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EFFECTS OF DDT AND MALATHION ON RAT MAMMARY TUMORS AND LEUKEMIA INDUCED BY DIMETHYLBENZ(a)ANTHRACENE

BY

KESTUTIS CHARLES SILINSKAS

A Thesis
Submitted to the Faculty of Graduate Studies through the
Department of Biology in Partial Fulfillment
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ABSTRACT

Female Sprague-Dawley rats, age 36-days, were treated for 2 weeks either with 100 ppm p,p'-DDT or 250 ppm malathion in the diet. Starting at age 50-days they were given, via stomach tube, 21 consecutive daily doses of 0.714 mg 7,12-dimethylbenz(a)anthracene (DMBA). Pesticide diets and observations of the animals for mammary tumors continued for 230 days after the start of DMBA administration. The animals were examined for the presence of anemia and leukemia at 200 days and 230 days after the start of DMBA administration.

DDT-treated rats had a significantly lower incidence of mammary tumors and of leukemia, a prolonged tumor latency period, fewer tumors per rat and fewer actively growing tumors than the control group. Malathion-treated rats had a higher incidence of mammmary tumors and leukemia, shorter tumor latency period, more tumors per rat and more actively growing tumors than the control group. All leukemias were of the myelogenous variety. Chloromas were prevalent in leukemic rats. Anemia, decreased serum total protein, and albumin concentration were common among leukemic rats.

It is likely that DDT inhibits DMBA tumorigenesis and leukemogenesis by stimulating hepatic metabolism and excretion of DMBA so that less of the carcinogen is available to mammary and hemopoietic tissues. Malathion may potentiate DMBA induction of mammary tumors and leukemia by inhibiting the same enzyme systems induced by DDT.

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ABBREVIATIONS USED

AHH	aryl hydrocarbon ("benzo(a)pyrene") hydroxylase
BCG	Bacille Calmette-Guerin (tuberculosis vaccine)
BP	benzo(a)pyrene
cm	centimeter
DDT	1,1,1-trichloro-2,2 bis(p-chlrophenyl) ethane (p,p'-DDT)
DMBA	7,12-dimethylbenz(a)anthracene
EDTA	disodium ethylenediamine tetraacetic acid
l	litre
malathion	S-(1,2-dicarbethoxyethyl)-0,0 -dimethyldithiophosphate
MC	3-methylcholanthrene
mA	milliampere
mg	milligram
ml	millilitre
mm	millimeter
min	minute(s)
nm	nanometers
PCH	polycyclic hydrocarbon
p.o.	par oris (by mouth)
RBC	red blood cell
S.E.	standard error
S.D.	standard deviation
SD	Sprague-Dawley
TRIS	tris(hydroxymethyl) aminomethane
U.V.	ultraviolet
V	volt
WBC	white blood cell

CHAPTER I

MICROSOMAL ENZYME INDUCTION AND DMBA METABOLISM

Approximately 80% of tumors occurring in the population of North America today are induced by the environment, and thus are theoretically preventable (Higginson, 1972). Polycyclic hydrocarbons (PCH) represent a very real environmental hazard because, although it is difficult to obtain direct proof, the PCHs are almost certainly carcinogenic in man. PCHs are relatively chemically inert. They are formed during the partial combustion of organic material and are found as common contaminants of the urban environment and as constituents of tobacco smoke.

Why study mammary cancer in rats ?

Reasons which lend special interest to the study of rat mammary tumors include: 1. malignant mammary tumors can be induced easily at young ages, thus making it possible to study the factors influencing the growth rate of tumors, the production of metastases, and the control of tumor growth through the animal's life span; 2. induced rat tumors are curiously sensitive to hormonal manipulations, and in this respect resemble human mammary cancer; 3. no viral factors have so far been unequivocally demonstrated. Carcinogen-induced mammary cancers of the rat and cancers of the human breast respond in a similar manner to hormonal modifications (Dao, 1964).

Mammary cancers induced by oral administration of PCHs are predominantly adenocarcinomas. Tumors of other histological types such as

ear-duct tumors, sarcomas, and fibroadenomas, have also been observed. Carcinogen-induced mammary cancers grow progressively. If the tumor is allowed to grow without interference, it can reach an enormous size and the host usually succumbs from hemorrhaging and infections following necrosis (Figures 5-8). Mammary adenocarcinoma induced by PCHs may metastasize, but only occasionally, to lymph nodes, lungs, and liver (Huggins and Yang, 1962).

Many hundreds of tons of benzo(a)pyrene (BP) are emitted annually into the atmosphere of a large industrialized country, and cancer can be induced in experimental animals by just a few micrograms of this PCH. Concentrations of BP as high as 3.5 nanograms per cubic meter have been found in urban air (Mench et al., 1974). People are exposed to PCHs, nitrosamines, aflatoxins, and many other chemical carcinogens in their environment (Conney, 1967; Conney and Burns, 1972), so factors that inhibit or stimulate the metabolism of these substances may play a major role in the induction of human cancer. The cofactor requirements and the microsomal distribution of drug metabolizing enzymes in human liver are similar to those previously reported for rat liver (Conney, 1967).

Hormones and DMBA tumorigenesis.

In every experiment described (Dao, 1971), induction of mammary cancer was inhibited when the source of estrogen was removed at the time of DMBA administration. Evidence overwhelmingly suggests that interaction between DMBA and cellular constituents of mammary tissue is interfered with in estrogen-depleted rats. Estrogen probably regulates the interaction between a carcinogen and a cell by modifying the

mitotic activity of the mammary epithelial cells. It has been suggested that carcinogenic agents of different kinds have the common property of interfering with the nucleus of a dividing cell in a characteristic way, and that a cell is most affected by carcinogens during mitosis. Experimental evidence (Huggins et al., 1961; Dao, 1971) demonstrates that only at a certain rate of mitosis is a cell susceptible to injury by a carcinogen; cells undergoing either a too rapid rate or too slow a rate of mitosis are not vulnerable to the action of a carcinogen. Perhaps one function of estrogen in this mechanism is the maintenance of a critical rate of mitosis.

Estrogens are not tumorigenic or carcinogenic in themselves, but they put the tissue in a state of active metabolism so that it is susceptible to a variety of carcinogenic agents or factors (Jensen, 1972). It is increasingly apparent that one of the primary effects of estrogen on rat mammary tumor formation is to mediate the secretion of prolactin, which is one of the essential pituitary hormones for both rat and mouse tumorigenesis. Estrogen, however, probably also acts synergistically at the tissue level to make possible the nodule-to-tumor tansformation and tumor growth (Beuving and Bern, 1972). It is possible that large doses of estrogen alter the biochemical substrate of the parenchymal cells of the mammary gland so that prolactin can no longer exert a lactational effect. Large doses of estrogen may similarly inhibit the tumorigenic action of prolactin on the mammary gland in the presence of a carcinogen (Meites, 1972). Mammary tumors in animals and human breast cancer have the estrogen receptor; it is clear that mammary cancer may in a sense be a self-contained system. That is, the cancer

cell not only has the mechanism for synthesizing estrogen but also has the receptor giving it the capacity to respond to the estrogen (Jensen et al, 1972).

Mammary glands cultivated in a synthetic medium supplemented with insulin, estrogen, prolactin, and progesterone, and DMBA developed adenocarcinoma when transplanted to isologous hosts (Dao and Sinha, 1972).

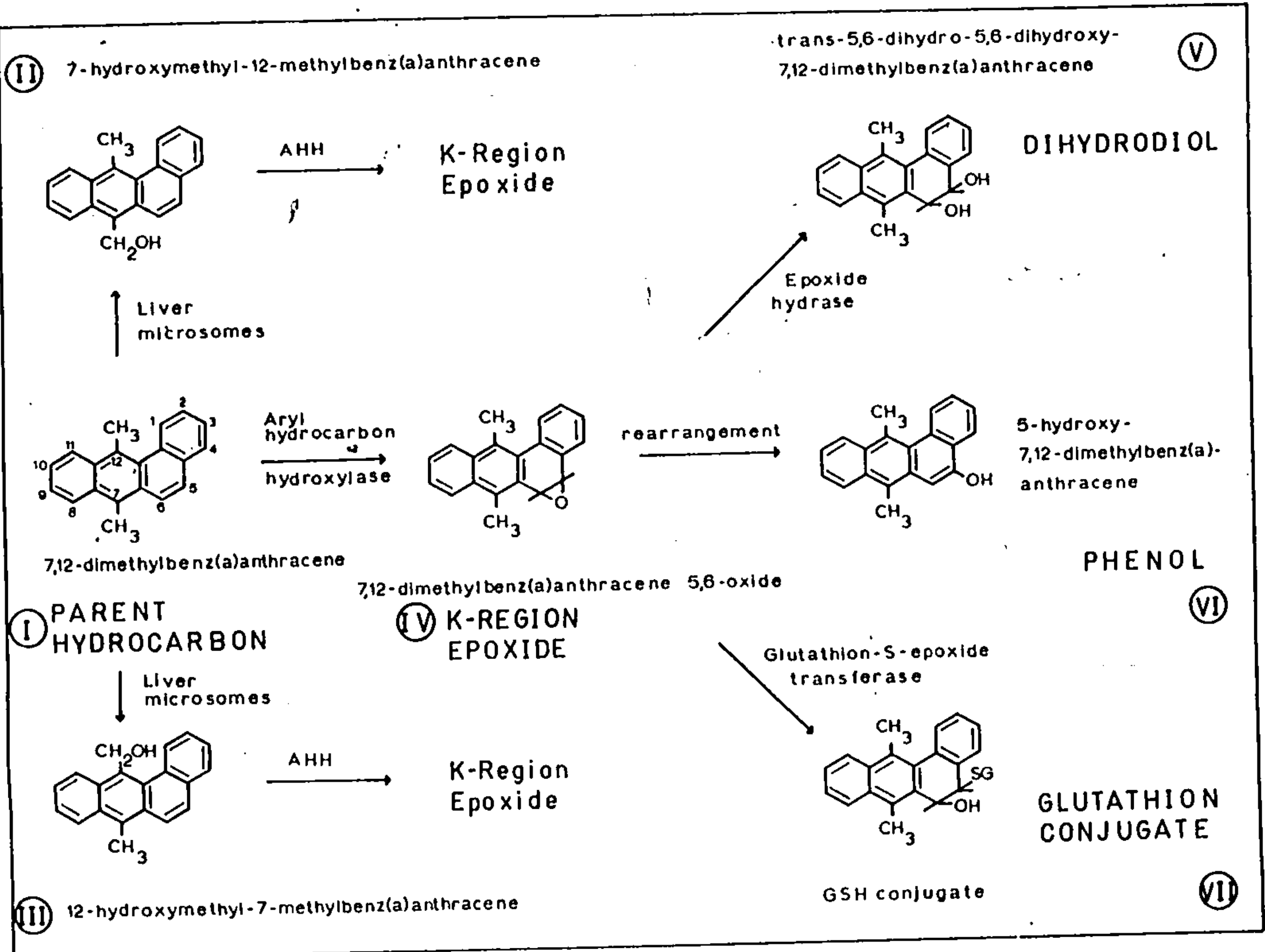
No single hormone is especially important for mammary tumorigenesis. Each essential hormone is important only to the extent that it contributes to the minimum effective hormonal milieu. Therefore Beuving and Bern (1972) view the role of hormones as supportive or permissive rather than inductive.

Metabolism of DMBA by hepatic microsomal enzyme systems.

Aryl hydrocarbon hydroxylase (also called benzpyrene hydroxylase) is an example of the mixed-function oxidases. In the presence of NADPH, NADH, molecular oxygen, and certain divalent cations, the membrane-bound enzyme (from disrupted cells) hydroxylates the substrate DMBA (or other PCHs) to more than a dozen products (Sims, 1973) Figure 1).

Of all the carcinogenic hydrocarbons, 7,12-dimethylbenz(a)anthracene (I, Figure 1) is one of the most widely studied, but earlier attempts to synthesize its K-region epoxide, 7,12-dimethylbenz(a)anthracene 5,6-oxide (IV), were unsuccessful. Since one of the pathways by which DMBA (I) is metabolized by hepatic microsomal systems involves the hydroxylation of the methyl groups, to yield 7-hydroxymethyl-12-methylbenz(a)anthracene (II) or 12-hydroxymethyl-7-methylbenz(a)anthracene (III) (Boyland and Sims, 1965; Flaks et al., 1972).the possibility that the K-region epoxides of the hydroxymethyl compounds are involved in the biological action of the parent hydrocarbon must also

Figure 1. Possible scheme of metabolism of 7, 12-dimethylbenz(a)anthracene at the K-region double bond.



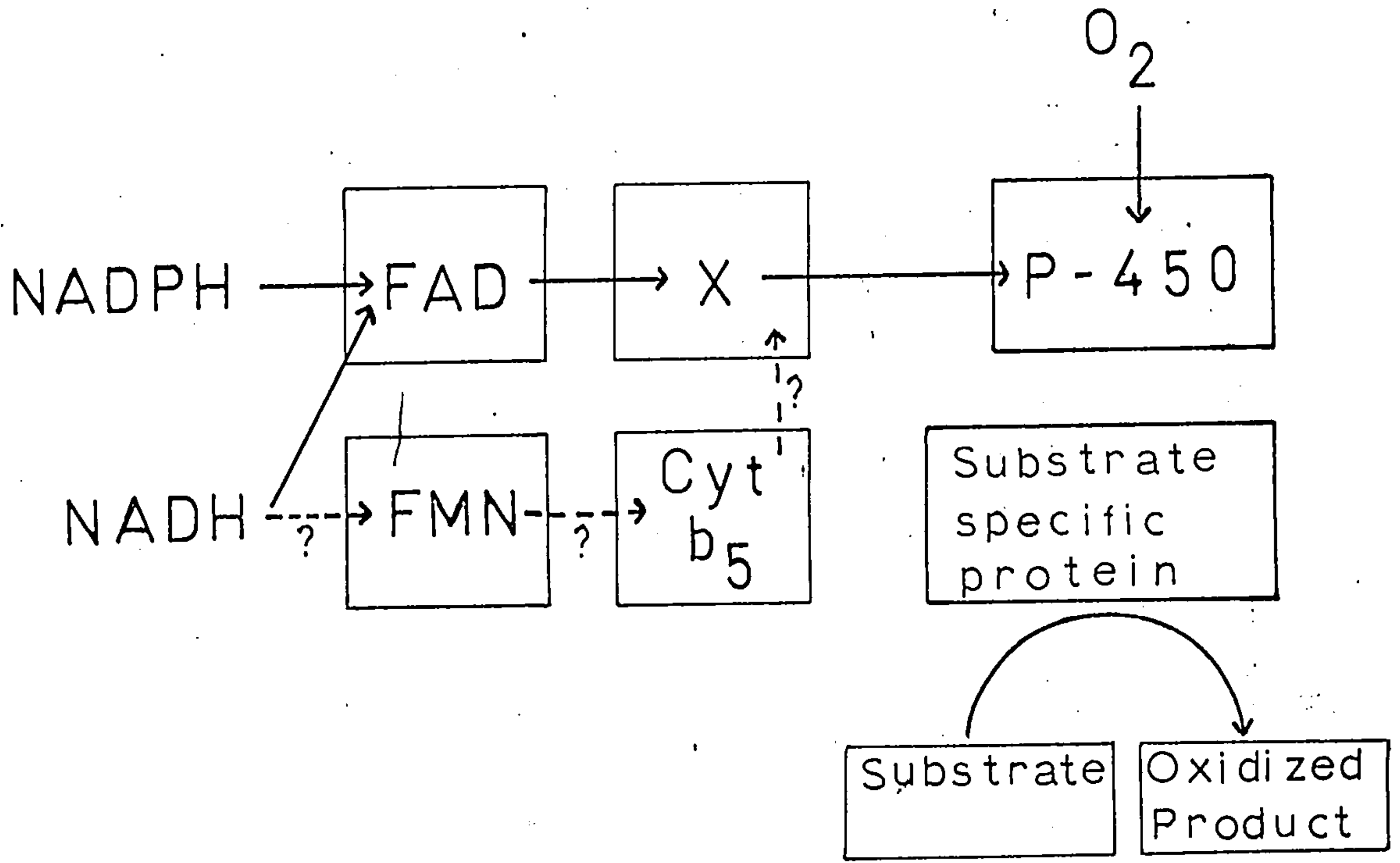
II 7-hydroxymethyl-12-methylbenz(a)anthracene
AHH
K-Region Epoxide
Liver microsomes
Aryl hydrocarbon hydroxylase
7,12-dimethylbenz(a)anthracene
I PARENT HYDROCARBON
Liver microsomes
AHH
K-Region Epoxide
III 12-hydroxymethyl-7-methylbenz(a)anthracene
7,12-dimethylbenz(a)anthracene 5,6-oxide
IV K-REGION EPOXIDE
Epoxide hydrase
rearrangement
Glutathion-S-epoxide transferase
trans-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz(a)anthracene
V
DIHYDRODIOL
5-hydroxy-7,12-dimethylbenz(a)-anthracene
PHENOL
VI
GLUTATHION CONJUGATE
GSH conjugate
VII

be considered. When the oxide (IV) was incubated with rat liver homogenate three products were detected. These were identical in their chromatographic properties to 5-hydroxy-7,12-dimethylbenz(a)anthracene (VI) trans-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz(a)anthracene (V) and the GSH conjugate (VII). There was no evidence for the formation of the cis-dihydrodiol (Sims, 1973).

