Figure 23: Incubation Time and Adrenal AHH Activity
Variation of adrenal microsomal protein concentration was tested for its effects on adrenal AHH activity, expressed as pmoles 3-hydroxybenzo(a)pyrene produced per minute. The straight line represents the linear regression fit of the first 5 data points; regression data: slope = 2.933, y-intercept = -0.06, correlation coefficient = 0.9965. Most adrenal microsomal preparations, used for other assays, had protein concentrations of approximately 2 mg/ml.

Figure 24: Microsomal Protein Concentration and Adrenal AHH
DDT, PB and DNF were tested for their ability to induce AHH in the adrenal, liver and small intestine of female Sprague-Dawley rats. DDT was given in the diet for two weeks at 10, 50 and 100 ppm. PB (80 mg/kg) was given as daily i.p. injections for 5 days prior to sacrifice. BNF (80 mg/kg) was given as daily i.p. injections for 2 days prior to sacrifice. AHH assay was as described in text, with incubation times of 60, 15, and 10 minutes for adrenal, liver, and small intestine respectively. AHH activity was expressed as pmoles 3-hydroxybenz[a]pyrene produced per mg microsomal protein per minute. Displayed are the mean and standard error of the mean for each treatment group; the number of animals in each group is shown in parentheses. Differences in liver and intestinal AHH levels among groups were significant ($p < 0.0005$, ANOVA). No significant differences in adrenal AHH activity between treatment groups was evident. The level of AHH present in adrenal, liver and intestine of controls differed significantly ($p < 0.01$, ANOVA, LSM and Duncan). The indicated significantly increased AHH levels are as compared to their respective controls.

Figure 25: Adrenal, Liver and Intestinal AHH Activity
### TABLE 6

**Adrenal, Liver and Intestinal AHH Activity**

Data in this table is a re-expression, as ratios, of data from Figure 25; see Figure 25 legend for specifics.

(a) **Comparison of Ratio of AHH Activity in Different Treatment Groups Compared to Their Respective Controls**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Adrenal</th>
<th>Liver</th>
<th>Small Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10 ppm DDT</td>
<td>0.9</td>
<td>1.6</td>
<td>1.3</td>
</tr>
<tr>
<td>50 ppm DDT</td>
<td>1.1</td>
<td>3.2</td>
<td>1.2</td>
</tr>
<tr>
<td>100 ppm DDT</td>
<td>1.2</td>
<td>3.5</td>
<td>1.4</td>
</tr>
<tr>
<td>PB</td>
<td>1.2</td>
<td>3.4</td>
<td>1.3</td>
</tr>
<tr>
<td>BNF</td>
<td>1.1</td>
<td>38.4</td>
<td>53.6</td>
</tr>
</tbody>
</table>

(b) **Comparison of ratio of AHH Activity in Different Treatment Groups and Tissues to that of the Control Liver**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Adrenal</th>
<th>Liver</th>
<th>Small Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.6</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>10 ppm DDT</td>
<td>2.4</td>
<td>1.6</td>
<td>0.2</td>
</tr>
<tr>
<td>50 ppm DDT</td>
<td>2.8</td>
<td>3.2</td>
<td>0.1</td>
</tr>
<tr>
<td>100 ppm DDT</td>
<td>3.1</td>
<td>3.5</td>
<td>0.2</td>
</tr>
<tr>
<td>PB</td>
<td>3.0</td>
<td>3.4</td>
<td>0.2</td>
</tr>
<tr>
<td>BNF</td>
<td>2.9</td>
<td>38.4</td>
<td>6.3</td>
</tr>
</tbody>
</table>
Chapter V
DISCUSSION

DDT effectively protects the rat adrenal gland from the cytotoxic effects of DMBA. This protection is effective with as little as 10 ppm DDT in the diet. Since adrenal and small intestinal AH activities were unchanged by this treatment, with only hepatic AH showing induction, it is speculated that this latter phenomenon is primarily responsible for the DMBA-protective action of DDT. Such a mechanism has been proposed previously for the prevention of both adrenocorticalosis (Dao and Tanaka, 1963a, b; Huggins and Pataki, 1965; Kovacs and Somogyi, 1969a, b; Somogyi and Kovacs, 1970; Somogyi et al., 1971; Szabo, Lazar and Kovacs, 1973) and mammary cancer (Dao and Tanaka, 1963a, b; Huggins and Pataki, 1965; Okey, 1972; Thorogardsson and Nebert, 1977; Silinskas and Okey, 1975) induction by DMBA. The decreased adrenal uptake of [3H]-DMBA following DDT exposure lends support to this, since it suggests a faster hepatic turnover and clearance of DMBA. The decreased pentobarbital anesthesia substantiates the increased metabolic ability of the liver following DDT exposure.