The arene oxide (IV) readily rearranges non-enzymatically to a phenol (VI). The conversion of the epoxide (IV) into trans-dihydrodiols (V) is presumably carried out by a microsomal "epoxide hydrase". The enzyme catalysing the conjugation of the oxide (IV) with GSH is presumably "glutathione-S-epoxide transferase" (Sims, 1973). The production of metabolites other than those outlined above is now under investigation. Keysell et al. (1972,1973) were able to positively show that an epoxide must be the precursor to dihydrodiol formation by using an epoxide hydrase inhibitor.

The number of necessary components in the aryl hydrocarbon hydroxylase system is not certain. The enzyme activity (Figure 2) measured as the rate of formation of the 5-hydroxy derivative of DMBA determined spectrophotoflurometrically is the result of a multicomponent electron chain similar in some ways to the oxidative phosphorylation chain involving mitochondial cytochromes (Sims, 1973; Nebert and Bausserman, 1971). An FAD-containing flavoprotein is reduced by NADPH and NADH; electrons are then passed, perhaps to cytochrome b_5 and other unknown structural or functional factors designated by X, and ultimately to the terminal oxidase, cytochrome P450, where the substrate is hydroxylated at the active heme site in the presence of active oxygen and a

Figure 2 Model depicting the microsomal electron pathway in substrate hydroxylation based on aryl hydrocarbon hydroxylase activity.


O2
NADPH
FAD
X
P-450
NADH
?
FMN
?
Cyt
b5
?
Substrate
specific
protein
Substrate
Oxidized
Product

theoretical "substrate-specific" protein. Cytochrome P450 is so named because its reduced complex with CO has an absorbance maximum at about 450 nm.

The sequence of events taking place during the induction of hydroxylase activity by PCHs in cell culture has been outlined by Nebert and Bausserman (1971). The rapid entry of PCHs into the cell is independent of temperature, and synthesis of an induction-specific RNA occurs during the first 30 min. Hydroxylase induction is dependent upon translation involving this RNA (Nebert and Bausserman, 1970; Nebert and Gelboin, 1970), and the appearance of a new, spectrally distinct, CO-binding cytochrome is also dependent on protein synthesis (Nebert, 1970). This RNA synthesis can occur during a block of protein synthesis. This requirement for early RNA synthesis suggests an inducer-mediated alteration of genetic expression. The addition of actinomycin D, puromycin, or cycloheximide simultaneously with inducer completely prevents the induction of AHH activity (Nebert and Gelboin, 1968). The induction of AHH activity possibly involves an activation of specific genes and changes in their transcription into messenger RNA.

The differential effects in vitro of 7,8-benzoflavone and organic solvents on AHH, from different tissues of rats, indicate the existance of at least two forms of this enzyme complex (Wiebel et al., 1971). One form is strongly inhibited by monohydroxy alcohols and is not inhibited by 7,8-benzoflavone. This type predominates in normal hepatic microsomes. A second form is considerably less susceptible to the influence of organic solvents, but is strongly inhibited by 7,8-benzoflavone. This type is predominant in microsomes of such extrahepatic

tissues as lung, kidney, and in homogenates of skin. It might be possible to inhibit or stimulate differentially various types of polycyclic hydrocarbon hydroxylating enzymes *in vivo* and in this way modify the host response to these compounds.

Genetic regulation of AHH induction.

The locus for aromatic hydrocarbon responsiveness (designated the *Ah* locus) is closely associated with formation of the newly synthesized CO-binding hemoprotein, cytochrome P448 and induction of certain monooxygenase activities. Liver cell cultures derived from genetically nonresponsive mice (low levels of AHH activity inducible only by excessive doses of inducer) treated with aromatic hydrocarbon and *in vivo* treatment of genetically nonresponsive mice with certain halogenated aromatic hydrocarbons produces rises in hydroxylase activity that are as high as those found in so-called genetically responsive mice; thus, aromatic hydrocarbon responsiveness appears to represent a threshold effect (Nebert *et al.*, 1973). In the genetically responsive rat or mouse, gene product(s) associated in some way with the *Ah* locus cause the formation of cytochrome P448.

Nebert and his associates (1973) have attributed the differences in increases of AHH and other monooxygenase activities between responsive and nonresponsive mice, to the production of several different types of cytochrome P450. Differences in these cytochromes are determined by the spin state of the cytochrome iron. The high spin state of cytochrome P450 has been renamed $P_1$450, P448 or P446. Several experiments, however, indicate that the substrate specificity and thus the enzyme activity of the hemoprotein P450 and P448 are not necessarily

determined by the spin state of the cytochrome iron. The prefered spin state of the iron (P448) may represent a configurational change near the enzyme active site, such as presence of a new apoenzyme or hemoprotein incorporation into the multicomponent monooxygenase system. The high spin cytochrome iron is easily lost with the consequent formation of cytochrome P420, a denatured form of cytochromes P450 and P448. Substrates for this monooxygenase activity are classified as type I or type II. Type I compounds convert the cytochrome iron from low spin to high spin when they bind covalently at binding sites near the cytochrome iron. Type II compounds convert the cytochrome iron from high spin to low spin. One should be able to detect different forms of cytochrome P450 or P448 by using selective inducing agents, preferential inhibition *in vitro* by enzyme inhibitors, and preferential destruction *in vitro*.

The interrelationship of hepatic monooxygenase and epoxide hydrase activities have recently been examined (Daly *et al.*, 1972). Both enzymes are localized in microsomal fractions and both are inducible in rodents by pretreatment of animals with 3-methylcholanthrene or phenobarbital. Soluble preparations of the monooxygenases, cytochrome P450 or P448, contain high levels of epoxide hydrase activity, suggestive of a close association of monooxygenases and epoxide hydrases in liver membranes (Daly *et al.*, 1972).

Mechanisms of action of chemical carcinogens.

Some xenobiotic compounds are converted *in vivo* to toxic or carcinogenic metabolites. The carcinogenicity of such PCHs, as DMBA, is the result of metabolic conversion to active species, such as

carbonium ions, radical cations or arene oxides (epoxides) (Daly et al., 1972). It is generally accepted that such compounds as PCHs to be carcinogenic must be enzymatically activated to forms that bind covalently with some tissue constituents. It is well known that several different positions of a chemical carcinogen are subject to attack by drug-metabolizing enzymes such as aryl hydrocarbon hydroxylase (AHH). Most of the resulting unbound metabolites are much less carcinogenic than the parent compound. In the act of converting these chemical carcinogens to more polar, unbound metabolites, some of the drug-metabolizing enzymes also activate the compounds to chemically reactive electrophilic forms that bind to macromolecules. It is likely that several sites on a molecule can be so activated, leading to several covalently bound forms (Corbett and Nettesheim, 1974).

Differences in the types of metabolites produced which indicate a particular pathway of metabolic activation could explain the variable susceptibility of different species and tissues to hydrocarbon carcinogenesis.

Since it seems that carcinogenic agents must induce neoplasia through interaction with tissue components, efforts to understand the mechanisms involved in chemical carcinogenesis have been directed primarily toward studies of the chemical reactions of the carcinogens and their metabolites. Determination of the active form(s) (ultimate carcinogens) of a chemical carcinogen comprises a logical first step in the elucidation of its mechanism of action (Miller, 1970).

PCHs are metabolized by intact rats and by rat liver slices, homogenates, and microsomes into K-region phenols, trans-dihydrodiols,

and glutathione conjugates (Heidelberger, 1973). Epoxides are the metabolically active forms (bind to cellular macromolecules) of at least those PCHs that lack methyl groups. However, it is possible that metabolic activation may involve free radical formation on methyl groups (Heidelberger, 1973).

Covalent interactions of chemical carcinogens with proteins of target tissues were first noted over 20 years ago (Miller and Miller, 1947, 1952), and the earliest studies on the reaction of nucleic acids with alkylating agents in vivo were made in 1957 (Wheeler and Skipper, 1957). Covalent bindings of carcinogen residues with macromolecules (DNA, RNA and / or proteins) in vivo have now been noted in all cases which have been adequately examined (Farber, 1968; Miller and Miller, 1966; Miller and Miller, 1969).

Epoxides are postulated to be obligatory intermediates in all microsomal aromatic ring hydroxylations. Polycyclic arene oxides are active alkylating agents that bind to and react with DNA, RNA and proteins. Certain polycyclic arene oxides are potent mutagens in strains of Salmonella, in bacteriophages and in a clone of chinese hamster cells, possibly because of interaction with DNA (Daly et al., 1972).

PCH K-region epoxides cause frameshift mutations in bacteria, but the hydrocarbons themselves and the hydroxylated derivatives are inactive. PCHs may be carcinogenic because of the mutagenicity of epoxide intermediates formed during their metabolism and that the mechanism of action may involve intercalation followed by covalent reaction (Ames et al., 1972). The K-region epoxides and cis-dihydrodiols derived from benz(a)anthracene and from dibenz(a,h)anthracene

have been found to be more active in the production of malignant transformation in hamster embryo cells than the hydrocarbons or the corresponding K-region phenols. The K-region epoxides derived from benz(a)-anthracene were also active in transforming a clone of ventral prostate cells from the C3H mouse that was not readily transformed by the parent hydrocarbon. The phenols were the most toxic compounds tested but did not transform cells; this confirms that toxicity and transformation are not directly related events. The results obtained support the view that metabolism of PCHs precedes toxicity and transformation in rodent cells in culture (Grover et al., 1971). In every case where metabolically activated forms are known and have been tested, they have been found to be mutagenic (Heidelberger, 1973; Miller and Miller, 1971).

Enzyme induction and inhibition of cancer

Several examples are now known in which enzyme induction blocks chemical carcinogenesis. Treatment of rats with 3-MC or certain other PCHs stimulates the liver microsomal metabolism of aminoazo dyes and 2-acetylaminofluorene to noncarcinogenic metabolites (Conney et al., 1956). These results explain why the hydrocarbons inhibit the ability of aminoazo dyes and 2-acetylaminofluorene to cause liver cancer, mammary cancer, and ear duct cancer. The administration of DMBA causes adrenal damage and mammary cancer in rats (Huggins et al., 1964), and both effects are inhibited by various PCHs and aromatic azo derivatives (Huggins and Pataki, 1965).

Pretreatment of rats with MC prior to DMBA-^{3}H administration resulted in markedly decreased concentrations of tritiated hydrocarbon in the adrenal gland, mammary gland, and fat (Levin and Conney, 1967).

MC pretreatment decreased the concentrations of DMBA, 7-OHM-12-MBA (II) and 12-OHM-7MBA (III) in these tissues. Boyland et al., (1965) found that 7-OHM-12-MBA is a metabolite of DMBA that causes adrenal necrosis and induces mammary cancer. The ability of one chemical to block cancer formation by another raises the possibility that cancer formation by environmental carcinogens in man might be blocked by suitable nontoxic enzyme inducers.

Most (possibly all) of the AHH activity of the small intestine and the lung is due to an exogenous inducer or inducers. These findings come from a variety of feeding and starvation experiments (Wattenberg, 1972). One of the experimental models which has been used to study the possible protection against carcinogenic effects of PCHs by inducing increased AHH activity is the formation of mammary tumors in rats given large oral doses of DMBA. In spite of the ability of AHH to activate PCHs to ultimate carcinogens *in vitro* as well as detoxifying PCHs, there are no reported data which clearly indicate that induction of increased mixed function oxidase activity causes an increase in carcinogenicity of a chemical carcinogen in an *in vivo* experiment. There are several possible explanations for this and these will be discussed in Chapter II.

Recently Wattenberg (1974) found that the sulfur-containing compounds: disulfiram, dimethyldithiocarbamate, and benzylthiocyanate, when added to the diet, inhibited DMBA-induced mammary tumor formation and adrenal necrosis in female SD rats. Wattenberg (1974) has made use of an experimental model using adrenal necrosis from DMBA as a rapid method of screening compounds for potential suppression of

DMBA-induced mammary tumor formation.

The ability to induce AHH may be beneficial, deleterious, or perhaps both, to the individual who is constantly exposed to hydrocarbons, insecticides, and drugs in his environment. AHH may be the carcinogen-activating enzyme. If this is so, the inhibition or control of this enzyme system, with respect to specific metabolite formation, may be an important approach to the chemoprophylaxis of PCH carcinogenesis (Nebert and Gelboin, 1969).

Microsomal enzymes and steroid metabolism

The hydroxylation of steroids by liver microsomes is influenced by the same factors that affect drug oxidations, which suggests that drugs and steroids are substrates for the same hydroxylating enzyme (Conney, 1967). Halogenated hydrocarbon insecticides (such as DDT) are potent stimulators of 17β-estradiol and estrone metabolism, and these inducers decrease the action of estrogens on the uterus. Compounds that inhibit drug-metabolizing enzymes in liver microsomes also inhibit steroid metabolism and augment the action of steroids in animals. Compounds that stimulate the hydroxylation of steroids by liver microsomes in animals also alter pathways of steroid metabolism in man (Kuntzman et al., 1964; Conney and Burns, 1972). Direct measurements of 17β-estradiol indicated reduced levels of the endogenous steroid in both mammary gland and uterus following DMBA treatment.

DDT has been shown to have both estrogenic (Bitman and Cecil, 1970; Clement and Okey, 1972; Heinrichs et al., 1971) and anti-estrogenic effects (Conney et al., 1967; Welch et al., 1967; Clement and Okey, 1972). In low concentrations of 100 ppm, DDT was significantly

antiestrogenic while in high concentrations in excess of 1000 ppm the effect was estrogenic (Clement and Okey, 1972).

Nebert and his associates (1970) found that in a cell-free *in vitro* system, both 17β-estradiol and testosterone competitively inhibited PCH hydroxylation, even at levels that are lower than that of the PCH substrate. Estradiol was generally more inhibitory than testosterone. This difference in inhibition between an estrogen and an androgen may be related to sex difference in the metabolism of drugs and in the susceptibility to carcinogenesis by PCHs.

Numerous attempts have been made to correlate the carcinogenicity of PCHs and their molecular structures. Steroids may be converted chemically to a number of PCHs, among which is the highly carcinogenic 3-methylcholanthrene. Numerous efforts to make this conversion *in vivo* have been unsuccessful. There is usually a direct increase in carcinogenicity as the hydrocarbons become sterically more similar to steroids. Methyl substituted benzanthracene and cholanthrene, which have the same molecular dimension as that of steroids, are among the most potent carcinogens known. Carcinogenesis by these hydrocarbons may be the result of their interference with normal steroid function (Yang *et al.*, 1961).

AHH activity in human tissues.

A cell culture system is suited for studies on microsmal enzyme induction in mammalian tissues because these investigations in the intact animal are subject to many variables, including age, sex and species differences, hormonal and nutritional variations, and exposure to a variety of environmental chemicals. Levin and coworkers (1972)

showed variation in the inducibility of benzo(a)pyrene in skin samples from different newborn infants to be due to genetic factors. AHH activity in human lung preparations has been shown (Grover et al., 1973) by forming the K-region epoxide of benz(a)anthracene. A method for determining AHH induction in human leukocytes was developed by Busbee et al. (1972). This cultured leukocyte system provides a simple method for studying the inducibility of AHH activity in the human population. Susceptibility to human bronchogenic carcinoma was associated with higher levels of inducible AHH activity in cultured leukocytes (Kellerman et al. (1973). Kellerman (1973) also found that when 3-MC was employed, the extents of induction of AHH and epoxide hydrase were under the same genetic control. Both enzymes may constitute a coupled monooxygenase-hydrase systems. The inducibility of AHH in humans may predict the ability of humans to metabolize environmental carcinogens.

DDT and enzyme induction

There are over 200 drugs and a number of insecticides, PCHs, and other compounds that can stimulate the activity of hepatic microsomal enzymes in animals and man (Conney, 1967). These compounds can be divided into several groups depending on a number of factors including: the number of microsomal systems stimulated or inhibited, the rate and time of onset and decline of increased activity, the species, the age, and the sex of animals affected, the effect on hepatocyte cytology and the postulated mechanisms of action. DDT has been shown to induce hepatic microsomal drug-metabolizing enzymes in a variety of animal species (Conney, 1967; Conney and Burns, 1972; Chhabra and Fouts, 1973).

In vivo administration of DDT into rats produces, in addition to an increase in liver weight, a net increase in total protein (Sanchez, 1967). In addition to increased RNA and protein synthesis, the microsomal enzymes involved in the metabolism of DDT are also activated. The stimulatory effects of DDT on enzyme activity are due to its causing a proliferation of smooth surfaced endoplasmic reticulum in the liver cell, with a consequent increase in the amount of the drug metabolizing enzymes (Hart and Fouts, 1963).

In extensive studies on human subjects exposed to large doses of DDT, as much as 1,250 times the average intake of the general population, no evidence of injury related to DDT was reported by the subjects or was found by careful medical examination (Hayes, *et al*., 1971; Laws *et al*., 1967; Morgan and Roan, 1974). In a study of workers exposed in their occupations to high levels of DDT for 10 to 20 years, no cases of cancer developed in some 1,300 man-years of exposure (Laws, 1971).

There are at least two known studies which have demonstrated the antitumorigenic effects of DDT (Laws, 1971; Okey, 1972).

The duration and intensity of action of many drugs are largely determined by the speed at which they are metabolized in the body by enzymes in liver microsomes. Inhibition of enzyme activity could lead to a dangerous accumulation of drugs, hormones, insecticides or carcinogens.

Malathion and enzyme inhibition

The inhibitory effects of malathion on microsomal enzyme activity (Conney, 1967) appear related to their ability to bind to cytochrome

P450 rather than their effects on the other components of the mixed function oxidase system. Organophosphate insecticides (eg. malathion) bind irreversibly to the enzyme in noncompetitive inhibition. Malathion binds covalently to the catalytic site of cytochrome P450 resulting in the production of cytochrome P421 and a significant loss in heme (Stevens, 1974).

Compounds that inhibit drug-metabolizing enzymes in liver microsomes also inhibit steroid metabolism in animals. Conney *et al*. (1967) found that organophosphate insecticides inhibit liver microsmal metabolism of several steroid hormones.

Inhibitors of microsmal enzyme activity inhibit the metabolism of benzo(a)pyrene *in vivo* and potentiate the acute toxicity of this polycyclic hydrocarbon in rodents (Conney and Burns, 1972; Weber *et al*., 1974).

CHAPTER II

MAMMARY TUMORS INDUCED IN RATS BY PROLONGED DMBA FEEDING: INHIBITION BY DDT ; POTENTIATION BY MALATHION

Pretreatment of female rats with DDT in the diet was previously found to significantly reduce their subsequent liability to mammary tumor induction by a single 15 mg p.o. dose of DMBA (Okey, 1972). Repeated, multiple doses are more effective in inducing tumors than single doses (Shimkin _et al_., 1969) and humans are more likely to injest carcinogens as chronic "trace" amounts than as single massive doses. Thus, the effect of DDT treatment on mammary tumor induction and growth during prolonged p.o. administration of DMBA was tested. Malathion, which was found to inhibit AHH activity (Conney and Burns, 1972), was tested for possible potentiation of DMBA tumorigenesis.

Materials and Methods

Female Sprague-Dawley rats were randomly assorted into 3 treatment groups, then housed individually in polypropylene cages at 24°C with 14 hours light: 10 hours dark diurnal cycles. Experimental diets were begun at age 36 - days; rats continued on these diets until necropsy. Group 1 (Control) rats were fed Purina Laboratory Chow meal. Group 2 received Chow containing 100 ppm p,p' - DDT. Group 3 received chow containing 250 ppm malathion (98.9% secondary standard). DDT and malathion were dissolved in 95% ethanol which then was blended with

chow in a mechanical mixer; an equal quantity of ethanol was added to chow for the Control group.

Beginning at age 50-days, each rat received 21 consecutive daily doses of 0.714 mg DMBA p.o. in 0.25 ml. corn oil (for a total dose of 15 mg DMBA per rat).

Each rat was weighed and examined for mammary tumor development weekly. Only nodules that were larger than 1 mm in diameter and which were palpable on 2 successive examinations were recorded as tumors. The growth of established tumors was followed by measurement of tumor diameters using the technique established by Dao and Sunderland (1959); Huggins _et al_. (1959) and Stevens _et al_. (1965). All rats still living 230 days after initiation of DMBA treatment were killed by ether overdose; they were examined for internal tumors, hidden subcutaneous tumors and the uterus, adrenals, ovaries and liver were removed, cleaned of extraneous tissue and weighed.

Results

DDT treatment significantly reduced (below control values) the percentage of rats with tumors (Table 1) and the number of tumors per rat (Table 2) while significantly lengthening the latent period for tumor appearance (Table 3). Tumor incidence was significantly higher than control in the malathion-treated rats during the period 95-130 days after the start of DMBA administration; by 230 days the difference in tumor incidence between malathion and control was not statistically significant (Table 1, Figure 3). The higher tumor incidence in the control group may have masked further tumor potentiation by malathion.

Table 1. Animals with mammary tumors (%) induced by DMBA

Treatment Group	Control (DMBA only)		DDT + DMBA		Malathion + DMBA	
Time after DMBA (Days)	Tumors No.	%	Tumors No.	%	Tumors No.	%
32	(0/28)	0	(0/29)	0	(0/29)	0
39	(0/28)	0	(0/29)	0	(1/29)	3.4
46	(3/28)	10.7	(0/29)	0	(4/29)	13.8
53	(8/28)	28.6	(0/29)	0*	(13/29)	44.8
60	(10/28)	35.7	(0/29)	0*	(15/29)	51.7
67	(14/28)	50.0	(2/29)	6.9**	(16/29)	55.2
74	(16/28)	57.1	(2/29)	6.9**	(20/29)	69.0
81	(16/28)	57.1	(2/29)	6.9**	(24/29)	82.8
88	(18/28)	64.3	(2/29)	6.9**	(24/29)	82.8
95	(18/28)	64.3	(2/29)	6.9**	(26/29)	89.7*
102	(20/28)	71.4	(3/29)	10.3**	(27/29)	93.1
109	(21/28)	75.0	(4/29)	13.8**	(28/29)	96.6*
116	(21/28)	75.0	(4/29)	13.8**	(28/29)	96.6*
123	(22/28)	78.6	(7/29)	24.1**	(28/29)	96.6
130	(23/28)	82.1	(7/29)	24.1**	(29/29)	100.0*
137	(25/28)	89.3	(7/29)	24.1**	(29/29)	100.0
144	(26/28)	92.9	(8/29)	27.6**	(29/29)	100.0
151	(26/28)	92.9	(10/29)	34.5**	(29/29)	100.0
⋮	⋮	⋮	⋮	⋮	⋮	⋮
230	(26/28)	92.9	(10/29)	34.5**	(29/29)	100.0

No new animals with tumors after 151 days.
* significantly different from control   * $p < 0.05$ ** $p < 0.0005$
(X^2 test, 2X2 contingency table)

Table 2. Tumors per rat (230 days after DMBA)

Treatment Group	No. rats	Total No. tumors	Tumors per rat (No. ± S.E.)
Control (DMBA only)	28	64	2.321 ± 0.27
DDT + DMBA	29	15	0.517* ± 0.15
Malathion + DMBA	29	82	2.827 ± 0.19

* significantly different from control $p < 0.001$ (student's "t" test)

Table 3. Mammary tumor latency period

Treatment Group	Time from DMBA until first tumor in group (days)	Mean tumor latency (days ± S.E.)	Range of tumor appearance (days)
Control (DMBA only)	44	80.73 ± 6.15	44-144
DDT + DMBA	65	116.00* ± 9.18	65-151
Malathion + DMBA	33	68.69 ± 3.99	33-130

* significantly different from control $p < 0.005$ (student's "t" test)

Table 4. Effect of DDT and malathion on tumor growth type

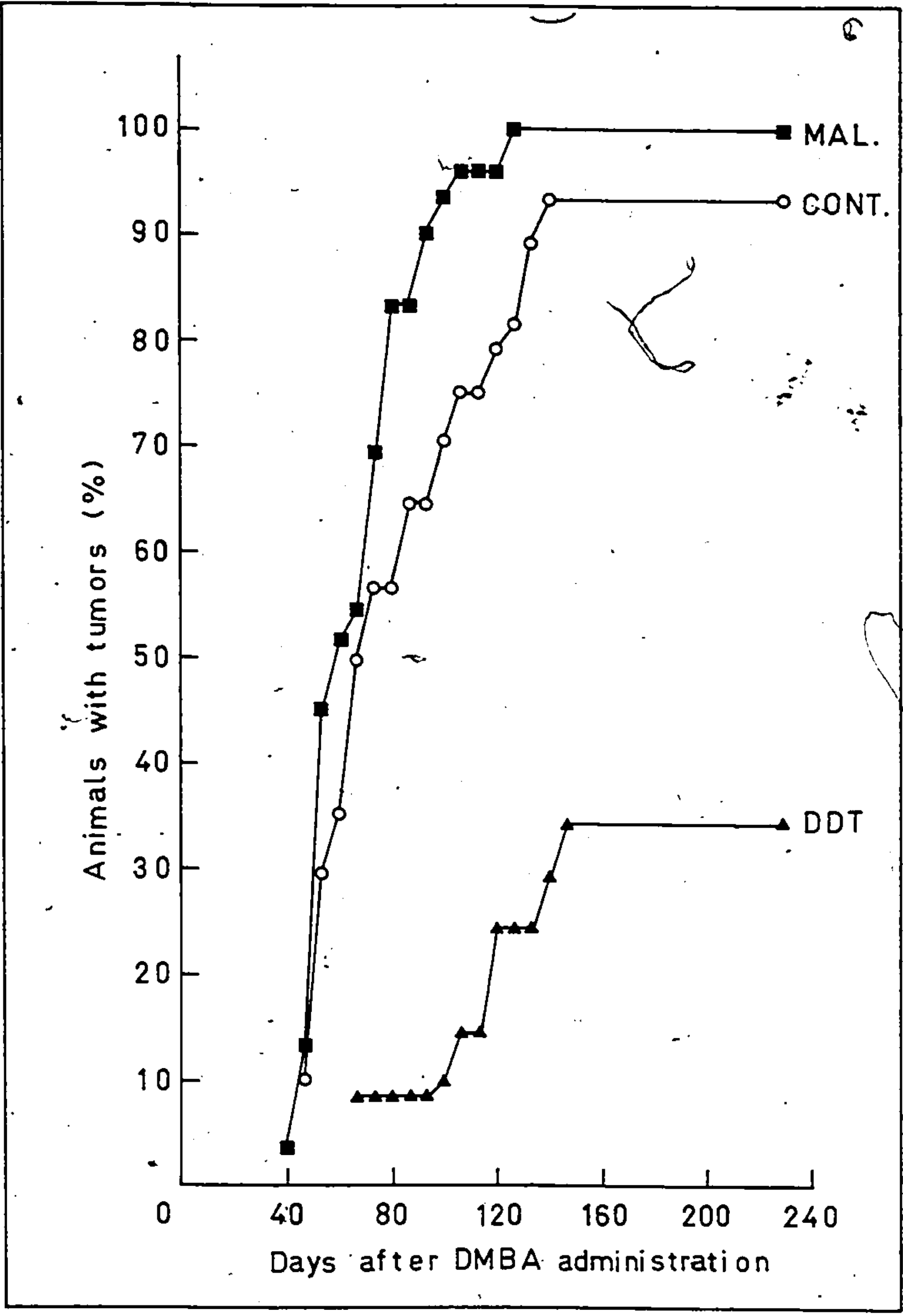
Treatment Group	Growing tumors		Stable tumors		Regressing tumors	
	No.	%	No.	%	No.	%
Control (DMBA only)	(20/65)	30.8	(18/65)	27.7	(27/65)	41.5
DDT + DMBA	(2/15)	13.3*	(9/15)	60.0	(4/15)	26.7
Malathion + DMBA	(49/82)	59.8**	(12/82)	14.6	(21/82)	25.6

* significantly different from malathion $p < 0.005$

** significantly different from control. $p < 0.001$

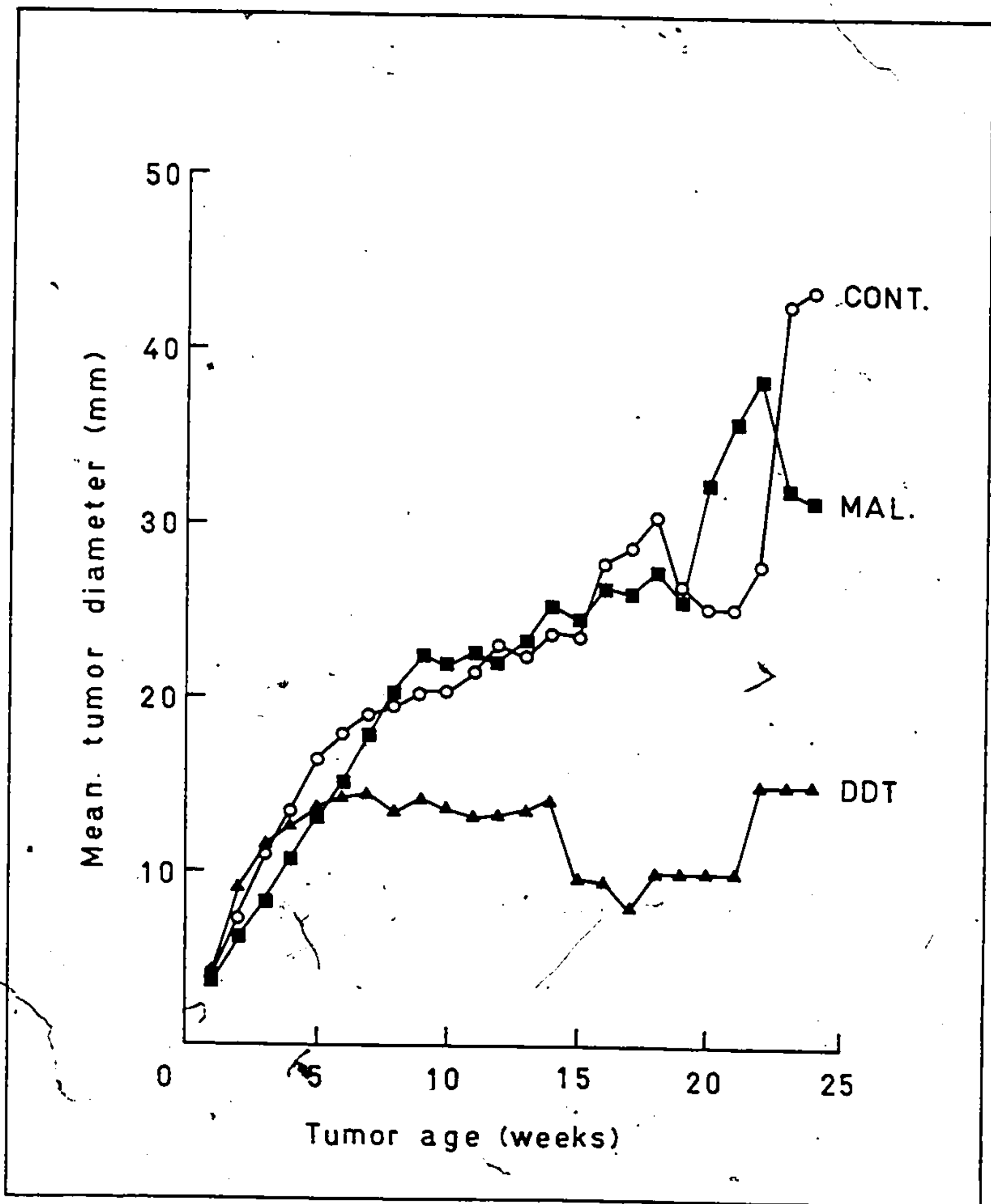
X^2 test, 2X2 contingency table

Figure 3. Effect of DDT and malathion on the incidence of DMBA-induced mammary tumors. No new animals developed tumors after 151 days. Control (○); DDT (▲); malathion (■).