Metabolites of DMBA are known to bind covalently to DNA, and other cellular macromolecules, in normal and
neoplastic tissues (Altmacher and Danz, 1975). Similarly, its binding to macromolecules in the adrenal is known (Marzocchi et al., 1978). Possibly, the DMBA-induced cytotoxic effects in the adrenal are mediated through this binding. A parallel observance in support of this is seen with bromobenzene. Bromobenzene is also metabolized by the P-450 oxidase system; its metabolites also bind to hepatic macromolecules. A direct correlation exists between the macromolecular binding of the bromobenzene metabolites and the hepatotoxic effect of this compound (Gillett, Mitchell and Brodie, 1974). If the toxic effects of DMBA are mediated through a similar means, then tissues showing high levels of AHH metabolic activity, with subsequent intracellular binding to macromolecules, should be most susceptible to its toxic effects. Such is the case for adrenal toxicity to DMBA, and probably bone marrow. These tissues, unlike the liver, probably have only marginal conjugating detoxifying enzyme systems.

It is interesting that the level of AHH activity in the adrenal gland is normally 2.5 times that found in the rat liver. Similar high levels in human and rat fetal adrenal glands have been reported (Juchau and Pedersen, 1973). This suggests that the metabolism of DMBA to its 'active' adreno-cortico-lytic form most probably occurs within the adrenal. It is significant that this AHH enzyme system appears to be of the P1-450 form, considered to be the carcinogen-activat-
ing form in the liver and peripheral tissues (Thorgeirsson and Nebert, 1977). Possibly the conversion of DMBA to its carcinogenic and adrenotoxic forms share a common pathway. If so, adrenal monitoring could possibly be a quick screening procedure for assessing DMBA carcinogenic studies. This has yet to be clarified.

Support for the activation of DMBA to its adrenotoxic form by the P1-450 system present in these cells is seen with the pesticide parathion. This insecticide is metabolized by the cytochrome P-450-mediated microsomal oxidase system (Eto, 1974). Treatment of rats with PB or DET, which both induce the P-450 system in the liver, promotes both desulfuration (insecticide activation) and dearylation (insecticide detoxification) of parathion. BP or MC pretreatment, which both induce the P1-450 system in the liver, however, preferentially enhance desulfuration (see Figure 2a). It is possible that the metabolites of the P-450 system have either a readier access to detoxifying systems or, are inherently less toxic than those of the P1-450 system.

Covalent binding of acetaminophen metabolites to cellular macromolecules is also considered to be a prerequisite for the hepatocellular toxicity of this drug. This effect is also thought to be mediated through the P-450 oxidase system. Covalent bonding of acetaminophen metabolites is however, not prevented by epoxide hydrolase. Thus,
the reactive metabolite is not likely an epoxide (Hinson et al., 1980; Atwood, 1980). Epoxides have long been thought of as the 'active' form of PAH carcinogens (Thorogarsson and Nebert, 1977). Such may not be the case for the toxic effects of these compounds, however. This tends to suggest that these two processes, toxicity and carcinogenicity, occur by metabolism if DMBA by different pathways. Characterization of the DMBA bound covalently to cellular macromolecules in the adrenal could help clarify the nature of the active toxic agent and hence its mode of formation.

Though the rat adrenal gland possess considerable AHH activity, it is not inducible. Thus, the adrenal-protecting ability of DDT, and other similar agents, is not due to increased metabolism of DMBA within the adrenal.

The onset of adrenocortical necrosis by DMBA is rapid. Its speed suggests that it is not mediated through alteration of the nuclear genome. However, recent electron microscopic and autoradiographic work (Belloni et al., 1978; Mazzocchi et al., 1978) has suggested that the adrenotoxicity of DMBA is mediated through alteration of the nuclear genome. These authors tend to feel that DMBA also, somehow, alters the 11-beta-hydroxylase system within the mitochondrial cristae. They base this assumption on the early, and dramatic, changes seen in the mitochondria following DMBA exposure, and the similarity in chemical structure of DMBA and corticosteroids. I, however, do not see how this could
account for cell toxicity. More plausible, I feel, is that DMBA and/or a metabolite of it, may indeed be a substrate for 11-beta-hydroxylase but that it also binds to, and alters, the mitochondrial DNA and/or RNA. This would more feasibly account for the rapid onset of adrenal damage by this chemical. The mitochondrial genome is probably more active than that of the nucleus, on a relative basis, in tissues of high metabolic activity such as the adrenal. Any impairment of mitochondrial functioning would adversely affect cellular functioning and could feasibly result in cell death if mitochondrial dysfunction becomes severe enough (Thompson, 1970).