MAL.
CONT.
DDT
Animals with tumors (%)
100
90
80
70
60
50
40
30
20
10
0
40
80
120
160
200
240
Days after DMBA administration

Figure 4. Effect of DDT and malathion on tumor growth rate of DMBA-induced mammary tumors. A tumor of age 1 week has been growing 1 week since it first appeared. A tumor of age 24 weeks has been growing for 24 weeks since it first appeared. All tumors of the same age in each treatment group were averaged. Decreases in mean tumor diameter occured at several points in DDT-treated animals due to regression of some tumors at a rate greater than the growth of other tumors in the group. Mean tumor diameter decreased during the last weeks of observation in the malathion-treated group because of death of several rats bearing large tumors. Control (○); DDT (▲); malathion (■).



CONT.
MAL.
DDT
Mean tumor diameter (mm)
Tumor age (weeks)
0
5
10
15
20
25
10
20
30
40
50

No new animals ~~developed~~ tumors after 151 days.

All tumors were classified into growth-type according to Stevens, Stevens and Currie (1965) (Table 4). The malathion group showed significantly more growing tumors than control.

Examination of the tumor growth data (Figure 4) shows a great deal of overlap among the three groups for the first 5 weeks of tumor growth. As the tumors in each group got older, the DDT group showed a distinct separation from the tumor growth rates of malathion and control groups. High points on the tumor growth curves (Figure 4) indicate periods in the life of the tumors when rapid growth is occurring, while low points indicate periods when tumors have stopped growing or have started to regress.

Many tumors were found to grow to a huge size (Figure 5). As the tumors grew they became necrotic internally and in many instances ruptured through the skin exposing the underlying tissue to infection and allowing considerable blood loss (Figure 6, 8). The only other external tumors were ear duct tumors which occurred in low frequencies (< 5%)(Figure 6).

The distribution of tumors among the 6 pairs of mammary glands showed a significant preference for the anterior 3 pairs over the posterior 3 pairs (Table 5). There were, however, equal numbers of tumors on left and right sides of the animal. Jabara <u>et al</u>; (1972) who also found this type of tumor distribution in some but not all of their different treatment groups attributed the results to a yet unexplained effect.

<u>Necropsy of surviving animals</u>

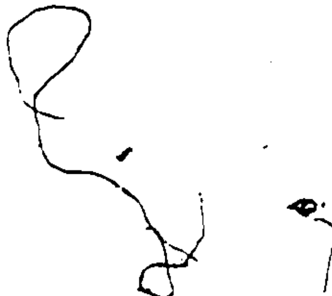
All DDT-treated rats were alive 230 days after the start of DMBA

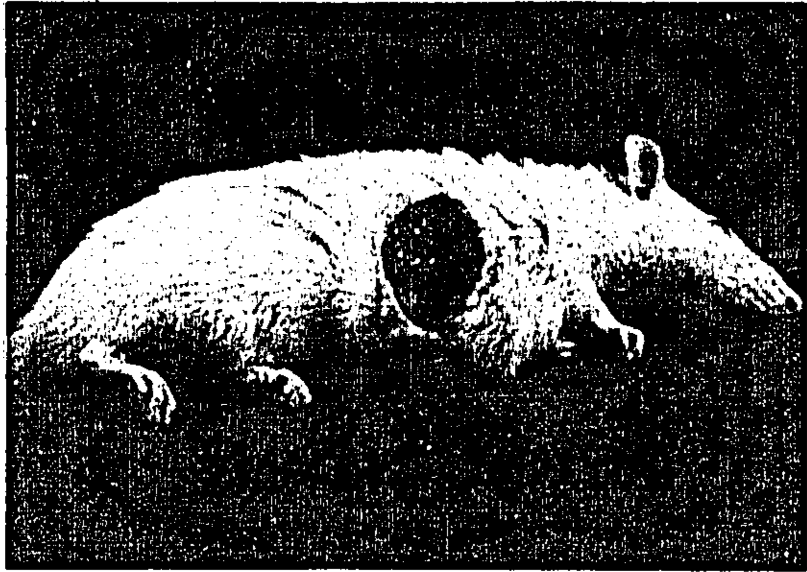
Figure 5. Animal with massive mammary tumor. This tumor in the left inguinal region was 100 mm in diameter. It is very unusual for a tumor to reach this size without rupturing through the skin.

Figure 6. Animal with tumor of the ear duct. Next to mammary tumors, ear duct tumors were the most commonly occurring tumors visible externally. The major part of this tumor was composed of dead tissue at this stage in its development.

Figure 7. Animal with ulcerated mammary tumor. When a tumor reaches a certain size it tends to rupture through the surface of the skin. Most rats will hollow out the tumor by eating the necrotic tissue.

Figure 8. Close-up view of necrotic tumor. This is a closer view of the tumor illustrated in Figure 7. Considerable hemorrhage takes place from such a tumor causing excessive blood loss.





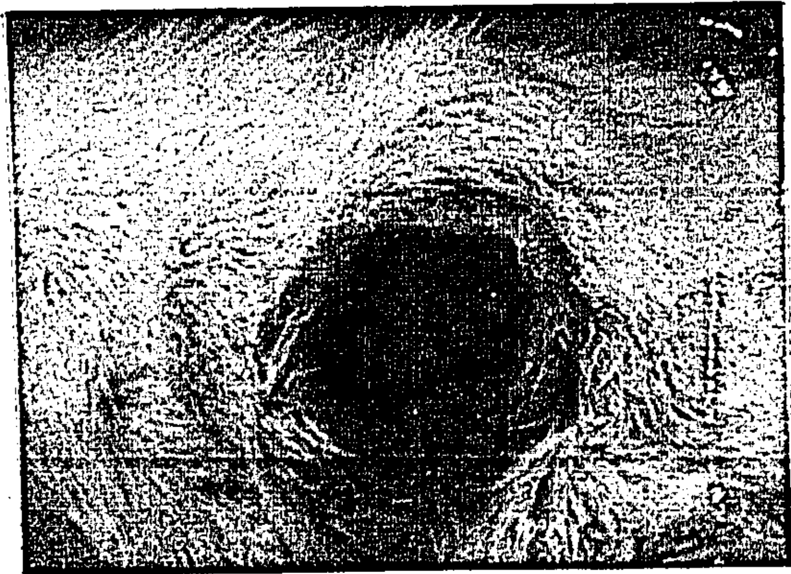


Table 5. Summary of DMBA-induced tumor distribution among the 6 pairs of mammary glands

Treatment Group	Tumor site: Right side	Left side	Upper 3 pairs	Lower 3 pairs
Control (DMBA only)	32/65	33/65	50/65*	15/65
DDT + DMBA	11/15	4/15	11/15	4/15
Malathion + DMBA	38/82	44/82	58/82*	24/82
Total No. tumors in all groups	81/162	81/162	119/162**	43/162

* significantly different from randon (50:50) distribution.

* $p < 0.01$ ** $p < 0.0001$ (X^2 test, 2X2 contingency table)

Table 6. Survival of animals (230 days after DMBA)

Treatment Group	No. rats	No. rats alive	% survival
Control (DMBA only)	28	20	71.4
DDT + DMBA	29	29	100.0**
Malathion + DMBA	29	12	41.4*

* significantly different from control * $p < 0.05$ ** $p < 0.01$

(X^2 test, 2X2 contingency table)

Figure 9. Effects of DDT, malathion and DMBA on animal survival. No deaths occured in the DDT group. No further deaths occured between 215 days and necropsy at 230 days. Control (○); DDT (▲); malathion (■).

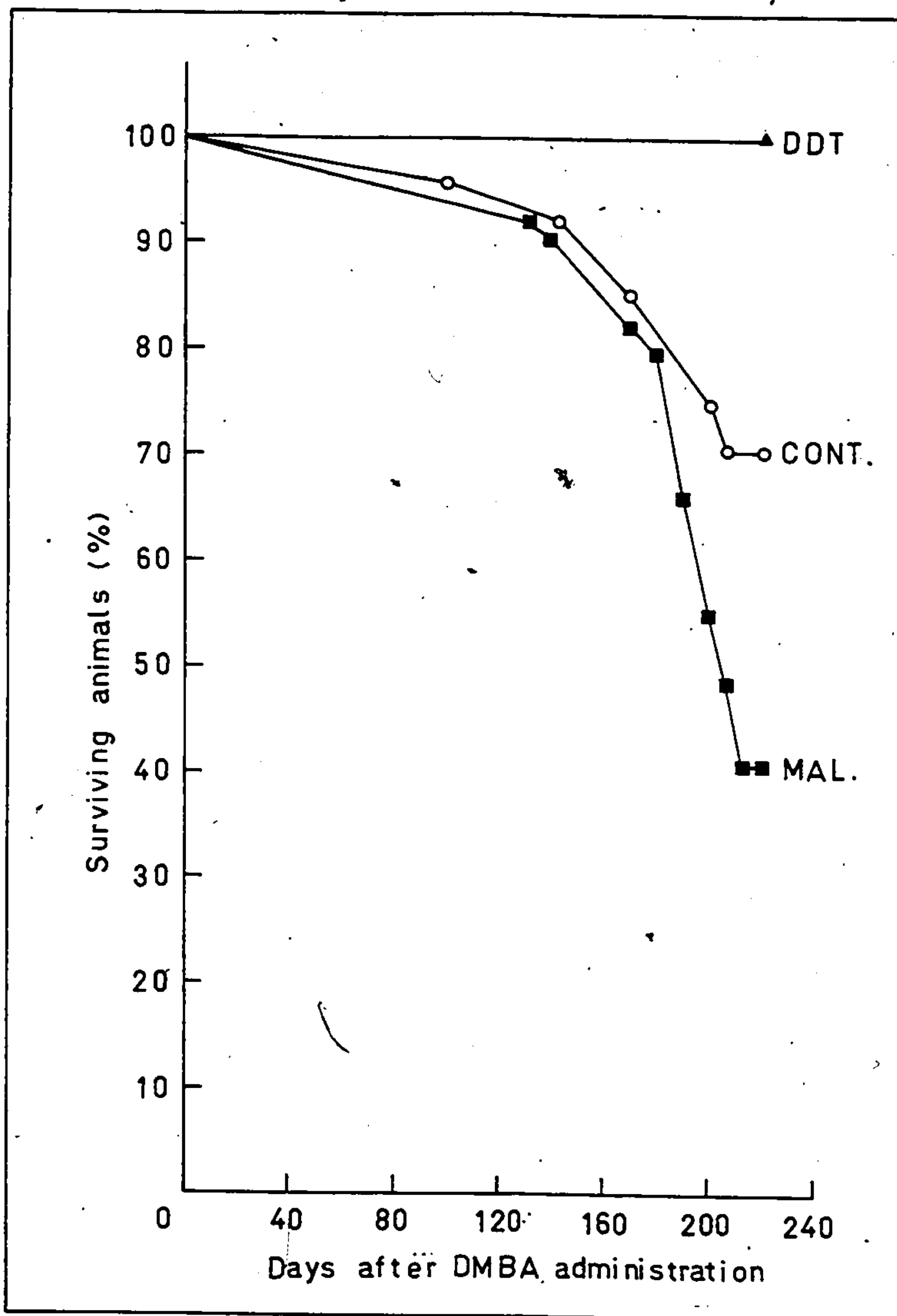

DDT
CONT.
MAL.
Surviving animals (%)
100
90
80
70
60
50
40
30
20
10
0
40
80
120
160
200
240
Days after DMBA administration

Figure 10. Body weight chart of animals treated with DDT, malathion and DMBA. Control (○); DDT (▲); malathion (■).

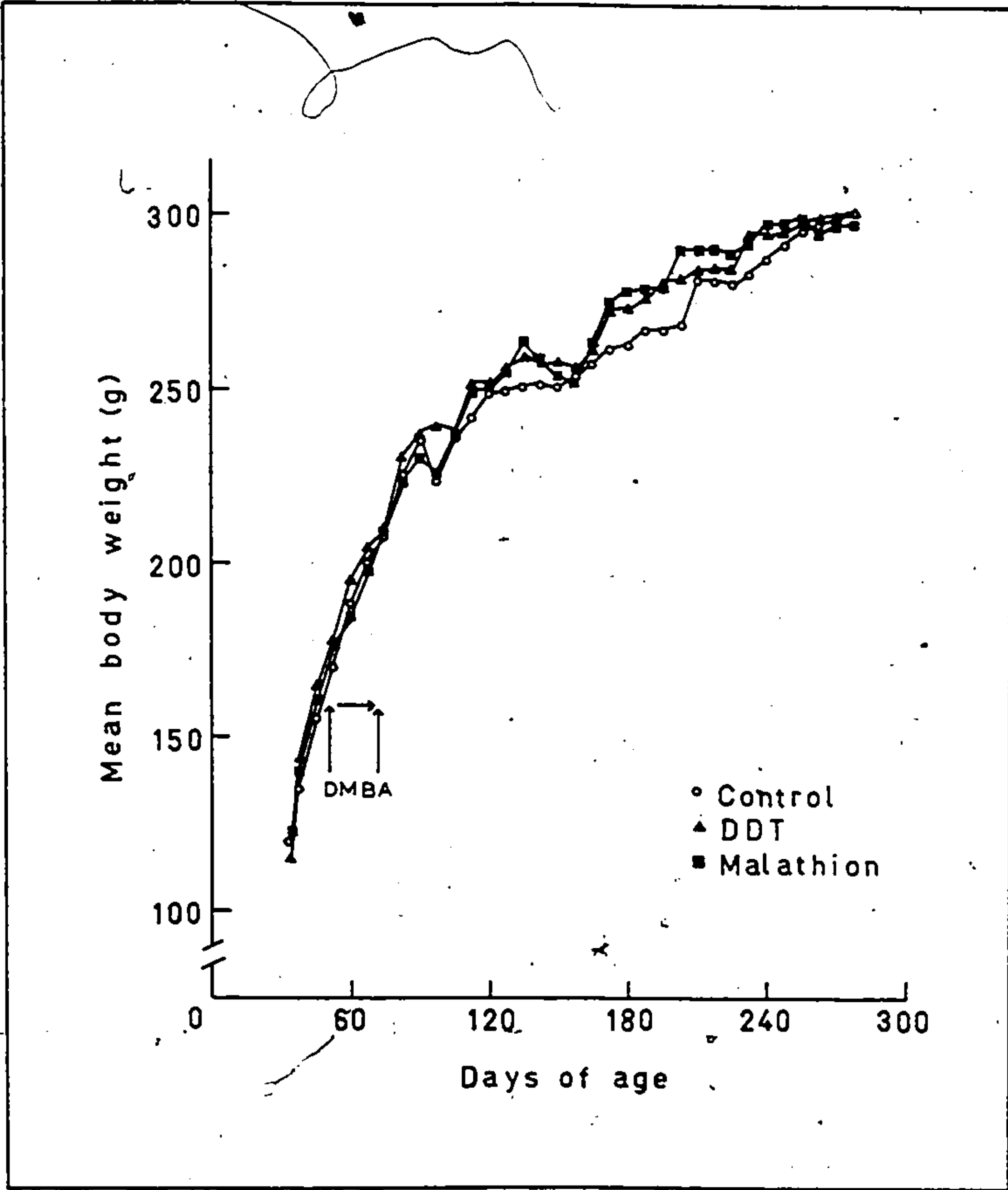

Mean body weight (g)
300
250
200
150
100
DMBA
Control
DDT
Malathion
0
60
120
180
240
300
Days of age

Table 7

Necropsy results of surviving animals (230 days after DMBA)

Treatment Group	No. rats	Body weight (g)	Adrenals (mg) (pair)	Uterus (mg)	Ovaries (mg)	Liver (g)
		Mean weights ± S.E.				
Control (DMBA only)	20	301.95 ± 4.83	63.94 ± 3.54	495.80 ± 43.59	71.83 ± 1.81	9.98 ± 0.14
DDT + DMBA	29	302.68 ± 3.86	67.07 ± 3.81	526.73 ± 27.30	83.26** ± 0.84	12.50** ± 0.13
Malathion + DMBA	12	298.00 ± 5.26	74.00 ± 6.36	633.25* ± 44.15	63.10* ± 1.16	10.10 ± 0.24

* significantly different from control * $p < 0.05$ ** $p < 0.001$ (student's "t" test)

Figure 11. Effects of DDT, malathion and DMBA on liver weight.
* significantly different from control $p < 0.001$.

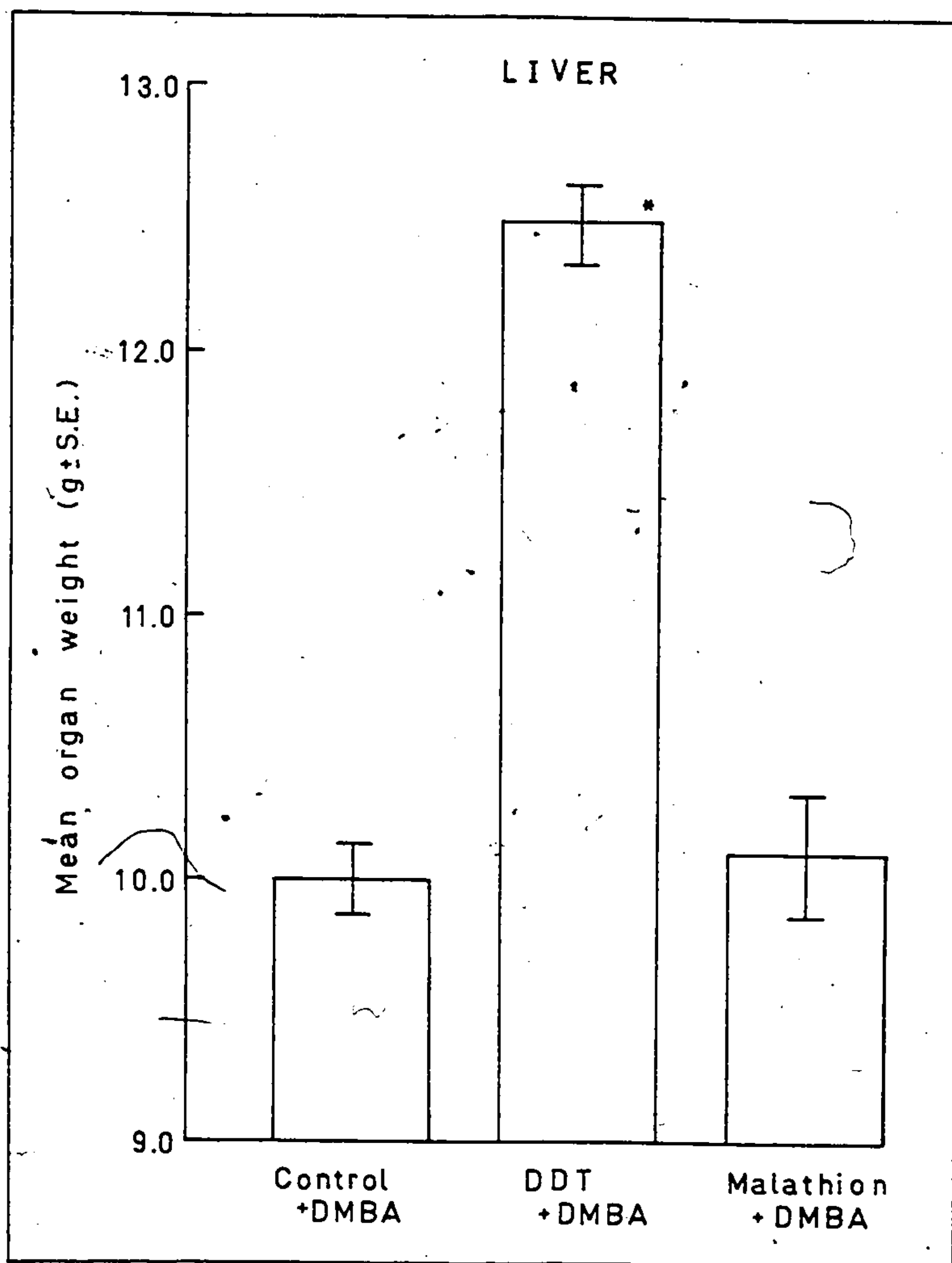
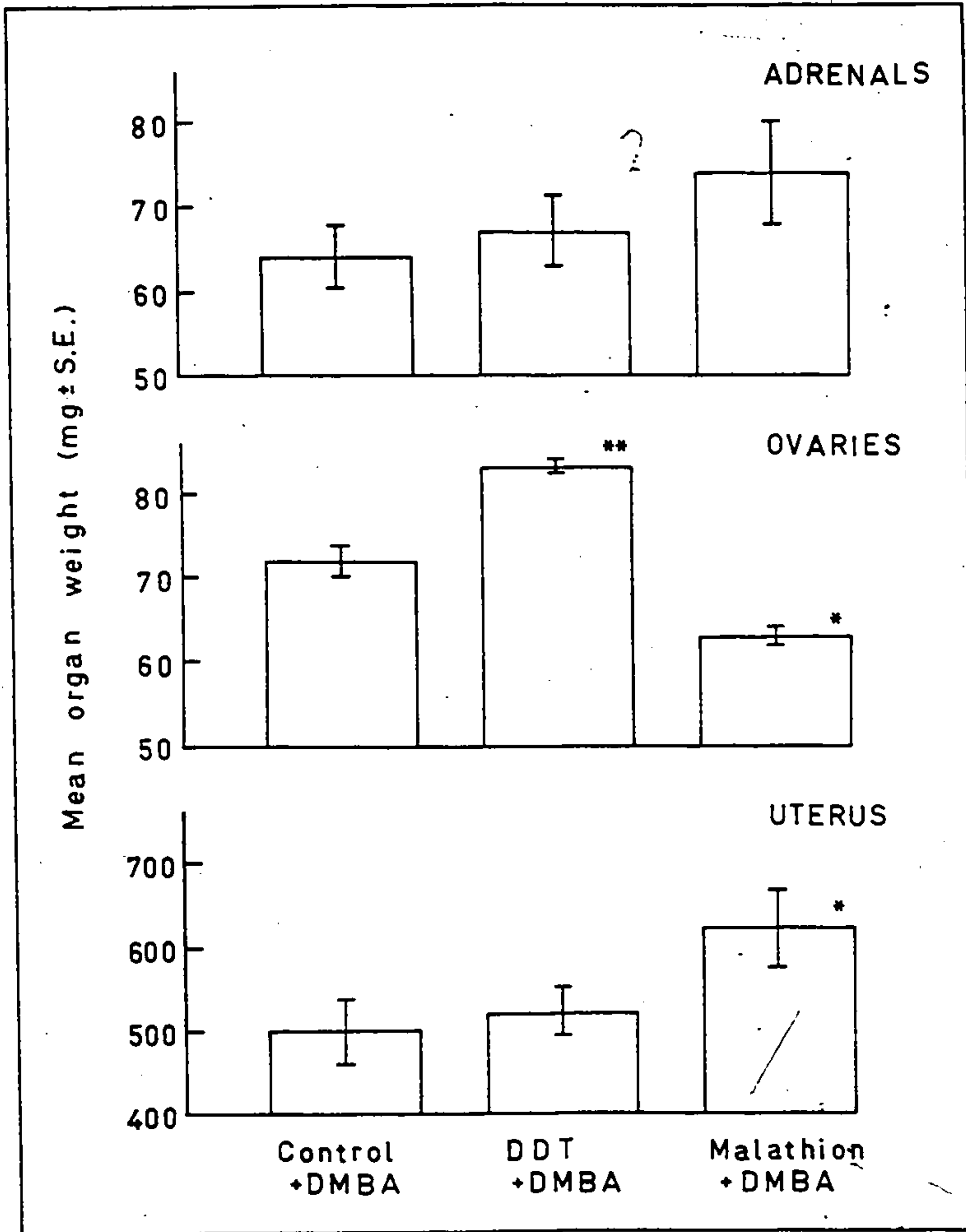

LIVER
13.0
12.0
11.0
10.0
9.0
Mean organ weight (g±S.E.)
*
Control
+DMBA
DDT
+DMBA
Malathion
+DMBA

Figure 12. Effects of DDT, malathion and DMBA on the weight of adrenals, ovaries and uterus.

* significantly different from control * $p < 0.05$

** $p < 0.001$


ADRENALS
OVARIES
UTERUS
Mean organ weight (mg ± S.E.)
80
70
60
50
700
600
500
400
**
*
Control
+DMBA
DDT
+DMBA
Malathion
+DMBA

while only 71.4% of control rats and 41.4% of malathion-treated rats survived that long (Table 6, Figure 9). DDT and malathion diets did not cause any observable toxic effects; body weight gain was equivalent in all three treatment groups throughout the experiment (Figure 10). Body weights in the last few weeks must be interpreted in light of the fact that large tumors accounted for a proportion of the animal's total body weight.

All animals surviving 230 days after the start of DMBA were necropsied. The adrenals, ovaries, uterus, spleen and liver were examined and weighed (Table 7). The livers of DDT - treated rats were significantly larger than control (Table 7, Figure 11) and the uteri of malathion - treated rats were significantly larger than control (Table 7, Figure 12). The ovaries of DDT - treated rats were significantly larger than control while the ovaries of malathion - treated rats were significantly smaller than control (Table 7, Figure 12). There were no significant differences in adrenal size among the three groups. The presence of internal tumors have been outlined under Results in Chapter III.

Discussion

Antitumorigenesis by enzymatic detoxification of DMBA

We propose that the principle mechanism by which DDT protects rats from DMBA tumorigenesis is enhanced degradation and excretion of the carcinogen. A number of studies have demonstrated that it is possible to protect against the carcinogenic effects of chemical carcinogens by inducing increased AHH activity (Wattenberg, 1972). Induction of

increased AHH activity by a number of different types of inducers prior to administration of DMBA will inhibit DMBA-induced mammary tumors. Effective inducers were found to be: PCHs, phenothiazines and flavons (Wattenberg, 1972). Substances that induce or inhibit the microsomal drug-metabolizing enzymes increase or decrease, respectively, the biliary excretion of metabolites after the injection of ^{3}H-DMBA. The principle route of excretion for PCHs in rodents is the bile. Before excretion these substances are converted to polar metabolites and little or no unchanged hydrocarbon appears in the bile (Levine, 1974). PCHs are converted in vitro in rat liver preparations into polar metabolites such as: epoxides, dihydrodiols, phenols, ketones, and quinones. Details of DMBA metabolism were outlined in Chapter I. The high lipid solubility of hydrocarbons is conducive for binding to microsomes, where hydrocarbon metabolism occurs. This step is a prerequisite for excretion since 96-97% of ^{3}H-DMBA appears in the bile as metabolites. (Levine, 1974). The liver (in which microsomal mixed-function oxidase activity is high) has traditionally been thought of as the principle site of drug metabolism. The mixed-function oxidase activity occurs, however, in a wide variety of other tissues including the lung, intestine and mammary gland. The results of feeding experiments indicate that most and possibly all of the AHH activity of the small intestine and lung is due to an exogenous inducer or inducers (Wattenberg, 1972). Previous experiments have shown that DDT doses as low as 10 ppm in the diet significantly reduced ^{3}H-DMBA uptake by mammary tissue (Okey, 1972). DDT must act by reducing cell transformation rather than altering the survival and selection of neoplastic cells since the protective effect

of DDT is significant only if DDT is given before DMBA (Okey, 1972).

Several investigators have demonstrated that the microsomal mixed function oxidases convert many chemical carcinogens to reactive carcinogenic forms (Miller, 1970) (see also chapter I). With some compounds the site of metabolism determines whether the detoxification or activation occurs (Wattenberg, 1972). The protective effect of DDT appears to be due to delivery of less carcinogen to macromolecules in target tissues.

7, 8-benzoflavone (BF) inhibits AHH activity and decreases DMBA tumorigenesis while increasing BP tumorigenesis when applied to mouse skin or in tissue culture (Gelboin et al., 1972). These results suggest that the pattern of activation and detoxification for DMBA and BP may be markedly different and the role of the enzyme system may be unique with each hydrocarbon. In one case the inhibition of AHH played a significant role in preventing DMBA activation while in the case of BP the BF may possibly have inhibited the carcinogenic deactivation pathway and enhanced the level of active carcinogen present. Wattenberg (1972) and Huggins et al. (1964) found that pretreatment of rats with BF or small amounts of PCHs decreased DMBA-induced tumorigenesis in rat lung and mammary gland. BF was also demonstrated to be a strong AHH inducer in liver, lung, and small intestine. The protective effects of pretreatment with inducers is possibly due to increased enzyme levels in the target tissue. Protective effects could operate either through a more rapid elimination of the carcinogen or through conversion to a less carcinogenic form. Induction of liver enzymes could lower the concentration of the carcinogen in the target tissue. Treatment

with inhibitors of AHH activity such as SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate-HCL) increases DMBA-induced mammary tumorigenesis probably due to an increase in the effective dose of the carcinogen in the target tissue (Gelboin, 1972).

The conflicting results which continously arise over the effects of enzyme activation or inhibition of PCH-induced tumorigenesis may be resolved when more is known about the effect of prior treatments or inhibitors on the specific bioactivation pathways and the way in which these pathways are coupled to the relevant deactivation pathways. The specific bioactivation with PCHs is presumably a monooxygenase-catalyzed conversion to one or more arene oxides, while the deactivation pathways include isomerization of the intermediate arene oxide to phenols, hydration to dihydrodiols, conjugation with glutathione, and further oxidation (Jerina and Daly, 1974). Competing pathways may be of great importance. Arene oxides may not be the only bioactivated intermediates responsible for the binding and the cytotoxic and carcinogenic effects of PCHs. Metabolites other than arene oxides cannot yet be excluded as active agents in carcinogenesis.

There is a tendency by some investigators to make rash assumptions concerning the protective action of enzyme inhibitors on chemical carcinogenesis based on metabolism data alone (without also performing *in vivo* tumor studies). In this experiment malathion, an organophosphate insecticide, which has well been established as an inhibitor of AHH activity, increased tumor incidence. A recent report by Weber *et al*. (1974) would suggest that enzyme inhibition by organophosphate insecticides should prevent carcinogenesis because of inhibition of

BP metabolism. It would appear that inhibition of BP or DMBA metabolism is hazardous since it leads to a dangerous accumulation of DMBA which can then attack the target tissues. Although metabolism data suggest the types of metabolites that may or may not be produced, the final test for the effect of enzyme inducers or inhibitors on PCH carcinogenesis must be the *in vivo* formation of tumors.

To date there are no reported data which clearly indicate that induction of increased mixed-function oxidase activity causes an increase in carcinogenicity of a chemical carcinogen in an *in vivo* experiment. Conflicting data on carcinogen activation may be explained by considering that in cases where both activation and detoxification occur, the detoxification reactions predominate; that slow activation of a carcinogen is as effective as rapid activation and that no proper *in vivo* experiments showing enzyme activation increases tumor incidence have yet been done (Wattenberg, 1972). Although the mixed-fuction oxidase system may produce some adverse effects its protective effects should be investigated further.

If we can characterize ultimate chemical carcinogens as strong electrophilic reactants then we can continue research on attempts to (I) inhibit or prevent generation *in vivo* of these strong electrophiles, (II) render these electrophiles harmless in reactions *in vivo* with low molecular weight nontoxic nucleophiles, and (III) predict the carcinogenicity of foreign molecules (eg., drugs, food additives, food-water-air contaminants, etc.) from measures of their conversion *in vivo* to reactive electrophiles.

Antitumorigenesis and steroid metabolism

DDT reduced the percentage of actively growing tumors while

malathion significantly increased the proportion of growing tumors. The majority of DMBA-induced mammary tumors are hormone-dependent, regress upon ovariectomy and resume growth with estrogen administration (Jensen et al., 1972). The hydroxylation of steroids by liver microsomes is influenced by the same factors (Chapter I) that effect drug oxidations, suggesting that drugs and steroids are substrates for the same hydroxylating enzymes (Conney and Burns, 1972). DDT is a potent stimulator of estradiol-17β metabolism, and it decreases the action of estrogens on the uterus (Conney and Burns, 1972; Kuntzman et al., 1964). DDT protects against DMBA-induced mammary tumors by stimulating steroid metabolism and thus decreasing the concentration of estradiol-17β which is a critical factor in the "initiation" and "promotion" of tumor development. The decrease in tumor incidence and growth rate in the DDT-treated group would support this postulate. The uterus in the malathion animals was significantly larger that the control. Large ovaries in the DDT group may be due to stimulation of follicle stimulating-hormone but as yet the cause of the enlargement remains unclear.