DDT might also be preventing DMBA-induced adrenal damage by acting as a competitive inhibitor of DMBA metabolism, resulting in a reduced conversion of DMBA to its toxic intermediate. Such a mechanism might seem more feasible operating in the adrenal, rather than the liver, given that adrenal AHH activity is unaltered by this pesticide. Also, the high lipid content of the adrenal cells would accommodate a high uptake of both DDT and DMBA, since they are both lipid soluble. Clarification of this might be evidenced by parallel feedings of DDT plus DMBA with no prior DDT pretreatment. This would help assess the competitive inhibition character of DDT towards DMBA metabolism by not allowing sufficient lead time for hepatic enzyme induction. Also, in vitro studies of the binding affinities of DDT and
DMBA for the AH enzyme system could help clarify the significance of this proposal.

The possibility of the liver metabolizing DMBA to an intermediate that is finally 'activated' within the adrenal to its toxic form also should be considered. Support for this proposal was seen in rats with impaired liver function, by either carbon tetrachloride poisoning or partial hepatectomy, being protected from the adrenal damaging effects of DMBA (Wheatley et al., 1966). One would initially expect impairment of liver functioning such as this, would result in an increased retention of DMBA resulting in an increased adrenal exposure to it, and thus increased severity in adrenal damage. This aspect needs closer scrutiny.

The aspect of cellular receptor sites and transport systems for PAHs has yet to be examined. To date, the cellular uptake of these compounds has been assumed to be passive due to their high lipid solubility, and hence relative ease of transfer through membranes. However, if a transport system is present, and if like the AH system it had relative nonspecific substrate recognition, DDT might then be able to compete and/or block this receptor or transport system. This would result in a decreased adrenal uptake of DMBA, as was seen experimentally, which would thus reduce adrenal exposure. Partial metabolism of DMBA within the liver resulting in increased polarity of the compound, could allow it to have a more ready access to a transport
system within the adrenal. The types of metabolites produced by the liver, and the availability of secondary detoxifying systems, could then predictably alter the amount of 'active' precursor reaching the adrenal.

In this study, the AMH activity in small intestine was low and not inducible by DDT. However, it was highly responsive to induction by 3-MF. Similar induction in this tissue was previously reported (Dao and Varela, 1966). This indicates that with certain xenobiotics, their ingestion could promote their own metabolism within the intestines. This could be an important mode of detoxification in cases of chronic exposure to such compounds.

At present, the following appears to be the most probable explanation of the adrenal-protecting phenomenon of DDT. If the adrenocorticolytic, and carcinogenic, metabolites of DMBA are chemically highly reactive then they would unlikely be able to escape the cells in which they were formed. Then pretreatment of animals with chemicals that induce liver enzymes, but not extrahepatic systems, for metabolizing DMBA would be expected to decrease the toxicity and carcinogenicity of this compound in extrahepatic tissues.
5.1 SUMMARY

1. p,p'-DDT in the diet, in concentrations as low as 10 ppm, effectively inhibits DMBA-induced adrenal necrosis in rats.

2. DDT exposure effectively increases the drug metabolizing capability of the rat liver, as evidenced by the decreased phenobarbital anesthesia. DDT also induces increased hepatic microsomal AHH enzyme activity. These changes appear to be DDT-dose-related.

3. The organophosphate pesticide malathion, which we had predicted should aggravate DMBA's toxic effects on the adrenal, showed no effects in this regard.

4. Adrenal microsomal AHH activity is typically 2.5 times that found in the normal rat liver. The adrenal AHH is noninducible in vivo by DDT, PB or RNF, and appears to be similar to the hepatic P450 form of this enzyme.

5. Intestinal microsomal AHH activity is normally very low, noninducible by DDT or PB, but is highly inducible in vivo by RNF.

6. DDT pretreatment significantly reduces adrenal uptake of [3H]-DMBA, though this effect is small in comparison to the protective action of DDT against DMBA-induced adrenal damage.
7. Prevention of DMBA-induced adrenal damage by DDT pretreatment most probably is due to the promotion of increased hepatic detoxification, and hence clearance of DMBA from the body; this due to DDT promoted increases in hepatic microsomal AH activity, and the parallel detoxification enzyme systems.
Metabolism of the insecticide parathion. Proposed activation and detoxification metabolic routes in the liver (Eto, 1974).

Figure 26: Metabolism of Parathion
\[
\begin{align*}
(C_2H_5O)_2PO\cdot(NO_2) \\
\text{parathion} \\
\text{desulfuration} \\
\text{"ACTIVATION"} \\
(C_2H_5O)_2PO\cdot(NO_2) \\
\text{paraoxon} \\
\text{dearylation} \\
\text{"DETOXIFICATION"} \\
S \text{P(OH)}_2 \\
\text{diethylphosphorothioic acid} \\
\text{+} \\
\text{p-nitrophenol}
\end{align*}
\]
Appendix A

ABSORPTION SPECTRA OF TISSUE HOMOGENATES

Lyophilized beef blood hemoglobin (Sigma Chem. Co.) was reconstituted with distilled water and its absorption spectrum determined against a water blank using a Beckman Model 25 spectrophotometer with chart recorder. Porcine adrenal gland was homogenized in water, centrifuged at 2800 x g for 7 hours at 4°C, and its absorption spectrum determined (see Figure 27).
See Appendix A for details.