Welch et al. (1967) found that the chronic administration of an organophosphate insecticide to immature rats inhibited the liver microsomal metabolism of estradiol-17β to highly polar metabolites. The chlorinated hydrocarbon insecticide, DDT, was less inhibitory than the organophosphates when added in vitro and markedly stimulated the metabolism of estradiol-17β by liver microsomes when given chronically to rats.

No evidence could be found that malathion alone was carcinogenic

at any dose. The observations presented in this chapter may be specific to DMBA only. Other hydrocarbons may behave differently with respect to detoxification and activation DDT and malathion. Suggesting that all PCHs would respond in a similar manner to DDT and malathion is a bit premature based on these data alone.

These observations and earlier studies suggest the theoretical possibility of using suitable enzyme inducers prophylactically to protect people from environmental carcinogens. The susceptibility of different persons to the carcinogenic effects of polycyclic hydrocarbons in our environment may be genetically determined (Wattenberg, 1966; Keellermann et al., 1973)

CHAPTER III

INHIBITION OF ANEMIA AND LEUKEMIA BY DDT IN RATS FED DMBA

The two tissues of the rat most susceptible to the induction of neoplasms by polycyclic aromatic hydrocarbons (PCH) are the mammary acini and hemopoietic stem cells (Huggins et al., 1972). Leukemia designates all malignant proliferative conditions of the reticuloendothelial system and is characterized by the production of abnormal and immature circulating white blood cells. Leukemia by PCHs was first observed by Shay et al.(1951) who used methylcholanthrene (MC) to induce lymphatic and myelogenous leukemia in Wistar rats. Huggins et al. (1972) found that multiple pulse-doses of DMBA can induce a high incidence of leukemia (55-73%) in Sprague-Dawley (SD) rats. Nowell et al.(1963) found abnormal chromosome numbers and morphology in cells in female Wistar rats with MC-induced leukemia. There are no cases on record of the development of spontaneous myelogenous leukemia in a rat of Wistar stock (shay et al., 1951) or at the SD breeding laboratories in Madison, Wisc. (Zipf, 1959).

It has been suggested by Shay et al. (1955) that PCH induced leukemia, specifically MC-induced chronic myelogenous chloroleukemia, be used as a screening tool for new therapy to be suggested for the treatment of this disease. This disease in the Wistar rat resembles chronic myelogenous leukemia in man very closely in its peripheral hematological and bone-marrow pictures, in its tissue

metastasis and in its response to chemotherapeutic agents.

Supported by earlier evidence of the anticancer effects of DDT (Laws, 1971; Okey, 1972) I tested the hypothesis that pretreatment of rats with DDT would protect them against DMBA-induced leukemia, and the anemia which invariably accompanies leukemia, by stimulating hepatic microsomal enzyme detoxification of DMBA. Malathion, an enzyme inhibitor (Conney, 1967; Conney and Burns, 1972), was tested as a possible potentiator of DMBA-induced leukemia.

Materials and Methods

Induction of anemia and leukemia

The animals used for the study of anemia and leukemia were the same animals that were used to study mammary cancer. These animals were fed a total of 15 mg of DMBA and were placed on control, DDT and malathion diets as described under methods in Chapter 2.

Collection of Blood Samples

Blood samples used for the diagnosis of anemia and leukemia were taken on two occasions. The first blood sample was taken 200 days after DMBA treatment started. The animals were placed under light ether anasthesia to prevent hematological changes due to stress. The caudal vein was exposed by cutting 3-5 mm off the tip of the rat's tail and 0.5-1.0 ml of blood were collected. The second blood sample was taken by cardiac puncture of an ether-anesthesized rat at necropsy (230 days after DMBA). This sample yielded 7-10 ml of blood.

Diagnosis of anemia

The diagnosis of anemia was based on the examination of blood using the following three tests: micro-hematocrit, hemoglobin concentration and red blood cell examination.

Micro-hematocrit

For this test both 200-day and 230-day blood samples were used. Heparinized capillary tubes were filled 3/4 full with blood, sealed with plastic clay and centrifuged at approximately 8,500 x g for 5 min in a microhematocrit centrifuge. This method which gives an accurate determination of packed red blood cells has been used as a screening proceedure for anemia.

Hemoglobin concentration

For this test the 200 day blood sample was used. Hemoglobin concentration was determined using the acid hematin method of Cohen and Smith (1919). A 0.2 ml sample of whole blood was added to 5.0 ml of 1 % HCl with a Sahli pipette. After mixing, the contents were allowed to stand for 60 min. The colorimeter was set at 525 nm and "blanked" with 1 % HCl. The concentration of hemoglobin in grams per 100 ml of blood was determined using the percent transmittance and a standard curve.

Red blood cell examination

For this examination both 200-day and 230-day blood samples were used. A blood sample was collected with a standard red cell pipette and diluted with Hayem's solution. Cells were counted in a hemacytometer. A blood smear stained with Wright's stain was used to examine red cells for morphological abnormalities.

Diagnosis of Leukemia

The diagnosis of leukemia was based on the examination of blood using the following tests: total white cell count, differential count, and serum protein electrophoresis. All animals were examined for internal tumors and tumors were examined microscopically for infestation with leukemic cells.

White blood cell examination

White cell total and differential counts were carried out on 200 day and 230 day blood samples. Blood was collected with a standard white cell pipette and diluted with 2 % acetic acid solution to which gentian violet had been added until the solution was a pale blue-violet. Cells were counted in a hemacytometer. A blood smear stained with Wright's stain was used for the differential leukocyte count. All leukocytes were classified as neutrophils, eosinophils, basophils, lymphocytes, monocytes, myelocytes, or lymphoblasts. Platelets were counted by comparing the number of platelets in one oil immersion field with the number of red blood cells. One thousand red cells were counted and the number of platelets were calculated from the known red cell count. Reticulocytes were counted in the same manner as platelets and expressed as a percentage of the normal red cells.

Serum protein elecrophoresis

A serum sample was prepared from a 200 day blood sample by allowing the blood to clot and centrifuging the clot at 5900 X g for 20 min. Total serum protein was determined using the biuret reaction. A 10 µl sample of undiluted serum was fractionated into its protein components with an LKB paper electrophoresis apparatus using the

method devised by Aronsson and Gronwall (1957). Serum components were separated using Whatman No. 1 filter paper and TRIS-borate buffer with pH 8.9 at 5.0 V/cm and 3.0 mA for 18 hours. The proteins were stained with Amido-Black 10 B and the strips were evaluated with a Photovolt integrating densitometer.

Necropsy examination

All rats surviving 230 days after DMBA feeding was started were killed by an overdose of ether and examined for internal tumors. Animals with green colored organs were illuminated with U.V. light to detect the presence of chloromas. The liver, spleen, and thymus from each animal was removed, cleaned of extraneous tissue, weighed and quick-frozen. Any organ that appeared to have a tumor was also removed and quick-frozen. "Touch" preparations (impression films) were made of all tissues suspected of being infiltrated with leukemic cells by pressing the cut surface of fresh tissue against a dry slide. Cells adhering to the surface of the slide were stained with Wright's stain and examined for the presence of leukemic cells.

Results

DDT pretreatment significantly reduced the incidence of both anemia and leukemia in rats fed DMBA. The diagnosis of anemia was based on a red cell count of less than 7.0×10^6 cells per mm^3; a hemoglobin concentration of less than 12 g per 100 ml of blood; and a hematocrit of less than 40%. These values fell below the range considered normal in the literature. There were no significant differences in red cell count, hematocrit or hemoglobin concentration

Table 8. Incidence of anemia in rats treated with DMBA

Treatment Group	Animals with anemia No.	Animals with anemia %	Time of sample (days after DMBA)	Erythrocyte count ($X10^6$ /mm^3 blood)	Hematocrit (% cells)	Hemoglobin concentration (g/100 ml blood)
				Mean ± S.E.		
Control (DMBA only)	13/21	61.9	200	5.32 ± 0.32	31.00 ± 2.12	8.81 ± 0.52
	12/20	60.0	230	4.88 ± 0.31	28.33 ± 2.34	
DDT + DMBA	4/29	13.8*	200	6.15 ± 0.13	36.75 ± 0.48	10.18 ± 0.25
	4/29	13.8*	230	5.50 ± 0.15	32.50 ± 0.29	
Malathion + DMBA	14/16	87.5	200	5.14 ± 0.27	30.07 ± 1.86	8.45 ± 0.48
	11/12	91.7	230	5.15 ± 0.21	30.20 ± 1.52	
Normal‡				9.4	52	15.8
				(7.0 - 10.0)	(42 - 61)	(12.0 - 17.5)

‡ Normal values were obtained from Creskoff et al., (1949)

* Significantly different from control $p < 0.005$ (X^2 test, 2 X 2 contingency table)

Table 9. Differential blood cell count of animals with DMBA-induced leukemia

Treatment Group	Time of blood sample (days after DMBA)	Control (DMBA only)	DDT + DMBA	Malathion + DMBA	Normal'
No. of leukemic rats	200	12/21 (57.1 %)	2/29* (6.9 %)	9/16 (56.3 %)	
	230	11/20 (55.0 %)	2/29* (6.9 %)	8/12 (66.7 %)	
White-cell count ($10^3/mm^3$)	200	136.67 ± 21.86‡	77.00 ± 13.00	261.56 ± 40.35	9.0+
	230	177.00 ± 15.94	199.00 ± 117.02	159.00 ± 36.96	(6.0 - 18.0)
Platelet count ($10^3/mm^3$)	200	268.33 ± 15.37	350.00 ± 50.01	269.22 ± 40.24	800
	230	272.55 ± 11.19	285.00 ± 45.01	276.88 ± 19.24	(500 - 1000)
Reticulocytes (% RBC)	200	2.98 ± 0.25	3.00 ± 1.00	3.00 ± 0.33	3-4
	230	2.91 ± 0.25	3.50 ± 0.71	2.50 ± 0.27	
Neutrophils (%)	200	33.67 ± 1.33	41.00 ± 1.00	37.67 ± 1.73	20
	230	37.02 ± 1.19	39.00 ± 1.00	33.25 ± 2.96	(2 - 25)
Basophils (%)	200	8.92 ± 0.38	8.50 ± 1.50	4.89 ± 0.99	< 1
	230	8.18 ± 0.30	7.00 ± 1.00	9.13 ± 2.03	(0 - 1)
Lymphocytes (%)	200	6.42 ± 0.73	14.00 ± 6.00	4.11 ± 0.75	78
	230	4.82 ± 0.32	10.50 ± 1.50	[illegible].25 ± 1.64	(55 - 96)
Myelocytes (%)	200	50.67 ± 0.84	31.50 ± 5.50	52.22 ± 1.23	< 1
	230	49.18 ± 0.90	43.50 ± 0.50	50.38 ± 1.71	
Eosinophils (%)	200	< 1	< 1	< 1	2
	230	< 1	< 1	< 1	(0 - 4)
Monocytes (%)	200	< 1	< 1	< 1	< 1
	230	< 1	< 1	< 1	(0 - 3)

+ Mean values and range ‡ Mean ± S.E. * Significantly different from control $p < 0.001$

' Normal values from Creskoff et al. (1949) X^2 test, 2 X 2 contingency table

Table 10. Differential blood cell count of non-leukemic animals

Treatment Group	Time of blood sample (days after DMBA)	Control (DMBA only)	DDT + DMBA	Malathion + DMBA	Normal'
No. of rats	200	9/21 (42.9 %)	27/29 (93.1 %)	7/16 (43.8 %)	
	230	9/20 (45.0 %)	27/29 (93.1 %)	4/12 (33.3 %)	
White-cell count ($10^3/mm^3$)	200	14.25 ± 2.29 ‡	10.32 ± 0.98	15.91 ± 2.11	9.0+
	230	16.72 ± 2.94	10.95 ± 1.51	16.54 ± 2.51	(6.0 - 18.0)
Platelet count ($10^3/mm^3$)	200	682.81 ± 6.84	715.12 ± 5.51	592.71 ± 7.92	800
	230	673.97 ± 7.19	721.37 ± 5.32	609.33 ± 5.57	(500-1000)
Reticulocytes (% RBC)	200	2.51 ± 0.11	2.72 ± 0.37	3.11 ± 0.29	3 - 4
	230	2.92 ± 0.27	2.85 ± 0.21	3.34 ± 0.35	
Neutrophils (%)	200	21.94 ± 1.21	15.89 ± 1.86	24.29 ± 1.43	20
	230	23.48 ± 1.71	17.15 ± 1.19	23.57 ± 1.61	(2 - 25)
Basophils (%)	200	<1	<1	<1	<1
	230	<1	<1	<1	(0 - 1)
Lymphocytes (%)	200	62.31 ± 0.75	75.23 ± 0.50	69.42 ± 0.98	78
	230	71.72 ± 0.52	74.51 ± 0.73	70.18 ± 0.63	(55-96)
Myelocytes (%)	200	<1	<1	<1	<1
	230	<1	<1	<1	
Eosinophils (%)	200	<1	<1	<1	2
	230	<1	<1	<1	(0 - 4)
Monocytes (%)	200	<1	<1	<1	<1
	230	<1	<1	<1	(0 - 3)

+ Mean values and range ‡ Mean ± S.E. ' Normal values from Creskoff *et al.*, (1949)

Table 11. Values of serum protein fractions in animals with DMBA-induced leukemia (200 days after DMBA)

Treatment Group	Animals with leukemia No.	(%)	Total Protein (g/100 ml serum)	Albumin (%)	Gamma globulin (%)
			Mean ± S.E.		
Control (DMBA only)	12/21	(57.1)	5.27 ± 0.16	37.06 ± 0.66	17.82 + 1.45
DDT + DMBA	2/29	(6.9)	4.80 ± 0.13	28.80 ± 2.70	14.50 ± 5.10
Malathion + DMBA	9/16	(56.3)	5.68 ± 0.14	37.49 ± 0.92	9.54* ± 0.64
Normal'	0/15		6.23 ± 0.32 (6.0 - 8.2)	58.0 ± 0.82 (47 - 71)	15.0 ± 0.70 (11 - 23)

* Significantly different from control $p < 0.001$ (student's "t" test)

' Normal values were determined by running serum samples of 15 untreated SD rats of approximately the same age as the experimental group.

Since the separation of the other globulin components was not perfect, no attempt was made to calculate the concentrations of the alpha and beta globulins.

Internal examination of rats at necropsy revealed numerous internal tumors. The most frequently affected organs were lymph nodes, spleen, liver, lung, stomach, thymus, kidney, adrenals, uterus, ovaries, and bones (Figure 14, 15). All animals with internal tumors, anemia or leukemia had also shown mammary tumors at some time during their life. Those tumors that appeared greenish in color (Figure 16) in daylight gave a redish-orange fluorescence under U.V. light which is characteristic of chloromas. Chloromas were found in 43% (9/21) of all leukemic rats at necropsy. No chloromas were found in rats that did not have leukemia. Another very striking morphological change that was observed was the huge spleens found in many of the leukemic rats (Table 12). Table 13 shows organ weights of non-leukemic rats. The largest spleen found (Figure 17) was 23 times as large as the average normal spleen. There were many instances of livers in leukemic animals that were a light brownish-yellow color (Figure 15). Several of the leukemic animals had accumulated large volumes of tissue fluid in the abdominal cavity. One animal contained over 150 ml of fluid in its abdominal cavity (Figure 18). This fluid contained a high concentration of epithelial-like cells. A conclusive diagnosis for ascites tumors, however, could not be made. None of the animals showed enlarged thymuses which are characteristic of lymphatic leukemia.