Figure 27: Absorption Spectra of Tissue Homogenates
Appendix D

[3H]-DMBA PURITY CHECK

Generally labelled [3H]-DMBA (Amersham/Searle Corp.), specific activity 500 mCi/mmole (1.95 mCi/mg), was dissolved in benzene, spotted on a silica gel TLC plate, and developed in benzene:ethanol (19:1). Unlabelled 7,12-DNBA was similarly spotted on the plate for fluorescent identification of DMBA migration. After development the plate was air-dried, observed under fluorescent light for spot identification, the gel was sectioned and scraped into scintillation vials. Scintillation cocktail was added and the vials mixed for 30 seconds, left to stand in the dark for eight hours, and counted. Counting efficiency was determined by external standard ratio method. An average counting efficiency of 40 percent was achieved. Under similar conditions Rf values for the following have been reported (Boylan and Sims, 1965; Levin and Conney, 1967):

<table>
<thead>
<tr>
<th>Rf</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.98</td>
<td>7,12-DNBA</td>
</tr>
<tr>
<td>0.55</td>
<td>12-OHM-7-DNBA</td>
</tr>
<tr>
<td>0.41</td>
<td>7-OHM-12-DNBA</td>
</tr>
</tbody>
</table>
Greater than 89 percent of all radioactivity was found to have a RF value > 0.85 in this system.
Appendix C

PREPARATION OF DIETS CONTAINING DDT AND MALATHION

The following diet formulations were prepared and used routinely in animal experiments:

1. Control diet
2. 10 ppm p,p'-DDT diet
3. 50 ppm p,p'-DDT diet
4. 100 ppm p,p'-DDT diet
5. 250 ppm Malathion diet

DDT and Malathion were dissolved in 95 percent ethanol. Appropriate volumes of these solutions were added to dry powdered Purina Rat Chow to give the desired pesticide level (calculated on a dry weight/weight basis). An equal volume of 95 percent ethanol was mixed with the Control feed. After these additions the feed was mixed in a mechanical mixer for at least 5 minutes.

Feeding was ad libitum from glass dishes. Monitoring of food intake was not done.
Appendix D

ANALYSIS OF VARIANCE

Comparing three or more groups with the t-test is both tedious and error prone. The probability of error increases with the number of tests. Thus, the total error probability can be excessive, invalidating the assumptions made with the t-tests.

Analysis of variance (ANOVA) measures both within group, and between group variations. If the natural variability within groups is great or greater than the variability between groups, then the treatment effects must be concluded as nonsignificant. However, if between group variability is significantly greater than the random within group variability, then the between group variability can be considered due to the treatments, at the level of significance tested.

It is assumed by ANOVA that error is randomly distributed. Thus randomization is required in experimental design. It is also assumed that the variable tested is normally distributed in the population. Slight deviations from normality are tolerable. Treatment effects are also assumed to be constant and additive. If treatment effects are multiplicative, rather than additive, then ANOVA should
not be used — at least on the raw data. Ideally, when three or more groups are involved, Bartlett's test for homogeneity of variance should be used to determine the degree of consistency among group variances; this was not done however, in these studies.

When ANOVA showed significant treatment effects existed in an experiment, group comparisons were done using Least Squares Means and Duncan's paired test (SAS, 1979). For further information regarding details of these statistical methods the reader should see a standard statistics text, or SAS (1979).
Appendix E
LOWRY TECHNIQUE FOR PROTEIN DETERMINATION

For determination of protein by the Lowry (1951) method the following solutions were prepared.

a) Solution A: 20 g sodium carbonate, 4 g sodium hydroxide, and 0.2 g sodium-potassium tartrate; diluted to 1 L with water.

b) Solution B: 0.5 % copper sulphate

c) Solution C: 50 ml of Solution A + 1.0 ml of Solution B.


Protein determination in microsomal preparations was determined in the following manner.

1. Microsomal preparations were diluted 1:2 or 1:3 with glycerol-phosphate buffer.

2. 100 ul Aliquots of these microsomal-buffer preparations were diluted with 900 ul water, and 5.0 ml Solution C added with mixing.

3. After 10 minutes 0.5 ml of the Folin Solution was added, and vortexed immediately.
4. Tubes were incubated for 30 minutes at room temperature, and the absorbance then read at 660 nm against a reagent blank.

5. A standard curve using bovine serum albumin (Sigma Chem. Co.) was prepared and run in parallel. The line of best fit, using linear least squares regression over the linear portion of the curve was determined.
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