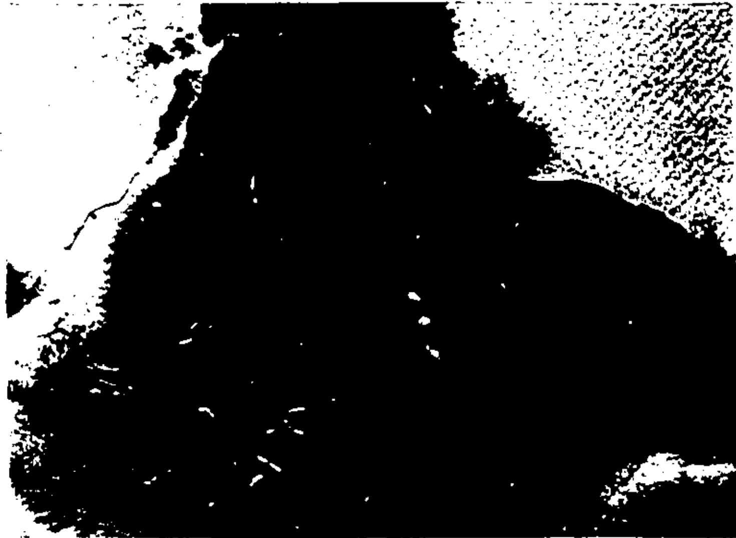
Slides made of tissues from various internal tumors showed the presence of myelogenous cells which indicated that leukemic cells had infiltrated many of the body organs.

Figure 13. A normal rat with thoracic and abdominal organs exposed. Note the color of the normal liver and the absence of internal tumors.

Figure 14. Animal with anemia and leukemia. Note white tumor masses in the lungs, green chloromas of the thymus, and cancer of the stomach.

Figure 15. Animal with anemia and leukemia showing tumors of the stomach, intestine, lymph nodes in the intestinal mesentary and lymph nodes in the inguinal region. The brownish-yellow liver was infiltrated with leukemic cells.

Figure 16. Ovaries from leukemic animal. Green color is typical of chloromas. These ovaries were 5-10 times normal size.



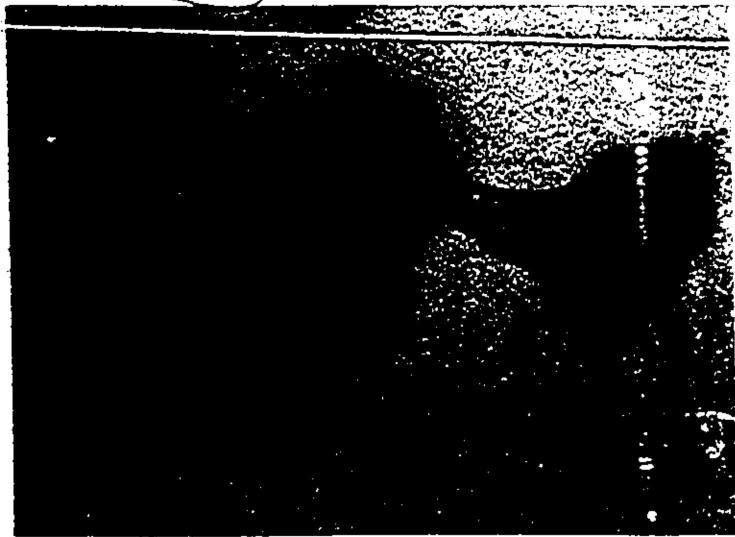


Figure 17. Spleen from leukemic rat compared to normal spleen (above). Lower spleen 23 times larger than upper (normal) spleen.

Figure 18. Bottle shows volume of fluid removed from abdominal cavity of leukemic rat. This animal also had tumors of liver, lung, stomach and lymph nodes.

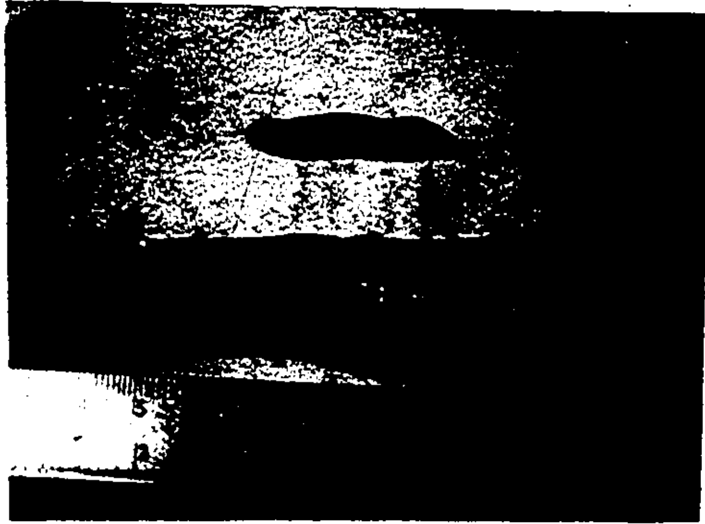


Table 12. Organ weights of leukemic animals at necropsy (230 days after DMBA)

Treatment Group	No. of leukemic animals	Thymus (mg)	Spleen (mg)	Liver (g)
			Mean ± S.E.	
Control (DMBA only)	12/21	147.73 ± 7.32	2745.00 ± 827.65	11.08 ± 0.75
DDT + DMBA	2/29	161.00 ± 8.00	7448.50 ± 6552.49	19.40 ± 5.90
Malathion + DMBA	9/16	153.38 ± 9.93	727.38 ± 89.25	10.18 ± 0.36
Normal'	0/15	145.6 ± 2.97 (112 - 243)	599.5 ± 4.21 (468 - 727)	9.71 ± 0.26 (7.8 - 12.4)

' Normal values were determined by removing the thymus, spleen and liver from 15 untreated SD rats of approximately the same age as the experimental group.

Table 13. Organ weights of non-leukemic animals at necropsy (230 days after DMBA)

Treatment Group	No. of animals	Thymus (mg)	Spleen (mg)	Liver (g)
		Mean ± S.E.		
Control (DMBA only)	9/20	158.44 ± 6.65	620.56 ± 7.26	9.02 ± 0.12
DDT + DMBA	27/29	148.72 ± 3.23	591.38 ± 5.98	12.39 ± 0.11
Malathion + DMBA	4/12	146.66 ± 6.11	588.75 ± 8.22	10.05 ± 0.32
Normal'	15/15	145.6 ± 2.97 (112 - 243)	599.5 ± 4.21 468 - 727)	9.71 ± 0.26 (7.8 - 12.4)

' Normal values were determined by removing the thymus, spleen and liver from 15 untreated SD rats of approximately the same age as the experimental group.

among anemic animals in the three treatment groups (Table 8). Control and DDT groups showed an increase (8-10%) in the severity of anemia from 200 days to 230 days after DMBA treatment started (Table 8). Microscopic examination revealed a hypochromic microcytic type of anemia in each of the anemic animals. Many of the red cells were slightly smaller in diameter than normal cells. A low hemoglobin concentration gave the cells a hypochromic appearence. The concentration of polychromatophilic cells or reticulocytes was well within the normal range. Irregular shaped red cells were found only in small numbers.

The diagnosis of leukemia was based on the presence of leukemic cells in excess of 1 % in the peripheral blood. Each leukemic animal showed a sharp increase in white cell count ranging from 5-46 times the normal average white cell count. There were increases in neutrophils and basophils and decreases in eosinophils and lymphocytes in the leukemic animals (Table 9). Table 10 shows the differential count of non-leukemic rats.

The predominant cell type in the leukocyte population was the myelocyte which is the key to the diagnosis of myelogenous leukemia. None of the other numerous types of leukemias were found in these animals. The platelet count averaged 64% below normal levels.

Examination of serum protein revealed an average drop of 9-23% in total serum protein level in the leukemic animals (Table 11). Albumin levels in the leukemic animals dropped an average of 35-50%. The mean concentration of gamma globulins remained within the normal range for control and DDT groups, however, there was a significant decrease in gamma globulin concentration in the malathion group ($p < 0.001$).

Discussion

It is rather unusual to identify only one type of leukemia in animals treated with the chemical carcinogens. Lymphatic leukemia can be induced just as easily as myelogenous leukemia which was the only variety of leukemia discovered in this experiment (Huggins and Sugiyama, 1966; Huggins et al., 1972; Shay et al., 1951). However, there is evidence from careful studies of survivors of the atomic destruction of Hirochima and Nagasaki that age at the time of exposure and possibly the dose, are among the several factors that determine the type of leukemia developed (Ackerman and del Regato, 1970). DMBA has been found to be a very potent leukemia-inducing chemical since it produces 100% incidence of leukemia even in low leukemic strain CFW mice (Ottonen and Ball, 1973). Huggins et al. (1966) have determined the best way to induce 100% leukemia in rats is to start with young animals (27 days old) and give many i. v. injections (4 or more) at two week intervals.

Each rat showing leukemia had most of the characteristics attributed to myelogenous leukemia such as: large spleen, enlarged lymph nodes, normal or small thymus, presence of green colored tumors infiltrated with leukemic cells, high white cell count and most important of all the presence of myelocytes in the peripheral blood in excess of one percent (Ackerman and del Regato, 1970; Zipf, 1959).

Hypochromic microcytic anemia which was the type of anemia found in all anemic rats is the type commonly found in rats with cancer. Characteristics common to hypochromic microcytic anemia are: low hemaglobin concentration, red cells less intensely stained, concentra-

tion of hemaglobin about the periphery of red cells and some distortion in the shape of the red cells (Daland, 1951). In anemic rats bearing different types of tumors a process of erythrocyte destruction is important in the pathogenesis of the anemia. As much as 10% of the circulating erythrocytes are lost in the hemorrhagic areas of the tumor (Price, 1959). This loss of blood leads to iron deficiency and an inability to produce adequate amounts of needed new hemaglobin. In advanced acute leukemia there are few platelets and most of the white blood cells are of the myelocyte type. Death is due to hemorrhage and anemia.

Green tumors, or chloromas, have been described in the past 100 years in man suffering from granulocytic leukemia (Schultz _et al_., 1954). The brilliant red color of metastatic lesions when illuminated with ultra violet light identified metastasis in tissues not observed in white light.

A porphyrin which appears to be a dicarboxylic acid and which has the solubility and spectrophotometric absorption characteristic of protoporphyrin has been responsible for at least part of the red fluorescence in chloromata (Shay _et al_., 1955). It is possible that the excess of porphyrin may be owing to the failure of the cell to incorporate iron in the porphyrin in the pathway of hemoprotein biosynthesis observed in certain anemias. The presence of excess porphyrin may be a fundamental characteristic of rapidly growing tissue (Schultz _et al_., 1954). Defects in the chain of reactions involved in porphyrin synthesis may be due to defects in bone marrow or liver (Kachmar, 1970).

Ascites tumors consist of isolated individual tumor cells suspended in serous fluid accumulated in the peritoneal cavity. Ascites tumors can be produced from solid tumors and ascites tumors may be reconverted to solid tumors. Ascites tumors represent and elegant testing system for anti-tumor agents, such as DDT, since the effectiveness of these may be readily followed by withdrawing ascites fluid and determining the change in cell count per unit volume (Arcos et al., 1968).

Enlargement of the spleen is quite common in blood diseases such as anemia and leukemia. The spleen can enlarge by proliferation, infiltration or both. A very large spleen is usually reddish-gray or chocolate-brown with innumerable gray-white or gray-yellow markings, corresponding to accumulation of lipoid substances. In making splenograms (impression slides) one can determine the origin of the leukemia from the type of cells prevalent in the spleen. During leukemia the liver becomes infiltrated with leukemic cells and enlarged but liver function is seldom significantly impaired (Bauer, 1963).

The liver produces albumins, alpha and beta and some non-immune gamma globulins; the spleen, bone marrow, and lymph nodes produce the immune gamma globulins and some beta globulins. If any protein fraction increases there may be an abnormal form of protein produced. Large quantities of proteins are lost with extensive bleeding and open wounds. Water is replaced by the body more rapidly than is protein, effecting a decrease in protein concentration (Kachmar, 1970). Winzler (1953) suggests that a general decrease in plasma albumin in cancer is possibly a result of a negative nitrogen balance. The growth of the tumor and the consequent withdrawal of nitrogen from the body pools

may result in a net loss of protein in spite of an overall nitrogen gain in tumor plus host. It is still unknown why serum albumin level is reduced more severely in cancer than are the other protein stores of the body. Serum albumin in malignant disease may be more labile and a negative nitrogen balance and hypoalbuminemia may be more closely related to increased protein breakdown than to decreased protein synthesis. The significance of increased alpha and beta globulins in cancer is still unknown. If an active immune response is fighting the cancer then gamma globulin levels should be high, however, a decrease in protein availability would tend to reduce gamma globulin concentration. Impaired synthesis in cancer giving low levels of gamma globulin would make the animal highly susceptible to infection and not show any antibody response (Petermann, 1960).

The arguments presented in Chapter 2 concerning the effects of DDT and malathion on the detoxification of DMBA and the probable role of estrogen in mammary tumor development certainly apply in this experiment to explain the induction of leukemia by DMBA. Kirschbaum (1951) was one of the first investigators to show that under certain conditions estrogen favors, and androgen inhibits leukemogenesis. However, there is no evidence that estrogen is itself leukemogenic.

There still remain some puzzling results, such as rapid growth of some mammary tumors, regression of other tumors and the low levels of gamma globulin in some of the animals, that can probably best be explained with a better understanding of the body's immune response to cancer and other diseases. Many studies have shown that successful immunotherapy in animals is only effective when the quantity of tumor

tissue is very small in the host (Powles, 1974). In leukemia, chemotherapy can often be given until no detectable disease remains, but after a variable disease-free period of remission the leukemia inevitably recurs. It is during this period of remission that immunotherapy is most likely to be effective. Injection of irradiated tumor cells with tubercle vaccine (BCG) has been shown (Powles, 1974) to be a very effective method for leukemia immunotherapy. BCG can reverse the immunosuppressive effects of certain carcinogens (Bast, et al., 1974).

An antiserum produced against DMBA-induced mammary tumors in rats was found to be effective in preventing growth of transplanted mammary tumor cells in 90% of all cases (Silinskas et al., 1974). These findings would suggest that an effective anti-leukemia vaccine could be produced to inhibit growth of chemically induced leukemias.

I am in agreement with many investigators who believe that the best attack on cancer will be a combination of chemotherapy and immunology.

CHAPTER IV

GENERAL CONCLUSIONS

1. DDT protects rats from DMBA-induced mammary tumors and leukemia.

2. This protective action is likely due to enhanced degradation of DMBA to non-carcinogenic metabolites which are then excreted, thus decreasing the concentration of DMBA available to the target tissue.

3. Degradation of DMBA can be brought about by an increase in AHH activity which can be stimulated by DDT.

4. DDT may also protect by stimulating steroid metabolism by AHH. A decrease in estradiol-17β may be necessary to avoid "initiation" and "promotion" of tumor development.

5. DDT stimulates enzyme activity by causing proliferation of smooth endoplasmic reticulum in liver cells, with a consequent increase in the amount of drug metabolizing enzymes. This was demonstrated in the DDT-treated animals which showed a significant increase in liver weight.

6. Leukemic animals showed a decrease in serum albumin levels possibly due to loss of large quantities of protein with extensive bleeding from open wounds or to a decrease in protein synthesis brought about by damage to the liver by DMBA.

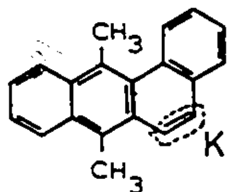
7. DMBA may impair synthesis of gamma globulin making the animal highly susceptible to infections and lacking any immune response.

Recommendations for further research.

There are many areas of chemical carcinogenesis that require more extensive studies. These areas include: (1) characterizing the active form(s) (ultimate carcinogens) which causes cell transformation; (2) understanding the genetic control of AHH inducibility: (3) determining the mechanism of AHH activity with respect to activation and detoxification of carcinogens; (4) determining the effects of hormones on tumor induction and growth; and (5) studying the role played by the immune system in recognizing and destroying newly transformed cells. The answers to these questions will take time. For the present we must develop a quick and reliable method for screening chemicals for possible carcinogenic activity in order to avoid contact with them until such time when we can protect ourselves against their lethal effects. Tests based on the assumption that a mutation occurs in a cell preceeding its transformation into a cancerous cell appear at this time to be the best screening test for the thousands of chemicals we are continuously exposed to in our environment.

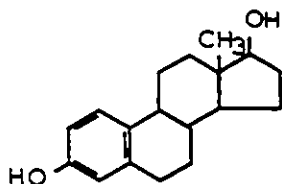
APPENDIX 1

STRUCTURAL FORMULAE

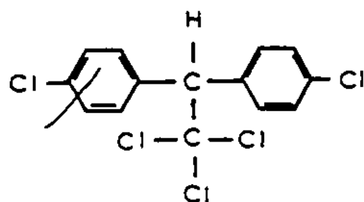


DMBA

7,12-Dimethylbenz(a)anthracene

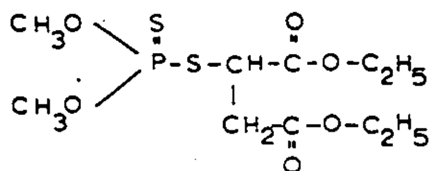


Estradiol-17β



p,p' - DDT

1,1,1-Trichloro-2,2bis(p-chlorophenyl)ethane



Malathion

S-(1,2-Dicarbethoxyethyl)-O,O-dimethyl-dithiophosphate

APPENDIX 2

CONTROL GROUP BODY WEIGHT CHART

AGE (DAYS)	MEAN BODY WEIGHT (g)	S.D.	S.E.	RANGE (g)
36	119.0	6.93	1.27	103-136
40	135.4	8.32	1.52	116-156
47	156.5	15.57	2.84	83-177
54	170.4	15.92	2.91	99-189
61	188.1	18.76	3.42	105-217
68	200.3	10.36	1.92	180-228
75	208.8	9.21	1.71	191-226
82	220.6	9.83	1.83	200-242
89	230.4	13.42	2.49	200-258
96	223.8	11.48	2.13	207-249
103	235.9	16.45	3.05	180-258
110	242.0	20.37	3.78	166-272
117	248.1	14.40	2.72	219-278
124	249.3	15.05	2.84	228-272
131	251.8	20.02	3.78	178-277
138	252.7	13.14	2.53	228-276
145	251.4	13.25	2.55	226-276
152	254.6	13.58	2.61	230-284
159	257.4	14.31	2.75	225-286
166	261.0	16.07	3.09	227-291
173	263.3	17.31	3.33	218-290
180	266.7	19.73	3.80	214-300
187	266.6	27.38	5.27	166-310
194	268.2	19.59	3.84	218-296
201	283.2	16.18	3.17	256-308
208	282.3	18.16	3.56	250-315
215	281.5	19.01	3.73	248-310
222	281.3	20.27	4.14	244-316
229	284.5	20.67	4.22	248-318
236	289.5	20.84	4.25	254-320
243	293.6	22.10	4.51	254-330
250	293.0	21.40	4.67	255-341
257	295.1	20.66	4.62	255-339
265	297.1	19.43	4.34	256-332
272	300.0	20.03	4.48	258-332
280	302.0	21.64	4.84	258-334

APPENDIX 3

DDT GROUP BODY WEIGHT CHART

AGE (DAYS)	MEAN BODY WEIGHT (g)	S.D.	S.E.	RANGE (g)
36	125.2	6.00	1.10	111-132
40	144.8	7.42	1.36	130-156
47	164.6	6.01	1.10	152-174
54	178.7	9.32	1.70	157-197
61	194.6	12.02	2.23	160-214
68	204.8	9.99	1.86	182-227
75	220.9	9.22	1.71	189-232
82	232.4	11.35	2.11	209-252
89	236.5	10.53	1.10	212-258
96	238.9	9.53	1.77	219-255
103	238.4	9.21	1.71	219-253
110	252.3	11.57	2.15	219-274
117	252.9	10.56	1.96	236-272
124	256.3	12.50	2.32	234-278
131	258.7	12.68	2.36	232-278
138	257.2	10.72	1.99	232-276
145	257.6	11.42	2.12	234-276
152	256.1	10.75	2.00	232-274
159	263.4	10.36	1.92	242-282
166	272.1	10.97	2.04	250-292
173	273.5	11.12	2.06	251-293
180	275.9	12.11	2.25	248-296
187	280.6	11.01	2.04	254-298
194	282.5	12.43	2.31	252-302
201	284.2	13.89	2.58	252-307
208	284.1	12.01	2.23	258-306
215	284.3	12.70	2.36	258-308
222	294.0	13.33	2.47	268-324
229	294.1	13.02	2.42	268-328
236	294.2	14.19	2.64	264-330
243	295.7	16.29	3.01	264-336
250	296.6	17.64	3.28	263-337
257	297.7	16.16	3.00	265-340
265	299.0	16.15	3.00	269-349
272	300.4	17.72	3.29	273-358
280	302.7	20.81	3.86	274-368

APPENDIX 4

MALATHION GROUP BODY WEIGHT CHART

AGE (DAYS)	MEAN BODY WEIGHT (g)	S.D	S.E	RANGE (g)
36	123.1	6.23	1.14	109-136
40	140.2	7.68	1.40	116-156
47	160.9	7.12	1.30	149-177
54	176.9	7.27	1.33	165-195
61	185.0	10.34	1.89	150-201
68	198.2	12.23	2.23	148-214
75	209.3	16.60	3.03	132-224
82	224.6	21.08	3.85	131-256
89	230.4	11.83	2.20	206-254
96	226.2	11.69	2.17	201-251
103	235.7	11.09	2.06	204-263
110	249.4	13.33	2.48	222-273
117	250.6	13.08	2.43	227-279
124	254.1	12.03	2.23	232-278
131	264.2	11.72	2.18	239-284
138	259.0	11.62	2.16	233-285
145	254.0	12.91	2.40	230-288
152	253.9	14.48	2.69	230-290
159	263.2	12.27	2.28	242-294
166	275.7	13.89	2.58	253-314
173	275.0	17.33	3.22	220-318
180	278.4	16.29	3.14	256-328
187	279.7	13.75	2.70	260-308
194	279.5	13.12	2.57	258-308
201	290.5	15.86	3.11	270-328
208	290.7	17.46	3.42	265-332
215	290.4	17.27	3.39	260-329
222	289.8	17.67	3.61	260-336
229	292.0	16.23	3.38	265-336
236	297.1	16.22	3.38	273-338
243	297.4	15.60	3.49	275-338
250	299.8	16.27	4.07	279-341
257	299.6	15.64	4.18	281-345
265	295.7	10.93	3.16	283-322
272	297.3	15.19	4.39	281-341
280	298.0	18.23	5.26	280-352

APPENDIX 5

CONTROL GROUP TUMOR GROWTH CHART

Tumor age (weeks)	Mean tumor size (mm)	S.D.	S.E.	No. of tumors	Range
1	4.12	2.40	0.30	65	1-10
2	7.36	5.09	0.64	64	2-25
3	10.96	9.25	1.25	55	2-50
4	13.94	10.60	1.51	49	2-35
5	16.51	13.04	1.90	47	2-50
6	17.91	13.74	2.07	44	2-50
7	19.05	14.40	2.20	43	2-50
8	19.44	13.63	2.18	39	2-50
9	20.22	13.12	2.16	37	2-45
10	20.43	14.23	2.41	35	2-50
11	21.30	14.65	2.55	33	2-50
12	23.10	14.81	2.66	31	2-50
13	22.55	15.00	2.78	29	2-50
14	23.67	15.92	3.06	27	2-50
15	22.86	15.59	3.32	21	2-50
16	27.79	17.89	4.78	14	4-60
17	28.55	17.20	5.18	11	4-55
18	30.40	19.04	6.02	10	4-60
19	26.38	18.66	6.60	8	1-50
20	25.14	19.64	7.42	7	1-50
21	25.14	19.64	7.42	7	1-50
22	27.67	20.14	8.22	6	1-50
23	42.50	14.79	7.40	4	20-60
24	43.33	20.55	11.86	3	20-70

APPENDIX 6

DDT GROUP TUMOR GROWTH CHART

Tumor age (weeks)	Mean tumor size (mm)	S.D.	S.E.	No. of tumors	Range
1	4.20	1.72	0.44	15	1-8
2	8.93	4.14	1.07	15	2-15
3	11.64	8.47	2.26	14	2-30
4	12.64	8.57	2.29	14	2-30
5	13.71	9.66	2.58	14	2-30
6	14.07	9.61	2.57	14	2-30
7	14.38	9.71	2.69	13	2-30
8	13.50	10.01	2.89	12	2-30
9	14.09	9.18	2.77	11	5-30
10	1364	8.74	2.64	11	5-30
11	13.18*	9.53	2.87	11	5-40
12	13.18	9.53	2.87	11	5-40
13	13.50	11.13	3.52	10	5-45
14	14.13	13.85	4.90	8	5-50
15	9.67*	2.75	1.12	6	6-15
16	9.50*	3.99	1.63	6	2-15
17	8.00*	3.74	1.87	4	2-12
18	10.00	5.00	3.54	2	5-15
19	10.00	5.00	3.54	2	5-15
20	10.00	5.00	3.54	2	5-15
21	10.00	5.00	3.54	2	5-15
22	15.00	0	0	1	15
23	15.00	0	0	1	15
24	15.00	0	0	1	15

* Significantly different from control (student's "t" test) $p < 0.05$

APPENDIX 7

MALATHION GROUP TUMOR GROWTH CHART

Tumor age (weeks)	Mean tumor size (mm)	S.D.	S.E.	No of tumors	Range
1	3.80	1.81	0.20	82	1-10
2	6.18	4.31	0.48	82	2-30
3	8.37	6.28	0.71	82	2-40
4	10.66	8.27	0.95	78	2-45
5	13.12	11.02	1.28	74	2-50
6	15.01	12.52	1.48	73	2-60
7	17.91	13.80	1.67	72	2-60
8	20.44	14.61	1.85	65	2-60
9	22.47	16.29	2.12	59	2-70
10	22.06	16.97	2.35	53	2-70
11	22.63	15.63	2.38	44	2-60
12	22.10	14.90	2.39	39	1-50
13	23.39	15.65	2.61	36	1-50
14	25.28	18.09	3.20	33	1-60
15	24.69	16.59	3.25	28	2-70
16	26.28	17.05	3.41	25	2-70
17	26.00	18.51	3.95	22	2-80
18	27.10	19.52	4.37	20	2-80
19	25.59	14.45	3.50	18	3-50
20	32.31	15.14	4.20	15	10-60
21	35.83	15.39	4.44	12	20-60
22	38.13	17.31	6.12	8	20-70
23	32.00	9.27	4.15	5	20-45
24	31.25	11.39	5.70	4	20-50

APPENDIX 8

Deaths Prior to Tumor Incidence

Treatment Group	Total No. of animals	Early deaths	No. survivors when tumors begin to appear
Control (DMBA only)	30	2	28
DDT + DMBA	30	1	29
Malathion + DMBA	30	1	29

Necropsy: Death in DDT animal due to punctured esophagus
death in malathion animal due to lung tubing
death in control animal - 1) due to lung tubing
2) unknown causes

None of the early deaths had visible tumors.

APPENDIX 9

(A) Two-by-two, Chi-square, X^2

From Wang 300 series program library, # 360.12-ST.

The value of X^2 is determined from the equation:

$$X^2 = \frac{N(/a_1b_2 - a_2b_1/ - \tfrac{1}{2}N)^2}{(a_1+b_1)(a_2+b_2)(a_1+a_2)(b_1+b_2)}$$

X^2 is corrected using Yate's correction (using $\log_e X$).

(B) Student's "t" test

Using Monroe model 1860 statistical programmable printing calculator.

The value of t is determined from the equation:

$$t_{ind} = \frac{\overline{X} - \overline{Y}}{\sqrt{\left(\frac{(N_x - 1)\ SD_x 2 + (N_y - 1)\ SD_y 2}{N_x + N_y - 2}\right)\left(\frac{1}{N_x} + \frac{1}{N_y}\right)}}$$

where: $\overline{X} = \frac{\sum X}{N_x}$ $\overline{Y} = \frac{\sum Y}{N_y}$

SD_x = Standard deviation of x

SD_y = Standard deviation of y

(C) Standard deviation; standard error

Using Monroe model 1860 calculator.

$$SD = \sqrt{\frac{\sum X^2 - \frac{(\sum X)^2}{N}}{N}} \qquad \overline{X} = \frac{\sum X}{N}$$

$$\text{Std error} = \frac{SD}{\sqrt{N}}$$

Appendix 10

Biuret Method for the Determination of Total Protein in Serum

The biuret method used was adapted from the method outlined by Reinhold (1953).

Biuret diluent:

KI, 0.5% (w/v) in 0.25 N NaOH using CO_2- free water.

Stock biuret reagent:

Dissolve 15.0 g $CuSO_4 \cdot 5H_2O$ in 80 ml distilled water. Prepare a solution of 45.0 g potassium sodium tartrate tetrahydrate in 700 ml of biuret diluent and slowly add the $CuSO_4$ solution. Add biuret diluent to a volume of 1000 ml. All solutions are prepared at room temperature. For a working biuret, dilute the stock biuret 1:5 with biuret diluent.

Procedure:

To a 0.10 ml sample of unknown add 2.0 ml of 0.85% saline, then add 8.0 ml of working biuret. Thoroughly mix the test-tube contents and allow the color to develop for 30 minutes. Measure the absorbances of the solutions at 550 nm using a spectrophotometer with the blank containing only 2.10 ml saline set at 100% T.

Construct a calibration curve using known concentrations of bovine serum albumin. The absorbance data can be converted to g of protein/100 ml serum.

Appendix 11

Serum Protein Electrophoresis

Serum proteins were separated using the method developed by Aronsson and Gronwall (1957).

Apparatus:

LKB 3276 B paper electrophoresis apparatus

Whatman No. 1 filter paper (20X410 mm)

Sample Applicator type 3276-SA (capacity 5 μl)

Buffer Solution:

Trishyroxymethylaminomethane (TRIS) 0.5 M; Ethylenediaminetetracetic acid (EDTA) 0.021 M; Boric acid 0.075 M at pH 8.9.

Electrophoresis Run:

Eight strips 20X410 mm were run simultaneously using a current of 3.0 mA and a potential of 5.0 V/cm (80 volts) for 18 hours. This combination of current and time gave the best separation of serum proteins.

Drying:

The paper strips were freely suspended in air on a wire support and dried in an oven at 105°C to total dryness.

Dyeing:

Dye solution 45 wt.% CH_3OH, 45% water, 10% CH_3COOH, and 7 g of Amido-black 10 B for 1 liter of solution. The washing solutions were prepared in the same way with the exception of Amido-black. Strips were dyed for 10 minutes and washed 4 times in fresh washing solution for 15, 30, 30 and 45 minutes respectively. The strips were dryed at room temperature cleared with mineral oil and evaluated

using a Densicord 542 densitometer, a Varicord 42 recorder and an Integraph Model 49 integrator. This combination of recording instruments gave an accurate numerical estimate of the quantity of protein in each fraction separated on the filter paper strips.

Appendix 12

Chemical and Equipment Suppliers

Acetic acid- Fisher, Toronto

Amido-Black 10B stain- Fisher, Toronto

Boric acid- Fisher, Toronto

Colorimeter (Spectronic 20)- Bausch and Lomb

DDT- Aldrich Chemical Co., Milwaukee, Wis.

Densitometer (Densicord 542)- Photovolt, New York, N. Y.

DMBA- Sigma Chemical Co., St. Louis, Mo.

Electrophoresis apparatus(LKB3276B)-LKB-Produkter,AB, Stockholm,Sweden

Ether (anesthesia grade)- Fisher, Toronto

Filter paper (Whatman No. 1)- Fisher, Toronto

Gentian Violet stain- Fisher, Toronto

Hayem's solution (Tablets)- BDH, Toronto

Hemacytometer (Spencer Bright line)- Fisher, Toronto

Hydrochloric acid- Fisher, Toronto

Integrator (Integraph Model 49)-Photovolt, New York, N. Y.

Laboratory Chow Meal- Ralston Purina Co.

Malathion (98.9% secondary standard)-American Cyanamid Co. Princton,N.J.

Rats (Sprague Dawley)- Holtzman Co., Madison, Wis.

Tris(Tris hydroxymethyl aminomethane)- Fisher, Toronto

Wright's stain- Fisher, Toronto

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VITA AUCTORIS

Name:

Kestutis Charles Silinskas

Born:
October 22, 1945, Kempten, Germany.

Elementary Education:

Vancouver, British Columbia.
Windsor, Ontario.

Secondary Education:

Walkerville Collegiate Institute, Windsor, Ontario.
1960-1966.

University Education:

University of Windsor, Windsor, Ontario
B.Sc. 1966 - 1969.
University of Western Ontario, London, Ontario
Diploma in Education 1969 - 1970.
University of Windsor, Windsor, Ontario
B.Sc. (Hons.) in Biology. 1971 - 1972.

Awards and Scholarships:

Ontario College of Education Fellowship. 1969 - 1970.

Employment:
Windsor Board of Education, High School Teacher,
1970 - 1971.
University of Windsor, Teaching Assistant, 1972 - 1973.
University of Windsor, Lecturer, 1973 - 1974.